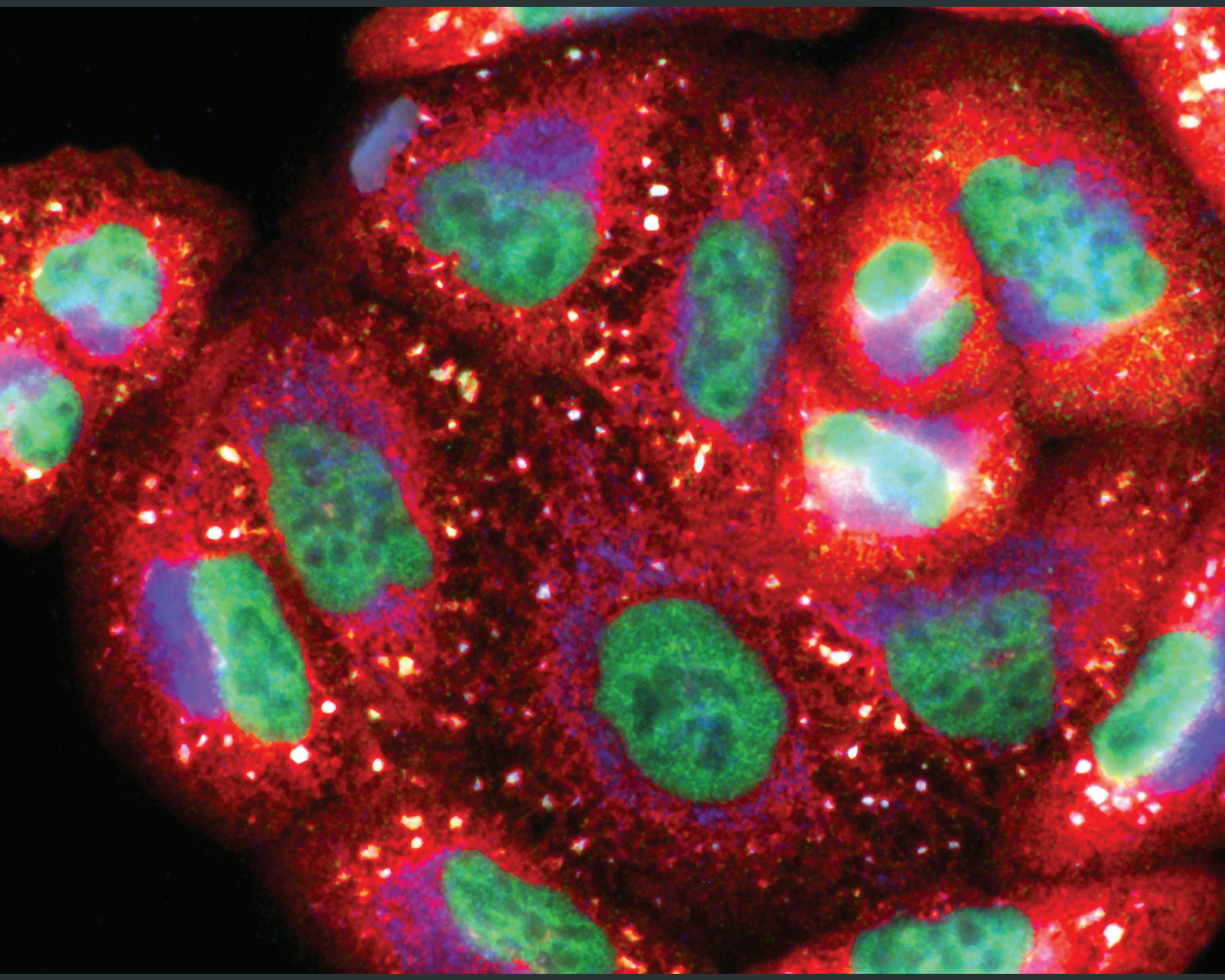
Oxidative Medicine and Cellular Longevity

Dietary Polyphenols and Their Effects on Cell Biochemistry and Pathophysiology 2014

Guest Editors: Cristina Angeloni, Tullia Maraldi, Dragan Milenkovic, and David Vauzour



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Editorial Board

Contents

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Editorial

Dietary Polyphenols and Their Effects on Cell Biochemistry and Pathophysiology 2014

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Epidemiological studies suggest that high dietary intake of phytochemicals and in particular of polyphenols is associated with decreased risk of a multitude of diseases states including cardiovascular disease, cancer, and neurodegenerative diseases.

As oxidative stress is involved in all these pathological conditions, the antioxidant properties of polyphenols and other natural compounds have attracted the interest of many authors.

M. Horvathova et al. investigated the effects of the intake of Robuvit, a water extract obtained from the wood of *Quercus robur*, showing that it is associated with decrease of markers of oxidative stress and increase of activity of antioxidant enzymes and total antioxidant capacity of plasma. As reactive oxygen species (ROS) are strongly associated also with skin aging, the study of K. Watanabe et al. investigated the antiatrophic effects of melinjo seed extract (MSE), containing transresveratrol and resveratrol derivatives, on age-related skin pathologies in $Sod1^{-/-}$ mice. Their results demonstrated that MSE and transresveratrol significantly reversed skin thinning via reduction of oxidative damage.

Even if many of the positive biological actions of flavonoids have been assigned to their antioxidant properties, there is an emerging view that they might play other protective activities such as acting as anti-inflammatory agents. The study by O. Farkas et al. strengthened this point of view demonstrating that the flavonoids apigenin and its trimethylated analogue attenuated LPS-induced inflammation in IPEC-J2 nontransformed intestinal epithelial cells. The anti-inflammatory, antioxidative, and cytoprotective effects of resveratrol, epigallocatechingallate, genistein, apigenin, (−)-epicatechin, and other polyphenols have been reviewed by A. Malhotra et al., J. Shay et al., and S. Upadhyay and M. Dixit.

As far as the lipid metabolism is concerned, I. Eseberri et al. investigated the effect of doses of quercetin on triacylglycerol accumulation in maturing preadipocytes and mature adipocytes. They concluded that quercetin, in the range of serum concentrations, is able to inhibit adipogenesis, but higher doses are needed to reduce fat accumulation in mature adipocytes.

Regarding glucose metabolism, D. M. P. H. J. Boesten et al. examined the protective role of three structurally related flavonoids (rutin, quercetin, and flavone) during high glucose conditions in human umbilical vein endothelial cells. They conclude that this protective effect of flavonoids is a combination of the flavonoids abilities to inhibit both PARP activation and aldose reductase enzyme activity. The review by L. Basheer and Z. Kerem focuses on interactions between dietary polyphenols and CYP3A4.

Among the fruits presenting chemopreventive and/or chemotherapeutic potential is pomegranate that has been shown to exert anticancer activity which is generally attributed to its high content of polyphenols. E. Turrini et al. reviewed pomegranate antiproliferative, anti-invasive, and antimetastatic effects focusing on apoptosis induction and the inhibition of inflammatory pathways.

Polyphenolic extracts from the edible part of artichoke (*Cynara scolymus* L.) have also been shown to be potential chemopreventive and anticancer dietary compounds as they induce apoptosis and decrease the invasive potential of the human breast cancer cells (A. M. Mileo et al.).

Among the polyphenols present in fruits are proanthocyanidins that are the most widely represented products of plants secondary metabolism. C. Minker et al. showed that they reach the colon practically intact, where they are able to locally exert their anticancer activities on colorectal precancerous and cancerous cells and can exert proapoptotic activities. Together with proanthocyanidins, it has been observed that other families of polyphenols can exert variety of biological properties, including both antioxidant and nonantioxidant functions, as described in the review article by J. Shay et al. Finally, J. Wang et al. review epidemiological studies or controlled clinical trials which employed biomarkers of exposure for polyphenols to help assess their anticarcinogenic role and concluded that more evidences should be collected before a conclusion can be made towards their protective roles.

Atherosclerosis is the primary cause of cardiovascular diseases and high levels of LDL cholesterol (LDL-C) and LDL oxidation have been regarded as two important risk factors for atherogenesis. H. Han et al. designed a study to evaluate the effects of flaxseed oil containing α-linolenic acid ester of plant sterols (ALA-PS) on apoE knock-out mice that received high fat diet alone or supplemented with flaxseed oil with or without ALA-PS for 18 weeks. Results demonstrated that flaxseed oil containing ALA-PS improved overall lipid levels, inhibited inflammation, and reduced oxidative stress. M. A. Rahman et al. demonstrated that the polyphenolic compounds present in the *Flammulina velutipes*, also known as golden needle mushroom, inhibited LDL oxidation and they ascribed this chain-breaking activity to protocatechuic acid (PCA), p-coumaric acid, and ellagic acid. The study by R. Varì et al. expanded this knowledge trying to define the molecular mechanism responsible for the protective effects of PCA against oxidative and proapoptotic damage exerted by oxLDL in J774 A.1 macrophages. They demonstrated the essential role of JNK/Nrf2 signalling pathway in the antiapoptotic activity exerted by PCA in oxidatively stressed macrophages by improving the endogenous cellular antioxidant system.

As hypertension is another important risk factor for cardiovascular disease, M. Micucci et al. focused their attention on the potential application of *Olea europaea* L. leaf extract (OEE) and of a *Hibiscus sabdariffa* L. flower extract (HSE) in the prevention/counteraction of hypertension, a pathological condition affecting a large number of populations. They showed the antioxidant and cytoprotective effects of OEE and HSE and their ability to modulate cardiac inotropy and chronotropy together with their relaxant activity on vascular smooth muscle.

In a review paper, A. N. Orekhov et al. addressed the important issue of identifying natural compound as an alternative strategy for atherosclerosis prevention as most of the synthetic drugs available have severe side effects and high cost of treatment. Moreover, they analyzed the achievements in the development and use of models based on primary cultures of human aortic cells to test the antiatherosclerotic activity of compounds of natural origin.

Neurodegenerative diseases are dramatically increasing worldwide and this is strictly correlated to the aging of the world population; therefore, natural compounds could be an effective and cheap preventive strategy to maintain cognitive health with age.

F. Jagla and O. Pechanova reviewed the potential protective effects of polyphenols on the development of neurodegenerative diseases, in particular, focusing on endothelial dysfunction implicated in cognitive decline. The review by E. Tellone et al. explored the neuroprotective role of resveratrol in counteracting different neurodegenerative disorders. M. Reinisalo et al. analyzed resveratrol positive effects on the most common aging-related diseases clarifying the molecular mechanisms involved in the stilbene-mediated protection against oxidative stress. Carqueja (*Baccharis trimera*) is a native plant found throughout South America and its hydroalcoholic extract (CHE) has been demonstrated to have a protective effect in a *C. elegans* model of Alzheimer's disease (F. A. Paiva et al.). A. Masci et al. showed that a raw broccoli sprout juice protects neuroblastoma cells against Aβ-induced cytotoxicity via the induction of antiapoptotic signals, such as increased Hsp70 mRNA levels, and the activation of Nrf2-ARE signalling pathway upregulation of Nrf2-dependent antioxidant capacity.

Besides neurodegenerative disease and ageing, there are other disorders related to brain damage such as psychiatric disorders including major depression, attention deficit hyperactivity disease (ADHD) and schizophrenia, and cognitive deficit and brain damage induced by chronic alcohol consumption. N. Phunchago et al. studied the effects of *Tiliacora triandra* extract on memory impairment, neuron density, cholinergic function, and oxidative stress in hippocampus of alcoholic rats demonstrating that the extract improved memory deficit partly via decreasing oxidative stress and the suppressing AChE. Polyphenolic compounds can be also involved in modulation of mental health including brain plasticity, behavior, mood, depression, and cognition. J. Trebatická and Z. Duracková analyzed the large number of studies on the effects of natural polyphenols on mental disorders, and they concluded that even if polyphenols in the diet have the potential to become medicaments in the field of mental health, the use in clinical practice is still a long way off.

In conclusion, this special issue provides new findings on the role that polyphenols and other natural compounds play in the prevention of chronic degenerative diseases and expands our knowledge on the new mechanisms of actions of these pleiotropic compounds.

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Research Article

Carqueja (*Baccharis trimera*) Protects against Oxidative Stress and β-Amyloid-Induced Toxicity in *Caenorhabditis elegans*

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Carqueja (*Baccharis trimera*) is a native plant found throughout South America. Several studies have shown that Carqueja has antioxidant activity *in vitro*, as well as anti-inflammatory, antidiabetic, analgesic, antihepatotoxic, and antimutagenic properties. However, studies regarding its antioxidant potential *in vivo* are limited. In this study, we used *Caenorhabditis elegans* as a model to examine the antioxidant effects of a Carqueja hydroalcoholic extract (CHE) on stress resistance and lifespan and to investigate whether CHE has a protective effect in a *C. elegans* model for Alzheimer's disease. Here, we show for the first time, using *in vivo* assays, that CHE treatment improved oxidative stress resistance by increasing survival rate and by reducing ROS levels under oxidative stress conditions independently of the stress-related signaling pathways (p38, JNK, and ERK) and transcription factors (SKN-1/Nrf and DAF-16/Foxo) tested here. CHE treatment also increased the defenses against β-amyloid toxicity in *C. elegans*, in part by increasing proteasome activity and the expression of two heat shock protein genes. Our findings suggest a potential neuroprotective use for Carqueja, supporting the idea that dietary antioxidants are a promising approach to boost the defensive systems against stress and neurodegeneration.

1. Introduction

Carqueja (*Baccharis trimera*) is a native plant found throughout South America. The plant has been used in popular alternative medicine to treat gastrointestinal and hepatic diseases as well as inflammatory processes and diabetes [1].

Several studies have shown that Carqueja has antioxidant activity *in vitro* [2, 3], as well as anti-inflammatory [2, 4, 5], antidiabetic [6], analgesic [5], antihepatotoxic [7], and antimutagenic [8] properties. More recently, Pádua et al. [9] showed that Carqueja also improves the *in vivo* antioxidant defense system in a rat inflammatory model induced by acetaminophen. Carqueja antioxidant activity has been related to its several phenolic compounds, including the polyphenols quercetin and rutin as well as phenolic acids such as caffeoylquinic acids [2, 3, 9, 10].

Phenolic compounds are widely found in various plant foods such as açaí [11], kiwi fruit [12], green tea, cocoa, and red wine [13], and they have many physiological and pharmacological functions. *In vitro* and *in vivo* studies have shown that phenolic compounds have powerful effects on biological responses by scavenging reactive oxygen species (ROS) and by activating cellular signaling pathways [11, 14]. Previous studies have also shown the capacity of phenolic

acids to prevent damage in neuronal cells and suggest that phenolic compounds are efficient as neuroprotective agents [15]. Additionally, caffeoylquinic acids have been shown to protect against β-amyloid toxicity in MC65 and SHSY5Y neuroblastoma cell lines [16] and to have a neuroprotective effect in the mouse brain. These acids have also been shown to improve spatial learning and memory in senescence accelerated mice (SAMP8) [17].

Altogether, these findings have prompted health care professionals to recommend the ingestion of foods rich in naturally occurring antioxidants as a way to prevent chronic diseases, such as atherosclerosis, cancers, diabetes, and neurodegenerative disorders, and to promote health and well-being in general [18].

Even though a number of investigations have shown the antioxidant activity of Carqueja, very few of them explored its antioxidant potentials *in vivo*.

In this study, we used *Caenorhabditis elegans* as a model to examine the antioxidant effects of a Carqueja hydroalcoholic extract (CHE) on stress resistance, survival rate, and lifespan and to investigate whether CHE has a protective effect in a *C. elegans* model for Alzheimer's disease. Our study is the first to show that not only does Carqueja have antioxidant proprieties *in vivo*, but it also has protective effects against β-amyloid-induced paralysis in *C. elegans*. Our results also suggest that Carqueja's antioxidant proprieties are not mediated by the action of stress-related signaling pathways but are rather the result of a direct effect of CHE. Finally, CHE's potential neuroprotective action against β-amyloid toxicity could be, in part, due to increased proteasome activity and heat shock protein gene expression.

2. Materials and Methods

2.1. Chemicals and Reagents. Methanol (MeOH), DPPH (2,2-diphenyl-1-picryl-hydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu (FC) reagent, gallic acid, *tert*-butyl hydrogen peroxide (t-BOOH), and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2′,7′-Dichlorodihydro-fluorescein diacetate (H_2DCF-DA) and Nile red were purchased from Invitrogen (Eugene, Oregon, USA). Hydrogen peroxide (H_2O_2) was purchased from VETEC (Duque de Caxias, Rio de Janeiro, Brazil). Streptomycin was purchased from Serva Eletrophoresis.

2.2. Plant Material. The aerial parts of *Baccharis trimera* were collected from Mariana, Minas Gerais, Brazil. The collected plants were authenticated and a voucher specimen (OUPR 24050) was deposited at the Herbarium José Badini (UFOP). After identification, the aerial parts of the plant were dried in a ventilated oven or incubator, pulverized, and stored in plastic bottles. The preparation of Carqueja hydroalcoholic extract (CHE) was conducted according to Pádua et al. [3], with some modifications. One hundred g of the dried plant powder was extracted with distilled water and 70% ethanol 1 : 1 for 24 hours (h). Solids were removed by vacuum filtration and the solvent was removed by a rotary evaporator.

2.3. Worm Strains: Maintenance and Treatment. Nematodes used were the wild-type strain N2, BA17, *fem-1(hc17)*; EU1, *skn-1(zu67)IV/nT1*; CF1038, *daf-16(mu86)*; AU3, *nsy-1 (ag3)*; KU4, *sek-1(km4)*; VC8, *jnk-1(gk7)*; AM1, *osr-1(rm1)*; KU25, *pmk-1(km25)*; MH37, *mpk-1(ku1)/unc-32(e189)*; MT2605, *unc-43(n498n1186)*; LD1171, *Is003 (gcs-1::GFP)*; CL2166, *dvls19[pAF15(gst-4::GFP::NLS)]*; CF1553, *muIs84 [pAD76(sod-3::GFP)]*; CL2070, *dvIs70[pCL25(hsp-16.2p::GFP)+pRF14(rol-6)]*; SJ4005, *zcls4[hsp-4::GFP]*; CL4176, *smg-1(cc546)*; *dvls27[pAF29(myo-3/Aβ_{1-42}/letUTR)]+pRF4[rol-6(su10069)]* and TJ356, *zIs356[pGP30(DAF-16::GFP)+pRF4 (rol-6)]*. All nematodes were cultivated on nematode growth medium (NGM) [19] at 20°C, except for the strain CL4176 which was maintained at 16°C. Synchronous L1 populations for all strains were obtained by hypochlorite treatment of gravid hermaphrodites except for *skn-1(zu67)* mutant and the transgenic lines expressing *gcs-1::GFP* (LD1171) and β-amyloid peptide 1–42 (CL4176) which were obtained by egg laying.

For all the experimental procedures, CHE was diluted in basal solution (0.1 M NaCl, 50 mM KPO_4 buffer) to 0.5, 5, and 50 mg/mL and filter-sterilized. Basal solution (control) or CHE plus basal solution (treatment) was then mixed with an *E. coli* (OP50) pellet at OD600 of 1 and seeded onto NGM plates. In experiments conducted with dead bacteria, NGM plates seeded with *E. coli* OP50, with or without CHE, were treated with 10 mM kanamycin (KAN-treated).

RNA interference (RNAi) was carried out using the feeding method described previously, with empty pL4440 as the control [20]. Briefly, RNAi clones were grown with 12.5 μg/mL tetracycline and 100 μg/mL ampicillin. On the following day, cultures were diluted in LB supplemented with 60 μg/mL ampicillin and grown to an OD600 of 1. This culture was used to seed plates containing ampicillin and 1 mM IPTG and left to dry for 1 day at room temperature. Synchronized L1 larvae were then placed at 20°C on *E. coli* HT115 that expressed *daf-16* and/or *skn-1* or control RNAi for 46 h, until they reached the L4 stage. *skn-1* RNAi efficiency was verified by the absence of F1 larvae. For *daf-16*, RNAi efficiency was confirmed by the suppression of GFP emission on *DAF-16::GFP* transgenic line (TJ356).

2.4. Polyphenol Dosage and DPPH Radical Scavenging Assay. Total polyphenol content of the CHE was determined by the Folin-Ciocalteu method as described by Georgé et al. [21]. In brief, 2.5 mL of Folin reagent was added to 500 μL of sample or standard solution of gallic acid. After incubation at room temperature for 2 minutes (min), 2 mL of sodium carbonate 7.5% was added and incubated at 50°C for 15 min, after which the mixture was placed in an ice bath. Absorbance was determined at 760 nm. Total polyphenol content was expressed as gallic acid equivalents (GAE) per milliliter of extract. Two measurements were performed in triplicate and the mean values were calculated.

DPPH radical scavenging activity of CHE was determined as described by Brand-Williams et al. [22]. In short, 100 μL of the CHE and standard Trolox antioxidant were added to 3.9 mL of 60 μM DPPH dissolved in methanol

80%. The mixture was homogenized and incubated at room temperature in the dark for 30 min and the absorbance at 515 nm was determined. The percentage of inhibition was determined according to the formula = (1 − Abs Sample 515/Abs Control 515) × 100. The results were expressed as mean ± SEM (standard error of the mean) from 3 replicated measurements.

2.5. Bacterial Growth Assay. *E. coli* OP50 growth was evaluated over 5 h in the presence of CHE. All OD readings at 600 nm were normalized to the OD of the control group at time zero. Bacterial growth was measured in three individual experiments.

2.6. Body Length and Brood Size Assays. To measure body length, twenty L1 wild-type animals were treated or not with 50 mg/mL CHE until being 1-day-old adult and were then photographed using a Zeiss Axio Imager Z2 (NY, USA) microscope. Body length was determined by measuring along the animal axis using Axio Vision Rel. 4.8 software.

To assay brood size, ten L4 wild-type animals were individually transferred daily to new NGM plates containing or not 50 mg/mL CHE, until the completion of the egg laying period. The total progeny numbers for each plate were counted and divided by the number of animals. Three experiments were performed for both assays.

2.7. Pharyngeal Pumping Rate. Pharyngeal pumping rate was quantified using L4 wild-type animals treated or not with 50 mg/mL CHE since L1. For each condition, 10 animals were placed individually on agar pads with *E. coli* OP50 and allowed to acclimate for 5 min. Pharyngeal pumping was recorded for 2 min using an Olympus CX21 microscope (PA, USA) connected to a camera (Videolabs AmScope FMA050, MN, USA). The recording was digitized and slowed using Windows Movie Maker software (Microsoft Corp., 2002). The number of pumps was determined as an average of three independent counts for each animal over a time span of 20 seconds. A pump was defined as the movement of the pharyngeal grinder. This experiment was performed twice.

2.8. Lifespan and Oxidative Stress Assay. We used the strain *fem-1(hc17)* whose lifespan is similar to those of wild-type animals [23]. This strain is a temperature-sensitive mutant developing into semifertile adults at 20°C and sterile adults at 25°C which facilitates its growth and reproduction. The animals were synchronized at L1 stage and then maintained throughout their lifetime in plates containing or not 0.5, 5, and 50 mg/mL CHE. Approximately 90 animals were divided into 3 plates with 30 animals each. Nematodes were checked every day and scored as dead when they did not move even after repeated taps with a pick. Three experiments were performed.

Oxidative stress was induced by an acute, lethal concentration of *tert*-butyl hydrogen peroxide (t-BOOH) that is thermally more stable and less sensitive to contamination by metal than H_2O_2. About 50 worms treated or not with CHE on either regular or KAN-treated OP50 for 48 h were transferred to 7.5 mM t-BBOH while those treated or not with CHE on RNAi bacteria for 48 h were transferred to 12 mM t-BOOH. Viability was assessed after 6, 9, and 12 h. Three experiments were performed.

2.9. Measurement of Intracellular ROS in C. elegans. Intracellular ROS in *C. elegans* were measured using the fluorescent probe 2′,7′-dichlorodihydro-fluorescein diacetate (H_2DCF-DA). Synchronized L1 wild-type animals were treated or not with 50 mg/mL CHE for 48 h and then transferred to 5 mM H_2O_2 or not for 1 h. Approximately 10 worms were collected into 1 mL of PBS with 1% Tween-20 (PBST), washed twice, and transferred into the wells of a 96-well microtiter plate containing 50 μM H_2DCF-DA. Samples were read every 30 min for 6 h in a multilabel microplate reader (Perkin Elmer VICTOR X3, MA, USA) at 37°C using excitation at 485 nm and emission at 535 nm. Three experiments were performed. Three measurements were performed in triplicate and the mean values were calculated.

2.10. Reporter Genes Analysis. The transgenic strains *gcs-1::GFP*, *gst-4::GFP*, and *sod-3::GFP* were treated or not with CHE for 48 h and then transferred or not to 7.5 mM t-BOOH for 1 h followed by a 1 h recovery period. The transgenic strains *hsp-16.2::GFP* and *hsp-4::GFP* were treated or not with 50 mg/mL CHE and then transferred or not to 35°C for 1 h followed by a 1 h and 30 min recovery period. Twenty worms from each group were photographed on a fluorescence microscope (Zeiss Axio Imager Z2, NY, USA) using a 10x ocular lens. GFP fluorescence signals were measured using NIH Image J software. Three experiments were performed.

2.11. Bioassays for β-Amyloid-Induced Paralysis. To determine if CHE suppresses or delays the onset of β-amyloid-induced progressive paralysis in CL4176 expressing muscle-specific Aβ1–42, freshly laid eggs were transferred to NGM containing 50 mg/mL CHE or control solution and incubated for 40 h at 16°C. To initiate amyloid-induced paralysis, the worms were up-shifted from 16°C to 25°C. Scoring was performed at 2 h intervals, typically after 24 h at 25°C. The worms were scored as "paralyzed" based either on the failure of the worms to move their body at the touch of a platinum loop or on the formation of a halo on the bacterial lawn, indicating a paralyzed condition. Each experiment was performed using at least 40 worms. These experiments were performed three times using living bacteria and two times using KAN-treated bacteria.

2.12. Proteasome Activity Quantification. *In vitro* 26S proteasome activity assays were performed as described by Bonomo et al. [11]. Approximately 5,000 N2 wild-type animals were treated with control solution (S basal) or 50 mg/mL CHE for 48 h since L1. L4 worms were then harvested and sonicated. The lysates were centrifuged at 20,000 ×g for 20 min at 4°C. Protein extract was quantified using the QuantiPro BCA Assay Kit (Sigma Aldrich, St. Louis, MO, USA). To measure the chymotrypsin-like activity of the proteasome, the peptide succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (SLLVY-MCA) (Sigma-Aldrich, St. Louis, MO, USA)

Table 1: Carqueja hydroalcoholic extract (CHE) and Trolox antioxidant capacity *in vitro* by DPPH assay.

	% inhibition (mean ± SEM)		
	—	+ OP50	+ OP50 + KAN
CHE			
0.5 mg/mL	19.95 ± 0.25	7.17 ± 0.45	20.82 ± 0.05
5 mg/mL	74.27 ± 0.35	71.38 ± 0.29	73.43 ± 0.22
50 mg/mL	82.86 ± 0.25	85.35 ± 0.21	87.57 ± 0.71
Trolox[a]			
100 μM	18.94 ± 0.46	n.d.	n.d.
300 μM	38.05 ± 1.24	n.d.	n.d.
400 μM	54.04 ± 0.46	n.d.	n.d.
700 μM	81.37 ± 0.31	n.d.	n.d.

[a] Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.
n.d. Not determined.

was used in both presence and absence of 20 μM MG-132, a proteasome inhibitor. Enzyme kinetics were monitored in a VICTOR X3 (Perkin Elmer, Massachusetts, USA) temperature-controlled microplate reader, every 15 min for 1 h at 37°C; the excitation and emission wavelengths were 380 and 460 nm, respectively. Relative proteasome activity was calculated as the difference between the total activity and the remaining activity in the presence of 20 μM MG-132. The proteasome activity quantification was conducted three times.

2.13. Nile Red Staining. NGM plates were prepared containing 100 ng/mL Nile red and allowed to dry overnight at 37°C. Plates were seeded with *E. coli* OP50 mixed or not with 50 mg/mL CHE, and L1 worms were allowed to grow for 48 h. Twenty worms from each group were photographed on a fluorescence microscope (Zeiss Axio Imager Z2, NY, USA) using a 10x ocular lens. Fluorescence signals were measured using NIH Image J software. Three experiments were performed.

2.14. Statistical Analyses. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (CA, USA) and SAS JMP Statistical Discovery version 10.0 (NC, USA). The results were plotted as the mean ± SEM of three individual experiments. Data were subjected to the Kolmogorov-Smirnov test for normality. For data with a normal distribution, Student's t-test was used to compare pairs of groups, whereas a one-way ANOVA followed by Tukey's posttest was used to compare three or more groups. Nonparametric data were analyzed using the Mann-Whitney test when comparing two groups and the Kruskal-Wallis test followed by Dunn's posttest for comparing three or more groups. All survival curves were analyzed by the log-rank (Mantel-Cox) test. The statistical significance was determined as $P < 0.05$.

3. Results

3.1. The Carqueja Hydroalcoholic Extract (CHE) Tested Has In Vitro Antioxidant Capacity. To confirm that the CHE we produced has antioxidant properties, we quantified the total amount of polyphenols and measured the scavenging activity of the CHE through removal of the DPPH radical. The CHE total phenolic content was 0.085 ± 0.001 mg of gallic acid equivalent (GAE) per L of extract in hydroalcohol. We next determined the antioxidant capacity of 0.5, 5, and 50 mg/mL CHE by the DPPH radical scavenging activity method. We observed that CHE displays increasing *in vitro* antioxidant capacity in a dose-dependent manner (Table 1). At 50 mg/mL CHE, the highest concentration tested, the DPPH inhibition was 82.86%, which is equivalent to 700 mM Trolox, the reference standard, which shows 81.37% inhibition. We also evaluated the effect of bacteria metabolism on CHE's antioxidant capacity. The percentage of DPPH inhibition for 0.5 mg/mL CHE was reduced from 19.95% to 7.17% when the extract was mixed with *E. coli* OP50. Pretreating the bacteria with KAN before mixing with CHE restored the DPPH inhibition to 20.82% (Table 1). The percentage of DPPH inhibition for 5 and 50 mg/mL CH was not significantly altered when the extracts were mixed with bacteria (Table 1). These results indicate that bacteria metabolism interferes with the CHE antioxidant capacity at lower but not at higher concentrations of CHE.

3.2. CHE Increases Oxidative Stress Resistance in C. elegans. We first tested whether CHE would affect the lifespan of *C. elegans* under standard laboratory conditions. Lifespan assays were performed in animals treated with three different CHE concentrations (0.5, 5, and 50 mg/mL) at 25°C. The mean lifespan of all treated animals did not differ significantly from controls lifespan ($P = 0.42$ for 0.5 mg/mL, 0.82 for 5 mg/mL, and 0.69 for 50 mg/mL) (Table 2). These findings indicate that, under standard conditions, CHE treatments at these concentrations did not alter *C. elegans* lifespan (Figure 1(a)).

It has been shown [2, 3, 9] that CHE has antioxidant activities *in vitro*; however, no *in vivo* oxidative models have been used to investigate these properties in animals under stress conditions. To explore whether CHE antioxidant properties have a protective effect *in vivo*, oxidative stress assays were performed in animals treated with three different concentrations of CHE (0.5, 5, and 50 mg/mL) for 48 h. We observed

Table 2: Effect of Carqueja hydroalcoholic extract (CHE) treatment on lifespan of *C. elegans fem-1* strain at 25°C.

	Survival rate (days)			P versus control (log-rank)[b]	n[a]
	Mean	Median	Maximal		
Control	13.09	11	18.1		88 (2)
0.5 mg/mL CHE	13.75	12	18.3	0.42	77 (2)
5 mg/mL CHE	13.08	13	19.5	0.82	93 (2)
50 mg/mL CHE	13.21	13	18.4	0.69	108 (2)

[a] Total number of animals analyzed. The number in parentheses indicates the number of independent trials.
[b] Comparisons were performed using the log-rank (Mantel-Cox) test.

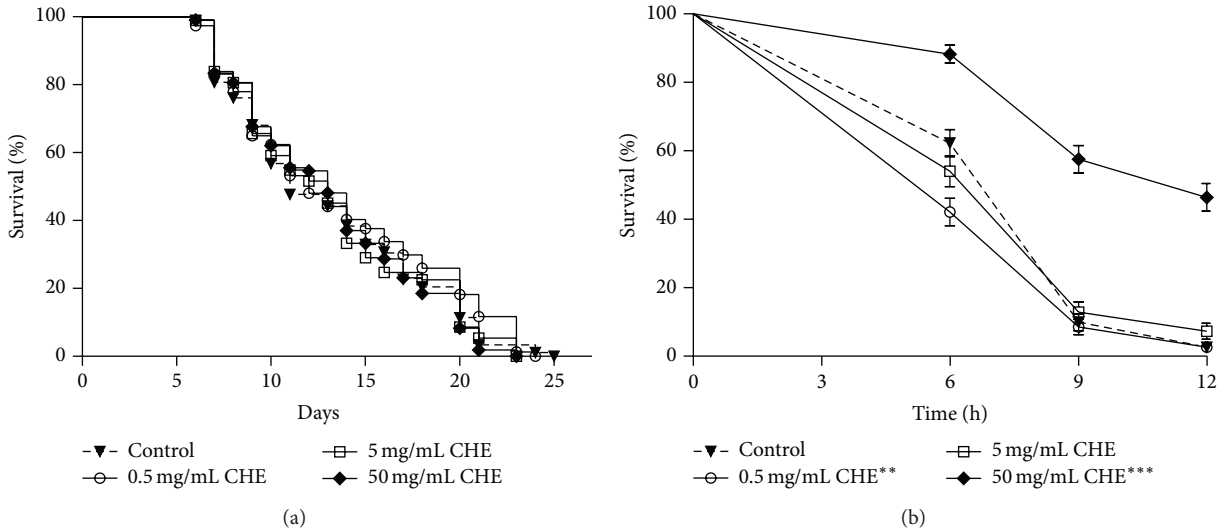

Figure 1: Effect of Carqueja hydroalcoholic extract (CHE) on *C. elegans* survival under standard laboratory and stress conditions. (a) Lifespan. *fem-1(hc17)* mutants animals were treated or not with three different CHE concentrations (0.5, 5, and 50 mg/mL) at 25°C beginning at L1. Nematodes were checked daily until all nematodes had died. Log-rank (Mantel-Cox) analysis showed no significant difference between the curves. (b) Stress resistance. Wild-type animals were treated or not with three different CHE concentrations (0.5, 5, and 50 mg/mL) from L1 to L4 and then submitted to 7.5 mM t-BOOH in M9. Survival was measured at 6, 9, and 12 h. The survival curves show that only 50 mg/mL CHE treatment increased *C. elegans* oxidative stress resistance, while 0.05 mg/mL CHE decreased oxidative stress resistance. $^{***}P < 0.001$ and $^{**}P = 0.009$ by the log-rank (Mantel-Cox) test.

that animals treated with 50 mg/mL CHE showed increased oxidative stress resistance, when compared to controls ($P < 0.0001$). Surprisingly, animals treated with 0.5 mg/mL CHE showed a decreased oxidative stress resistance ($P = 0.009$), whereas in those treated with 5 mg/mL CHE there was no change in oxidative stress resistance (Figure 1(b), Table 3).

3.3. Increased Oxidative Stress Resistance Induced by CHE Remains in C. elegans Fed with Dead Bacteria. It is known that *E. coli* has a pathogenic effect on *C. elegans,* which affects its lifespan and stress resistance [24]. Therefore, we investigated whether the CHE protective effect we observed (Figure 1(b)) could result from inhibition of bacterial growth. When we assayed *E. coli* OP50 growth over 5 h in the presence of CHE, we observed an antimicrobial effect. This effect was observed after 80 min of incubation with 50 mg/mL CHE and after 120 and 200 min with 5 and 0.5 mg/mL CHE, respectively (Figure 2(a)).

However, in *C. elegans* fed with KAN-treated *E. coli,* treatment with 50 mg/mL CHE still increased the oxidative stress resistance ($P < 0.0001$), indicating that the CHE bacteriostatic propriety is not the only factor affecting oxidative stress resistance in these animals (Figure 2(b), Table 3). The treatment with 0.5 mg/mL CHE did not affect the oxidative stress resistance ($P = 0.251$) while with 5 mg/mL significantly decreased stress resistance ($P < 0.0001$). This result prompted us to perform the following assays using 50 mg/mL of CHE.

3.4. CHE Treatment Does Not Redirect Resources for Growth and Reproduction or Alter Feeding Behavior. It is known [25] that, in response to adverse environmental factors, energetic resources can be redirected to different developmental processes such as growth or reproduction. To verify whether CHE treatment would affect *C. elegans* growth and reproduction, we evaluated the body length and the progeny size of animals treated with 50 mg/mL CHE. The body length of treated animals was not significantly different from that of controls ($P = 0.100$) (Figure 3(a)). We did not observe any difference in the total progeny between controls and treated

Table 3: Effect of Carqueja hydroalcoholic extract (CHE) treatment on oxidative stress resistance of *C. elegans* wild-type fed or not with kanamycin-treated *E. coli* OP50.

	Mean survival rate (hours)	*P* versus control (log-rank)[b]	*n*[a]
E. coli OP50			
Control	8.16		151 (3)
0.5 mg/mL CHE	7.52	0.0090	152 (3)
5 mg/mL CHE	7.98	0.9668	124 (3)
50 mg/mL CHE	10.37	<0.0001	153 (3)
E. coli OP50 + KAN			
Control	10.27		132 (3)
0.5 mg/mL CHE	10.45	0.251	91 (3)
5 mg/mL CHE	8.53	<0.0001	84 (3)
50 mg/mL CHE	11.19	<0.0001	138 (3)

[a]Total number of animals analyzed. The number in parentheses indicates the number of independent trials.
[b]Comparisons were performed using the log-rank (Mantel-Cox) test.

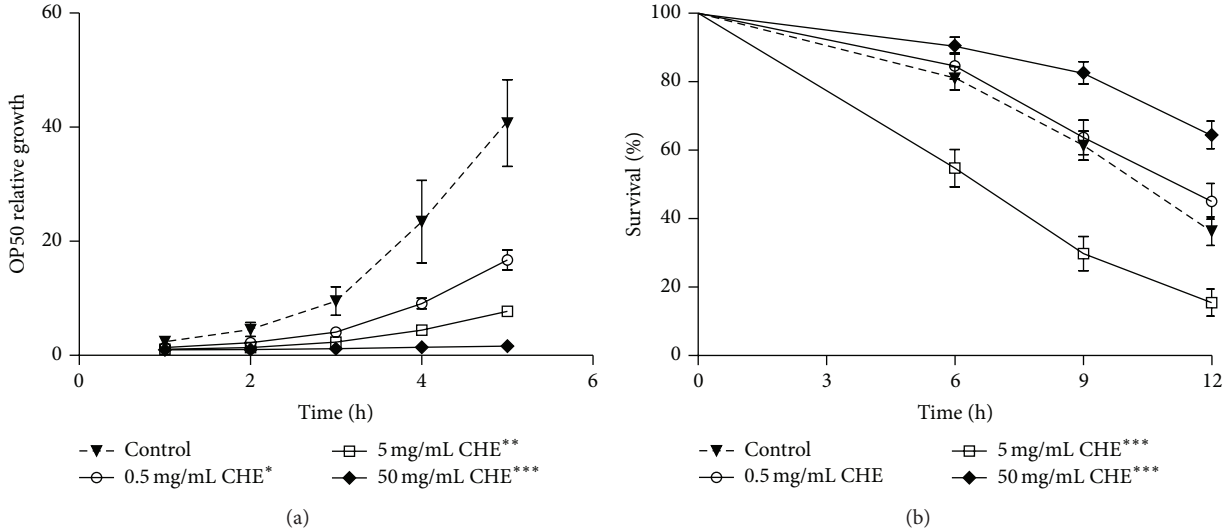

Figure 2: Effect of Carqueja hydroalcoholic extract (CHE) on *E. coli* growth and oxidative stress resistance of *C. elegans* fed with kanamycin-treated *E. coli*. (a) *E. coli* growth. *E. coli* OP50 growth was evaluated over 5 h in the presence of three different CHE concentrations (0.5, 5, and 50 mg/mL). The OD of the control group at time zero was used to normalize all other OD readings. *Treatment of 0.5 mg/mL CHE decreased bacteria growth after 200 min. **Treatment of 5 mg/mL CHE decreased bacteria growth after 120 min. ***Treatment of 50 mg/mL CHE decreased bacteria growth after 80 min. $P < 0.05$, determined by a two-tailed Student's *t*-test. (b) Stress resistance assay on bacteria killed with KAN. Wild-type animals were treated or not with three different CHE concentrations (0.5, 5, and 50 mg/mL) mixed with either *E. coli* OP50 or *E. coli* OP50 treated with 10 mM KAN from L1 to L4 and then submitted to 7.5 mM t-BOOH in M9. The survival was measured at 6, 9, and 12 h. The survival curves show that 50 mg/mL CHE treatment increased *C. elegans* oxidative stress resistance independent of its antibacterial effect. ***$P < 0.001$ related to the respective controls by the log-rank (Mantel-Cox) test.

animals ($P = 1.000$) (data not shown). Moreover, 50 mg/mL CHE did not alter the egg-laying profile (Figure 3(b)). We also measured the pharyngeal pumping rate in L4 animals in order to verify whether CHE alters feeding behavior. The pumping frequency of 50 mg/mL CHE-treated animals was not significantly different compared to untreated animals (Figure 3(c)).

Taken together, these results suggest that CHE does not interfere with growth, reproduction, and food intake in *C. elegans*.

3.5. CHE Increases Oxidative Stress Resistance In Vivo by Reducing ROS. Antioxidant phytochemicals can function either directly by scavenging ROS or indirectly through modulation of signaling pathways. To investigate whether the protective effect of 50 mg/mL CHE in treated *C. elegans* acts directly or indirectly, we measured ROS production and gene expression of *sod-3*, *gcs-1*, and *gst-4* antioxidant enzymes. CHE treatment reduced ROS production in animals under both standard and stress conditions (Figure 4(a)). CHE treatment reduced *sod-3::GFP* expression, if compared to

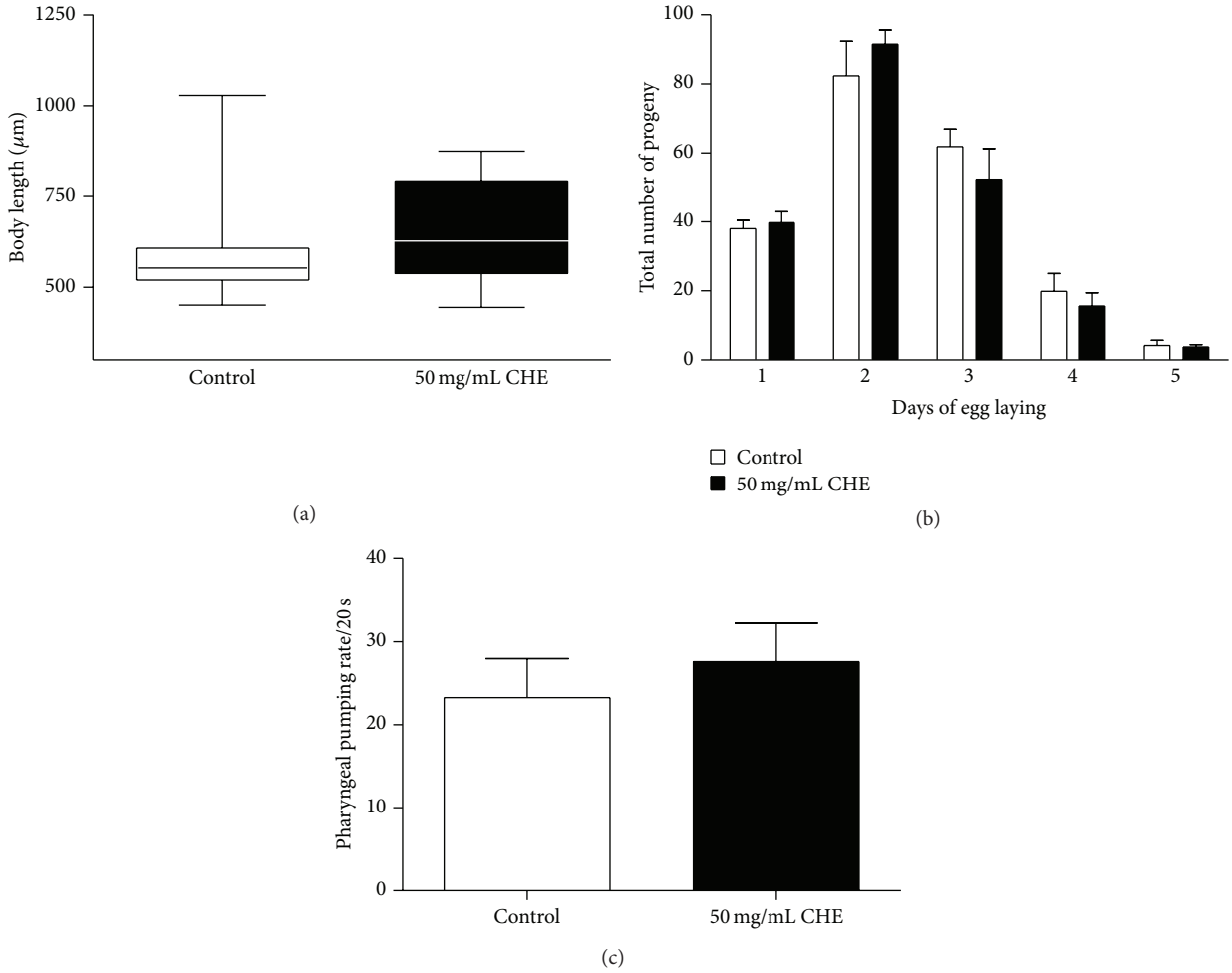

Figure 3: Effect of Carqueja hydroalcoholic extract (CHE) on wild-type *C. elegans* growth, reproduction and pharyngeal pumping. (a) Body length. L1 animals were treated or not with 50 mg/mL CHE for 48 h. Images were captured of L4 animals, and body length was measured along the animal axis using Axio Vision Rel. 4.8 software. There was no significant difference between groups, as determined by two-tailed Student's *t*-test. (b) Brood size. Progeny profiles were measured in animals treated or not with 50 mg/mL CHE. Animals were transferred individually to NGM plates and moved daily until the end of the reproductive period. The results were plotted as the mean ± SEM for each day. (c) Pharyngeal pumping. Pharyngeal pumping rate was quantified using L4 wild-type animals treated or not with 50 mg/mL CHE. There was no significant difference between groups, as determined by two-tailed Student's *t*-test.

untreated animals under standard conditions, but no significant differences were observed between treated and untreated groups under stress conditions (Figure 4(b)). Under stress conditions, CHE treatment reduced *gcs-1::GFP* expression, if compared to untreated animals, but no significant differences were observed between treated and untreated groups under standard conditions (Figure 4(c)). Expression of *gst-4::GFP* increased in the CHE-treated group, if compared to that observed in untreated animals under standard conditions. In animals under stress condition, no significant differences were observed between treated and untreated groups (Figure 4(d)). These results suggest that CHE treatment reduces ROS production and inhibits *sod-3::GFP* under standard conditions and *gcs-1::GFP* under stress conditions while it activates *gst-4::GFP* expression under standard conditions.

A number of genes and pathways have been identified in *C. elegans* to modulate lifespan and stress resistance. These highly conserved pathways include mitogen-activated protein kinase (MAPK) p38, JNK, and ERK MAPK pathways [26–29], which in turn may activate several transcription factors, including DAF-16 and SKN-1 [30–32]. To investigate whether these stress response signaling pathways would have a role in CHE-antioxidant activity, we performed the oxidative stress resistance assay in wild-type and mutant animals for DAF-16 and SKN-1 transcription factors and for the three MAPK pathways. For each stress-related signaling pathway, different mutants were tested (see Table 4 for details). All mutants treated with 50 mg/mL CHE showed increased oxidative stress resistance compared to their respective controls (Table 4). Since CHE has an antimicrobial effect on the bacteria, we repeated the stress assay for *nsy-1*, *jnk-1*, and *mpk-1* mutants that showed the smallest increase in median survival time. Still, even after being fed with CHE mixed with KAN-treated bacteria, these mutants exhibited

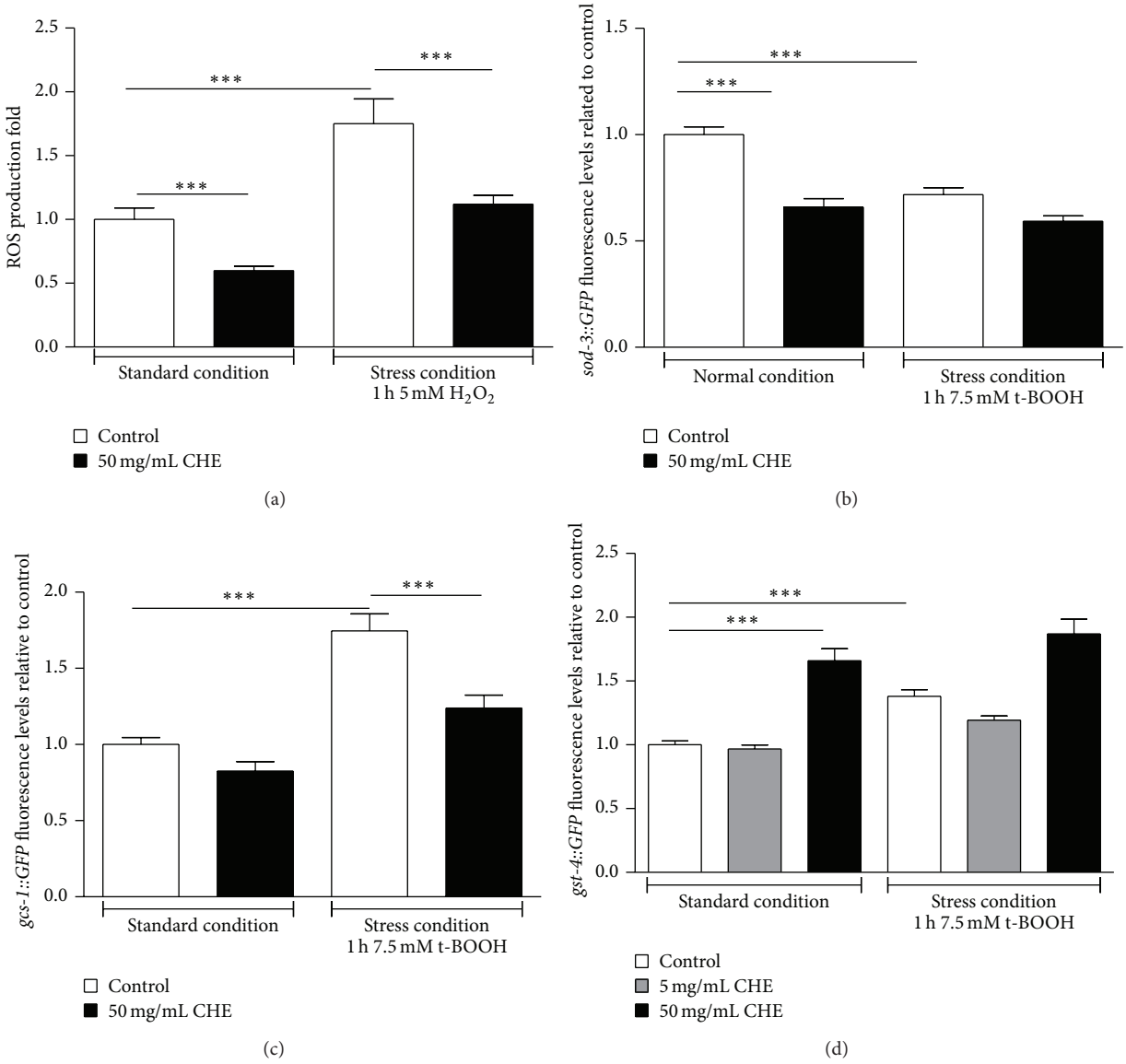

Figure 4: Effect of Carqueja hydroalcoholic extract (CHE) on redox status in wild-type *C. elegans*. (a) ROS production. *C. elegans* was treated or not with 50 mg/mL CHE for 48 h and then submitted to the presence or absence of 5 mM H_2O_2 for 1 h. CHE reduced ROS production under both standard and stress conditions. $^{***}P < 0.0001$ by Kruskal-Wallis test followed by Dunn's posttest. Analysis of *sod-3::GFP* (b), *gcs-1::GFP* (c), and *gst-4::GFP* (d). Transgenic worms were treated or not with 50 mg/mL CHE for 48 h beginning at L1 and then with or without the oxidative stress condition. After a 1 h recovery period, photographs were taken on a fluorescence microscope and GFP fluorescence signals were measured using NIH Image J software. (b) Expression of *sod-3::GFP* is reduced in the 50 mg/mL CHE-treated group under standard conditions (c). CHE treatment reduced *gcs-1::GFP* expression under stress conditions. (d) Expression of *gst-4::GFP* increased in the 50 mg/mL CHE-treated group under standard conditions. $^{***}P < 0.0001$ by Kruskal-Wallis test followed by Dunn's posttest.

increased oxidative stress resistance (Table 4). Therefore, the notion of possible antimicrobial effect induced oxidative stress resistance was rejected again. These results indicate that the oxidative stress resistance induced by CHE is neither mediated by these stress-related signaling pathways nor by the transcription factors tested here.

In order to rule out that a nonfunctional pathway could compensate by a functional one, we tested the double knockdown of SKN-1 and DAF-16 simultaneously. We observed that *skn-1(RNAi); daf-16(RNAi)* animals treated with 50 mg/mL CHE still showed increased oxidative stress resistance compared to their respective controls (Table 4).

3.6. CHE Treatment Delays Paralysis Induced by β-Amyloid (Aβ) Expression. The oxidative-stress state has an important role in the development of neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's. Accumulation of ROS and deposition of toxic amyloid species have been proposed to exacerbate the symptoms observed in AD patients [33, 34].

Table 4: Effect of 50 mg/mL Carqueja hydroalcoholic extract (CHE) treatment on oxidative stress resistance of *C. elegans* wild-type and mutants.

	Mean survival rate (hours)		P versus control (log-rank)[b]	n[a]	
	Control	Treated		Control	Treated
E. coli OP50					
N2	9.6	10.8	<0.0001	669 (13)	645 (13)
nsy-1(ag3)	7.6	8.8	<0.0001	154 (3)	160 (3)
pmk-1(ku25)	7.3	8.7	<0.0001	152 (3)	154 (3)
sek-1(km4)	7.1	9.0	<0.0001	147 (3)	158 (3)
skn-1(zu67)	6.5	9.6	<0.0001	131 (3)	133 (3)
daf-16(mu86)	8.2	10.0	<0.0001	159 (3)	155 (3)
jnk-1(gk7)	9.0	9.9	<0.0001	153 (3)	152 (3)
osr-1(rm-1)	8.4	9.7	<0.0001	154 (3)	154 (3)
unc-43(n498n1186)	8.5	10.4	<0.0001	149 (3)	130 (3)
mpk-1(ku1)	7.5	9.3	<0.0001	158 (3)	161 (3)
N2 *control(RNAi)*	10.45	11.26	<0.0001	146 (3)	150 (3)
N2 *skn-1(RNAi)*; *daf-16(RNAi)*	8.12	10.40	<0.0001	134 (3)	146 (3)
E. coli OP50 + KAN					
N2	10.32	11.15	<0.0001	306 (6)	283 (6)
nsy-1(ag3)	9.39	10.67	<0.0001	100 (2)	99 (2)
jnk-1(gk7)	9.89	10.51	0.0024	98 (2)	99 (2)
mpk-1(ku1)	8.64	9.83	0.0014	76 (2)	94 (2)

[a] Total number of hermaphrodites analyzed. The number in parentheses indicates the number of independent trials.
[b] Comparisons were performed using the log-rank (Mantel-Cox) test.

In *C. elegans*, oxidative stress strongly correlates with Aβ toxicity, and it has been shown that a number of natural products that reduce ROS are neuroprotective [35, 36].

The observation that CHE has antioxidant properties *in vivo* led us to ask whether CHE treatment would have a protective effect against β-amyloid toxicity in a *C. elegans* model for the Alzheimer's disease. The expression of human Aβ1–42 in the muscle of transgenic CL4176 strain promotes a paralysis that can be monitored over time. We observed that the onset of paralysis was significantly delayed in 50 mg/mL CHE-treated animals ($P = 0.0029$). We observed that, after 24 h of induced Aβ1–42 expression, all CHE-treated animals were responsive to touch. After 32 h, this effect was still observed in 20% of the CHE-treated animals, compared to only 9% of the animals in the control group (Figure 5(a)). This effect was also independent of CHE antimicrobial effect. After 36 h, 50% of the CHE-treated animals were still responsive to touch, compared to 30% of the animals in the control group ($P = 0.0353$) (Figure 5(b)).

The disruption of protein homeostasis underlies the pathologies of most neurodegenerative disorders [37]. To evaluate whether CHE treatment alters protein homeostasis, we measured proteasome activity in animals treated with 50 mg/mL CHE. Proteasome chymotrypsin-like activity was monitored by SLLVY-MCA digestion in L4 worm extracts containing equal amounts of total protein. CHE increased proteasome degradation activity by 5-fold relative to the controls ($P < 0.05$) (Figure 6(a)).

Increasing the expression levels of heat shock proteins (HSPs) is known to enhance stress resistance and have a neuroprotective effect in *C. elegans* [38, 39]. Here, we tested whether 50 mg/mL CHE treatment could modulate the gene expression of *hsp-16.2* and *hsp-4* heat shock proteins. Under standard conditions, CHE treatment reduced *hsp-16.2::GFP* expression if compared to that observed in untreated animals. Animals under stress conditions showed increased *hsp-16.2::GFP* expression in the treated versus the untreated group (Figure 6(b)). Expression of *hsp-4::GFP* increased in the CHE-treated group, if compared to that observed in untreated animals under standard conditions. In animals under stress condition, no significant differences were observed between treated and untreated groups (Figure 6(c)).

Interestingly, HSP-4 is an essential endoplasmic reticulum (ER) chaperone that senses nutritional status and stimulates the expression of inducible lipases [40]. This activation is correlated with reduced levels of lysosome-related organelles (LROs) [40]. LROs are the site of concentration of lipophilic Nile red dye as well as age-associated autofluorescence. Therefore, we investigated whether CHE treatment would also cause LRO Nile red reduction. We observed that animals treated with 50 mg/mL CHE showed lower levels of Nile red fluorescence when compared to untreated animals (Figure 6(d)).

These results suggest that CHE treatment increases the defenses against Aβ toxicity by increasing proteasome activity and chaperone expression. Moreover, the increased *hsp-4* expression induced by CHE is associated with reduced LRO Nile red fluorescence.

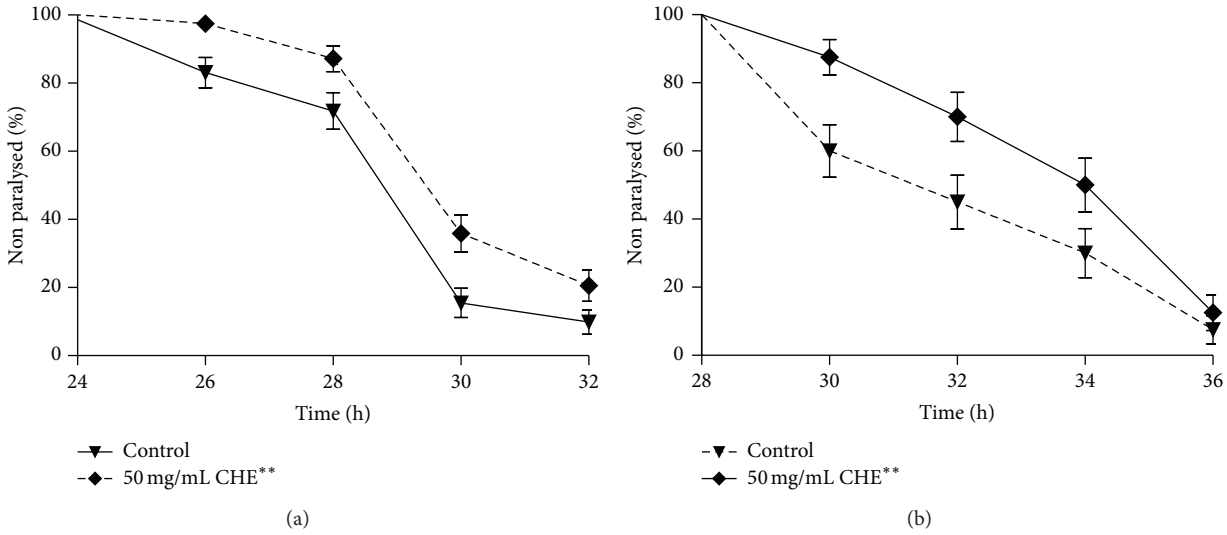

Figure 5: Effect of Carqueja hydroalcoholic extract (CHE) in transgenic *C. elegans* model for Alzheimer's disease. CL4176 transgenic strains treated or not with 50 mg/mL CHE were incubated for 40 h at 16°C. To initiate the β-amyloid-induced paralysis, the worms were up-shifted from 16°C to 25°C. The paralysis was verified each 2 h interval after 24 h at 25°C. The survival curves show that CHE alleviates β-amyloid-induced paralysis. (a) Paralysis profile of CL4176 transgenic animals fed with 50 mg/mL of CHE. $^{**}P = 0.0029$ by the log-rank (Mantel-Cox) test. (b) Paralysis profile of CL4176 transgenic animals fed with 50 mg/mL of CHE mixed with KAN-treated bacteria. $^{*}P = 0.0353$ by the log-rank (Mantel-Cox) test.

4. Discussion

In popular medicine, infusions of the aerial parts of Carqueja are commonly used to treat gastrointestinal, liver, and a variety of inflammatory diseases [41]. Carqueja has been widely studied for its anti-inflammatory, antidiabetic, analgesic, antihepatotoxic, and antimutagenic properties [2, 4–8]. Although it contains the polyphenols quercetin and rutin as well as phenolic acids such as caffeoylquinic acids, there are no descriptions of its use against neurodegenerative disorders. Here, we show, for the first time, using *in vivo* assays, that treatment with CHE not only improved oxidative stress resistance by increasing survival rate and reducing ROS levels under oxidative stress conditions, but also increased defense against Aβ toxicity in *C. elegans*.

Our results show that CHE has *in vitro* (Table 1) and *in vivo* (Figure 1(b), Table 3) antioxidant properties, which were assessed through DPPH scavenging analysis and stress resistance assays. DPPH scavenging activity with 0.5 mg/mL CHE was low (19.95%). With 5 and 50 mg/mL CHE, higher antioxidant activities were found (74.27% and 82.86%, resp.). We also observed that bacteria metabolism interferes with the CHE antioxidant capacity at 0.5 mg/mL but not at 5 and 50 mg/mL (Table 3). Only the 50 mg/mL CHE treatment improved the survival rate of animals under stress (Figure 1(b)). Grünz et al. [42] showed by chemical and fluorescence analyses that flavonoids, such as quercetin, are taken up by *C. elegans* and are metabolized through conjugation in a dose-dependent manner. These findings may explain the fact that animals treated with lower CHE doses (0.5 and 5 mg/mL) failed to exhibit increased stress resistance (Figure 1(b)).

It is well known that bacteria used as a food source for *C. elegans* can be pathogenic and therefore may affect the animal's lifespan and response to oxidative stress [43]. Indeed, it has been suggested that nematodes may experience health benefits if the food source is composed of bacteria in conjunction with antimicrobial substances [44]. In this study, we observed that the 50 mg/mL CHE treatment improved the survival rate of *C. elegans* under stress even when the bacterial source was previously treated with KAN, indicating that the observed higher survival rate was not fully caused by a bacteriostatic effect of CHE (Figure 2(b)). Antimicrobial effects were also observed when working with isolated polyphenols and açaí extract [11, 44] without compromising the beneficial properties of these compounds in *C. elegans*. These findings strongly support an antioxidant effect for CHE itself.

Studies done in several animal models have shown a strong correlation between oxidative stress resistance and increased lifespan, which can be explained by a reduction in oxidative damage to biomolecules [42, 45, 46]. Although CHE increased stress resistance, we did not observe increased lifespan (Figure 1(a)). This lack of association between stress resistance and lifespan has been reported by other studies showing that the antioxidant effect is not predictive of increased lifespan [42, 46]. Although CHE did not increase lifespan, the fact that CHE did not interfere with growth and reproduction indicates that CHE is not toxic to *C. elegans* (Figures 3(a) and 3(b)).

The fact that ROS production was lower in *C. elegans* treated with CHE under stress and standard conditions suggests that the radical scavenging properties of CHE's phenolic compounds directly neutralize ROS production (Figure 4(a)). Moreover, we observed that CHE treatment reduced the expression of *sod-3::GFP* under standard conditions (Figure 4(b)). The radical scavenging activity of CHE probably leads

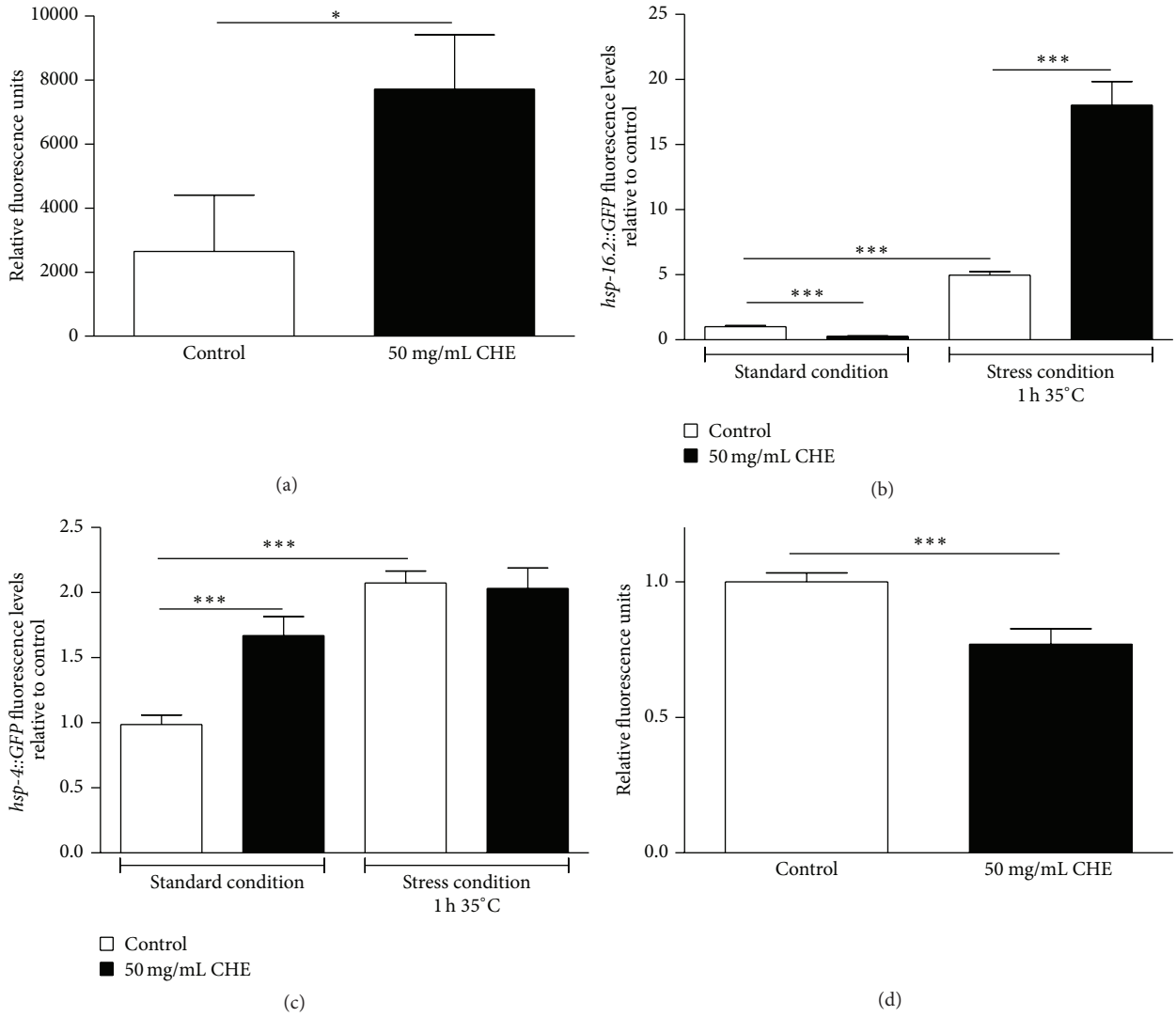

Figure 6: Effect of Carqueja hydroalcoholic extract (CHE) in protein homeostasis and Nile red staining. (a) Proteasome activity is increased by CHE treatment. Animals were treated or not with 50 mg/mL CHE from L1 to L4. Proteasome chymotrypsin-like activity was monitored by SLLVY-MCA digestion in L4 worm extracts containing equal amounts of total protein. $^{***}P < 0.05$ by two-tailed Student's t-test. Transgenic worms *hsp-16-2::GFP* (b) and *hsp-16-2::GFP* (c) were treated or not with 50 mg/mL CHE for 48 h beginning at L1 and then transferred or not to 35°C for 1 h. After a 1 h and 30 min recovery period, photographs were taken on a fluorescence microscope and GFP fluorescence signals were measured using NIH Image J software. (b) CHE treatment increased *hsp-16-2::GFP* expression under stress conditions. (c) Expression of *hsp-4::GFP* increased in the 50 mg/mL CHE-treated group under standard conditions. $^{***}P < 0.0001$ by Kruskal-Wallis test followed by Dunn's posttest. (d) Nile red accumulation reduction induced by CHE. Wild-type animals treated or not with 50 mg/mL CHE for 48 h beginning at L1 were maintained in plates containing Nile red. Subsequently, photographs were taken on a fluorescence microscope and GFP fluorescence signals were measured using NIH Image J software. CHE treatment reduced lipid fat deposit. $^{***}P < 0.0001$ by Mann-Whitney test.

to an improvement of cellular oxidative burden resulting in a repression of SOD-3. Quercetin treatment of *C. elegans* resulted in similar decreased expression of *sod-3::GFP* [47]. Cells under stress conditions use glutathione (GSH), the major cellular redox agent, in order to counteract the increased ROS production [48, 49]. The fact that the expression of *gcs-1::GFP* (γ-glutamylcysteine synthetase), which is the enzyme that synthetizes GSH, was lower under stress conditions in CHE-treated animals indicates that CHE was able to avert the need to synthesize more GSH (Figure 4(c)).

Glutathione S-transferases (GSTs) are considered one of the major players in phase II detoxification of both endogenous products of oxidative stress and electrophilic xenobiotics [50]. GST binds with GSH and catalyzes GSH conjugation with target substrates in order to facilitate their excretion from the cell. Several authors have reported that *gst-4* expression is induced by oxidative stressors and phytochemicals [51, 52]. We observed that 50 mg/mL CHE, but not 5 mg/mL CHE, significantly increased the expression of *gst-4::GFP* under standard conditions (Figure 4(d)). These results

suggest that CHE may be xenobiotic and therefore it may function as a mild stressor.

Taking together our data on DPPH inhibition analysis, oxidative stress assays, and *gst-4::GFP* expression, we hypothesize that CHE's antioxidant capacity is counteracted by its xenobiotic properties and that bacteria metabolism plays a role in it. First, the percentage of DPPH inhibition for 0.5 mg/mL CHE was reduced from 19.95% to 7.17% when the extract was mixed with *E. coli* OP50. Pretreating the bacteria with KAN restored the CHE DPPH inhibition to 20.82% (Table 1). This reduction could explain why stress resistance of 0.5 mg/mL CHE-treated animals compared to control group is decreased (Figure 1(b) and Table 3) but it is not altered when CHE is mixed with KAN-treated bacteria (Figure 2(b) and Table 3). The percentage of DPPH inhibition for 5 and 50 mg/mL of CHE was not altered (74.27% and 82.86%, resp.) when the extracts were mixed neither with living bacteria (71.38% and 85.35%, resp.) nor with KAN-treated bacteria (73.43% and 87.57%, resp.). Since the quantity of bacteria added to the extract was always the same (OD600 = 1), these results indicate that bacteria metabolism interferes with the CHE antioxidant capacity at lower but not at higher concentrations. Based on *gst-4::GFP* expression, our results also indicate that 50 mg/mL CHE may be xenobiotic but 5 mg/mL is not (Figure 4(d)). Moreover, the survival rate under stress conditions of animals fed with 5 mg/mL CHE was not significantly different from control group (Figure 1(b), Table 3) but it was actually significantly lower than that of controls when the animals received 5 mg/mL CHE mixed with KAN-treated bacteria (Figure 2(b), Table 3). These results suggest that the bacteria metabolism could be reducing any CHE's xenobiotic capacity at 5 mg/mL without altering its antioxidant content. Finally, it is possible that the antioxidant effect of 50 mg/mL CHE overcomes any potential xenobiotic effect that might be observed with lower doses of CHE (5 mg/mL), but not with the lowest dose tested (0.5 mg/mL). If this is true, one would expect that, at some dose between 5 mg/mL and 50 mg/mL, CHE proprieties would shift from xenobiotic to antioxidant. Further studies should shed light on this interesting hypothesis.

It is known that dietary antioxidants may work directly through ROS scavenging, or indirectly through activation of stress-related signaling pathways [15, 53]. Mitogen-activated protein kinase (MAPK) signal transduction pathways are conserved throughout eukaryotic animals [26–28]. These pathways transduce signals in response to a variety of extracellular stimuli, such as oxidative and thermal stress and exposure to heavy metals and drugs [52, 54, 55]. Here we individually tested at least one mutant for each of the three subgroups of MAPKs that have been identified, namely, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinases [26, 29] (see Table 4 for more details). We also tested individual mutants for two transcription factors (SKN-1 and DAF-16) and the double knockdown *skn-1(RNAi); daf-16(RNAi)* that are MAPKs pathways' targets (Table 2). SKN-1/Nrf is activated by the p38 and ERK MAPK pathways in response to oxidative stress or xenobiotics [31, 32]. DAF-16/FOXO has also been reported as a target of the JNK MAPK pathway [30]. Studies have shown the potential of antioxidants to activate either SKN-1 and DAF-16 in *C. elegans*, suggesting that these transcription factors mediate beneficial responses induced by antioxidants, such as increased oxidative stress resistance and lifespan [56, 57]. Considering that CHE-induced increased stress resistance was still observed in these mutants, our results suggest that none of these pathways is involved with this beneficial effect. Moreover, CHE-induced increased stress resistance was still observed in animals submitted to double knockdown of SKN-1 and DAF-16 simultaneously. This finding further supports a direct mode of action for CHE.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by formation of plaques composed primarily of insoluble Aβ. The toxic nature of Aβ1–42 makes it a marker of AD progression and a target for new therapeutic treatments. Epidemiological studies have reported that consumption of phenolic compounds may reduce the risk of developing AD [58]. Many compounds such as derivatives of quinic acid and polyphenols such as the flavonoid quercetin have been identified in Carqueja extracts of different polarities [10, 59]. Interestingly, various studies have shown that the protective effect of phenolic compounds, including quercetin and caffeoylquinic acids, against amyloid-induced neurotoxicity is due to their antioxidant properties and to reduced generation of toxic Aβ species [33, 60]. These studies agree with our findings in which CHE alleviates β-amyloid-induced paralysis in *C. elegans* (Figure 5). Therefore, this result might be explained by CHE antioxidant action.

Increased proteasome activity and chaperone activation are among the highly conserved responses employed by eukaryotic cells to maintain protein-folding homeostasis (proteostasis) [61, 62]. The failure of these mechanisms to adequately respond to proteotoxic imbalances results in the accumulation of misfolded proteins, which can lead to neurodegeneration diseases such as Alzheimer's. To investigate whether CHE could be involved with protein homeostasis, we evaluated proteasome activity and expression of the chaperone genes *hsp-16.2* and *hsp-4*. In *C. elegans*, HSP-4, an ER chaperone, plays a central role in response to stress associated with heat shock and protein misfolding [63]. HSP-16.2 has been shown to prevent unfolded proteins from aggregating and to interact with intracellular human Aβ1–42 peptide [64, 65]. We observed that 50 mg/mL CHE treatment increases proteasome degradation activity and *hsp-4::GFP* expression under standard conditions (Figures 6(a) and 6(c)). Moreover, we observed that CHE significantly increased the expression of *hsp-16.2::GFP* under heat stress conditions (Figure 6(b)). These results suggest that CHE alleviated β-amyloid-induced toxicity, in part, by increasing proteasome activity and expression of two heat shock protein genes.

HSP-4 also stimulates expression of fasting-induced lipases, which reduces LRO Nile red fluorescence upon fasting in *C. elegans* [40]. The observation that CHE treatment increased *hsp-4* expression prompted us to ask whether CHE treatment would reduce LRO Nile red accumulation. We observed that the level of LRO staining by Nile red was reduced when compared to untreated animals (Figure 6(d)). In *C. elegans*, LROs, like the lysosome, play a key role in trafficking of proteins and degradation products around

the cell [66]. They are also the site of microscopic autofluorescence that accumulates as animals age [66, 67]. Therefore, a decrease in Nile red may serve as an indicator of increased stress resistance and lifespan, since the fluorescence is lower in long-lived mutants (e.g., *daf-2*) compared to short-lived mutants (e.g., *daf-16*) [68]. Our results are in agreement with this assumption, since CHE increased oxidative stress resistance (Figure 1(b)). Finally, our results suggest that the neuroprotective effect against Aβ1–42 induced by CHE is also a result of increased protein homeostasis.

5. Conclusions

Our data reveal that CHE improves the oxidative stress response of *C. elegans*, which is in line with previous *in vitro* findings [2, 3]. Our findings also suggest a potential neuroprotective use for Carqueja, supporting the idea that dietary antioxidants are a promising approach to boost defense systems against stress and neurodegeneration [55]. Characterization of the CHE tested here may provide additional information on its compounds and may help with a better understanding of how this powerful phytochemical works and how to use it to improve human health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] L. G. Verdi, I. M. C. Brighente, and M. G. Pizzolatti, "The *baccharis* genus (asteraceae): chemical, economic and biological aspects," *Química Nova*, vol. 28, no. 1, pp. 85–94, 2005.

[2] C. B. de Oliveira, L. N. Comunello, A. Lunardelli et al., "Phenolic enriched extract of *Baccharis trimera* presents anti-inflammatory and antioxidant activities," *Molecules*, vol. 17, no. 1, pp. 1113–1123, 2012.

[3] B. C. Pádua, L. D. Silva, J. V. Rossoni et al., "Antioxidant properties of *Baccharis trimera* in the neutrophils of fisher rats," *Journal of Ethnopharmacology*, vol. 129, no. 3, pp. 381–386, 2010.

[4] N. P. A. Nogueira, P. A. Reis, G. A. T. Laranja et al., "*In vitro* and *in vivo* toxicological evaluation of extract and fractions from *Baccharis trimera* with anti-inflammatory activity," *Journal of Ethnopharmacology*, vol. 138, no. 2, pp. 513–522, 2011.

[5] R. M. Gené, C. Cartañá, T. Adzet, E. Marín, T. Parella, and S. Cañigueral, "Anti-inflammatory and analgesic activity of *Baccharis trimera*: identification of its active constituents," *Planta Medica*, vol. 62, no. 3, pp. 232–235, 1996.

[6] M. Trojan-Rodrigues, T. L. S. Alves, G. L. G. Soares, and M. R. Ritter, "Plants used as antidiabetics in popular medicine in rio grande do sul, southern Brazil," *Journal of Ethnopharmacology*, vol. 139, no. 1, pp. 155–163, 2012.

[7] H. Soicke and E. Leng-Peschlow, "Characterisation of flavonoids from *Baccharis trimera* and their antihepatotoxic properties," *Planta Medica*, vol. 53, no. 1, pp. 37–39, 1987.

[8] C. R. F. Rodrigues, J. H. Dias, R. N. de Mello, M. F. Richter, J. N. Picada, and A. B. F. Ferraz, "Genotoxic and antigenotoxic properties of *Baccharis trimera* in mice," *Journal of Ethnopharmacology*, vol. 125, no. 1, pp. 97–101, 2009.

[9] B. C. Pádua, J. V. R. Júnior, C. L. de Brito Magalhães et al., "Baccharis trimera improves the antioxidant defense system and inhibits inos and nadph oxidase expression in a rat model of inflammation," *Current Pharmaceutical Biotechnology*, vol. 14, no. 11, pp. 975–984, 2014.

[10] C. A. Simões-Pires, E. F. Queiroz, A. T. Henriques, and K. Hostettmann, "Isolation and on-line identification of antioxidant compounds from three *Baccharis* species by HPLC-UV-MS/MS with post-column derivatisation," *Phytochemical Analysis*, vol. 16, no. 5, pp. 307–314, 2005.

[11] L. F. Bonomo, D. N. Silva, P. F. Boasquivis et al., "Açaí (*Euterpe oleracea* Mart.) modulates oxidative stress resistance in *Caenorhabditis elegans* by direct and indirect mechanisms," *PLoS ONE*, vol. 9, no. 3, Article ID e89933, 2014.

[12] H. Iwasawa, E. Morita, S. Yui, and M. Yamazaki, "Anti-oxidant effects of kiwi fruit *in vitro* and *in vivo*," *Biological and Pharmaceutical Bulletin*, vol. 34, no. 1, pp. 128–134, 2011.

[13] N. Nicod, G. Chiva-Blanch, E. Giordano, A. Dávalos, R. S. Parker, and F. Visioli, "Green tea, cocoa, and red wine polyphenols moderately modulate intestinal inflammation and do not increase high-density lipoprotein (HDL) production," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 10, pp. 2228–2232, 2014.

[14] Y. P. Hwang, H. J. Yun, H. K. Chun et al., "Protective mechanisms of 3-caffeoyl, 4-dihydrocaffeoyl quinic acid from *Salicornia herbacea* against *tert*-butyl hydroperoxide-induced oxidative damage," *Chemico-Biological Interactions*, vol. 181, no. 3, pp. 366–376, 2009.

[15] L. Rossi, S. Mazzitelli, M. Arciello, C. R. Capo, and G. Rotilio, "Benefits from dietary polyphenols for brain aging and Alzheimer's disease," *Neurochemical Research*, vol. 33, no. 12, pp. 2390–2400, 2008.

[16] N. E. Gray, J. Morre, J. Kelley et al., "Caffeoylquinic acids in centella asiatica protect against amyloid-beta toxicity," *Journal of Alzheimer's Disease*, vol. 40, no. 2, pp. 359–373, 2014.

[17] K. Sasaki, J. Han, H. Shimozono, M. O. Villareal, and H. Isoda, "Caffeoylquinic acid-rich purple sweet potato extract, with or without anthocyanin, imparts neuroprotection and contributes to the improvement of spatial learning and memory of samp8 mouse," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 21, pp. 5037–5045, 2013.

[18] F. Giampieri, J. M. Alvarez-Suarez, and M. Battino, "Strawberry and human health: effects beyond antioxidant activity," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 18, pp. 3867–3876, 2014.

[19] S. Brenner, "The genetics of *Caenorhabditis elegans*," *Genetics*, vol. 77, no. 1, pp. 71–94, 1974.

[20] R. P. Oliveira, J. P. Abate, K. Dilks et al., "Condition-adapted stress and longevity gene regulation by *Caenorhabditis elegans* SKN-1/Nrf," *Aging Cell*, vol. 8, no. 5, pp. 524–541, 2009.

[21] S. Georgé, P. Brat, P. Alter, and M. J. Amiot, "Rapid determination of polyphenols and vitamin C in plant-derived products," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 5, pp. 1370–1373, 2005.

[22] W. Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of a free radical method to evaluate antioxidant activity," *LWT—Food Science and Technology*, vol. 28, no. 1, pp. 25–30, 1995.

[23] G. A. Nelson, K. K. Lew, and S. Ward, "Intersex, a temperature-sensitive mutant of the nematode *Caenorhabditis elegans*," *Developmental Biology*, vol. 66, no. 2, pp. 386–409, 1978.

[24] D. H. Kim, "Bacteria and the aging and longevity of *Caenorhabditis elegans*," *Annual Review of Genetics*, vol. 47, pp. 233–246, 2013.

[25] D. Vilchez, I. Morantte, Z. Liu et al., "RPN-6 determines *C. elegans* longevity under proteotoxic stress conditions," *Nature*, vol. 489, no. 7415, pp. 263–268, 2012.

[26] A. Sakaguchi, K. Matsumoto, and N. Hisamoto, "Roles of map kinase cascades in *Caenorhabditis elegans*," *The Journal of Biochemistry*, vol. 136, no. 1, pp. 7–11, 2004.

[27] L. Chang and M. Karin, "Mammalian MAP kinase signalling cascades," *Nature*, vol. 410, no. 6824, pp. 37–40, 2001.

[28] J. M. Kyriakis and J. Avruch, "Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation," *Physiological Reviews*, vol. 81, no. 2, pp. 807–869, 2001.

[29] M. A. Ermolaeva, A. Segref, A. Dakhovnik et al., "DNA damage in germ cells induces an innate immune response that triggers systemic stress resistance," *Nature*, vol. 501, no. 7467, pp. 416–420, 2013.

[30] S. W. Oh, A. Mukhopadhyay, N. Svrzikapa, F. Jiang, R. J. Davis, and H. A. Tissenbaum, "JNK regulates lifespan in *Caenorhabiditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 12, pp. 4494–4499, 2005.

[31] H. Inoue, N. Hisamoto, H. A. Jae et al., "The *C. elegans* p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response," *Genes and Development*, vol. 19, no. 19, pp. 2278–2283, 2005.

[32] T. Okuyama, H. Inoue, S. Ookuma et al., "The ERK-MAPK pathway regulates longevity through SKN-1 and insulin-like signaling in *Caenorhabditis elegans*," *The Journal of Biological Chemistry*, vol. 285, no. 39, pp. 30274–30281, 2010.

[33] S. J. Choi, J. K. Kim, S. H. Suh et al., "Ligularia fischeri extract protects against oxidative-stress-induced neurotoxicity in mice and pc12 cells," *Journal of Medicinal Food*, vol. 17, no. 11, pp. 1222–1231, 2014.

[34] H. R. Jeong, Y. N. Jo, J. H. Jeong, H. J. Kim, M.-J. Kim, and H. J. Heo, "Blueberry (*Vaccinium virgatum*) leaf extracts protect against Aβ-induced cytotoxicity and cognitive impairment," *Journal of Medicinal Food*, vol. 16, no. 11, pp. 968–976, 2013.

[35] Y. Y. Cao, L. Wang, H. Ge et al., "Salvianolic acid A, a polyphenolic derivative from *Salvia miltiorrhiza* bunge, as a multifunctional agent for the treatment of Alzheimer's disease," *Molecular Diversity*, vol. 17, no. 3, pp. 515–524, 2013.

[36] J. S. Sangha, X. Sun, O. S. D. Wally et al., "Liuwei dihuang (lwdh), a traditional chinese medicinal formula, protects against beta-amyloid toxicity in transgenic *Caenorhabditis elegans*," *PLoS ONE*, vol. 7, no. 8, Article ID e43990, 2012.

[37] F. U. Hartl, A. Bracher, and M. Hayer-Hartl, "Molecular chaperones in protein folding and proteostasis," *Nature*, vol. 475, no. 7356, pp. 324–332, 2011.

[38] S. L. Rea, D. Wu, J. R. Cypser, J. W. Vaupel, and T. E. Johnson, "A stress-sensitive reporter predicts longevity in isogenic populations of *Caenorhabditis elegans*," *Nature Genetics*, vol. 37, no. 8, pp. 894–898, 2005.

[39] G. A. Walker and G. J. Lithgow, "Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals," *Aging Cell*, vol. 2, no. 2, pp. 131–139, 2003.

[40] H. Jo, J. Shim, J. H. Lee, and J. B. Kim, "Ire-1 and hsp-4 contribute to energy homeostasis via fasting-induced lipases in *C. elegans*," *Cell Metabolism*, vol. 9, no. 5, pp. 440–448, 2009.

[41] S. R. M. Grance, M. A. Teixeira, R. S. Leite et al., "*Baccharis trimera*: effect on hematological and biochemical parameters and hepatorenal evaluation in pregnant rats," *Journal of Ethnopharmacology*, vol. 117, no. 1, pp. 28–33, 2008.

[42] G. Grünz, K. Haas, S. Soukup et al., "Structural features and bioavailability of four flavonoids and their implications for lifespan-extending and antioxidant actions in *C. elegans*," *Mechanisms of Ageing and Development*, vol. 133, no. 1, pp. 1–10, 2012.

[43] C. Portal-Celhay, E. R. Bradley, and M. J. Blaser, "Control of intestinal bacterial proliferation in regulation of lifespan in *Caenorhabditis elegans*," *BMC Microbiology*, vol. 12, article 49, 2012.

[44] K. Pietsch, N. Saul, S. Chakrabarti, S. R. Stürzenbaum, R. Menzel, and C. E. W. Steinberg, "Hormetins, antioxidants and prooxidants: defining quercetin-, caffeic acid- and rosmarinic acid-mediated life extension in *C. elegans*," *Biogerontology*, vol. 12, no. 4, pp. 329–347, 2011.

[45] G. J. Lithgow and G. A. Walker, "Stress resistance as a determinate of *C. elegans* lifespan," *Mechanisms of Ageing and Development*, vol. 123, no. 7, pp. 765–771, 2002.

[46] P. B. L. Pun, J. Gruber, S. Y. Tang et al., "Ageing in nematodes: do antioxidants extend lifespan in *Caenorhabditis elegans*?" *Biogerontology*, vol. 11, no. 1, pp. 17–30, 2010.

[47] A. Kampkötter, C. Timpel, R. F. Zurawski et al., "Increase of stress resistance and lifespan of *Caenorhabditis elegans* by quercetin," *Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology*, vol. 149, no. 2, pp. 314–323, 2008.

[48] S. C. Lu, "Regulation of glutathione synthesis," *Molecular Aspects of Medicine*, vol. 30, no. 1-2, pp. 42–59, 2009.

[49] V. H.-C. Liao and C.-W. Yu, "Caenorhabditis elegans gcs-1 confers resistance to arsenic-induced oxidative stress," *BioMetals*, vol. 18, no. 5, pp. 519–528, 2005.

[50] R. Masella, R. Di Benedetto, R. Varì, C. Filesi, and C. Giovannini, "Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes," *Journal of Nutritional Biochemistry*, vol. 16, no. 10, pp. 577–586, 2005.

[51] C.-W. Yu, C.-C. Wei, and V. H.-C. Liao, "Curcumin-mediated oxidative stress resistance in *Caenorhabditis elegans* is modulated by *age-1, akt-1, pdk-1, osr-1, unc-43, sek-1, skn-1, sir-2.1, and mev-1*," *Free Radical Research*, vol. 48, no. 3, pp. 371–379, 2014.

[52] K. Hasegawa, S. Miwa, K. Isomura, K. Tsutsumiuchi, H. Taniguchi, and J. Miwa, "Acrylamide-responsive genes in the nematode *Caenorhabditis elegans*," *Toxicological Sciences*, vol. 101, no. 2, pp. 215–225, 2008.

[53] A. Kampkötter, T. Pielarski, R. Rohrig et al., "The Ginkgo biloba extract EGb761 reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione S-transferase 4 in *Caenorhabditis elegans*," *Pharmacological Research*, vol. 55, no. 2, pp. 139–147, 2007.

[54] B. Onken and M. Driscoll, "Metformin induces a dietary restriction-like state and the oxidative stress response to extend *C. elegans* healthspan via AMPK, LKB1, and SKN-1," *PLoS ONE*, vol. 5, no. 1, Article ID e8758, 2010.

[55] A. Kampkötter, C. G. Nkwonkam, R. F. Zurawski et al., "Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism *Caenorhabditis elegans*," *Archives of Toxicology*, vol. 81, no. 12, pp. 849–858, 2007.

[56] Y. B. Yu, L. Dosanjh, L. Lao, M. Tan, B. S. Shim, and Y. Luo, "Cinnamomum cassia bark in two herbal formulas increases life span in *Caenorhabditis elegansvia* insulin signaling and stress response pathways," *PLoS ONE*, vol. 5, no. 2, Article ID e9339, 2010.

[57] N. Saul, K. Pietsch, R. Menzel, and C. E. W. Steinberg, "Quercetin-mediated longevity in *Caenorhabditis elegans*: is DAF-16 involved?" *Mechanisms of Ageing and Development*, vol. 129, no. 10, pp. 611–613, 2008.

[58] C. B. Pocernich and D. A. Butterfield, "Elevation of glutathione as a therapeutic strategy in alzheimer disease," *Biochimica et Biophysica Acta: Molecular Basis of Disease*, vol. 1822, no. 5, pp. 625–630, 2012.

[59] A. L. Aboy, M. A. Apel, S. Debenedetti, L. Francescato, M. A. Rosella, and A. T. Henriques, "Assay of caffeoylquinic acids in *Baccharis trimera* by reversed-phase liquid chromatography," *Journal of Chromatography A*, vol. 1219, pp. 147–153, 2012.

[60] C. Regitz, L. M. Dußling, and U. Wenzel, "Amyloid-beta ($A\beta_{1--42}$)-induced paralysis in *Caenorhabditis elegans* is inhibited by the polyphenol quercetin through activation of protein degradation pathways," *Molecular Nutrition & Food Research*, vol. 58, no. 10, pp. 1931–1940, 2014.

[61] C. Voisine, J. S. Pedersen, and R. I. Morimoto, "Chaperone networks: tipping the balance in protein folding diseases," *Neurobiology of Disease*, vol. 40, no. 1, pp. 12–20, 2010.

[62] V. Prahlad and R. I. Morimoto, "Integrating the stress response: lessons for neurodegenerative diseases from *C. elegans*," *Trends in Cell Biology*, vol. 19, no. 2, pp. 52–61, 2009.

[63] W. J. Kapulkin, B. G. Hiester, and C. D. Link, "Compensatory regulation among ER chaperones in *C. elegans*," *FEBS Letters*, vol. 579, no. 14, pp. 3063–3068, 2005.

[64] V. Fonte, D. R. Kipp, J. Yerg III et al., "Suppression of in vivo β-amyloid peptide toxicity by overexpression of the HSP-16.2 small chaperone protein," *The Journal of Biological Chemistry*, vol. 283, no. 2, pp. 784–791, 2008.

[65] V. Fonte, V. Kapulkin, A. Taft, A. Fluet, D. Friedman, and C. D. Link, "Interaction of intracellular β amyloid peptide with chaperone proteins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 14, pp. 9439–9444, 2002.

[66] A. A. Soukas, C. E. Carr, and G. Ruvkun, "Genetic regulation of *Caenorhabditis elegans* lysosome related organelle function," *PLoS Genetics*, vol. 9, no. 10, Article ID e1003908, 2013.

[67] B. Gerstbrein, G. Stamatas, N. Kollias, and M. Driscoll, "*In vivo* spectrofluorimetry reveals endogenous biomarkers that report healthspan and dietary restriction in *Caenorhabditis elegans*," *Aging Cell*, vol. 4, no. 3, pp. 127–137, 2005.

[68] E. J. O'Rourke, A. A. Soukas, C. E. Carr, and G. Ruvkun, "*C. elegans* major fats are stored in vesicles distinct from lysosome-related organelles," *Cell Metabolism*, vol. 10, no. 5, pp. 430–435, 2009.

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Review Article

Biomarkers of Dietary Polyphenols in Cancer Studies: Current Evidence and Beyond

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Polyphenols, commonly contained in fruits and vegetables, have long been associated with a protective role against multiple diseases and adverse health effects. Generally, *in vitro* and animal experiments have provided strong positive evidence, whereas evidence from *in vivo* and human epidemiological studies is not strong enough. Most epidemiological studies to date use food frequency questionnaire based dietary intake estimations, which inevitably incur imprecision. Biomarkers of polyphenol have the potential to complement and enhance current studies. This review performed a literature search of all epidemiological studies or controlled clinical/intervention trials which employed biomarkers of exposure for polyphenols to help assess their anticarcinogenic role, using studies on green tea polyphenols as a study model. Currently, studies on this topic are still limited; breast cancer and prostate cancer were the only widely studied cancer types. Isoflavone is the only widely studied polyphenol. In addition to associations between polyphenols and cancer risks, factors such as host genetic susceptibility, epigenetic modification, and gut microbiome patterns may also impact on the protective roles of polyphenols. More evidence should be collected by utilizing biomarkers of exposure for polyphenols in future epidemiological studies before a clear conclusion can be made.

1. Introduction

Extensive evidence has documented diets rich in fruits and vegetables protect human against multiple chronic diseases. Polyphenols, common constitutes in fruits and vegetables, are suggested to play a major protective role in disease prevention [1]. Evidences on beneficial effects of polyphenols are mainly derived from *in vitro* or animal experiments, as well as some human epidemiological studies [2]. Currently, epidemiological studies tend to accept a protective role of polyphenol containing foods on cardiovascular disease [3, 4]. Preventive effects of polyphenols on other chronic diseases, such as cancer, are generally less convincing [3, 5], with the exception of green tea polyphenols [6–8].

Part of the difficulties in assessing the beneficial effects of polyphenols in *in vivo* or epidemiological studies is related to poor understanding of their bioavailability and metabolism in human physiological system [5]. The bioavailability of typical polyphenols is rather low upon oral administration, due to their poor water solubility, rapid biotransformation, membrane permeability, and molecular size [9, 10]. In addition, most *in vitro* or animal studies examined aglycones of polyphenols [11]; however, most polyphenols exist as glycosides in natural food sources [3, 12], which are known to be less bioactive as compared to their aglycones [3, 11]. Moreover, although human gut microbiota is capable of transforming polyphenol glycosides into their aglycone, intestine barrier and first-pass metabolism will transform polyphenols into methoxylated, glucuronidated, sulfated, or other conjugated forms [4]. Therefore, aglycones of polyphenols rarely exist in systemic circulation [12].

Another difficulty in assessing the role of polyphenol in *in vivo* and epidemiological studies is the lack or inaccuracies in the measurement of bioavailable and effective polyphenols, which may lead to weaker or even spurious correlations between exposure and disease risks [5, 13]. In epidemiological studies, food frequency questionnaire (FFQ) based estimation of dietary intake of specific polyphenols is the most commonly used surrogate of polyphenol exposure. This method suffers from imprecision [14, 15]. Since the mid-1990s,

the analytical methods for specific polyphenolic compounds in body fluids have become available [14]. Biomarkers as a result of these developments, such as measurement of urinary excretion or plasma level of polyphenols, could complement or even replace questionnaire based dietary assessment [2, 4–6].

There have been numerous reviews and meta-analysis on the chemopreventive effects of polyphenols on different cancer types [16–24]. All these reports concluded that existing evidence is insufficient to support a chemopreventive role of polyphenols. However, studies reviewed or summarized within abovementioned reports are mostly questionnaire based. The purpose of this review is to focus on epidemiological studies which employed biomarkers of polyphenol exposure, using green tea polyphenols as an example, and to examine whether using more accurate measurements of polyphenol biomarkers could reveal the beneficial role of polyphenol compounds as suggested in *in vitro* and animal studies. This review will start from a brief summary of bioavailability studies on common polyphenol compounds that have been suggested to have an anticarcinogenic role, followed by a detailed summary of epidemiological studies or clinical trials using polyphenol biomarkers and a summary of studies on green tea polyphenols as a study model.

2. Bioavailability of Common Polyphenols

A previous review [25] has comprehensively examined bioavailability and bioefficacy of polyphenols by summarizing 97 studies. Based on that report and new evidences thereafter, we briefly discuss the human dietary intake, the absorption, and pharmacokinetics of common polyphenols. The reason of summarizing bioavailability studies is that biomarkers of polyphenol exposure are most likely to be either the direct bioavailable forms of a specific polyphenol or their metabolites. Therefore, understanding the bioavailability of these compounds could help us assess the validity of biomarkers applied in existing studies and suggest potential biomarkers.

2.1. Anthocyanins. Anthocyanins are one of the most widespread polyphenols and exist in large amount in some diets [25]. An earlier estimate suggested that the daily intake of anthocyanin can be as high as 215 mg/day/person [26]. More recent reports have suggested a lower but still substantial dietary intake. In a study in Finland, FINDIET 2002 Survey, mean dietary intake of anthocyanins was estimated to be 47 mg/day/person [27]. Another report based on the SU.VI. MAX study in France estimated mean dietary intake of anthocyanins to be 57 mg/day/person [28]. An estimate from the European Prospective Investigation into Cancer and Nutrition (EPICN) Spanish subcohort reported a much lower intake of about 18.88 mg/day/person [29]. This estimate is in the same magnitude of another study conducted among US adults (12.5 mg/day/person) [30].

Previous evidence has shown that anthocyanins are absorbed as intact glycosides or as metabolized conjugates in stomach or intestine [31, 32]; no aglycones could be recovered either in plasma or in urine [33]. Animal and human studies further demonstrated that anthocyanins could be rapidly absorbed [33]; the plasma concentration reaches its peak concentration in 0.5–4 hours in both human and animal studies [31]. However, the bioavailability of anthocyanins is very low, with a majority of studies reporting the urinary recovery less than 1% [33, 34]. Some recent studies have argued that the bioavailability of anthocyanins may not be as low as it is used to be believed because a variety of metabolites of anthocyanins may be absorbed in gastrointestinal tract [31].

2.2. Flavonols/Quercetin. Flavonols are another group of widely distributed polyphenols. Quercetin is one of the most commonly dietary available flavonols, which can be found in onions, tea, and wine. The daily intake of quercetin, however, might be low because of relatively low dietary content [25]. Average dietary intake of quercetin in a Netherland study is 16 mg/day/person [55]. More recent reports from other European studies have shown daily intakes of flavonols to be 5.4 mg/day/person in Finland [27], 51 mg/day/person in France [28], and 18.7 mg/day/person in Spain [29]. A recent US study based on NHANES 1999–2002 estimated the average daily intake of flavonols to be 12.8 mg/day/person [56].

Although earlier researchers used to believe that quercetin cannot be absorbed by humans [25], Hollman et al. firstly showed that quercetin could indeed be absorbed in small intestine as mainly glucosides (52% of absorbed quercetin) and as quercetin aglycone (24%) or quercetin-3-rutinoside (17%) [55]. More recent studies [57–59] confirmed this finding, but current consensus is that quercetin aglycone cannot be absorbed into systemic circulation and it is quercetin glucuronides instead of glucosides that are present in plasma or urine after oral intake of quercetin. Graefe et al. found that different sugar moiety of quercetin glycosides influenced their bioavailability [58]. For example, glucosides of quercetin (the major form in onions) could be rapidly absorbed, and the plasma quercetin reached its peak concentration, 2.1–2.3 μg/mL (about 100 mg equivalent quercetin intake), about 0.7 hours after oral administration. But rutin forms of quercetin (the major form in tea) were slowly absorbed, reaching its peak concentration, 0.3–0.6 μg/mL (100 mg equivalent quercetin intake), about 4.3 to 7 hours after administration with a much lower relative peak concentration. Urinary recovery of quercetin is low: 48-hours after oral intake, about 6.4% of quercetin was recovered if taking glucosides, but less than 1% could be recovered if taking rutin form [58].

2.3. Flavanones. Flavanones represent a small group of polyphenols. They almost exclusively exist in citrus fruits and in some aromatic herbs such as mint [25]. Although dietary sources of flavanones are limited, their daily intakes can be high because citrus fruits are commonly consumed in different forms [60]. In a study in Finland, hesperetin, a major flavanone compound, was reported to be the most highly consumed flavonoid [61]. Other European studies have reported the daily intake of total flavanones (including eriodictyol, hesperetin, and naringenin) to be 27 mg/day/person in Finland [27], 50 mg/day/person in Spain [29], and 26 mg/day/person

in France [28]. The study among US adults reported daily intake of 13.3 to 15.5 mg/day/person [56].

Among a few studies on the bioavailability of flavanones in human subjects, Manach et al. conducted a crossover study among five healthy volunteers who consumed two different doses of orange juice and reported that no free flavanone aglycone existed in plasma after each dosing [60]. Further analysis suggested that plasma flavanone is glucuronidated or sulfoglucuronidated [60]. The two compounds, hesperetin and naringenin, tested in this study were absorbed much slower and at much lower level as compared to other previously mentioned polyphenols. Plasma hesperetin reached its peak concentration, about 0.14 μg/mL after 222 mg oral intake and 0.39 μg/mL after 440 mg oral intake, about 5-6 hours after administration. Naringenin reached its peak concentration, about 0.054 μg/mL after 96 mg intake and 0.016 μg/mL after 48 intake, about 5.4 hours after administration [60]. In addition, about 4–6% of hesperetin intake and about 7% of naringenin intake could be recovered from urine, and not surprisingly both compounds were in glucuronidated or sulfoglucuronidated form [60].

2.4. Isoflavones. Isoflavones are only found in soybean-derived products [25]. The natural isoflavones, such as daidzein or genistein, are usually in glucose-conjugated form [62]. The intake of isoflavone also varies greatly by different countries. The recent report among US adults estimated the daily intake of isoflavones was the lowest among all flavonoids, only about 1.2 mg/day [56]. Similar results were obtained from a Finnish study (0.9 mg/day) [27] and nearly none in a Spanish study [29], whereas in Asia, a study in Japan [63] among 1232 healthy participants has reported that the median intake of daidzein was from 9.5 to 12.1 mg/day and the intake of genistein was from 14.9 to 19.6 mg/day, more than ten times as compared to western countries. The median intake of isoflavone in another study [64] among 60 healthy Chinese women was as high as 39.3 mg/day.

The bioavailability of isoflavones is well documented. An early study [65] recruited eight volunteers and measured the plasma isoflavones concentrations after two doses (high and low) of either isoflavone aglycone or glycosides. This study found that plasma isoflavones reached peak concentrations 2–4 hours after intake of aglycone, which was faster than correspondent glycosides (about 4–8 hours after intake); the peak concentrations for aglycones were also higher than glycosides. Based on these findings, it was concluded that isoflavone aglycones were more efficiently absorbed than glycosides. Another study [66] revealed similar pattern, although much slower: for genistein and daidzein glycosides (genistein and daidzein), the mean time of reaching peak plasma concentration was around 9 hours, whereas for genistein and daidzein aglycone, the mean time was around 5-6 hours. The peak concentrations measured in this later study for daidzein and genistein were 194 ng/mL and 341 ng/mL, whereas the peak concentrations for daidzein and genistein were 394 ng/mL and 341 ng/mL. These findings further suggested that the absorption of glycosides was higher as compared to their respective aglycones based on the area under pharmacokinetic curves [66]. The discrepancy between these two studies might be because the first study [65] did not measure isoflavone level between 6 and 24 hours; it was possible that the glycosides levels were still rising after 6 hours and therefore were not captured.

The longer time for isoflavone to reach a peak plasma concentration after intake of glycosidic form was attributed to the fact that glycosides cannot be directly absorbed in intestine in both abovementioned studies; they have to be firstly hydrolyzed [65, 66]. A later study confirmed that no glycosides could be absorbed by human volunteers and found that the circulating forms of isoflavone in plasma were mainly conjugated forms [12]. The urinary excretion of isoflavone is typically high. As shown in one study [67], 30% of daidzein and 9% of genistein could be recovered in urine after a single dose of each isoflavone among healthy premenopausal women.

2.5. Flavanols/Catechins. Catechins, one of a major group in flavanols or flavan-3-ols, are the principle components in tea, apple, chocolate, pear, grape, and red wine [25]. Since its discovery more than 5000 years ago, tea has grown to be the most widely consumed beverage in the world [13]. The habit of tea consumption varies by different geographic regions: black tea is principally consumed in Europe, North America, and North Africa while green tea is consumed throughout Asia. Oolong tea is particularly consumed in China and Southeastern Asia [68]. The three classes of tea were produced by different manufacturing processes: green tea without fermentation, oolong tea with partial fermentation, and black tea fully fermented. Fermentation converts tea polyphenols into theaflavins, thearubigins, theasinensins, and proanthocyanidin polymers that are abundant in black tea [69]. Green tea is most abundant for tea catechins [70] which include (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (EG), and (−)-epigallocatechin gallate (EGCG) [13]. Due to the long history of tea drinking and its long recognized beneficial effects, catechins are probably the most extensively studied polyphenols.

The recent report in US [56] estimated that the daily consumption of flavan-3-ols was the highest among all flavonoids, as high as 156.5 mg/day among US adults. The European studies reported average flavan-3-ols intake to be 15.67–22.06 mg/day in Spain [29] and 99 mg/day in France [28]. Although intakes of flavanols by Asian populations are speculated to be very high, there is few quantitative data reporting the amount of their flavanols daily intakes available.

Multiple lines of evidence have documented bioavailability of tea catechins. A human study found that after intake of about 1.2 g decaffeinated green tea (DGT, containing 88 mg of EGCG, 82 mg of EGC, 33 mg of ECG, and 32 mg of EC), EGC, EGCG, and EC, but not ECG, were detected in volunteers plasma samples [71]. However, more recent studies did detect ECG after dosing either pure ECG or green tea to healthy volunteers [72, 73]. The discrepancy might be because of the different conditions of analytical methods to detect ECG [72]. Regarding the chemical speciation of catechins after intake of tea polyphenols, EGC was originally reported to present mostly in glucuronidated form (57–71%) and sulfate form (23–36%); only a small portion (3–13%) of free form

was presented, whereas EC was exclusively in the conjugated form, with approximately 67% as glucuronidated form and 33% as sulfated form [71]. These results were confirmed by a more recent study [74]. For EGCG, a more recent study [74] found that EGCG were presented mostly in its free form.

Yang et al. [13] demonstrated that, after ingestion of DGT, plasma concentrations of EGCG, ECG, and EC reached their peak concentrations about 1.5–2.5 hours after intake [13]. The plasma concentrations for EGCG, ECG, and EC were found to be dose dependent at 1.4–5.5 hours after ingestion [13]. The peak concentrations of EGCG, EGC, and EC after intake of 3.0 g DGT were 0.326 μg/mL, 0.508 μg/mL, and 0.189 μg/mL [13], suggesting that EGCG has a lower absorbability. These results were confirmed by later studies [72–74]. In addition, multiple evidence has showed that no EGCG could be detected in urine after oral intake of catechins [13, 71, 72, 74]. One possible explanation is that EGCG or its metabolites are mainly excreted in bile as found in rat [13].

3. Epidemiological Studies Employing Biomarkers of Polyphenol Exposure

For the search of epidemiological studies or controlled clinical trials which employed biomarkers of polyphenol exposure to assess their anticarcinogenic role, we included all available literature from articles of original research to meeting proceedings since 1995 because analytical techniques have been available since the mid-1990s [14]. We also performed a manual search using reference lists of original articles, meta-analysis, and relevant reviews. Out of 315 articles, 20 were deemed relevant to the scope of this review after carefully examining the titles and abstracts and therefore summarized. Information on the 20 articles is presented in Table 1.

Among the 20 articles, the vast majority of studies (16 out of 20) assessed the beneficial effects of isoflavone using either plasma isoflavone concentrations or urinary excretion as biomarkers of exposure (Table 1). This might partly be because plasma and urinary excretion of isoflavone have been recognized as valid biomarkers of exposure [44, 46, 75] while other polyphenols remain to be elicited. The other three studies assessed the effects of tea catechins. A recent biomarker validation study confirmed that blood ECG and EGCG could reflect the diet intake of green tea polyphenol and therefore suggested their use in population level studies [6].

Breast cancer (8 out of 20) and prostate cancer (6 out of 20) are the most frequently studied cancer types; two other studies examined colorectal cancer; three examined gastric cancer; one examined liver cancer; and one examined lung cancer. It was also interesting to see that the majority of the studies were focused on Asian population (10 out of 20) and European population (6 out of 20); only two studies were conducted in United States; another one was conducted in Jamaica and one in Australia.

3.1. Breast Cancer. All eight studies assessed the role of isoflavone on breast cancer risk. Urinary excretion rates of daidzein and its metabolite equol were used as biomarkers of isoflavone exposure to assess the risk of breast cancer among a group of Australian women [35]. Increased excretion of equol (indicating a higher bioavailable amount) was reported to be significantly associated with a reduced risk. Subjects among the highest quartile of equol excretion had a 73% lower risk of developing breast cancer as compared to reference level (lowest quartile) (odds ratio ($OR_{\text{high versus ref.}}$) = 0.27, 95% confidence interval (CI) was 0.10–0.69); the dose-response relationship was also significant for equol (P-trend = 0.009). However, no significant association with increased daidzein excretion (OR = 0.47, 95% CI = 0.17–1.33) was found [35]. Nevertheless, two European studies [36, 37] failed to find a significant association between isoflavone exposure and breast cancer risk, although the risk tended to be reduced. Another study [38] reported that higher plasma genistein level (but not daidzein) was associated with a significantly reduced risk ($OR_{\text{high versus ref.}}$ = 0.68, 95% CI 0.47–0.98). One possible reason for the null association suggested by these authors is that the intakes of isoflavone among European population were mainly from dietary additions and therefore relatively low [36, 38]. It is true that the urinary excretions from all these studies were below 1.0 nmol/mg creatinine for isoflavones and the median plasma levels were all below 10–15 nmol/L. Other possible reasons might be the biases employed due to the study design. For example, in one study [37], blood samples were taken after the cases were diagnosed; the median time between subject participation (when blood samples were taken) and diagnosis was 2 months. It was possible that patients might have changed their diet after diagnosis.

Moreover, a positive association was reported when examining EPICN-Norfolk (U.K.) subcohort using both plasma level and urinary excretion of isoflavones; exposure to all isoflavone was associated with increased breast cancer risk, significantly so for daidzein and equol [39]. In addition to the consistently low levels of isoflavone among study subjects as in other European countries, interpretation of results from this study should be cautious. A $\log_2$ OR was reported in this study [39], willing to present the relative risk associated with doubling of the exposure. However, their calculation might be problematic because $\log_2$ transformation should be made on exposure variable (plasma or urinary level of isoflavones), not the OR itself [76].

Three studies among Asian populations reported relatively consistent results [40–42]. In a study using Shanghai Breast Cancer Study cohort samples, a lower but insignificant risk between urinary excretion of daidzein and genistein and breast cancer risk was found [40]. A relative small sample size (60 cases and 60 controls) may contribute to the results. A later study from the same group [41], using more samples from 250 cases and 250 controls and a more sensitive technique (LC/MS instead of spot urine analysis), found significant lower risk from subjects with the highest tertile of urinary daidzein level ($OR_{\text{high versus ref.}}$ = 0.54, 95% CI = 0.34–0.85). A significant dose-response relationship was also found (P-trend < 0.01). Another study with Japan Public Health Center cohort samples reported that plasma genistein concentrations were significantly associated with breast cancer risk and $OR_{\text{high versus ref.}}$ = 0.34 (95% CI = 0.16–0.74) [42].

Table 1: Epidemiological studies/clinical trials employed biomarkers of polyphenol exposure.

Reference	Location	Types of study	Cancer type	Number of subjects	Polyphenol	Plasma level[1] nmol/L	Urinary excretion[1] nmol/mg creatinine[2]
[35]	Australia	Case-control	Breast cancer	Cases: 144 Controls: 144	Equol Daidzein		nmol/24 h Cases: 97 Controls: 108.8 nmol/24 h Cases: 782.9 Controls: 913.4
[36]	Netherland	Nested case-control	Breast cancer	Cases: 88 Controls: 268	Genistein		Cases: 0.70 Controls: 0.81
[37]	Germany	Case-control	Breast cancer	Cases: 220 Controls: 237	Genistein	Cases: 4.5 Controls: 3.7	
[38]	Netherland	Nested case-control	Breast cancer	Cases: 383 Controls: 383	Daidzein	Cases: 9.20–11.25 Controls: 10.31–12.86	
[39]	UK	Nested case-control	Breast cancer	Plasma Cases: 97 Controls: 187 Urinary Cases: 114 Controls: 219	Daidzein Genistein Equol	All: 8.7 All: 12.9 All: 0.4	All: 0.60 All: 0.23 All: 0.0036
[40]	China	Nested case-control	Breast cancer	Cases: 60 Controls: 60	Daidzein Genistein		Cases: 2.15 Controls: 108.8 Cases: 0.76 Controls: 2.17
[41]	China	Nested case-control	Breast cancer	Cases: 250 Controls: 250	Daidzein Genistein		Mean Cases: 4.95 Controls: 9.90 Mean Cases: 2.19 Controls: 4.37
[42]	Japan	Nested case-control	Breast cancer	Cases: 144 Controls: 288	Daidzein Genistein	Cases: 65.7 Controls: 70.4 Cases: 487.7 Controls: 534.7	
[43]	Japan	Nested case-control	Prostate cancer	Cases: 201 Controls: 402	Daidzein Genistein Equol	Cases: 145.5 Controls: 139.6 Cases: 330.4 Controls: 319.0 Cases: 65.7 Controls: 70.4	
[44]	UK	Nested case-control	Prostate cancer	Cases: 193 Controls: 828	Daidzein Genistein Equol	All: 9.8 All: 25.5 All: 0.04	All: 0.51 All: 0.25 All: 0.009

Table 1: Continued.

Reference	Location	Types of study	Cancer type	Number of subjects	Polyphenol	Plasma level[1] nmol/L	Urinary excretion[1] nmol/mg creatinine[2]
[45]	EU	Nested case-control	Prostate cancer	Cases: 950 Controls: 1042	Daidzein Genistein Equol	All: 4.3 All: 6.7 All: 0.8	
[46]	US	Nested case-control	Prostate cancer	Cases: 249 Controls: 404	Daidzein Genistein Equol		Cases: 0.173 Controls: 0.291 Cases: 0.048 Controls: 0.078 Cases: 0.005 Controls: 0.004
[47]	Jamaica	Case-control	Prostate cancer	Cases: 175 Controls: 194	Daidzein Genistein Equol		Cases: 0.199 Controls: 0.221 Cases: 0.280 Controls: 0.273 Cases: 0.072 Controls: 0.067
[48]	US	Clinical trial	Prostate cancer		Catechin		
[49]	China	Nested case-control	Colorectal cancer	Cases: 162 Controls: 806	EGC 4′-MeEGC		Geometric mean Nondrinker: 1.11 Tea drinkers: −4.47 Geometric mean Nondrinker: 4.30 Tea drinkers: −17.40
[44]	UK	Nested case-control	Colorectal cancer	Cases: 221 Controls: 889	Daidzein Genistein Equol	All: 8.7 All: 23.1 All: 0.04	All: 0.53 All: 0.20 All: 0.002
[50]	China	Nested case-control	Gastric cancer	Cases: 190 Controls: 772	Catechins		Unclear
[51]	Korea	Nested case-control	Gastric cancer	Cases: 131 Controls: 393	Daidzein	Cases: 80.5 Controls: 131.2	
[52]	Japan	Nested case-control	Gastric cancer	Cases: 483 Controls: 483	Daidzein Genistein	Cases: ~161 Controls: ~155 Cases: 17 Controls: 18	
[50]	China	Nested case-control	Esophageal cancer	Cases: 42 Controls: 772	Catechins		Unclear

Table 1: Continued.

Reference	Location	Types of study	Cancer type	Number of subjects	Polyphenol	Plasma level[1] nmol/L	Urinary excretion[1] nmol/mg creatinine[2]
[53]	Japan	Nested case-control	Lung cancer	Cases: 126 Controls: 252	Daidzein	Cases: 115.2 Controls: 125.1	
					Genistein	Cases: 266.4 Controls: 267.9	
					Equol	Cases: 11.6 Controls: 14.4	
[54]	China	Clinical trial	Liver cancer		Catechin		

[1] All values are median, unless otherwise noted.
[2] All units are nmol/mg creatinine, unless otherwise noted.

3.2. Prostate Cancer. Isoflavone levels were measured in five of the six studies assessing the prostate cancer risk with dietary intake of polyphenols. In a nested case-control study [43] with the previously mentioned Japan Public Health Center cohort samples, highest tertile for equol exposure was significantly associated with a decreased risk of total prostate cancer ($OR_{\text{high versus ref.}} = 0.60$; 95% CI = 0.36–0.99; P-trend = 0.03); this association was even stronger in localized cases ($OR_{\text{high versus ref.}} = 0.43$; 95% CI = 0.22–0.82; P-trend = 0.02). Plasma genistein level also tended to be inversely associated with total prostate cancer ($OR_{\text{high versus ref.}} = 0.54$; 95% CI = 0.29–1.01; P-trend = 0.03). However, the association could not be seen in advanced cases.

Similar to breast cancer, the association between dietary isoflavone intake and prostate cancer risk was not obvious in several European studies. A study using EPICN-Norfolk subcohort samples with 193 cases and 828 controls did not find any association between the plasma or urinary biomarkers for isoflavone exposure and prostate cancer risk [44]. Another study using the whole EPICN cohort samples with 950 cases and 1042 controls also failed to find sufficient evidence, although plasma genistein levels tended to be associated with reduced risk ($OR_{\text{high versus ref.}} = 0.74$; 95% CI = 0.54–1.00) [45]. However, another nested case-control study with samples from multiethnic population in Hawaii and California found that urinary excretion of daidzein was associated with reduced risk of prostate cancer ($OR_{\text{high versus ref.}} = 0.55$; 95% CI = 0.31–0.98) [46]. The Jamaican study [47] analyzed spot urinary isoflavones and also found urinary equol level was significantly associated with reduced risk of total prostate cancer ($OR_{\text{high versus ref.}} = 0.48$; 95% CI = 0.26–0.87) and high-grade disease ($OR_{\text{high versus ref.}} = 0.29$; 95% CI = 0.13–0.60). No associations between urinary excretion of daidzein and genistein and the risk of prostate cancer were observed.

The only study that assessed the beneficial effects of polyphenols other than isoflavone is a clinical trial conducted in US [48]. This randomized, double blinded, and placebo controlled trial studied polyphenol E (a drug that contains EGCG) in men with prostate cancer scheduled to conduct radio prostatectomy. Volunteers were dosed with polyphenol E that contains about 800 mg EGCG, and no significant reduction of prostate cancer tissue biomarker was found and EGCG levels in prostate tissue were rather low.

3.3. Colorectal Cancer. Only two studies assessing colorectal cancer risk adopted biomarkers of polyphenol exposure. In a nested case-control study with Shanghai cohort samples [49], the effects of tea catechins were assessed, and individuals with high prediagnostic urinary EGC levels and 4′-MeEGC were associated with reduced risk of colon cancer. $OR_{\text{high versus ref.}} = 0.40$ (95% CI, 0.19–0.83) for EGC; a dose-response trend was also presented (P-trend = 0.02); $OR_{\text{high versus ref.}} = 0.41$ (95% CI, 0.20–0.84; P-trend = 0.006) for 4′-MeEGC. No association between tea polyphenols and rectal cancer risk was observed.

In the previously mentioned EPICN-Norfolk subcohort, no associations were observed between plasma or urinary levels of daidzein, genistein, and equol and colorectal cancer risk [44].

3.4. Gastric Cancer. Studies on beneficial effects of polyphenol on gastric cancer risk were mainly conducted in Asian countries, probably because Eastern Asia region has the highest gastric cancer incidence worldwide [77]. A study using the previous mentioned Shanghai cohort samples found that urinary tea catechin (EGC) positivity was associated with reduced risk of gastric cancer (OR = 0.52; 95% CI = 0.28–0.97) [50]. Another nested case-control study in South Korea [51] also found significant reductions in gastric cancer risk: $OR_{\text{high versus ref.}} = 0.21$ (95% CI = 0.08–0.58) for daidzein, $OR_{\text{high versus ref.}} = 0.54$ (95% CI = 0.31–0.93) for genistein, and $OR_{\text{high versus ref.}} = 0.50$ (95% CI = 0.27–0.90) for equol, respectively [50]. Another Japanese study [52], however, failed to find such a significant relationship.

3.5. Other Cancers. In a study of the association between tea catechins and esophageal cancers conducted in China, no significant association was found [50]. A Japanese nested case-control study [53] assessed the relationship between isoflavone and lung cancer and found that plasma genistein concentration was significantly associated with reduced risk of lung cancer ($OR_{\text{high versus ref.}} = 0.31$; 95% CI = 0.12–0.86). Daidzein and equol, however, did not present similar associations. The association between dietary intake of tea polyphenols and liver cancer risks will be summarized in Section 4 as part of illustration of green tea polyphenol study model.

3.6. Summary. Currently, studies employing measurements of body fluid polyphenol concentration as biomarkers of exposure to assess the protective roles against cancer risk are still very limited. Breast cancer and prostate cancer are the most commonly studied cancer types. It is interesting to find that most studies among Asian populations presented a reduced risk, whereas studies in other parts of the world are still inconsistent. One probable reason for this striking contrast might be because Asian populations ingest a much higher amount of isoflavones as compared to other regions, which was evidenced in the studies summarized and data from Table 1.

Most of the studies summarized here adopted case-control design, which usually employs a single measurement for evaluation of efficacy, which may not accurately reflect the actual continuous exposure. The analytical methods and sampling procedures employed in this type of studies may also have some limitations; for example, in all isoflavones related studies, samples were analyzed with pretreatment with β-glucuronidase and sulfatase which transformed nearly all conjugated forms into their respective aglycones. However, as previously mentioned, conjugated forms of polyphenol do not have the same effectivity as their aglycones; in fact, in most cases conjugated forms are less bioactive. In addition, some experimental conditions may have significant impacts on the study results. For example, time of sample collection is crucial; if samples are collected after the meal, the concentrations are usually higher than those collected in the early morning [49].

4. Green Tea Polyphenol as a Study Model

With strong evidence from *in vitro* and animal studies as well as historical perspective, but relatively less convincing evidence from previous epidemiological studies, green tea polyphenol (GTP, mainly catechins) attracts much attention. To bridge discrepancy between animal data and human studies, simultaneously accounting for the limitations in epidemiological studies and variations in individual response, a series of human perspective studies incorporating biomarkers as surrogate endpoints have been conducted to elucidate GTP's role in human health with better accuracy and precision and therefore are summarized here as a study model.

4.1. Phase II Clinical Trial with Green Tea Polyphenols in Southern Guangxi, China. A randomized, double blinded, and placebo controlled, phase IIa chemoprevention trial was conducted in Guangxi, China, to validate GTP biomarkers in human body fluids, study the modulative effects of GTPs on carcinogen biomarkers, and directly examine the possible adverse effect of GTPs in human subjects [78, 79]. The study screened 1,200 blood samples for hepatitis B virus (HBV) and aflatoxin (AF) biomarkers and recruited 124 residents who were both HBsAg and AF-albumin adducts positive, aged 20–55 with normal liver function test, serum alpha-fetoprotein negative, no personal history of cancer, and no use of prescribed medications. These subjects were regarded as having higher risk of liver cancer. Two doses of GTP (500 mg and 1000 mg, equivalent to two and four cups of tea drink, resp.) and placebo were administrated to three groups. Initial studies did not find significant differences on adverse effects and parameters representing liver and kidney function among three groups, indicating the relative safety of GTP in human subjects [78, 79].

4.2. Validation of Green Tea Polyphenols Biomarkers in Human Population Levels. Several follow-up studies [6, 80, 81] were conducted to validate the use of GTP biomarkers in human studies based on previously mentioned phase II clinical trial. A total of 340 urine samples collected at day 0 (baseline) and 1 and 3 months of the clinical trial were analyzed. Urinary excretion of GTPs was adjusted by creatinine levels. Trace amounts of GTP components were detectable for all 3 groups at baseline with no statistical significance ($P = 0.92$). Analysis of urine samples collected at 1 and 3 months revealed that levels of urinary EGC and EC were dose-dependently elevated in GTP-treated groups. Significant differences between times and groups of treatment ($P < 0.05$) were also found. A total of 343 plasma samples collected at the same time points were analyzed. Trace amounts of GTP components were detectable for all 3 groups at baseline with no statistical significance ($P > 0.33$). Analysis of samples collected at 1 and 3 months revealed a similar pattern as in urine sample: plasma levels of EGCG and ECG were dose-dependently elevated in GTP-treated groups. Significant differences between times and groups of treatment ($P < 0.05$) were also found. These results demonstrated that urinary excretion of EGC and EC and plasma levels of EGCG and ECG can serve as valid biomarkers for green tea consumption and may be applicable in future intervention and epidemiologic studies [81]. Luo et al. further confirmed previous results by a metabolomics study in which 106 metabolites of GTP were identified, and 56 of them were chosen to construct discriminant functions (DFs) based on the data at 1 and 3 months. Overall this study found metabolic profiles effective in discriminating different GTP dosages [80].

4.3. Modulation of Carcinogen Biomarkers by Green Tea Polyphenols. Reactive oxygen species (ROS), produced in the process of carcinogen metabolism, inflammation, and aerobic respiration, can attack all macromolecules including lipids, proteins, and DNA. Urinary 8-hydroxydeoxyguanosine (8-OHdG) generally reflects the whole body's oxidative DNA damage and repair and becomes a putative biomarker for oxidative stress [82]. Detection of urinary 8-OHdG provides a sensitive and noninvasive means to evaluate the efficacy of chemoprevention. Urinary excretion of GTPs and 8-OHdG was therefore analyzed in samples collected from the phase II clinical trial [54]. At the end of 3-month intervention, 8-OHdG levels decreased significantly in both GTP-treated groups, suggesting that clinical intervention with GTPs was effective in diminishing oxidative DNA damage [54].

The mediation effects of GTP among subjects with positive HBsAg and AF-albumin adducts (high liver cancer risk subjects) were also assessed in above phase II clinical trial [83]. The AF-albumin adduct levels in serum samples collected at 1 month of the intervention were significantly decreased in the high-dose group ($P < 0.05$) as compared with level in the placebo group. The levels of AF-albumin adducts in serum samples collected at 3 months of the intervention showed significant decrease in the low- and the high-dose groups as compared with the levels of the baseline values ($P < 0.05$). Levels of AFM_1 and AFB_1-mercapturic acid (AFB_1-NAC) in urine samples collected at 3 months of the intervention showed that treatment of GTP for up to three months significantly decreased levels of AFM_1 in both low-dose and high-dose intervention groups. Treatment of GTP elevated levels of AFB_1-NAC in GTP-treated groups [83]. These results demonstrate that treatment with GTP effectively inhibits phase I metabolic enzyme activities and induces phase II metabolic enzyme activities.

4.4. Long-Term Clinical Trial with Green Tea Polyphenols in Southern Guangxi, China. Current short-term evidence showed that taking GTPs does not have obvious negative effects, elevates plasma and urinary GTP components, and mediates oxidative stress and biomarkers of high liver cancer risk. Long-term clinical trial was therefore warranted. Yu et al. [84] initiated a 3-year randomized, double blinded, and placebo control study in 2004. This study screened 10,000 adult people and recruited 1,826 HBsAg positive adults with normal liver function test, negative serum alpha-fetoprotein, no personal history of cancer, and no use of prescribed medications, in Guangxi, China. Two capsules (500 mg GTP or placebo) were instructed to be taken twice daily after meal. The efficacy of the trial is measured as the ability of GTPs to decrease the levels of serum and urinary AF biomarkers and urinary 8-OHdG within the first year. The efficacy of

GTPs will be eventually measured as the ability to reduce actual incidence of hepatocellular carcinoma (HCC) in the studied population at the end of three years of intervention. The HCC incidence rate for years 2 and 3 was significantly reduced in GTPs-treated group (443.46/100,000 person year) as compared to that of placebo group (1092.39/100,000 person year) ($P_{\text{one-sided}} = 0.039$) [84].

5. Prospective

5.1. Interplay between Dietary Intakes, Genetic Factors, Epigenetic Mechanisms, and Postgenomic Considerations. Adopting biomarker-based methods to improve the accuracy could complement and enhance studies on the beneficial role of dietary polyphenol on human health. This method allows researcher to gain more insights on specific polyphenol compounds on certain diseases. For example, equol, the metabolite for daidzein, was considered the most bioactive isoflavone but could not be accurately measured using traditional methods [85]. Although more knowledge has been obtained since involving biomarkers of exposure for polyphenol intake, there are still many questions regarding the relationship between polyphenols and cancer risks waiting to be answered, from the perspectives of large interpersonal and interethnic variations in absorption, metabolism, and excretion of polyphenols [85]. For example, genetic variation on metabolizing enzymes may play important roles in determining the reduced risks of polyphenols, as evidenced by several studies [86–91]. Besides, epigenetic regulation in physiological environment has been widely considered to play an important role in biological functions. Their potential role in the relationship between polyphenol and cancer risk has also been suggested [92]. For example, Fang et al. suggested dietary polyphenols may play a role in DNA methylation by inhibiting DNA methyltransferases [93].

From dietary intake, to biomarkers, to genetic and epigenetic factors, and even to metabolomics [94], all focused on the subject itself when assessing the role of polyphenol in disease prevention. This scheme has been challenged in recent years since people have gained more understanding of the environments surrounding and inside us. Gut microflora has long been suggested to play an important role in human metabolism of polyphenols; however their role on disease prevention is only recently suggested [94]. With the rapid development of high throughput second-generation or even third-generation sequencing techniques, researchers can now directly measure the interplay between dietary nutrients including polyphenols intakes and gut microflora changes: how its community structure changes (microbiome study) and how its function changes (bacteria metabolomics), which may lead to another round of scheme shift.

5.2. Future Directions. Given current evidences and potential opportunities, research works in following aspects are suggested to improve in the future.

(i) Epidemiological evidence on beneficial role of specific polyphenols on specific cancer risk should continuously be collected, especially on polyphenols such as tea polyphenols, in multiple cancer types with different stage of carcinogenesis.

(ii) When measuring a specific biomarker, more attention should be paid to the chemical speciation of that biomarker.

(iii) The metabolomics study should be conducted to screen more sensitive and useful biomarkers and generate hypotheses driven measurement. With these hypotheses, the interplays between genetic and epigenetic factors and polyphenols on cancer risk should be further examined.

(iv) Microbiome changes in response to polyphenol exposure and their interplay with other factors should be elicited.

6. Conclusions

This review performed a literature search of all epidemiological studies or controlled clinical trials which employed biomarkers of exposure for polyphenols to help assess their anticarcinogenic role, using studies on green tea polyphenols as a study model. Currently, studies on this topic are still very limited. Breast cancer and prostate cancer were the only widely studied cancer types. Isoflavone is the only widely studied polyphenol. Protective roles of isoflavones against breast cancer and prostate cancer were consistently reported among Asian populations. Evidences on other populations and cancer sites are still insufficient. Prospective studies on green tea polyphenols have suggested a protective role against carcinogen biomarkers of liver cancer risks. In addition to associations between polyphenols and cancer risks, factors such as host genetic susceptibility, epigenetic modification, and gut microbiome patterns may also impact on the protective roles of polyphenols. More evidences should be collected by utilizing biomarkers of exposure for polyphenols in epidemiological studies and extended to molecular level before a conclusion can be made towards their protective roles.

Conflict of Interests

The authors declare no conflict of interests in this paper.

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References

[1] A. Scalbert, C. Manach, C. Morand, C. Rémésy, and L. Jiménez, "Dietary polyphenols and the prevention of diseases," *Critical Reviews in Food Science and Nutrition*, vol. 45, no. 4, pp. 287–306, 2005.

[2] A. Scalbert, I. T. Johnson, and M. Saltmarsh, "Polyphenols: antioxidants and beyond," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, supplement, pp. 215S–217S, 2005.

[3] J. M. Landete, "Updated knowledge about polyphenols: functions, bioavailability, metabolism, and health," *Critical Reviews in Food Science and Nutrition*, vol. 52, no. 10, pp. 936–948, 2012.

[4] I. C. W. Arts and P. C. H. Hollman, "Polyphenols and disease risk in epidemiologic studies," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, supplement, pp. 317S–325S, 2005.

[5] F. Visioli, C. A. de la Lastra, C. Andres-Lacueva et al., "Polyphenols and human health: a prospectus," *Critical Reviews in Food Science and Nutrition*, vol. 51, no. 6, pp. 524–546, 2011.

[6] J.-S. Wang, H. Luo, P. Wang et al., "Validation of green tea polyphenol biomarkers in a phase II human intervention trial," *Food and Chemical Toxicology*, vol. 46, no. 1, pp. 232–240, 2008.

[7] C.-L. Shen, M.-C. Chyu, and J.-S. Wang, "Tea and bone health: steps forward in translational nutrition," *The American Journal of Clinical Nutrition*, vol. 98, no. 6, pp. 1694S–1699S, 2013.

[8] C. L. Shen, M. C. Chyu, J. K. Yeh et al., "Effect of green tea polyphenols and Tai Chi exercise on bone health in postmenopausal women with low bone mass: a 24-week placebo-controlled randomized trial," *The FASEB Journal*, vol. 25, p. 594.3, 2011.

[9] O. I. Parisi, F. Puoci, and D. Restuccia, "Polyphenols and their formulations: different strategies to overcome the drawbacks associated with their poor stability and bioavailability," in *Polyphenols in Human Health and Disease*, R. R. Watson, V. R. Preedy, and S. Zibadi, Eds., chapter 4, pp. 29–45, Academic Press, San Diego, Calif, USA, 2014.

[10] A. Scalbert and G. Williamson, "Dietary intake and bioavailability of polyphenols," *Journal of Nutrition*, vol. 130, no. 8, supplement, pp. 2073S–2085S, 2000.

[11] P. A. Kroon, M. N. Clifford, A. Crozier et al., "How should we assess the effects of exposure to dietary polyphenols in vitro?" *The American Journal of Clinical Nutrition*, vol. 80, no. 1, pp. 15–21, 2004.

[12] K. D. R. Setchell, N. M. Brown, L. Zimmer-Nechemias et al., "Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability," *The American Journal of Clinical Nutrition*, vol. 76, no. 2, pp. 447–453, 2002.

[13] C. S. Yang, L. Chen, M. J. Lee, D. Balentine, M. C. Kuo, and S. P. Schantz, "Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers," *Cancer Epidemiology Biomarkers & Prevention*, vol. 7, no. 4, pp. 351–354, 1998.

[14] J. Linseisen and S. Rohrmann, "Biomarkers of dietary intake of flavonoids and phenolic acids for studying diet-cancer relationship in humans," *European Journal of Nutrition*, vol. 47, supplement 2, pp. 60–68, 2008.

[15] V. Kipnis, A. F. Subar, D. Midthune et al., "Structure of dietary measurement error: results of the OPEN Biomarker Study," *American Journal of Epidemiology*, vol. 158, no. 1, pp. 14–21, 2003.

[16] C. Nagata, T. Mizoue, K. Tanaka et al., "Soy intake and breast cancer risk: an evaluation based on a systematic review of epidemiologic evidence among the Japanese population," *Japanese Journal of Clinical Oncology*, vol. 44, no. 3, pp. 282–295, 2014.

[17] H. D. Woo and J. Kim, "Dietary flavonoid intake and risk of stomach and colorectal cancer," *World Journal of Gastroenterology*, vol. 19, no. 7, pp. 1011–1019, 2013.

[18] H. D. Woo and J. Kim, "Dietary flavonoid intake and smoking-related cancer risk: a meta-analysis," *PLoS ONE*, vol. 8, no. 9, Article ID e75604, 2013.

[19] S. Malerba, C. Galeone, C. Pelucchi et al., "A meta-analysis of coffee and tea consumption and the risk of glioma in adults," *Cancer Causes and Control*, vol. 24, no. 2, pp. 267–276, 2013.

[20] Z.-H. Hu, Y.-W. Lin, X. Xu et al., "No association between tea consumption and risk of renal cell carcinoma: a meta-analysis of epidemiological studies," *Asian Pacific Journal of Cancer Prevention*, vol. 14, no. 3, pp. 1691–1695, 2013.

[21] C. Hui, X. Qi, Z. Qianyong, P. Xiaoli, Z. Jundong, and M. Mantian, "Flavonoids, flavonoid subclasses and breast cancer risk: a meta-analysis of epidemiologic studies," *PLoS ONE*, vol. 8, no. 1, Article ID e54318, 2013.

[22] Z.-H. Wang, Q.-Y. Gao, and J.-Y. Fang, "Green tea and incidence of colorectal cancer: evidence from prospective cohort studies," *Nutrition and Cancer*, vol. 64, no. 8, pp. 1143–1152, 2012.

[23] M. G. M. Braem, N. C. Onland-Moret, L. J. Schouten et al., "Coffee and tea consumption and the risk of ovarian cancer: a prospective cohort study and updated meta-analysis," *The American Journal of Clinical Nutrition*, vol. 95, no. 5, pp. 1172–1181, 2012.

[24] N.-P. Tang, H. Li, Y.-L. Qiu, G.-M. Zhou, and J. Ma, "Tea consumption and risk of endometrial cancer: a metaanalysis," *American Journal of Obstetrics and Gynecology*, vol. 201, no. 6, pp. 605.e1–605.e8, 2009.

[25] C. Manach, G. Williamson, C. Morand, A. Scalbert, and C. Rémésy, "Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, supplement, pp. 230S–242S, 2005.

[26] J. Kühnau, "The flavonoids. A class of semi-essential food components: their role in human nutrition," *World Review of Nutrition and Dietetics*, vol. 24, pp. 117–191, 1976.

[27] M.-L. Ovaskainen, R. Törrönen, J. M. Koponen et al., "Dietary intake and major food sources of polyphenols in Finnish adults," *Journal of Nutrition*, vol. 138, no. 3, pp. 562–566, 2008.

[28] J. Pérez-Jiménez, L. Fezeu, M. Touvier et al., "Dietary intake of 337 polyphenols in French adults," *The American Journal of Clinical Nutrition*, vol. 93, no. 6, pp. 1220–1228, 2011.

[29] R. Zamora-Ros, C. Andres-Lacueva, R. M. Lamuela-Raventós et al., "Estimation of dietary sources and flavonoid intake in a Spanish adult population (EPIC-Spain)," *Journal of the American Dietetic Association*, vol. 110, no. 3, pp. 390–398, 2010.

[30] X. Wu, G. R. Beecher, J. M. Holden, D. B. Haytowitz, S. E. Gebhardt, and R. L. Prior, "Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 11, pp. 4069–4075, 2006.

[31] G. Mazza and C. D. Kay, "Bioactivity, absorption, and metabolism of anthocyanins," in *Recent Advances in Polyphenol Research*, pp. 228–262, Wiley-Blackwell, 2009.

[32] I. Fernandes, A. Faria, C. Calhau, V. de Freitas, and N. Mateus, "Bioavailability of anthocyanins and derivatives," *Journal of Functional Foods*, vol. 7, no. 1, pp. 54–66, 2014.

[33] T. K. McGhie and M. C. Walton, "The bioavailability and absorption of anthocyanins: towards a better understanding," *Molecular Nutrition and Food Research*, vol. 51, no. 6, pp. 702–713, 2007.

[34] R. M. de Ferrars, C. Czank, Q. Zhang et al., "The pharmacokinetics of anthocyanins and their metabolites in humans," *British Journal of Pharmacology*, vol. 171, no. 13, pp. 3268–3282, 2014.

[35] D. Ingram, K. Sanders, M. Kolybaba, and D. Lopez, "Case-control study of phyto-oestrogens and breast cancer," *The Lancet*, vol. 350, no. 9083, pp. 990–994, 1997.

[36] I. den Tonkelaar, L. Keinan-Boker, P. V. Veer et al., "Urinary phytoestrogens and postmenopausal breast cancer risk," *Cancer Epidemiology Biomarkers and Prevention*, vol. 10, no. 3, pp. 223–228, 2001.

[37] R. Piller, J. Chang-Claude, and J. Linseisen, "Plasma enterolactone and genistein and the risk of premenopausal breast cancer," *European Journal of Cancer Prevention*, vol. 15, no. 3, pp. 225–232, 2006.

[38] M. Verheus, C. H. van Gils, L. Keinan-Boker, P. B. Grace, S. A. Bingham, and P. H. M. Peeters, "Plasma phytoestrogens and subsequent breast cancer risk," *Journal of Clinical Oncology*, vol. 25, no. 6, pp. 648–655, 2007.

[39] P. B. Grace, J. I. Taylor, Y.-L. Low et al., "Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European Prospective Investigation of Cancer and Nutrition-Norfolk," *Cancer Epidemiology Biomarkers and Prevention*, vol. 13, no. 5, pp. 698–708, 2004.

[40] W. Zheng, Q. Dai, L. J. Custer et al., "Urinary excretion of isoflavonoids and the risk of breast cancer," *Cancer Epidemiology Biomarkers and Prevention*, vol. 8, no. 1, pp. 35–40, 1999.

[41] Q. Dai, A. A. Franke, F. Jin et al., "Urinary excretion of phytoestrogens and risk of breast cancer among Chinese women in Shanghai," *Cancer Epidemiology Biomarkers and Prevention*, vol. 11, no. 9, pp. 815–821, 2002.

[42] M. Iwasaki, M. Inoue, T. Otani et al., "Plasma isoflavone level and subsequent risk of breast cancer among Japanese women: a nested case-control study from the Japan public health center-based prospective study group," *Journal of Clinical Oncology*, vol. 26, no. 10, pp. 1677–1683, 2008.

[43] N. Kurahashi, M. Iwasaki, M. Inoue, S. Sasazuki, and S. Tsugane, "Plasma isoflavones and subsequent risk of prostate cancer in a nested case-control study: the Japan Public Health Center," *Journal of Clinical Oncology*, vol. 26, no. 36, pp. 5923–5929, 2008.

[44] H. Ward, G. Chapelais, G. G. C. Kuhnle, R. Luben, K.-T. Khaw, and S. Bingham, "Lack of prospective associations between plasma and urinary phytoestrogens and risk of prostate or colorectal cancer in the European Prospective into Cancer-Norfolk study," *Cancer Epidemiology Biomarkers and Prevention*, vol. 17, no. 10, pp. 2891–2894, 2008.

[45] R. C. Travis, E. A. Spencer, N. E. Allen et al., "Plasma phytoestrogens and prostate cancer in the European Prospective Investigation into Cancer and Nutrition," *British Journal of Cancer*, vol. 100, no. 11, pp. 1817–1823, 2009.

[46] S.-Y. Park, L. R. Wilkens, A. A. Franke et al., "Urinary phytoestrogen excretion and prostate cancer risk: a nested case-control study in the Multiethnic Cohort," *British Journal of Cancer*, vol. 101, no. 1, pp. 185–191, 2009.

[47] M. D. Jackson, N. D. McFarlane-Anderson, G. A. Simon, F. I. Bennett, and S. P. Walker, "Urinary phytoestrogens and risk of prostate cancer in Jamaican men," *Cancer Causes and Control*, vol. 21, no. 12, pp. 2249–2257, 2010.

[48] M. M. Nguyen, F. R. Ahmann, R. B. Nagle et al., "Randomized, double-blind, placebo-controlled trial of polyphenon E in prostate cancer patients before prostatectomy: evaluation of potential chemopreventive activities," *Cancer Prevention Research*, vol. 5, no. 2, pp. 290–298, 2012.

[49] J.-M. Yuan, Y.-T. Gao, C. S. Yang, and M. C. Yu, "Urinary biomarkers of tea polyphenols and risk of colorectal cancer in the Shanghai Cohort Study," *International Journal of Cancer*, vol. 120, no. 6, pp. 1344–1350, 2007.

[50] C. L. Sun, J. M. Yuan, M. J. Lee et al., "Urinary tea polyphenols in relation to gastric and esophageal cancers: a prospective study of men in Shanghai, China," *Carcinogenesis*, vol. 23, no. 9, pp. 1497–1503, 2002.

[51] K.-P. Ko, S. K. Park, B. Park et al., "Isoflavones from phytoestrogens and gastric cancer risk: a nested case-control study within the Korean Multicenter Cancer Cohort," *Cancer Epidemiology Biomarkers & Prevention*, vol. 19, no. 5, pp. 1292–1300, 2010.

[52] A. Hara, S. Sasazuki, M. Inoue et al., "Plasma isoflavone concentrations are not associated with gastric cancer risk among japanese men and women," *Journal of Nutrition*, vol. 143, no. 8, pp. 1293–1298, 2013.

[53] T. Shimazu, M. Inoue, S. Sasazuki et al., "Plasma isoflavones and the risk of lung cancer in women: a nested case-control study in Japan," *Cancer Epidemiology Biomarkers and Prevention*, vol. 20, no. 3, pp. 419–427, 2011.

[54] H. Luo, L. Tang, M. Tang et al., "Phase IIa chemoprevention trial of green tea polyphenols in high-risk individuals of liver cancer: modulation of urinary excretion of green tea polyphenols and 8-hydroxydeoxyguanosine," *Carcinogenesis*, vol. 27, no. 2, pp. 262–268, 2006.

[55] P. C. H. Hollman, J. H. M. de Vries, S. D. van Leeuwen, M. J. B. Mengelers, and M. B. Katan, "Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers," *The American Journal of Clinical Nutrition*, vol. 62, no. 6, pp. 1276–1282, 1995.

[56] K. C. Ock, J. C. Sang, and W. O. Song, "Estimated dietary flavonoid intake and major food sources of U.S. adults," *Journal of Nutrition*, vol. 137, no. 5, pp. 1244–1252, 2007.

[57] A. J. Day, F. Mellon, D. Barron, G. Sarrazin, M. R. A. Morgan, and G. Williamson, "Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin," *Free Radical Research*, vol. 35, no. 6, pp. 941–952, 2001.

[58] E. U. Graefe, J. Wittig, S. Mueller et al., "Pharmacokinetics and bioavailability of quercetin glycosides in humans," *The Journal of Clinical Pharmacology*, vol. 41, no. 5, pp. 492–499, 2001.

[59] J. H. Moon, R. Nakata, S. Oshima, T. Inakuma, and J. Terao, "Accumulation of quercetin conjugates in blood plasma after the short-term ingestion of onion by women," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 279, no. 2, pp. R461–R467, 2000.

[60] C. Manach, C. Morand, A. Gil-Izquierdo, C. Bouteloup-Demange, and C. Rémésy, "Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two dose of orange juice," *European Journal of Clinical Nutrition*, vol. 57, no. 2, pp. 235–242, 2003.

[61] J. T. Kumpulainen, "Intake of flavonoids, phenolic acids and lignans in various populations," in *Proceedings of the 3rd International Conference on Natural Antioxidants and Anticarcinogens in Food, Health and Disease*, Kuopion University Publications D. Medical Sciences, Kuopio, Finland, 2001.

[62] M. Kano, T. Takayanagi, K. Harada, S. Sawada, and F. Ishikawa, "Bioavailability of isoflavones after ingestion of soy beverages in healthy adults," *Journal of Nutrition*, vol. 136, no. 9, pp. 2291–2296, 2006.

[63] K. Wakai, I. Egami, K. Kato et al., "Dietary intake and sources of isoflavones among Japanese," *Nutrition and Cancer*, vol. 33, no. 2, pp. 139–145, 1999.

[64] Z. Chen, W. Zheng, L. J. Custer et al., "Usual dietary consumption of soy foods and its correlation with the excretion rate of isoflavonoids in overnight urine samples among Chinese women in Shanghai," *Nutrition and Cancer*, vol. 33, no. 1, pp. 82–87, 1999.

[65] T. Izumi, M. K. Piskula, S. Osawa et al., "Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans," *Journal of Nutrition*, vol. 130, no. 7, pp. 1695–1699, 2000.

[66] K. D. R. Setchell, N. M. Brown, P. Desai et al., "Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements," *Journal of Nutrition*, vol. 131, no. 4, supplement, pp. 1362S–1375S, 2001.

[67] K. D. R. Setchell, M. S. Faughnan, T. Avades et al., "Comparing the pharmacokinetics of daidzein and genistein with the use of ^{13}C-labeled tracers in premenopausal women," *The American Journal of Clinical Nutrition*, vol. 77, no. 2, pp. 411–419, 2003.

[68] D. L. McKay and J. B. Blumberg, "The role of tea in human health: an update," *Journal of the American College of Nutrition*, vol. 21, no. 1, pp. 1–13, 2002.

[69] D. A. Balentine, S. A. Wiseman, and L. C. M. Bouwens, "The chemistry of tea flavonoids," *Critical Reviews in Food Science and Nutrition*, vol. 37, no. 8, pp. 693–704, 1997.

[70] O. J. Park and Y.-J. Surh, "Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies," *Toxicology Letters*, vol. 150, no. 1, pp. 43–56, 2004.

[71] M. J. Lee, Z. Y. Wang, H. Li et al., "Analysis of plasma and urinary tea polyphenols in human subjects," *Cancer Epidemiology Biomarkers and Prevention*, vol. 4, no. 4, pp. 393–399, 1995.

[72] J. M. M. Van Amelsvoort, K. H. Van Het Hof, J. N. J. J. Mathot, T. P. J. Mulder, A. Wiersma, and L. B. M. Tijburg, "Plasma concentrations of individual tea catechins after a single oral dose in humans," *Xenobiotica*, vol. 31, no. 12, pp. 891–901, 2001.

[73] M.-J. Lee, P. Maliakal, L. Chen et al., "Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability," *Cancer Epidemiology Biomarkers and Prevention*, vol. 11, no. 10, pp. 1025–1032, 2002.

[74] H.-H. S. Chow, Y. Cai, D. S. Alberts et al., "Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E," *Cancer Epidemiology Biomarkers & Prevention*, vol. 10, no. 1, pp. 53–58, 2001.

[75] J. Pérez-Jiménez, J. Hubert, L. Hooper et al., "Urinary metabolites as biomarkers of polyphenol intake in humans: a systematic review," *The American Journal of Clinical Nutrition*, vol. 92, no. 4, pp. 801–809, 2010.

[76] T. J. Key, "Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies," *Journal of the National Cancer Institute*, vol. 94, no. 8, pp. 606–616, 2002.

[77] J. Ferlay, I. Soerjomataram, M. Ervik et al., *GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11*, International Agency for Research on Cancer, Lyon, France, 2012, http://globocan.iarc.fr.

[78] T. Huang, J. Yu, L. Tang et al., "Phase IIa chemoprevention trial of green tea polyphenols in high-risk population of liver cancer: I. Design, clinical outcome, and baseline biomarker data," *International Journal of Cancer Prevention*, vol. 1, no. 4, pp. 269–280, 2004.

[79] J.-S. Wang, T. Huang, and J. Yu, "Chemoprevention trial of green tea polyphenols in high-risk population of liver cancer," *Proceedings of American Association of Cancer Research*, vol. 44, 2003.

[80] H. Luo, S. B. Cox, W.-M. Gao, J. Yu, L. Tang, and J.-S. Wang, "Metabolic profiling in validation of plasma biomarkers for green tea polyphenols," *Metabolomics*, vol. 2, no. 4, pp. 235–241, 2006.

[81] H. Luo, L. Tang, M. Tang et al., "Validation of green tea polyphenol biomarkers in high-risk population of liver cancer," in *Proceedings of the 95th Annual Meeting of American Association of Cancer Research*, vol. 45, p. 931, Orlando, Fla, USA, March 2004.

[82] A. Pilger, D. Germadnik, K. Riedel, I. Meger-Kossien, G. Scherer, and H. Rüdiger, "Longitudinal study of urinary 8-hydroxy-2′-deoxyguanosine excretion in healthy adults," *Free Radical Research*, vol. 35, no. 3, pp. 273–280, 2001.

[83] L. Tang, M. Tang, L. Xu et al., "Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention," *Carcinogenesis*, vol. 29, no. 2, pp. 411–417, 2008.

[84] J. Yu, T. Huang, L. Zhang et al., "Chemoprevention trial of green tea polyphenols in high-risk population of liver cancer in southern Guangxi, China," in *Proceedings of the American Association of Cancer Research*, vol. 2006, p. 1148, 2006.

[85] Y. L. Low, J. I. Taylor, P. B. Grace et al., "Polymorphisms in the CYP19 gene may affect the positive correlations between serum and urine phytoestrogen metabolites and plasma androgen concentrations in men," *Journal of Nutrition*, vol. 135, no. 11, pp. 2680–2686, 2005.

[86] M. Inoue, K. Robien, R. Wang, D. J. Van Den Berg, W.-P. Koh, and M. C. Yu, "Green tea intake, MTHFR/TYMS genotype and breast cancer risk: the Singapore Chinese Health Study," *Carcinogenesis*, vol. 29, no. 10, pp. 1967–1972, 2008.

[87] M. Iwasaki, G. S. Hamada, I. N. Nishimoto et al., "Isoflavone, polymorphisms in estrogen receptor genes and breast cancer risk in case-control studies in Japanese, Japanese Brazilians and non-Japanese Brazilians," *Cancer Science*, vol. 100, no. 5, pp. 927–933, 2009.

[88] T. K. Lam, M. Rotunno, J. H. Lubin et al., "Dietary quercetin, quercetin-gene interaction, metabolic gene expression in lung tissue and lung cancer risk," *Carcinogenesis*, vol. 31, no. 4, pp. 634–642, 2010.

[89] I.-H. Lin, M.-L. Ho, H.-Y. Chen et al., "Smoking, green tea consumption, genetic polymorphisms in the insulin-like growth factors and lung cancer risk," *PLoS ONE*, vol. 7, no. 2, Article ID e30951, 2012.

[90] J. J. Yang, L. Y. Cho, K.-P. Ko et al., "Interaction effects between genes involved in the AKT signaling pathway and phytoestrogens in gastric carcinogenesis: a nested case-control study from the Korean Multi-Center Cancer Cohort," *Molecular Nutrition and Food Research*, vol. 56, no. 11, pp. 1617–1626, 2012.

[91] J.-M. Yuan, W.-P. Koh, C.-L. Sun, H.-P. Lee, and M. C. Yu, "Green tea intake, ACE gene polymorphism and breast cancer risk among Chinese women in Singapore," *Carcinogenesis*, vol. 26, no. 8, pp. 1389–1394, 2005.

[92] A. Link, F. Balaguer, and A. Goel, "Cancer chemoprevention by dietary polyphenols: promising role for epigenetics," *Biochemical Pharmacology*, vol. 80, no. 12, pp. 1771–1792, 2010.

[93] M. Fang, D. Chen, and C. S. Yang, "Dietary polyphenols may affect DNA methylation," *Journal of Nutrition*, vol. 137, no. 1, supplement, pp. 223S–228S, 2007.

[94] D. del Rio, L. G. Costa, M. E. J. Lean, and A. Crozier, "Polyphenols and health: what compounds are involved?" *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 20, no. 1, pp. 1–6, 2010.

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Review Article

Interactions between CYP3A4 and Dietary Polyphenols

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The human cytochrome P450 enzymes (P450s) catalyze oxidative reactions of a broad spectrum of substrates and play a critical role in the metabolism of xenobiotics, such as drugs and dietary compounds. CYP3A4 is known to be the main enzyme involved in the metabolism of drugs and most other xenobiotics. Dietary compounds, of which polyphenolics are the most studied, have been shown to interact with CYP3A4 and alter its expression and activity. Traditionally, the liver was considered the prime site of CYP3A-mediated first-pass metabolic extraction, but *in vitro* and *in vivo* studies now suggest that the small intestine can be of equal or even greater importance for the metabolism of polyphenolics and drugs. Recent studies have pointed to the role of gut microbiota in the metabolic fate of polyphenolics in human, suggesting their involvement in the complex interactions between dietary polyphenols and CYP3A4. Last but not least, all the above suggests that coadministration of drugs and foods that are rich in polyphenols is expected to stimulate undesirable clinical consequences. This review focuses on interactions between dietary polyphenols and CYP3A4 as they relate to structural considerations, food-drug interactions, and potential negative consequences of interactions between CYP3A4 and polyphenols.

1. Introduction

Cytochrome P450 enzymes (P450s) are responsible for the metabolism of a wide range of endogenous compounds (steroid hormones, lipids, and bile acids), as well as xenobiotics including drugs, environmental pollutants, and dietary products [1–4]. P450 enzymes are widely distributed among the phylogenetic trees [5] and considered as a significant player in the world around us, where life and the earth itself would be visibly different and diminished without cytochrome P450s [6]. A direct impact on humans is mediated especially through our own set of 57 P450s [7]. CYP is an abbreviation for cytochrome P450; the gene family is then indicated by a number following the letters "CYP." Subfamilies are represented by a letter that is followed by yet another number to indicate the specific gene. For example, for the enzyme CYP3A4, "3" stands for the gene family, "A" for the subfamily, and "4" defines the gene that encodes a specific polypeptide [8].

Among this large family of oxidizing enzymes, CYP3A4 is recognized as the main enzyme involved in the metabolism of drugs in the liver and, no less importantly, in the gut. Hence, potential interactions between promising new drugs and CYP3A4 are assessed starting at the early stages of their development [9–11]. CYP3A4 is most abundant P450 in the human liver, accounting for 30% of the total P450 protein content but is also expressed in the prostate, breast, gut, colon, small intestine, and brain [12–17]. In the small intestine, CYP3A enzymes represent the principle drug-metabolizing system and account for approximately 80% of total P450 content [18–20]. Although the total amount of CYP3A expressed in the human small intestine represents approximately 1% of the amount expressed in the liver [21, 22], substantial drug extraction takes place during the absorption of orally administered drugs [23–26]. Orally administered substrates must pass through enterocytes while they can bypass hepatocytes by remaining in the sinusoidal blood before reaching the systemic circulation. The remarkably lower blood flow to the intestinal mucosa as compared to the liver allows for prolonged exposure to the intestinal metabolizing enzymes and lead to relatively high enterocytic drug concentrations. The predominance of CYP3A4 in human intestine and its high

capacity enable it to can act several-fold more efficiently in the intestine than in the liver [20, 27, 28]. Furthermore, the intestine receives not only dietary compounds, but also phase I and II metabolites that have been excreted back into the intestine through the enterohepatic cycle [29, 30]. All these facts indicate the importance of intestinal CYP3A4 activity in the metabolism of dietary constituents. In rodents, the isofrom CYP3A is expressed predominantly in the liver, with only scant expression observed in the intestine [31–33]. The different isoforms and distinct expression levels and patterns for P450s in the intestine between humans and rodents limit the suitability of rodents as a model to predict drug metabolism or oral bioavailability in human [34]. This points the importance of studying the effects of ingested polyphenols and other dietary substrates on the metabolism of intestinal CYP3A4 in humans or in models other than rodents' intestine. The latter include cell cultures, microsomes, and microorganisms that express the specific P450 of interest or a whole array of P450s [35–39].

The active site of a substrate-free cytochrome P450 contains one-heme iron center anchored by the four bonds of the heme group, fifth proximal ligand of the conserved cysteine, and water molecule as the sixth distal ligand [1]. The catalytic mechanisms of P450 enzymes are thoroughly investigated in the literature, as demonstrated in a scheme based on previous publications (Figure 1) [1, 40–42]. Like most other P450 enzymes, CYP3A4 acts as a monooxygenase (e.g., it catalyzes the insertion of one atom of oxygen into an organic substrate while another oxygen atom is reduced to water) [43]. The substrate chemical characteristics and the preferred position of hydroxyl insertion change from one family of P450 to another [3, 44–46]. P450 enzymes play a major role in phase I metabolism of dietary xenobiotics, including polyphenols, whereby a hydroxyl group is introduced to the molecule. These metabolic products are more water-soluble and become available to phase II enzymes. The latter include UDP-glucuronosyl transferases and sulfotransferases that add to the increased water solubility of the hydroxylated polyphenols, producing glucuronides and sulfates, which are then eliminated from the body [29, 47, 48].

In recent studies, evidence has accumulated to indicate potent interactions between CYP3A4 and edible phytochemicals. These compounds, some of which are abundant in our diet, belong to the large and diverse family of polyphenolics, including flavonoids, phenolic acids, phenolic alcohol, stilbenoids, and lignans [49–53]. It is commonly accepted that the powerful antioxidant activity of polyphenolic compounds is due to their free-radical scavenging capacity and their iron-chelating activity [54–56]. Reviews of the health benefits of polyphenols demonstrate that these compounds have numerous therapeutic effects against several diseases (e.g., atherosclerosis, certain forms of carcinogenic processes, and cardiovascular and neurodegenerative diseases) [57–60]. Among the therapeutic implications of polyphenols on human health, the interactions between polyphenols and cytochrome P450 have been recently reviewed [56, 61–64]. These interactions were highlighted following the increased use of herbal medicines and supplements. As many of the herbs used in these preparations are known to be rich in polyphenolics, their interaction with the major enzyme of presystemic metabolism has attracted significant research attention [56, 65–67]. Since cytochrome P450 enzymes are responsible for the metabolism of a wide range of drugs and polyphenols, which might also change their antimicrobial potential and human toxicity, the simultaneous consumption of drugs, herbals, and plant foods raises concerns. The coadministration of active constituents derived from food or herbs and prescribed drugs may lead to undesirable clinical effects, which may include increased toxicity and/or treatment failure [67–69].

Here, we focus on the interactions of polyphenols with CYP3A4, the major enzyme in the gut and liver metabolism of drugs and xenobiotics. The effects of several subcategories of polyphenols on the expression and activity of CYP3A4 (inhibition or induction) are reviewed (Table 1). Structural and physicochemical considerations that define these interactions are also reviewed.

2. CYP3A4 and Food-Drug Interactions

Drug-metabolizing P450s such as CYP3A4 have relaxed selectivity and are able to bind and metabolize a large array of substrates of different size, shapes, and chemical properties, for example, many dietary polyphenols. Crystal structures, biophysical studies, and molecular dynamics have provided important insights into how drug-metabolizing P450s, especially CYP3A4, structurally adapt to a variety of inhibitors and substrates [70]. Indeed, CYP3A4 is involved in the metabolism of over 50% of marketed drugs that undergo metabolic elimination [71]. The high level of CYP3A4 expression in the intestine, as well as its broad substrate specificity may explain the accumulating data regarding its susceptibility to modulation by food constituents [38, 61, 72–75]. Examples of metabolic food-drug interactions involving the modulation of CYP3A4 activity by components from dietary and herbal sources are accumulating, including those of grapefruit with over 85 drugs, for example, cyclosporine and felodipine [27, 76–78], and those of St. John's wort [54, 79, 80], and red wine [38, 75, 81] with cyclosporine. In most of these cases, components in foods, drinks, food additives, and orally administered medicines were shown to inhibit CYP3A4 activity and, as a result, increase the actual dose of the drug that reaches the blood circulation in its active form, which often causes unfavorable and long-lasting interactions and probably fatal toxicity [82, 83]. Continuous exposure to these compounds, especially those that activate the xenobiotic nuclear receptor PXR (pregnane X receptor), may lead, in a feedback fashion, to increased expression of CYP3A4 in the intestine, making the food-drug interaction even more complex during extended periods of use [84–87]. Drug-drug, food-drug, and herb-drug interactions in the liver have been well documented in the literature [72, 88–90]. An intensive CYP3A4-dependent intestinal metabolism of low-absorbed compounds such as most polyphenols might be expected [29, 54, 91–93]. However, to the best of our knowledge the research in this area is limited and additional data are needed.

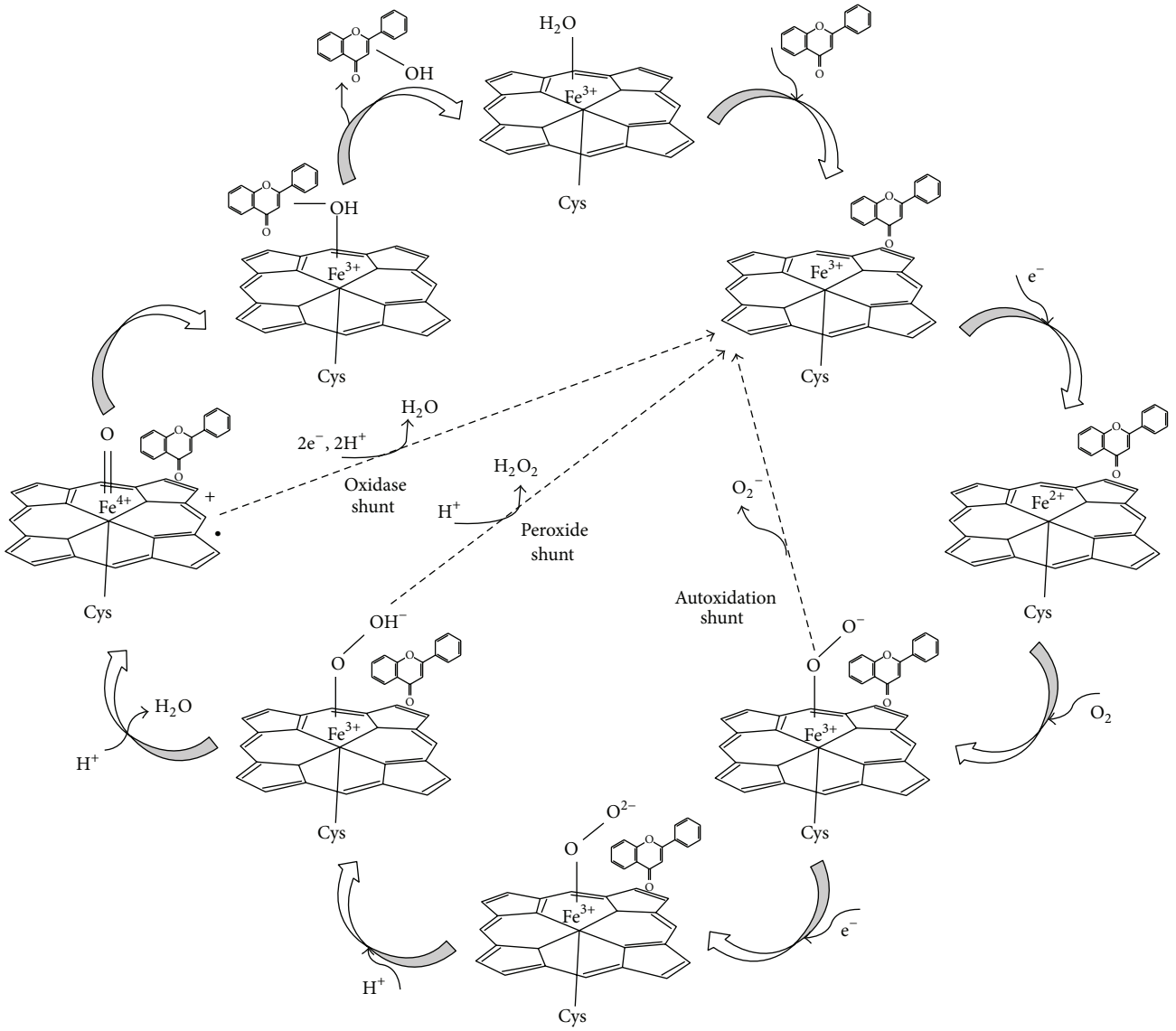

Figure 1: The catalytic cycle of P450s: a flavonoid structure was selected to represent dietary polyphenols.

3. Polyphenols

As reviewed in other works in this issue, polyphenols constitute a large and diverse family of compounds that is commonly divided into subfamilies that share similar chemistry: flavonoids, flavonols, phenolic acids, phenolic alcohols, stilbenoids, tannins, and lignans (Figure 2). Polyphenols are found in several foods, fruits, vegetables, and herbs [52, 94, 95]. In general, the total intake of polyphenols is approximated at 1 g/individual/day and polyphenols are considered by many to be the major source of antioxidants in our diet [51, 95–97]. However, this estimate varies depending on the type of diet. For example, total polyphenol intake in the Finnish diet is 817–919 mg/individual/day [98]. In the Vietnamese diet, it is 595 mg/individual/day [99], and in the Mediterranean diet, polyphenol intake ranges between 1800 and 3000 mg/individual/day [100]. Still, and due to their low absorption, it has been suggested that their major sites of antioxidant activity are the stomach [101] and the intestine [102]. Whether acting in the gastrointestinal tract or in the liver, the potent antioxidant effects of polyphenols are widely accepted as health promoting [103–105]. Antivira, antibacterial, anti-inflammatory, neuroprotective, and anticarcinogenic effects have also been attributed to polyphenols [106–109]. Medicinal herbs such as St. John's wort (*Hypericum perforatum*), ginseng (*Panax ginseng*), black cohosh (*Actaea racemosa*), echinacea (*Echinacea purpurea*), cranberry (*Vaccinium macrocarpon*), and ginger (*Zingiber officinale*) are rich sources of a vast array of polyphenolic compounds [74, 110–115]. The biochemical mechanisms underlying metabolic herb-drug interaction were well described in a recent review [72]. These herbal sources of polyphenols deserve special attention when the activity of P450s is discussed, due to the dramatic increase in the use of herbal medicines and

Table 1: Potential interactions of polyphenols with CYP3A4.

Category of polyphenols	Subcategory of polyphenols	Polyphenols in category	Interaction with CYP3A4	References
Flavonoids	Flavonols	Kaempferol, galangin	Inhibition	[87, 148, 149]
		Quercetin	Inhibition	[87, 148–150]
			Induction of CYP3A4 mRNA expression *in vivo* and in prolonged-exposure assays	[87, 151, 153]
	Flavones	Apigenin, chrysin, amentoflavone	Inhibition	[73]
		Luteolin, diosmetin	Inhibition	[156]
		Flavone, tangeretin	Activation	[156]
		α-Naphthoflavone	Activation	[11]
	Flavonols	EGCG, ECG	Inhibition	[73, 121, 139, 162]
	Flavanones	Naringin, naringenin	Inhibition	[73, 165]
	Isoflavones	Genestein	Inhibition	[169–171]
			Activation (modest activation in clinical trials)	[172, 173]
	Anthocyanins	Anthocyanins (and anthocyanins aglycones)	Inhibition	[174]
Nonflavonoids	Stilbenes	Resveratrol (and resveratrol derivatives)	Inhibition	[38, 175–179]
	Lignans	Gomisins (B and C)	Inhibition	[182]
		Silymarin mixture	Inhibition (with slight activation at low concentrations)	[183, 184]
	Tannins	Tannic acid	Inhibition	[185]
Phenolic acids	Hydroxycinnamic acid	Caffeic acid	Inhibition	[186]
	Hydroxybenzoic acid	Gallic acid	Inhibition	[187, 188]

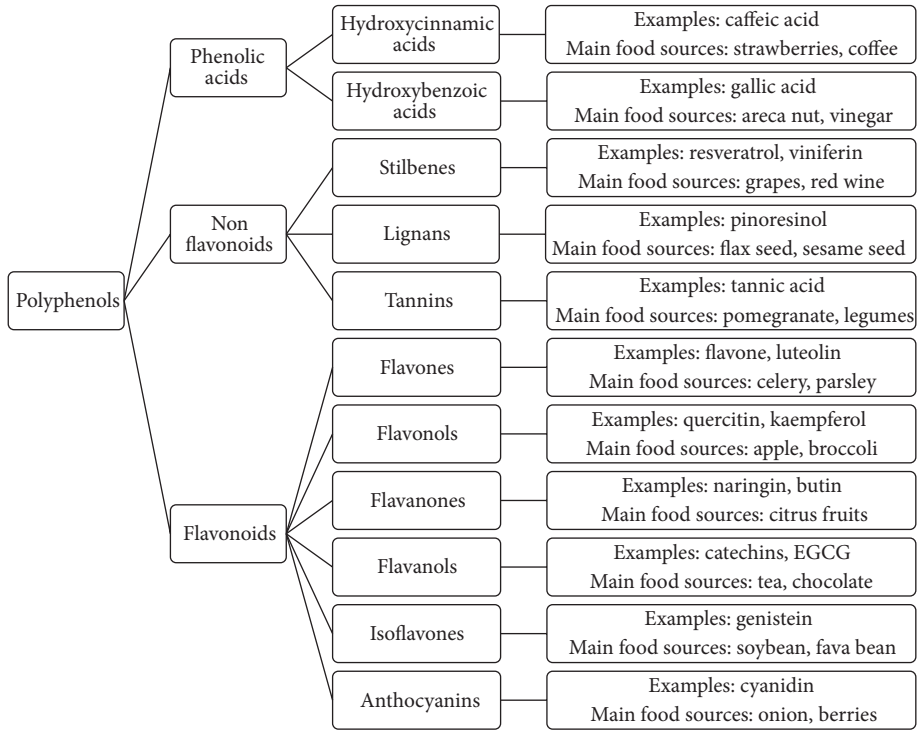

Figure 2: Classification of polyphenols.

supplements [65, 66]. Recent surveys suggest that one in three Americans use dietary supplements daily and among cancer patients the rate is much higher [54]. Moreover, medicinal herbs are not inspected by regulatory authorities such as the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA) [72]. Indeed, medical doctors as well as pharma professionals should be aware of the many interactions of polyphenolics with drugs and tools should be developed to assess the potential of individual polyphenolics to enter the active sites of P450 enzymes and become substrates, competitive inhibitors, or other types of inhibitors of these enzymes in the intestine and the liver. CYP3A4 should be a major point of focus in studies of the undesirable clinical consequences of the timed use of prescribed drugs and herbs [74].

4. Metabolism of Polyphenols by P450s

4.1. Metabolism of Polyphenols by P450 Enzymes. The metabolic fate of polyphenols is largely dictated by their chemical structure and depends on several parameters, including their functional groups (i.e., benzene or flavone derivatives), molecular weight, stereostructure, glycosylation, polymerization, and conjugation with other phenolics [97, 116, 117]. Flavonoids, which are the largest subgroup of polyphenols, have been identified as substrates of P450 enzymes [118, 119]. Flavonoids are hydroxylated and/or *o*-demethylated by various hepatic P450 enzymes prior to their elimination [67]. Jančová and coworkers showed that silybin, a flavono-lignan found in silymarin, is metabolized to *o*-demethylated product by CYP2C8 and CYP3A4 *in vitro* [120]. Meanwhile it has been reported that flavonoids rich with hydroxyl group such as green tea catechins are fairly water soluble and are not likely to be good substrates for P450 enzymes [121, 122]. This is consistent with findings that have demonstrated the importance of ligand hydrophobicity for interactions with these enzymes [38, 123, 124]. Paradoxically, inhibitory effects of green tea catechins on several P450 enzymes have been reported in *in vivo* trials [125, 126]. Another intensively studied polyphenol is the stilbene *t*-resveratrol (trans-3,4′,5-trihydroxystilbene), a polyphenol found in grape skins and red wine, peanuts, and a limited number of other plants, and its effects on CYP3A4 will be discussed later (Section 5.2.1). It exhibits a high level of membrane permeability and is categorized as a class-II compound in the Biopharmaceutical Classification System (BCS) [127]. *t*-Resveratrol has a low bioavailability (less than 1%) due to the low water solubility (a log *P* of 3.1), and the extensive first-pass metabolism by CYP3A4 in the intestine and in the liver, which extended by the enterohepatic recirculation. Further metabolism leads to the formation of the glucuronide and the sulfate metabolites of *t*-resveratrol [128, 129]. Recently, Singh and Pai reported the success of a systematically optimized nanoparticulate drug delivery system to increase the oral bioavailability of *t*-resveratrol in rats [130]. In a similar context in *in vitro* study, Seljak et al. developed a mixed lipid–mixed surfactant self-microemulsifying drug delivery system (SMEDDS) to improve the biopharmaceutical, pharmacokinetic, and toxicological characteristics of resveratrol, suggesting a way to lower the applied dose of resveratrol, to reduce toxicity while maintaining a sufficient pharmacological response [131].

4.2. Involvement of Microbiota in the Metabolism of Polyphenols. There is accumulating evidence to suggest that gut microbiota play a significant role in the metabolism, bioavailability, and bioactivity of dietary polyphenols [132–134]. The involvement of microbiota in the metabolism of these compounds generally starts with the hydrolysis of polymeric, glycosylated and/or esterified polyphenols by brush border and/or microbial enzymes, which is a prerequisite for the absorption and bioactivity of most compounds [134–136]. These biotransformations affect the structural characteristics of polyphenols and may generate metabolites with altered bioactivity profiles [30, 134]. Considering the water soluble green tea catechins, which should be very poor substrates for CYPs, their biotransformation by human gut microbiota could lead to the formation of better CYP substrates, as was demonstrated *in vitro* by Stoupi et al. [137]. Taken together, we suggest that gut microbiota may play a role in the formation of polyphenol-derived metabolites that are more likely to interact with P450 enzymes. The role of intestinal microbiota in the metabolism and bioavailability of dietary polyphenols has been examined [30, 132–136], but, unfortunately, data on the three-way interactions between polyphenols, microbiota and P450s are scarce.

5. Modulation of CYP3A4 Activity by Polyphenols

Interactions between polyphenols and CYP3A4 are important due to their potential implications for drug metabolism. These interactions can modulate the activity or expression of the enzyme. Kimura et al. demonstrated inhibitory effects of polyphenols on human CYP3A4 and CYP2C9 activity *in vitro* [73]. These inhibitory effects generally involve the formation of a covalent bond between the polyphenol and the CYP3A4 molecule, which leads to the inactivation of the enzyme, or reversible binding that causes reversible inhibition [138]. In some cases, the inhibition of P450 enzymes by polyphenols may have a chemopreventative effect, due to the potential activation of carcinogens by P450 enzymes within the course of their natural metabolic activity [81, 139–142]. The inhibition of xenobiotic-metabolizing phase I enzymes (i.e., P450 enzymes) could be one target of the chemopreventive effects of naturally occurring polyphenols. Alternatively, it could be the induction of phase II conjugation enzymes, such as UDP-glucuronosyl transferase and glutathione S-transferase, which are responsible for the detoxification of carcinogens [54].

5.1. Interactions between Flavonoids and CYP3A4. In large, flavonoids account for about two-thirds of the total intake of dietary polyphenols and phenolic acids account for the remaining one-third [33]. Flavonoids, which are found primarily in fruits, vegetables, and beverages such as tea and wine are bioactive compounds that carry several benefits for human health [142–144]. Flavonoids are known to modulate several P450 enzymes, including CYP1A1, CYP1A2, CYP1B1,

CYP2C9, CYP3A4, and CYP3A5 [145, 146]. Hence, their interactions with CYP3A4 are studied in more systems than most other polyphenols and provide evidence for various interactions of polyphenols with this enzyme. There is accumulated evidence that within the family of polyphenols, flavonoids especially can modulate drug metabolism, and in several modes: by altering the expression and/or activity of P450 enzymes, by affecting the P-glycoprotein-mediated cellular efflux of drugs and/or by inhibiting the intestinal glucuronidation of the drug. This evidence indicates that the use of flavonoid-containing dietary supplements concurrent with conventional pharmacotherapeutic regimens should be considered in order to avoid drug-flavonoid interactions [54, 72, 143–146]. In this direction, studies are being conducted to develop methods for evaluating food-drug interactions. For example, Koe and coworkers recently developed a novel multiplex RT-qPCR *in vitro* assay to examine the P450 enzyme-induction properties of herb-derived compounds [147].

5.1.1. Flavonols. The flavonols kaempferol, quercetin, and galangin inhibit CYP3A4-mediated metabolism of xenobiotics *in vitro* [87, 148, 149]. Studies performed *in vivo* have shown conflicting modulation of CYP3A activity by quercetin. Choi et al. reported that oral administration of quercetin to rats led to inhibition of CYP3A, which caused a significant enhancement in the doxorubicin concentration in the plasma. On the contrary, Yu et al. reported an activation of the enzyme that resulted in a reduction in the plasma concentration of cyclosporine in a similar model. The latter observation suggests that this enzyme is not activated by the flavonols, but by their sulfated or glucorunidated products [150, 151]. No *in vivo* inhibition of CYP3A4-mediated metabolism of nifedipine was observed following the ingestion of a high dose of quercetin by others [152]. Interestingly, prolonged exposure to quercetin leads to a significant increase in CYP3A4 mRNA expression levels in cell cultures [87, 153]. We suggest that these findings might be related to the well-established induction of CYP3A4 in response to consumption of St. John's wort extract, which is a rich source of quercetin, in addition to another recognized inducer, the nonphenolic hyperforin [54, 85, 86, 154]. Kaempferol and quercetin have been found to inhibit intestinal UDP-glucuronyl transferase *in vitro* at clinically achievable concentrations, which may lead to an increase in the bioavailability of several drugs [146]. A recent study conducted on rats found that oral administration of morin, a flavonol found in many fruits and herbal medicines, increased the plasma half-life ($t_{1/2}$) of febuxostat, a drug used to treat gout 2.5-fold as compared with the control group, leading to significantly higher bioavailability. One suggested mode of action was that morin could be effective in inhibiting CYP1A1, CYP1A2 and CYP3A mediated metabolism of febuxostat [143].

5.1.2. Flavones. The flavones apigenin and chrysin have a marked inhibitory effect on CYP3A4 activity *in vitro*, with IC_{50} values of 0.4 μM and 0.9 μM, respectively. Amentoflavone (a dimer of apigenin) has even a stronger inhibitory effect, with an IC_{50} value of 0.07 μM [73]. Calculations of the lipophilicity of the two compounds provide support for previous suggestions that higher lipophilicity may contribute to stronger binding of the substrate. It is also possible that the larger stereodimensions of the dimer may lead to irreversible binding of the hydroxylation product to the enzyme, thereby achieving inhibition via a suicidal mode of action [12, 155]. A recent study in rats suggests that the coadministration of apigenin would be very useful for improving the bioavailability of paclitaxel in chemotherapeutic applications, due to the inhibitory effects of apigenin on CYP3A and P-glycoprotein, leading to higher concentration of paclitaxel in the plasma [144]. The ability of apigenin to inhibit intestinal UGT activity has also been investigated *in vitro* [146]. In a study designed to reveal structure-activity relationships, flavones possessing more than two hydroxyl groups (e.g., luteolin and diosmetin) were shown to inhibit the biotransformation of midazolam *in vitro*, whereas flavones that do not have hydroxyl groups in their A and B rings (e.g., flavone and tangeretin) stimulated midazolam metabolism [156]. These results may support the activation effect of α-naphthoflavone (a flavone with no hydroxyl groups) on CYP3A4 and two other CYP3A enzymes, CYP3A5 and CYP3A7 [11]. In addition, α-naphthoflavone represents an interesting case of heterotropic cooperativity in CYP3A4, as it interacts with a peripheral ligand binding site, located at the distal surface of the enzyme and surrounded by the F/F9 and G/G9 loops, resulting in allosteric mechanism [157–161].

5.1.3. Flavonols. Green tea flavonols epigallocatechingallate (EGCG) and epicatechingallate (ECG) inhibit the mutagenic action of aflatoxin B_1 (AFB_1) and 1′-hydroxylation of midazolam *in vitro*. Both actions are known to be mediated by CYP3A4 [139]. Inhibitory effects of catechins on CYP3A4 have been reported in several additional *in vitro* and *in vivo* studies, but no specific mode of action has been suggested [73, 121, 125, 126, 162].

5.1.4. Flavanones. The inhibition of CYP3A4 by grapefruit juice is probably the most well-known example of food-drug inhibition [76, 163]. It was suggested that the flavanone naringin, the predominant flavanone in grapefruit, might be responsible for the observed interaction effect [164]. However, naringin appears to be a weak inhibitor of CYP3A4, while its aglycone, naringenin, may be a more potent inhibitor. The IC_{50} value of naringin is 10-fold greater than that of naringenin *in vitro* and this difference is attributed to the lack of a hydroxyl group on ring A of naringin [73, 165]. This is in agreement with the finding of Shimada and coauthors regarding the importance of the hydroxylation of ring A flavones for the inhibition of CYP3A4 [148]. However, the most potent inhibitor of CYP3A4 in grapefruit has been suggested to be bergapten, a furanocoumarin derivative [165], that does not belong to the polyphenol family, but has a relatively similar structure. The inhibitory effects of other furanocoumarins on CYP3A4 activity *in vitro* are also well established [166–168].

5.1.5. Isoflavones. Isoflavones such as genistein and daidzein are found in soybean and hence are very abundant in many processed food products. Isoflavones differ from flavones in

the location of their phenyl group. It has been suggested that isoflavones may act as phytoestrogens and they appear potential substrates or inhibitors of P450 enzymes. Conflicting data have been presented in several works describing *in vitro* and *in vivo* studies. For instance, soy isoflavones have been found to inhibit CYP3A4 metabolism [169–171], whereas the administration of genistein resulted in a modest induction of CYP3A enzymes among healthy participants [172, 173].

5.1.6. Anthocyanins. Dreiseitel et al. found that anthocyanins and their aglycones are weak inhibitors of CYP3A4 *in vitro* [174]. The IC_{50} values of anthocyanin derivatives ranged from 12.2 to 7,842 μM; whereas ketoconazole, a synthetic CYP3A4 inhibitor that is often used as a reference, has an IC_{50} value of 18.4 nM. Measurement of the IC_{50} values of the different aglycones revealed an inverse relationship between the number of sugar moieties per compound and the ability of anthocyanins to inhibit CYP3A4 [174]. This provides further support for the accumulating data pointing to the importance of lipophilicity for interaction with CYP3A4 [38, 123, 124]. We recently reached a similar conclusion using software to study docking of polyphenols, in which we observed a correlation between the log *P* values of ligands and their docking energies with CYP3A4 (CDOCKER energy expressed in Kcal/mole; Basheer and Kerem, unpublished data).

5.2. Interaction between Nonflavonoids and CYP3A4

5.2.1. Stilbenoids. The inhibitory effects of *t*-resveratrol on CYP3A4 *in vitro* and *in vivo* are well established, and it has been suggested that resveratrol might act as an irreversible, mechanism-based inactivator of this enzyme [38, 175–179]. This inhibition occurs when a CYP3A4 substrate/inhibitor forms a reactive intermediate at the CYP3A4 active site, leading to enzyme inactivation by modification to the heme or the apoprotein [180, 181]. Chan and Delucchi suggested that an electron-rich unsaturated molecule like resveratrol could be a substrate for CYP3A4 and might, in turn, inactivate CYP3A4 during the course of catalysis [175]. Clinical and rat trials have found that the administration of resveratrol increases the area under the plasma concentration-time curve (AUC) for several drugs [81, 177]. Thus, consuming large amounts of resveratrol could theoretically increase the bioavailability of and risk of toxicity from drugs that undergo extensive first-pass metabolism by CYP3A4 [179]. *In vitro* study of the effect of lipophilicity on the interactions of resveratrol derivatives with CYP3A4 revealed that methoxy-stilbenes have lower IC_{50} values and greater affinity for CYP3A4, as compared to the parent resveratrol and its glucosides [38]. CYP3A-mediated aromatic hydroxylation and epoxidation of resveratrol is possible and results in a reactive p-benzoquinone methide metabolite that is capable of binding covalently to CYP3A4, leading to inactivation and potential drug interactions [175].

5.2.2. Lignans. The lignans gomisins B and C, components of Schisandra fruit (*Schisandra chinensis*) extract, have been identified as potent inhibitors of CYP3A4 *in vitro* [182]. Other evidence for the inhibitory effects of plant lignans on CYP3A4 is provided by silymarin, a mixture of flavonolignans extracted from milk thistle (*Silybum marianum*). Silymarin (0.1 mM and 0.25 mM) significantly reduced the activity of CYP3A4 in human hepatocyte cultures by 50 and 100%, respectively, as determined by the formation of 6-β-hydroxy testosterone [183]. Studying the effects of selected lignans from silymarin (silybin, dehydrosilybin, silydianin and silycristin) on CYP3A4 activity as determined *in vitro* by nifedipine oxidation revealed that CYP3A4 activity is inhibited as the concentration of each flavonolignan increases. However, a slight increase in activity was also observed in the presence of low flavonolignan concentrations (0.1–1 μM) [184].

5.2.3. Tannins. Tannic acid, a type of hydrolysable tannin commonly found in plant foods, inhibited testosterone 6-β-hydroxylation (CYP3A4) in human- and rat-liver microsomes with IC_{50} values of 20.2 μM and 16.8 μM, respectively [185].

5.3. Interactions between Phenolic Acids and CYP3A4. Phenolic acids do not all belong to the polyphenols, but are commonly discussed together. The interaction of phenolic acids with CYP3A4 and their potential metabolism by the enzyme would be of high relevance as the research of the more multi-member interactions of CYP3A4, polyphenols and gut microbiota advances, due to the high antimicrobial activity of phenolic acids.

5.3.1. Hydroxycinnamic Acids. Caffeic acid (3,4-dihydrocinnamic acid), which do belong to the polyphenols, is one of the most common phenolic acids found in fruits, coffee, olive oil and dietary supplements. Caffeic acid has been shown to inhibit CYP3A4 activity in human liver microsomes by noncompetitive inhibition, with an IC_{50} of 0.72 μM. In addition, ester and amide analogues of caffeic acid have been found to act as competitive inhibitors, with IC_{50} values ranging from 0.31 μM to 0.82 μM [186].

5.3.2. Hydroxybenzoic Acids. Gallic acid (3,4,5-trihydroxybenzoic acid), also a member of the polyphenols and is abundant in many beverages, for example, wine, tea, pomegranate juice and olive oil, has an inhibitory effect on androstenedione 6-β-hydroxylase activity *in vitro* (apparent K_i value 70 μM), which is regarded as a marker for CYP3A enzyme activity [187]. In another study, Stupans and coworkers provided additional evidence for the inhibition of CYP3A activity by gallic acid. In that study, they showed that preincubation of human liver microsomes with 100 μM gallic acid before the assay of androstenedione 6-β-hydroxylase activity significantly increased the inhibitory effects of the gallic acid. In addition, they reported that the removal of gallic acid-derived products from the incubation mixture completely restored CYP3A activity [188].

6. Structure-Activity Relationships

Various interactions have been demonstrated between compounds belonging to the large family of polyphenols and P450 enzymes. While members of this family share many

structural and functional features, existing reports do not provide sufficient information to allow us to fully understand the rules that determine the nature of these interactions. The number of hydroxyl groups, stereostructure, molecular weight and lipophilicity all seem to have some sort of effect on individual results. Up to date, the protein data bank (PDB) contains 18 crystal structure of human CYP3A4. One of the most prominent characteristics reported was the large, highly ordered hydrophobic core of phenylalanine residues above the active site [189, 190]. A recent review concluded that the CYP3A4 active site is considerably larger than the active site of any other P450 isoform [191].

CYP3A4 substrates form hydrogen bonds with the Asn74 residue of CYP3A4. Structural requirements of CYP3A4 substrates have been suggested to include a hydrogen-bond acceptor atom located 5.5–7.8 Å from the site of metabolism and 3 Å from the oxygen molecule associated with the heme [192]. A three-dimensional pharmacophore based on 38 substrates of CYP3A4 possessed two hydrogen bond acceptors, one hydrogen bond donor, and one hydrophobic region [193]. Inhibitor pharmacophores include three hydrophobes at distances of 5.2 to 8.8 Å from a hydrogen-bond acceptor, three hydrophobes at distances of 4.2 to 7.1 Å from a hydrogen-bond acceptor and at an additional 5.2 Å from another hydrogen-bond acceptor, or one hydrophobe at a distance of 8.1 to 16.3 Å from the two furthest of three hydrogen-bond acceptors [194].

Substrates or inhibitors can bind to CYP3A4 at multiple sites due to the flexible structure of this enzyme's active site [195–197]. For example, a study of the crystal structures of human CYP3A4 in complex with two well characterized drugs, ketoconazole and erythromycin, revealed that the enzyme undergoes dramatic conformational changes upon ligand binding, with an increase in the volume of the active site of more than 80%. These structures represent two distinct open conformations of CYP3A4 because ketoconazole and erythromycin induce different types of coordinate shifts [198]. CYP3A4, like many of P450 enzymes, have large and flexible substrate binding pockets capable of accommodating large substrates or alternatively two or three smaller molecules [199]. Examples on CYP3A4 cooperativity and its non-Mechaelis-Menten kinetics are found in several studies [195, 200, 201]. However, recent studies demonstrate a very complex allosteric mechanism of P450's including overlay of a multiple substrate-binding space-filling mechanism, enzyme conformational changes induced by ligands and modulation of protein-protein interactions in the enzyme oligomers [158, 202]. Allosteric behavior includes homotropic and heterotropic activation and inhibition effects depending on thermodynamic factors as demonstrated by Denisov and Sligar. The latter suggest that "functional cooperativity" best describes P450s fold that includes remote binding sites which may serve for the allosteric regulation of equilibrium and/or kinetic functional properties, including substrate binding and product dissociation, stability of oxy-complex and autoxidation [203].

6.1. Quantitative Structure-Activity Relationship (QSAR). Didziapetris and coworkers developed a structure-activity relationship model to predict the probability that a compound can inhibit human CYP3A4, based on data for more than 800 compounds from various literature sources. Their model is based on GALAS methodology, which involves QSAR (quantitative structure-activity relationship) and local similarity-based corrections. The findings of the GALAS model revealed that increasing the size of the molecule via the incorporation of hydrophobic aliphatic or aromatic residues enhances the ability of the compound to inhibit CYP3A4, while a strong acidic or basic group in the molecule reduces its inhibition potential. This model emphasizes the importance of lipophilicity and the presence of hydrophobic groups on the inhibition potency of compounds, which is consistent with the phenylalanine residues already seen at the active site [123]. An additional QSAR study based on five statistical tools identified a strong correlation between the *n*-octanol/water partition coefficient (log *P*) and the binding affinity of compounds for CYP3A4 [124]. In line with these findings, a study on the influence of lipophilicity on the interactions of hydroxystilbenes with CYP3A4 revealed that methoxy-stilbenes had lower IC_{50} values and greater affinity for CYP3A4, as compared to the parent resveratrol and its glucosides. These results support the hypothesized role of lipophilicity in the interaction of polyphenols with CYP3A4 [38]. Other QSAR analyses conducted by Lewis and coworkers rationalized the lipophilicity relationships in CYP3A4 inhibitors in terms of typical active-site interactions such as hydrogen bonding and π–π stacking, whereas the multiple binding sites in the heme environment could lead to variation in gradients [204, 205].

Mao et al. showed that the traditional QSAR model applied to one data set does not lead to predictive models that would be useful for *in silico* filtering of chemical libraries and presents a multiple pharmacophore hypothesis (MPH) that is a conceptual extension of the conventional QSAR approach. Their study was based on 2,400 marketed drugs and made use of pair-wise comparisons of IC_{50} activity values for different substrates of CYP3A4. The substrates were then characterized according to the proximal and distal binding relative. MPH provides us with structural insight into how multiple substrates of CYP3A4 may interact with the enzyme (e.g., the extent to which their binding sites may lie in close proximity to one another or even overlap) [206].

7. Concluding Remarks

A number of studies in recent years have highlighted the potential risk inherent in the uncontrolled use of herbal medicines concurrent with conventional therapeutic regimens and emphasized the need for regulation in this field based on a set of evaluation criteria [207–211]. We propose here that it is the polyphenols in the herbal preparations that interact with CYP3A4, modify the metabolism of xenobiotics and drugs, and consequently change the active doses of prescribed medicines and the nature of the prescribed compounds. The abundance of polyphenols in many food products, the abundance of CYP3A4 in the intestine, its broad ranges of substrates/inhibitors and cooperativity, the potential involvement of gut microbiota in polyphenol-CYP3A4

interactions and *vice versa*, the extended exposure of the intestinal enzyme to polyphenol metabolites through the enterohepatic cycle and the short-term inhibition, and long-term induction of CYP3A4 by some phenolic compounds all contribute to the interest in the polyphenol-CYP3A4 interactions and their outcomes and underscore the need for further research in this area.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] I. G. Denisov, T. M. Makris, S. G. Sligar, and I. Schlichting, "Structure and chemistry of cytochrome P450," *Chemical Reviews*, vol. 105, no. 6, pp. 2253–2277, 2005.

[2] T. L. Domanski, Y. A. He, K. K. Khan, F. Roussel, Q. Wang, and J. R. Halpert, "Phenylalanine and tryptophan scanning mutagenesis of CYP3A4 substrate recognition site residues and effect on substrate oxidation and cooperativity," *Biochemistry*, vol. 40, no. 34, pp. 10150–10160, 2001.

[3] D. W. Nebert and D. W. Russell, "Clinical importance of the cytochromes P450," *The Lancet*, vol. 360, no. 9340, pp. 1155–1162, 2002.

[4] P. R. O. de Montellano, *Cytochrome P450: Structure, Mechanism, and Biochemistry*, Springer, 3rd edition, 2005.

[5] D. R. Nelson, "The cytochrome P450 homepage," *Human Genomics*, vol. 4, no. 1, pp. 59–65, 2009.

[6] D. R. Nelson, "A world of cytochrome P450s," *Philosophical transactions of the Royal Society of London, Series B: Biological Sciences*, vol. 368, no. 1612, Article ID 20120430, 2013.

[7] D. W. Nebert, K. Wikvall, and W. L. Miller, "Human cytochromes P450 in health and disease," *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, vol. 368, no. 1612, Article ID 20120431, 2013.

[8] D. W. Nebert, D. R. Nelson, M. J. Coon et al., "The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature," *DNA and Cell Biology*, vol. 10, no. 1, pp. 1–14, 1991.

[9] U. M. Zanger and M. Schwab, "Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation," *Pharmacology & Therapeutics*, vol. 138, no. 1, pp. 103–141, 2013.

[10] F. P. Guengerich, "Cytochrome P450 and chemical toxicology," *Chemical Research in Toxicology*, vol. 21, no. 1, pp. 70–83, 2008.

[11] S. Ekins, D. M. Stresser, and J. A. Williams, "*In vitro* and pharmacophore insights into CYP3A enzymes," *Trends in Pharmacological Sciences*, vol. 24, no. 4, pp. 161–166, 2003.

[12] K. S. Lown, D. G. Bailey, R. J. Fontana et al., "Grapefruit juice increases felodipine oral availability in humans by decreasing intestinal CYP3A protein expression," *The Journal of Clinical Investigation*, vol. 99, no. 10, pp. 2545–2553, 1997.

[13] J. C. Kolars, K. S. Lown, P. Schmiedlin-Ren et al., "CYP3A gene expression in human gut epithelium," *Pharmacogenetics and Genomics*, vol. 4, no. 5, pp. 247–259, 1994.

[14] C. S. Ferguson and R. F. Tyndale, "Cytochrome P450 enzymes in the brain: emerging evidence of biological significance," *Trends in Pharmacological Sciences*, vol. 32, no. 12, pp. 708–714, 2011.

[15] C. Ghosh, N. Marchi, N. K. Desai et al., "Cellular localization and functional significance of CYP3A4 in the human epileptic brain," *Epilepsia*, vol. 52, no. 3, pp. 562–571, 2011.

[16] F. P. Guengerich, "Cytochrome P-450 3A4: regulation and role in drug metabolism," *Annual Review of Pharmacology and Toxicology*, vol. 39, pp. 1–17, 1999.

[17] Z. Huang, M. J. Fasco, H. L. Figge, K. Keyomarsi, and L. S. Kaminsky, "Expression of cytochromes P450 in human breast tissue and tumors," *Drug Metabolism and Disposition*, vol. 24, no. 8, pp. 899–905, 1996.

[18] J. H. Lin, M. Chiba, and T. A. Baillie, "Is the role of the small intestine in first-pass metabolism overemphasized?" *Pharmacological Reviews*, vol. 51, no. 2, pp. 135–158, 1999.

[19] M. F. Paine, H. L. Hart, S. S. Ludington, R. L. Haining, A. E. Rettie, and D. C. Zeldin, "The human intestinal cytochrome P450 'pie'," *Drug Metabolism & Disposition*, vol. 34, no. 5, pp. 880–886, 2006.

[20] A. Galetin, M. Gertz, and J. B. Houston, "Contribution of intestinal cytochrome P450-mediated metabolism to drug-drug inhibition and induction interactions," *Drug Metabolism and Pharmacokinetics*, vol. 25, no. 1, pp. 28–47, 2010.

[21] J. Yang, G. T. Tucker, and A. Rostami-Hodjegan, "Cytochrome P450 3A expression and activity in the human small intestine," *Clinical Pharmacology and Therapeutics*, vol. 76, no. 4, article 391, 2004.

[22] M. F. Paine, M. Khalighi, J. M. Fisher et al., "Characterization of interintestinal and intraintestinal variations in human CYP3A-dependent metabolism," *Journal of Pharmacology and Experimental Therapeutics*, vol. 283, no. 3, pp. 1552–1562, 1997.

[23] F. H. Karlsson, S. Bouchene, C. Hilgendorf, H. Dolgos, and S. A. Peters, "Utility of *in vitro* systems and preclinical data for the prediction of human intestinal first-pass metabolism during drug discovery and preclinical development," *Drug Metabolism and Disposition*, vol. 41, no. 12, pp. 2033–2046, 2013.

[24] S. Siissalo and A. T. Heikkinen, "*In vitro* methods to study the interplay of drug metabolism and efflux in the intestine," *Current Drug Metabolism*, vol. 14, no. 1, pp. 102–111, 2013.

[25] M. Gertz, J. D. Davis, A. Harrison, J. B. Houston, and A. Galetin, "Grapefruit juice-drug interaction studies as a method to assess the extent of intestinal availability: utility and limitations," *Current Drug Metabolism*, vol. 9, no. 8, pp. 785–795, 2008.

[26] S. D. Hall, K. E. Thummel, P. B. Watkins et al., "Molecular and physical mechanisms of first-pass extraction," *Drug Metabolism and Disposition*, vol. 27, no. 2, pp. 161–166, 1999.

[27] D. G. Bailey, G. Dresser, and J. M. O. Arnold, "Grapefruit-medication interactions: forbidden fruit or avoidable consequences?" *Canadian Medical Association Journal*, vol. 185, no. 4, pp. 309–316, 2013.

[28] M. Gertz, A. Harrison, J. B. Houston, and A. Galetin, "Prediction of human intestinal first-pass metabolism of 25 CYP3A substrates from in vitro clearance and permeability data," *Drug Metabolism and Disposition*, vol. 38, no. 7, pp. 1147–1158, 2010.

[29] J. van Duynhoven, E. E. Vaughan, D. M. Jacobs et al., "Metabolic fate of polyphenols in the human superorganism," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 1, pp. 4531–4538, 2011.

[30] G. Williamson and M. N. Clifford, "Colonic metabolites of berry polyphenols: the missing link to biological activity?" *British Journal of Nutrition*, vol. 104, supplement 3, pp. S48–S66, 2010.

[31] L. S. Kaminsky and Q.-Y. Zhang, "The small intestine as a xenobiotic-metabolizing organ," *Drug Metabolism and Disposition*, vol. 31, no. 12, pp. 1520–1525, 2003.

[32] M. Martignoni, G. Groothuis, and R. de Kanter, "Comparison of mouse and rat cytochrome P450-mediated metabolism in liver and intestine," *Drug Metabolism and Disposition*, vol. 34, no. 6, pp. 1047–1054, 2006.

[33] M. Lindell, M. Lang, and H. Lennernäs, "Expression of genes encoding for drug metabolising cytochrome P450 enzymes and P-glycoprotein in the rat small intestine; comparison to the liver," *European Journal of Drug Metabolism and Pharmacokinetics*, vol. 28, no. 1, pp. 41–48, 2003.

[34] X. Cao, S. T. Gibbs, L. Fang et al., "Why is it challenging to predict intestinal drug absorption and oral bioavailability in human using rat model," *Pharmaceutical Research*, vol. 23, no. 8, pp. 1675–1686, 2006.

[35] C. Awortwe, P. S. Fasinu, and B. Rosenkranz, "Application of Caco-2 cell line in herb-drug interaction studies: current approaches and challenges," *Journal of Pharmacy and Pharmaceutical Sciences*, vol. 17, no. 1, pp. 1–19, 2014.

[36] N. Holmstock, F. J. Gonzalez, M. Baes, P. Annaert, and P. Augustijns, "PXR/CYP3A4-humanized mice for studying drug-drug interactions involving intestinal P-glycoprotein," *Molecular Pharmaceutics*, vol. 10, no. 3, pp. 1056–1062, 2013.

[37] K. Schroer, M. Kittelmann, and S. Lütz, "Recombinant human cytochrome P450 monooxygenases for drug metabolite synthesis," *Biotechnology and Bioengineering*, vol. 106, no. 5, pp. 699–706, 2010.

[38] G. Regev-Shoshani, O. Shoseyov, and Z. Kerem, "Influence of lipophilicity on the interactions of hydroxy stilbenes with cytochrome P450 3A4," *Biochemical and Biophysical Research Communications*, vol. 323, no. 2, pp. 668–673, 2004.

[39] L. Tang, L. Ye, C. Lv, Z. Zheng, Y. Gong, and Z. Liu, "Involvement of CYP3A4/5 and CYP2D6 in the metabolism of aconitine using human liver microsomes and recombinant CYP450 enzymes," *Toxicology Letters*, vol. 202, no. 1, pp. 47–54, 2011.

[40] Y. V. Grinkova, I. G. Denisov, M. A. McLean, and S. G. Sligar, "Oxidase uncoupling in heme monooxygenases: human cytochrome P450 CYP3A4 in Nanodiscs," *Biochemical and Biophysical Research Communications*, vol. 430, no. 4, pp. 1223–1227, 2013.

[41] D. Hamdane, H. Zhang, and P. Hollenberg, "Oxygen activation by cytochrome P450 monooxygenase," *Photosynthesis Research*, vol. 98, no. 1–3, pp. 657–666, 2008.

[42] B. Meunier, S. P. de Visser, and S. Shaik, "Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes," *Chemical Reviews*, vol. 104, no. 9, pp. 3947–3980, 2004.

[43] P. R. O. de Montellano and J. J. de Voss, "Substrate oxidation by cytochrome P450 enzymes," in *Cytochrome P450*, pp. 183–245, Springer, New York, NY, USA, 3rd edition, 2005.

[44] S.-F. Zhou, J.-P. Liu, and B. Chowbay, "Polymorphism of human cytochrome P450 enzymes and its clinical impact," *Drug Metabolism Reviews*, vol. 41, no. 2, pp. 89–295, 2009.

[45] M. H. Choi, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum, "Characterization of testosterone 11β-hydroxylation catalyzed by human liver microsomal cytochromes P450," *Drug Metabolism and Disposition*, vol. 33, no. 6, pp. 714–718, 2005.

[46] N. Yasui-Furukori, M. Hidestrand, E. Spina, G. Facciolá, M. G. Scordo, and G. Tybring, "Different enantioselective 9-hydroxylation of risperidone by the two human CYP2D6 and CYP3A4 enzymes," *Drug Metabolism & Disposition*, vol. 29, no. 10, pp. 1263–1268, 2001.

[47] Z. Liu and M. Hu, "Natural polyphenol disposition via coupled metabolic pathways," *Expert Opinion on Drug Metabolism and Toxicology*, vol. 3, no. 3, pp. 389–406, 2007.

[48] C. Manach, J. Hubert, R. Llorach, and A. Scalbert, "The complex links between dietary phytochemicals and human health deciphered by metabolomics," *Molecular Nutrition and Food Research*, vol. 53, no. 10, pp. 1303–1315, 2009.

[49] M. P. Corcoran, D. L. McKay, and J. B. Blumberg, "Flavonoid basics: chemistry, sources, mechanisms of action, and safety," *Journal of Nutrition in Gerontology and Geriatrics*, vol. 31, no. 3, pp. 176–189, 2012.

[50] G. F. Ferrazzano, I. Amato, A. Ingenito, A. Zarrelli, G. Pinto, and A. Pollio, "Plant polyphenols and their anti-cariogenic properties: a review," *Molecules*, vol. 16, no. 2, pp. 1486–1507, 2011.

[51] A. Scalbert and G. Williamson, "Dietary intake and bioavailability of polyphenols," *Journal of Nutrition*, vol. 130, no. 8, pp. 2073S–2085S, 2000.

[52] J. D. Lambert, S. Sang, and C. S. Yang, "Biotransformation of green tea polyphenols and the biological activities of those metabolites," *Molecular Pharmaceutics*, vol. 4, no. 6, pp. 819–825, 2007.

[53] J. D. Lambert, S. Sang, and C. S. Yang, "Possible controversy over dietary polyphenols: benefits vs risks," *Chemical Research in Toxicology*, vol. 20, no. 4, pp. 583–585, 2007.

[54] S. Wanwimolruk and V. Prachayasittikul, "Cytochrome P450 enzyme mediated herbal drug interactions (part 1)," *EXCLI Journal*, vol. 13, pp. 347–391, 2014.

[55] K. E. Heim, A. R. Tagliaferro, and D. J. Bobilya, "Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships," *Journal of Nutritional Biochemistry*, vol. 13, no. 10, pp. 572–584, 2002.

[56] I. Rodeiro, M. T. Donato, A. Lahoz, G. Garrido, R. Delgado, and M. J. Gómez-Lechón, "Interactions of polyphenols with the P450 system: possible implications on human therapeutics," *Mini-Reviews in Medicinal Chemistry*, vol. 8, no. 2, pp. 97–106, 2008.

[57] L. M. Blanco-Colio, M. Valderrama, L. A. Alvarez-Sala et al., "Red wine intake prevents nuclear factor-κB activation in peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia," *Circulation*, vol. 102, no. 9, pp. 1020–1026, 2000.

[58] L. Hooper, P. A. Kroon, E. B. Rimm et al., "Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials," *The American Journal of Clinical Nutrition*, vol. 88, no. 1, pp. 38–50, 2008.

[59] J. P. E. Spencer, "Flavonoids and brain health: multiple effects underpinned by common mechanisms," *Genes and Nutrition*, vol. 4, no. 4, pp. 243–250, 2009.

[60] Y. J. Surh, "Cancer chemoprevention with dietary phytochemicals," *Nature Reviews Cancer*, vol. 3, no. 10, pp. 768–780, 2003.

[61] L. Rodríguez-Fragoso, J. L. Martínez-Arismendi, D. Orozco-Bustos, J. Reyes-Esparza, E. Torres, and S. W. Burchiel, "Potential risks resulting from fruit/vegetable-drug interactions: effects on drug-metabolizing enzymes and drug transporters," *Journal of Food Science*, vol. 76, no. 4, pp. R112–R124, 2011.

[62] L. Korkina, C. De Luca, and S. Pastore, "Plant polyphenols and human skin: friends or foes," *Annals of the New York Academy of Sciences*, vol. 1259, no. 1, pp. 77–86, 2012.

[63] Y. D. Muthiah, C. E. Ong, S. A. Sulaiman, S. C. Tan, and R. Ismail, "In-vitro inhibitory effect of Tualang honey on cytochrome P450 2C8 activity," *Journal of Pharmacy and Pharmacology*, vol. 64, no. 12, pp. 1761–1769, 2012.

[64] D. Schwarz, P. Kisselev, W.-H. Schunck, and I. Roots, "Inhibition of 17β-estradiol activation by CYP1A1: genotype- and regioselective inhibition by St. John's Wort and several natural polyphenols," *Biochimica et Biophysica Acta—Proteins and Proteomics*, vol. 1814, no. 1, pp. 168–174, 2011.

[65] J. M. Lillywhite, J. E. Simonsen, and V. Wilson, "Growing Chinese medicinal herbs in the United States: understanding practitioner preferences," *Agriculture and Human Values*, vol. 29, no. 2, pp. 151–159, 2012.

[66] A. Jain, R. Sharma, N. Gahalain, J. Chaudary, and G. K. Gupta, "Herbal plants used in diabetic complications: an overview," *Journal of Pharmacy Research*, vol. 4, no. 4, pp. 986–988, 2011.

[67] S. Zhou, Y. Gao, W. Jiang, M. Huang, A. Xu, and J. W. Paxton, "Interactions of herbs with cytochrome P450," *Drug Metabolism Reviews*, vol. 35, no. 1, pp. 35–98, 2003.

[68] A. A. Izzo, "Herb-drug interactions: an overview of the clinical evidence," *Fundamental and Clinical Pharmacology*, vol. 19, no. 1, pp. 1–16, 2005.

[69] P. H. Marathe and A. D. Rodrigues, "*In vivo* animal models for investigating potential CYP3A- and Pgp-mediated drug-drug interactions," *Current Drug Metabolism*, vol. 7, no. 7, pp. 687–704, 2006.

[70] T. L. Poulos, "Cytochrome P450 dynamics," in *Fifty Years of Cytochrome P450 Research*, pp. 75–94, Springer, Tokyo, Japan, 2014.

[71] A. R. Topletz, J. B. Dennison, R. J. Barbuch, C. E. Hadden, S. D. Hall, and J. L. Renbarger, "The relative contributions of CYP3A4 and CYP3A5 to the metabolism of vinorelbine," *Drug Metabolism & Disposition*, vol. 41, no. 9, pp. 1651–1661, 2013.

[72] S. J. Brantley, A. A. Argikar, Y. S. Lin, S. Nagar, and M. F. Paine, "Herb-drug interactions: challenges and opportunities for improved predictions," *Drug Metabolism and Disposition*, vol. 42, no. 3, pp. 301–317, 2014.

[73] Y. Kimura, H. Ito, R. Ohnishi, and T. Hatano, "Inhibitory effects of polyphenols on human cytochrome P450 3A4 and 2C9 activity," *Food and Chemical Toxicology*, vol. 48, no. 1, pp. 429–435, 2010.

[74] I. Rodeiro, M. T. Donato, N. Jimenez et al., "Inhibition of human P450 enzymes by natural extracts used in traditional medicine," *Phytotherapy Research*, vol. 23, no. 2, pp. 279–282, 2009.

[75] R. Z. Harris, G. R. Jang, and S. Tsunoda, "Dietary effects on drug metabolism and transport," *Clinical Pharmacokinetics*, vol. 42, no. 13, pp. 1071–1088, 2003.

[76] D. G. Bailey, J. Malcolm, O. Arnold, and J. D. Spence, "Grapefruit juice-drug interactions," *British Journal of Clinical Pharmacology*, vol. 46, no. 2, pp. 101–110, 1998.

[77] Y.-M. Ku, D. I. Min, and M. Flanigan, "Effect of grapefruit juice on the pharmacokinetics of microemulsion cyclosporine and its metabolite in healthy volunteers: does the formulation difference matter?" *Journal of Clinical Pharmacology*, vol. 38, no. 10, pp. 959–965, 1998.

[78] D. I. Min, Y.-M. Ku, P. J. Perry et al., "Effect of grapefruit juice on cyclosporine pharmacokinetics in renal transplant patients1," *Transplantation*, vol. 62, no. 1, pp. 123–125, 1996.

[79] J. S. Markowitz, J. L. Donovan, C. L. deVane et al., "Effect of St. John's Wort on drug metabolism by induction of cytochrome P450 3A4 enzyme," *Journal of the American Medical Association*, vol. 290, no. 11, pp. 1500–1504, 2003.

[80] G. W. Barone, B. J. Gurley, B. L. Ketel, M. L. Lightfoot, and S. R. Abul-Ezz, "Drug interaction between St. John's wort and cyclosporine," *Annals of Pharmacotherapy*, vol. 34, no. 9, pp. 1013–1016, 2000.

[81] H.-H. S. Chow, L. L. Garland, C.-H. Hsu et al., "Resveratrol modulates drug- and carcinogen-metabolizing enzymes in a healthy volunteer study," *Cancer Prevention Research*, vol. 3, no. 9, pp. 1168–1175, 2010.

[82] S. F. Zhou, C. C. Xue, X. Q. Yu, C. Li, and G. Wang, "Clinically important drug interactions potentially involving mechanism-based inhibition of cytochrome P450 3A4 and the role of therapeutic drug monitoring," *Therapeutic Drug Monitoring*, vol. 29, no. 6, pp. 687–710, 2007.

[83] X.-W. Chen, K. B. Sneed, S.-Y. Pan et al., "Herb-drug interactions and mechanistic and clinical considerations," *Current Drug Metabolism*, vol. 13, no. 5, pp. 640–651, 2012.

[84] F. Qiu, J. Jiang, Y. Ma et al., "Opposite effects of single-dose and multidose administration of the ethanol extract of danshen on CYP3A in healthy volunteers," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 730734, 8 pages, 2013.

[85] R. Rahimi and M. Abdollahi, "An update on the ability of St. John's wort to affect the metabolism of other drugs," *Expert Opinion on Drug Metabolism and Toxicology*, vol. 8, no. 6, pp. 691–708, 2012.

[86] H. G. Xie and R. B. Kim, "St. John's wort-associated drug interactions: Short-term inhibition and long-term induction?" *Clinical Pharmacology & Therapeutics*, vol. 78, no. 1, pp. 19–24, 2005.

[87] J. Patel, B. Buddha, S. Dey, D. Pal, and A. K. Mitra, "*In vitro* interaction of the HIV protease inhibitor ritonavir with herbal constituents: changes in P-gp and CYP3A4 activity," *The American Journal of Therapeutics*, vol. 11, no. 4, pp. 262–277, 2004.

[88] T. M. Vijayakumar, R. M. Kumar, A. Agrawal, G. P. Dubey, and K. Ilango, "Comparative inhibitory potential of selected dietary bioactive polyphenols, phytosterols on CYP3A4 and CYP2D6 with fluorometric high-throughput screening," *Journal of Food Science and Technology*, 2014.

[89] S. J. Brantley, T. N. Graf, N. H. Oberlies, and M. F. Paine, "A systematic approach to evaluate herb-drug interaction mechanisms: investigation of milk thistle extracts and eight isolated constituents as CYP3A inhibitors," *Drug Metabolism and Disposition*, vol. 41, no. 9, pp. 1662–1670, 2013.

[90] I. Rodeiro, M. J. Gómez-Lechón, G. Perez et al., "*Mangifera indica* L. extract and mangiferin modulate cytochrome P450 and UDP-glucuronosyltransferase enzymes in primary cultures of human hepatocytes," *Phytotherapy Research*, vol. 27, no. 5, pp. 745–752, 2013.

[91] J. I. Boullata, "Drug and nutrition interactions: not just food for thought," *Journal of Clinical Pharmacy and Therapeutics*, vol. 38, no. 4, pp. 269–271, 2013.

[92] O. A. Fahmi, T. S. Maurer, M. Kish, E. Cardenas, S. Boldt, and D. Nettleton, "A combined model for predicting CYP3A4 clinical net drug-drug interaction based on CYP3a4 inhibition, inactivation, and induction determined *in vitro*," *Drug Metabolism and Disposition*, vol. 36, no. 8, pp. 1698–1708, 2008.

[93] Y.-H. Wang, D. R. Jones, and S. D. Hall, "Prediction of cytochrome P450 3A inhibition by verapamil enantiomers and their metabolites," *Drug Metabolism and Disposition*, vol. 32, no. 2, pp. 259–266, 2004.

[94] J. A. Ross and C. M. Kasum, "Dietary flavonoids: bioavailability, metabolic effects, and safety," *Annual Review of Nutrition*, vol. 22, pp. 19–34, 2002.

[95] A. Scalbert, I. T. Johnson, and M. Saltmarsh, "Polyphenols: antioxidants and beyond," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, pp. 215S–217S, 2005.

[96] P. Chabert, C. Auger, J. Pincemail, and V. B. Schini-Kerth, "Overview of plant-derived antioxidants," in *Systems Biology of Free Radicals and Antioxidants*, pp. 4005–4022, Springer, Berlin, Germany, 2014.

[97] J. Pérez-Jiménez, M. E. Díaz-Rubio, and F. Saura-Calixto, "Non-extractable polyphenols, a major dietary antioxidant: occurrence, metabolic fate and health effects," *Nutrition Research Reviews*, vol. 26, no. 2, pp. 118–129, 2013.

[98] M.-L. Ovaskainen, R. Törrönen, J. M. Koponen et al., "Dietary intake and major food sources of polyphenols in Finnish adults," *Journal of Nutrition*, vol. 138, no. 3, pp. 562–566, 2008.

[99] N. N. Thu, C. Sakurai, H. Uto et al., "The polyphenol content and antioxidant activities of the main edible vegetables in Northern Vietnam," *Journal of Nutritional Science and Vitaminology*, vol. 50, no. 3, pp. 203–210, 2004.

[100] F. Saura-Calixto, J. Serrano, and I. Goñi, "Intake and bioaccessibility of total polyphenols in a whole diet," *Food Chemistry*, vol. 101, no. 2, pp. 492–501, 2007.

[101] J. Kanner and T. Lapidot, "The stomach as a bioreactor: dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants," *Free Radical Biology & Medicine*, vol. 31, no. 11, pp. 1388–1395, 2001.

[102] Z. Kerem, D. Chetrit, O. Shoseyov, and G. Regev-Shoshani, "Protection of lipids from oxidation by epicatechin, trans-resveratrol, and gallic and caffeic acids in intestinal model systems," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 26, pp. 10288–10293, 2006.

[103] C. J. Dufresne and E. R. Farnworth, "A review of latest research findings on the health promotion properties of tea," *Journal of Nutritional Biochemistry*, vol. 12, no. 7, pp. 404–421, 2001.

[104] F. Shahidi and Y. Zhong, "Novel antioxidants in food quality preservation and health promotion," *European Journal of Lipid Science and Technology*, vol. 112, no. 9, pp. 930–940, 2010.

[105] N. Khan and H. Mukhtar, "Cancer and metastasis: prevention and treatment by green tea," *Cancer and Metastasis Reviews*, vol. 29, no. 3, pp. 435–445, 2010.

[106] B. B. Aggarwal and S. Shishodia, "Molecular targets of dietary agents for prevention and therapy of cancer," *Biochemical Pharmacology*, vol. 71, no. 10, pp. 1397–1421, 2006.

[107] G. Garrido, D. González, Y. Lemus et al., "*In vivo* and in *vitro* anti-inflammatory activity of *Mangifera indica* L. extract (VIMANG)," *Pharmacological Research*, vol. 50, no. 2, pp. 143–149, 2004.

[108] S. N. Nichenametla, T. G. Taruscio, D. L. Barney, and J. H. Exon, "A review of the effects and mechanisms of polyphenolics in cancer," *Critical Reviews in Food Science and Nutrition*, vol. 46, no. 2, pp. 161–183, 2006.

[109] N. T. Zaveri, "Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications," *Life Sciences*, vol. 78, no. 18, pp. 2073–2080, 2006.

[110] N. Kalogeropoulos, K. Yannakopoulou, A. Gioxari, A. Chiou, and D. P. Makris, "Polyphenol characterization and encapsulation in β-cyclodextrin of a flavonoid-rich Hypericum perforatum (St John's wort) extract," *LWT—Food Science and Technology*, vol. 43, no. 6, pp. 882–889, 2010.

[111] H. J. Lee, H.-S. Lee, H. J. Cho, S. Y. Kim, and H. J. Suh, "Utilization of hydrolytic enzymes for the extraction of ginsenosides from Korean ginseng leaves," *Process Biochemistry*, vol. 47, no. 3, pp. 538–543, 2012.

[112] M. Maizura, A. Aminah, and W. M. W. Aida, "Total phenolic content and antioxidant activity of kesum (*Polygonum minus*), ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) extract," *International Food Research Journal*, vol. 18, no. 2, pp. 529–534, 2011.

[113] B. Jiang, F. Kronenberg, M. J. Balick, and E. J. Kennelly, "Stability of black cohosh triterpene glycosides and polyphenols: potential clinical relevance," *Phytomedicine*, vol. 20, no. 6, pp. 564–569, 2013.

[114] S. S. Georgieva, V. L. Christova-Bagdassarian, and M. S. Atanassova, "Comparative evaluation of the polyphenol composition and antioxidant capacity of propolis and *Echinacea purpurea*," *Journal of Experimental and Integrative Medicine*, vol. 4, no. 1, pp. 51–56, 2014.

[115] F. F. Anhê, D. Roy, G. Pilon et al., "A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased *Akkermansia* spp. population in the gut microbiota of mice," *Gut*, 2014.

[116] A. R. Rechner, G. Kuhnle, P. Bremner, G. P. Hubbard, K. P. Moore, and C. A. Rice-Evans, "The metabolic fate of dietary polyphenols in humans," *Free Radical Biology and Medicine*, vol. 33, no. 2, pp. 220–235, 2002.

[117] L. Bravo, "Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance," *Nutrition Reviews*, vol. 56, no. 11, pp. 317–333, 1998.

[118] H. Doostdar, M. D. Burke, and R. T. Mayer, "Bioflavonoids: selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1," *Toxicology*, vol. 144, no. 1–3, pp. 31–38, 2000.

[119] C. Rice-Evans, "Flavonoid antioxidants," *Current Medicinal Chemistry*, vol. 8, no. 7, pp. 797–807, 2001.

[120] P. Jančová, E. Anzenbacherová, B. Papoušková et al., "Silybin is metabolized by cytochrome P450 2C8 in vitro," *Drug Metabolism & Disposition*, vol. 35, no. 11, pp. 2035–2039, 2007.

[121] C. S. Yang and E. Pan, "The effects of green tea polyphenols on drug metabolism," *Expert Opinion on Drug Metabolism and Toxicology*, vol. 8, no. 6, pp. 677–689, 2012.

[122] S. Sang, J. D. Lambert, C.-T. Ho, and C. S. Yang, "The chemistry and biotransformation of tea constituents," *Pharmacological Research*, vol. 64, no. 2, pp. 87–99, 2011.

[123] R. Didziapetris, J. Dapkunas, A. Sazonovas, and P. Japertas, "Trainable structure-activity relationship model for virtual screening of CYP3A4 inhibition," *Journal of Computer-Aided Molecular Design*, vol. 24, no. 11, pp. 891–906, 2010.

[124] K. Roy and P. P. Roy, "QSAR of cytochrome inhibitors," *Expert Opinion on Drug Metabolism and Toxicology*, vol. 5, no. 10, pp. 1245–1266, 2009.

[125] S. Misaka, K. Kawabe, S. Onoue et al., "Green tea extract affects the cytochrome P450 3A activity and pharmacokinetics of simvastatin in rats," *Drug Metabolism and Pharmacokinetics*, vol. 28, no. 6, pp. 514–518, 2013.

[126] M. Nishikawa, N. Ariyoshi, A. Kotani et al., "Effects of continuous ingestion of green tea or grape seed extracts on the pharmacokinetics of midazolam," *Drug Metabolism and Pharmacokinetics*, vol. 19, no. 4, pp. 280–289, 2004.

[127] A. Amri, J. C. Chaumeil, S. Sfar, and C. Charrueau, "Administration of resveratrol: what formulation solutions to bioavailability limitations?" *Journal of Controlled Release*, vol. 158, no. 2, pp. 182–193, 2012.

[128] T. Walle, "Bioavailability of resveratrol," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 9–15, 2011.

[129] J.-F. Marier, P. Vachon, A. Gritsas, J. Zhang, J.-P. Moreau, and M. P. Ducharme, "Metabolism and disposition of resveratrol in

rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model," *Journal of Pharmacology and Experimental Therapeutics*, vol. 302, no. 1, pp. 369–373, 2002.

[130] G. Singh and R. S. Pai, "*In-vitro*/*in-vivo* characterization of trans-resveratrol-loaded nanoparticulate drug delivery system for oral administration," *Journal of Pharmacy and Pharmacology*, vol. 66, no. 8, pp. 1062–1076, 2014.

[131] K. B. Seljak, K. Berginc, J. Trontelj, A. Zvonar, A. Kristl, and M. Gašperlin, "A self-microemulsifying drug delivery system to overcome intestinal resveratrol toxicity and presystemic metabolism," *Journal of Pharmaceutical Sciences*, vol. 103, no. 11, pp. 3491–3500, 2014.

[132] S. G. Parkar, T. M. Trower, and D. E. Stevenson, "Fecal microbial metabolism of polyphenols and its effects on human gut microbiota," *Anaerobe*, vol. 23, pp. 12–19, 2013.

[133] S. G. Parkar, D. E. Stevenson, and M. A. Skinner, "The potential influence of fruit polyphenols on colonic microflora and human gut health," *International Journal of Food Microbiology*, vol. 124, no. 3, pp. 295–298, 2008.

[134] S. Bolca, T. van de Wiele, and S. Possemiers, "Gut metabotypes govern health effects of dietary polyphenols," *Current Opinion in Biotechnology*, vol. 24, no. 2, pp. 220–225, 2013.

[135] C. Morand, C. Dubray, D. Milenkovic et al., "Hesperidin contributes to the vascular protective effects of orange juice: a randomized crossover study in healthy volunteers," *The American Journal of Clinical Nutrition*, vol. 93, no. 1, pp. 73–80, 2011.

[136] J. W. Lampe, "Interindividual differences in response to plant-based diets: implications for cancer risk," *The American Journal of Clinical Nutrition*, vol. 89, no. 5, pp. 1553S–1557S, 2009.

[137] S. Stoupi, G. Williamson, J. W. Drynan, D. Barron, and M. N. Clifford, "A comparison of the *in vitro* biotransformation of (-)-epicatechin and procyanidin B2 by human faecal microbiota," *Molecular Nutrition and Food Research*, vol. 54, no. 6, pp. 747–759, 2010.

[138] G. K. Dresser, J. D. Spence, and D. G. Bailey, "Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition," *Clinical Pharmacokinetics*, vol. 38, no. 1, pp. 41–57, 2000.

[139] S. Muto, K.-I. Fujita, Y. Yamazaki, and T. Kamataki, "Inhibition by green tea catechins of metabolic activation of procarcinogens by human cytochrome P450," *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 479, no. 1-2, pp. 197–206, 2001.

[140] C. R. Wolf, "Chemoprevention: increased potential to bear fruit," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 6, pp. 2941–2943, 2001.

[141] S. Parys, S. Kehraus, A. Krick et al., "*In vitro* chemopreventive potential of fucophlorethols from the brown alga *Fucus vesiculosus* L. by anti-oxidant activity and inhibition of selected cytochrome P450 enzymes," *Phytochemistry*, vol. 71, no. 2-3, pp. 221–229, 2010.

[142] M. Athar, J. H. Back, X. Tang et al., "Resveratrol: a review of preclinical studies for human cancer prevention," *Toxicology and Applied Pharmacology*, vol. 224, no. 3, pp. 274–283, 2007.

[143] K. Sahu, A. A. Siddiqui, M. Shaharyar, and S. Malik, "Pharmacokinetic interaction between febuxostat and morin in rats," *Expert Opinion on Drug Metabolism and Toxicology*, vol. 10, no. 3, pp. 307–312, 2014.

[144] K. K. Kumar, L. Priyanka, K. Gnananath, P. R. Babu, and S. Sujatha, "Pharmacokinetic drug interactions between apigenin, rutin and paclitaxel mediated by P-glycoprotein in rats," *European Journal of Drug Metabolism and Pharmacokinetics*, 2014.

[145] A. Ferreira, S. Pousinho, A. Fortuna, A. Falcão, and G. Alves, "Flavonoid compounds as reversal agents of the P-glycoprotein-mediated multidrug resistance: biology, chemistry and pharmacology," *Phytochemistry Reviews*, 2014.

[146] B. T. Gufford, G. Chen, P. Lazarus, T. N. Graf, N. H. Oberlies, and M. F. Paine, "Identification of diet-derived constituents as potent inhibitors of intestinal glucuronidation," *Drug Metabolism & Disposition*, vol. 42, no. 10, pp. 1675–1683, 2014.

[147] X. F. Koe, T. S. T. Muhammad, A. S. C. Chong, H. Abdul Wahab, and M. L. Tan, "Cytochrome P450 induction properties of food and herbal-derived compounds using a novel multiplex RT-qPCR *in vitro* assay, a drug-food interaction prediction tool," *Food Science & Nutrition*, vol. 2, no. 5, pp. 500–520, 2014.

[148] T. Shimada, K. Tanaka, S. Takenaka et al., "Structure-function relationships of inhibition of human cytochromes P450 1A1, 1A2, 1B1, 2C9, and 3A4 by 33 flavonoid derivatives," *Chemical Research in Toxicology*, vol. 23, no. 12, pp. 1921–1935, 2010.

[149] M. A. Sarkar, G. Scambia, F. O. Ranelletti et al., "Quercetin not only inhibits P-glycoprotein efflux activity but also inhibits CYP3A isozymes," *Cancer Chemotherapy and Pharmacology*, vol. 36, no. 5, pp. 448–449, 1995.

[150] J.-S. Choi, Y.-J. Piao, and K. W. Kang, "Effects of quercetin on the bioavailability of doxorubicin in rats: role of CYP3A4 and P-gp inhibition by quercetin," *Archives of Pharmacal Research*, vol. 34, no. 4, pp. 607–613, 2011.

[151] C.-P. Yu, P.-P. Wu, Y.-C. Hou et al., "Quercetin and rutin reduced the bioavailability of cyclosporine from Neoral, an immunosuppressant, through activating P-glycoprotein and CYP 3A4," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 9, pp. 4644–4648, 2011.

[152] J. Rashid, C. McKinstry, A. G. Renwick, M. Dirnhuber, D. G. Waller, and C. F. George, "Quercetin, an *in vitro* inhibitor of CYP3A, does not contribute to the interaction between nifedipine and grapefruit juice," *British Journal of Clinical Pharmacology*, vol. 36, no. 5, pp. 460–463, 1993.

[153] J. L. Raucy, "Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products," *Drug Metabolism and Disposition*, vol. 31, no. 5, pp. 533–539, 2003.

[154] W. C. Lau, T. D. Welch, T. Shields, M. Rubenfire, U. S. Tantry, and P. A. Gurbel, "The effect of St John's Wort on the pharmacodynamic response of clopidogrel in hyporesponsive volunteers and patients: increased platelet inhibition by enhancement of CYP3A4 metabolic activity," *Journal of Cardiovascular Pharmacology*, vol. 57, no. 1, pp. 86–93, 2011.

[155] A. Kamel and S. Harriman, "Inhibition of cytochrome P450 enzymes and biochemical aspects of mechanism-based inactivation (MBI)," *Drug Discovery Today: Technologies*, vol. 10, no. 1, pp. e177–e189, 2013.

[156] L. Quintieri, P. Palatini, A. Nassi, P. Ruzza, and M. Floreani, "Flavonoids diosmetin and luteolin inhibit midazolam metabolism by human liver microsomes and recombinant CYP 3A4 and CYP3A5 enzymes," *Biochemical Pharmacology*, vol. 75, no. 6, pp. 1426–1437, 2008.

[157] E. V. Sineva, J. A. O. Rumfeldt, J. R. Halpert, and D. R. Davydov, "A large-scale allosteric transition in cytochrome P450 3A4 revealed by luminescence resonance energy transfer (LRET)," *PLoS ONE*, vol. 8, no. 12, Article ID e83898, 2013.

[158] D. R. Davydov, N. Y. Davydova, E. V. Sineva, I. Kufareva, and J. R. Halpert, "Pivotal role of P450-P450 interactions in CYP3A4

allostery: the case of α-naphthoflavone," *Biochemical Journal*, vol. 453, no. 2, pp. 219–230, 2013.

[159] D. R. Davydov, J. A. O. Rumfeldt, E. V. Sineva, H. Fernando, N. Y. Davydova, and J. R. Halpert, "Peripheral ligand-binding site in cytochrome P450 3A4 located with fluorescence resonance energy transfer (FRET)," *Journal of Biological Chemistry*, vol. 287, no. 9, pp. 6797–6809, 2012.

[160] D. J. Frank, I. G. Denisov, and S. G. Sligar, "Analysis of heterotropic cooperativity in cytochrome P450 3A4 using α-naphthoflavone and testosterone," *Journal of Biological Chemistry*, vol. 286, no. 7, pp. 5540–5545, 2011.

[161] A. G. Roberts and W. M. Atkins, "Energetics of heterotropic cooperativity between α-naphthoflavone and testosterone binding to CYP3A4," *Archives of Biochemistry and Biophysics*, vol. 463, no. 1, pp. 89–101, 2007.

[162] M. I. Netsch, H. Gutmann, C. B. Schmidlin, C. Aydogan, and J. Drewe, "Induction of CYP1A by green tea extract in human intestinal cell lines," *Planta Medica*, vol. 72, no. 6, pp. 514–520, 2006.

[163] D. G. Bailey, J. M. O. Arnold, and J. D. Spence, "Grapefruit juice and drugs. How significant is the interaction?" *Clinical Pharmacokinetics*, vol. 26, no. 2, pp. 91–98, 1994.

[164] U. Fuhr and A. L. Kummert, "The fata of naringin in humans: a key to grapefruit juice-drug interactions?" *Clinical Pharmacology and Therapeutics*, vol. 58, no. 4, pp. 365–373, 1995.

[165] P.-C. Ho, D. J. Saville, and S. Wanwimolruk, "Inhibition of human CYP3A4 activity by grapefruit flavonoids, furanocoumarins and related compounds," *Journal of Pharmacy & Pharmaceutical Sciences*, vol. 4, no. 3, pp. 217–227, 2001.

[166] K. Iwanaga, M. Hayashi, Y. Hamahata et al., "Furanocoumarin derivatives in Kampo extract medicines inhibit cytochrome P450 3A4 and P-glycoprotein," *Drug Metabolism and Disposition*, vol. 38, no. 8, pp. 1286–1294, 2010.

[167] K. M. VanderMolen, G. R. Ainslie, M. F. Paine, and N. H. Oberlies, "Labeled content of two furanocoumarins in dietary supplements correlates with neither actual content nor CYP3A inhibitory activity," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 98, pp. 260–265, 2014.

[168] Y.-F. Ueng, C.-C. Chen, H. Yamazaki et al., "Mechanism-based inhibition of CYP1A1 and CYP3A4 by the furanocoumarin chalepensin," *Drug Metabolism and Pharmacokinetics*, vol. 28, no. 3, pp. 229–238, 2013.

[169] B. C. Foster, S. Vandenhoek, J. Hana et al., "*In vitro* inhibition of human cytochrome P450-mediated metabolism of marker substrates by natural products," *Phytomedicine*, vol. 10, no. 4, pp. 334–342, 2003.

[170] E. S. Roberts-Kirchhoff, J. R. Crowley, P. F. Hollenberg, and H. Kim, "Metabolism of genistein by rat and human cytochrome P450s," *Chemical Research in Toxicology*, vol. 12, no. 7, pp. 610–616, 1999.

[171] L. M. Scott, P. Durant, S. Leone-Kabler et al., "Effects of prior oral contraceptive use and soy isoflavonoids on estrogen-metabolizing cytochrome P450 enzymes," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 112, no. 4-5, pp. 179–185, 2008.

[172] C.-Q. Xiao, R. Chen, J. Lin et al., "Effect of genistein on the activities of cytochrome P450 3A and P-glycoprotein in Chinese healthy participants," *Xenobiotica*, vol. 42, no. 2, pp. 173–178, 2012.

[173] Y. Chen, C.-Q. Xiao, Y.-J. He et al., "Genistein alters caffeine exposure in healthy female volunteers," *European Journal of Clinical Pharmacology*, vol. 67, no. 4, pp. 347–353, 2011.

[174] A. Dreiseitel, P. Schreier, A. Oehme, S. Locher, G. Hajak, and P. G. Sand, "Anthocyanins and their metabolites are weak inhibitors of cytochrome P450 3A4," *Molecular Nutrition and Food Research*, vol. 52, no. 12, pp. 1428–1433, 2008.

[175] W. K. Chan and A. B. Delucchi, "Resveratrol, a red wine constituent, is a mechanism-based inactivator of cytochrome P450 3A4," *Life Sciences*, vol. 67, no. 25, pp. 3103–3112, 2000.

[176] B. Piver, F. Berthou, Y. Dreano, and D. Lucas, "Inhibition of CYP3A, CYP1A and CYP2E1 activities by resveratrol and other non volatile red wine components," *Toxicology Letters*, vol. 125, no. 1–3, pp. 83–91, 2001.

[177] S.-P. Hong, D.-H. Choi, and J.-S. Choi, "Effects of resveratrol on the pharmacokinetics of diltiazem and its major metabolite, desacetyldiltiazem, in rats," *Cardiovascular Therapeutics*, vol. 26, no. 4, pp. 269–275, 2008.

[178] Y. C. Chi, S. P. Lin, and Y. C. Hou, "A new herb-drug interaction of Polygonum cuspidatum, a resveratrol-rich nutraceutical, with carbamazepine in rats," *Toxicology and Applied Pharmacology*, vol. 263, no. 3, pp. 315–322, 2012.

[179] P. Detampel, M. Beck, S. Krähenbühl, and J. Huwyler, "Drug interaction potential of resveratrol," *Drug Metabolism Reviews*, vol. 44, no. 3, pp. 253–265, 2012.

[180] M. A. Correia and P. R. O. de Montellano, "Inhibition of cytochrome P450 enzymes," in *Cytochrome P450*, pp. 247–322, Springer, Berlin, Germany, 3rd edition, 2005.

[181] Y. Sahali-Sahly, S. K. Balani, J. H. Lin, and T. A. Baillie, "*In vitro* studies on the metabolic activation of the furanopyridine L- 754,394, a highly potent and selective mechanism-based inhibitor of cytochrome P450 3A4," *Chemical Research in Toxicology*, vol. 9, no. 6, pp. 1007–1012, 1996.

[182] H. Iwata, Y. Tezuka, S. Kadota, A. Hiratsuka, and T. Watabe, "Identification and characterization of potent CYP3A4 inhibitors in Schisandra fruit extract," *Drug Metabolism and Disposition*, vol. 32, no. 12, pp. 1351–1358, 2004.

[183] R. Venkataramanan, V. Ramachandran, B. J. Komoroski, S. Zhang, P. L. Schiff, and S. C. Strom, "Milk thistle, a herbal supplement, decreases the activity of CYP3A4 and uridine diphosphoglucuronosyl transferase in human hepatocyte cultures," *Drug Metabolism and Disposition*, vol. 28, no. 11, pp. 1270–1273, 2000.

[184] R. Zuber, M. Modrianský, Z. Dvořák et al., "Effect of silybin and its congeners on human liver microsomal cytochrome P450 activities," *Phytotherapy Research*, vol. 16, no. 7, pp. 632–638, 2002.

[185] H.-T. Yao, Y.-W. Chang, S.-J. Lan, and T.-K. Yeh, "The inhibitory effect of tannic acid on cytochrome P450 enzymes and NADPH-CYP reductase in rat and human liver microsomes," *Food and Chemical Toxicology*, vol. 46, no. 2, pp. 645–653, 2008.

[186] C. Jaikang, K. Niwatananun, P. Narongchai, S. Narongchai, and C. Chaiyasut, "Inhibitory effect of caffeic acid and its derivatives on human liver cytochrome P450 3A4 activity," *Journal of Medicinal Plants Research*, vol. 5, no. 15, pp. 3530–3536, 2011.

[187] I. Stupans, G. Stretch, and P. Hayball, "Olive oil phenols inhibit human hepatic microsomal activity," *Journal of Nutrition*, vol. 130, no. 9, pp. 2367–2370, 2000.

[188] I. Stupans, H.-W. Tan, A. Kirlich, K. Tuck, P. Hayball, and M. Murray, "Inhibition of CYP3A-mediated oxidation in human hepatic microsomes by the dietary derived complex phenol, gallic acid," *Journal of Pharmacy and Pharmacology*, vol. 54, no. 2, pp. 269–275, 2002.

[189] J. K. Yano, M. R. Wester, G. A. Schoch, K. J. Griffin, C. D. Stout, and E. F. Johnson, "The structure of human microsomal

cytochrome P450 3A4 determined by X-ray crystallography to 2.05-Å resolution," *The Journal of Biological Chemistry*, vol. 279, no. 37, pp. 38091–38094, 2004.

[190] P. A. Williams, J. Cosme, D. M. Vinković et al., "Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone," *Science*, vol. 305, no. 5684, pp. 683–686, 2004.

[191] J. Sridhar, J. Liu, M. Foroozesh, and C. L. K. Stevens, "Insights on cytochrome P450 enzymes and inhibitors obtained through QSAR studies," *Molecules*, vol. 17, no. 8, pp. 9283–9305, 2012.

[192] D. F. V. Lewis and B. G. Lake, "Molecular modelling and quantitative structure-activity relationship studies on the interaction of omeprazole with cytochrome P450 isozymes," *Toxicology*, vol. 125, no. 1, pp. 31–44, 1998.

[193] S. Ekins, G. Bravi, J. H. Wikel, and S. A. Wrighton, "Three-dimensional-quantitative structure activity relationship analysis of cytochrome P-450 3A4 substrates," *Journal of Pharmacology and Experimental Therapeutics*, vol. 291, no. 1, pp. 424–433, 1999.

[194] S. Ekins, M. J. de Groot, and J. P. Jones, "Pharmacophore and three-dimensional quantitative structure activity relationship methods for modeling cytochrome p450 active sites," *Drug Metabolism and Disposition*, vol. 29, no. 7, pp. 936–944, 2001.

[195] A. Galetin, S. E. Clarke, and J. B. Houston, "Multisite kinetic analysis of interactions between prototypical CYP3A4 subgroup substrates: midazolam, testosterone, and nifedipine," *Drug Metabolism and Disposition*, vol. 31, no. 9, pp. 1108–1116, 2003.

[196] K. Ohkura, Y. Kawaguchi, Y. Watanabe, Y. Masubuchi, Y. Shinohara, and H. Hori, "Flexible structure of cytochrome P450: promiscuity of ligand binding in the CYP3A4 heme pocket," *Anticancer Research*, vol. 29, no. 3, pp. 935–942, 2009.

[197] K. E. Kenworthy, S. E. Clarke, J. Andrews, and J. B. Houston, "Multisite kinetic models for CYP3A4: simultaneous activation and inhibition of diazepam and testosterone metabolism," *Drug Metabolism and Disposition*, vol. 29, no. 12, pp. 1644–1651, 2001.

[198] M. Ekroos and T. Sjögren, "Structural basis for ligand promiscuity in cytochrome P450 3A4," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 37, pp. 13682–13687, 2006.

[199] I. G. Denisov, D. J. Frank, and S. G. Sligar, "Cooperative properties of cytochromes P450," *Pharmacology & Therapeutics*, vol. 124, no. 2, pp. 151–167, 2009.

[200] W. M. Atkins, "Non-Michaelis-Menten kinetics in cytochrome P450-catalyzed reactions," *Annual Review of Pharmacology and Toxicology*, vol. 45, pp. 291–310, 2005.

[201] J. M. Hutzler and T. S. Tracy, "Atypical kinetic profiles in drug metabolism reactions," *Drug Metabolism and Disposition*, vol. 30, no. 4, pp. 355–362, 2002.

[202] D. R. Davydov and J. R. Halpert, "Allosteric P450 mechanisms: multiple binding sites, multiple conformers of both?" *Expert Opinion on Drug Metabolism and Toxicology*, vol. 4, no. 12, pp. 1523–1535, 2008.

[203] I. G. Denisov and S. G. Sligar, "A novel type of allosteric regulation: functional cooperativity in monomeric proteins," *Archives of Biochemistry and Biophysics*, vol. 519, no. 2, pp. 91–102, 2012.

[204] D. F. Lewis, B. G. Lake, and M. Dickins, "Quantitative structure-activity relationships (QSARs) in inhibitors of various cytochromes P450: the importance of compound lipophilicity," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 22, no. 1, pp. 1–6, 2007.

[205] D. F. V. Lewis, B. G. Lake, and M. Dickins, "Quantitative structure-activity relationships (QSARs) in CYP3A4 inhibitors: the importance of lipophilic character and hydrogen bonding," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 21, no. 2, pp. 127–132, 2006.

[206] B. Mao, R. Gozalbes, F. Barbosa et al., "QSAR modeling of in vitro inhibition of cytochrome P450 3A4," *Journal of Chemical Information and Modeling*, vol. 46, no. 5, pp. 2125–2134, 2006.

[207] P. Matoušková, H. Bártíková, I. Boušová et al., "Effect of defined green tea extract in various dosage schemes on drug-metabolizing enzymes in mice *in vivo*," *Journal of Functional Foods*, vol. 10, pp. 327–335, 2014.

[208] U. F. Ezuruike and J. M. Prieto, "The use of plants in the traditional management of diabetes in Nigeria: pharmacological and toxicological considerations," *Journal of Ethnopharmacology*, vol. 155, no. 2, pp. 857–924, 2014.

[209] C. Bunchorntavakul and K. R. Reddy, "Review article: herbal and dietary supplement hepatotoxicity," *Alimentary Pharmacology and Therapeutics*, vol. 37, no. 1, pp. 3–17, 2013.

[210] E. J. Y. Kim, Y. Chen, J. Q. Huang et al., "Evidence-based toxicity evaluation and scheduling of Chinese herbal medicines," *Journal of Ethnopharmacology*, vol. 146, no. 1, pp. 40–61, 2013.

[211] V. S. Neergheen-Bhujun, "Underestimating the toxicological challenges associated with the use of herbal medicinal products in developing countries," *BioMed Research International*, vol. 2013, Article ID 804086, 9 pages, 2013.

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Research Article

Resveratrol Derivative-Rich Melinjo Seed Extract Attenuates Skin Atrophy in *Sod1*-Deficient Mice

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The oxidative damages induced by a redox imbalance cause age-related changes in cells and tissues. Superoxide dismutase (SOD) enzymes play a pivotal role in the antioxidant system and they also catalyze superoxide radicals. Since the loss of cytoplasmic SOD (SOD1) resulted in aging-like phenotypes in several types of murine tissue, SOD1 is essential for the maintenance of tissue homeostasis. Melinjo (*Gnetum gnemon* Linn) seed extract (MSE) contains trans-resveratrol (RSV) and resveratrol derivatives, including gnetin C, gnemonoside A, and gnemonoside D. MSE intake also exerts no adverse events in human study. In the present studies, we investigated protective effects of MSE on age-related skin pathologies in mice. Orally MSE and RSV treatment reversed the skin thinning associated with increased oxidative damage in the *Sod1*$^{-/-}$ mice. Furthermore, MSE and RSV normalized gene expression of *Col1a1* and *p53* and upregulated gene expression of *Sirt1* in skin tissues. *In vitro* experiments revealed that RSV significantly promoted the viability of *Sod1*$^{-/-}$ fibroblasts. These finding demonstrated that RSV in MSE stably suppressed an intrinsic superoxide generation *in vivo* and *in vitro* leading to protecting skin damages. RSV derivative-rich MSE may be a powerful food of treatment for age-related skin diseases caused by oxidative damages.

1. Introduction

Intrinsic skin aging induced by chronological or intrinsic factors leads to skin atrophy [1]. Skin collagen components show age-dependent reductions in both male and female subjects, resulting in age-related skin thinning in older individuals [2]. Accumulated evidence suggests that oxidatively modified proteins, DNA, and lipids in the skin and other organs during aging are progressively accumulated [3], indicating that reactive oxygen species (ROS) are strongly associated with skin aging. To attenuate oxidative damages, multiple antioxidative and repair systems exert in cells. Superoxide dismutase (SOD) plays a central role in the antioxidative systems due to its ability to catalyze cellular superoxide radicals ($O_2^{\bullet-}$) to H_2O_2. H_2O_2 is further degraded to O_2 and H_2O by catalase, glutathione peroxidases, and peroxiredoxins. Copper/zinc superoxide dismutase (SOD1) is localized to react intracellular $O_2^{\bullet-}$ in the cytoplasm. Our previous studies demonstrated that *Sod1*-deficient (*Sod1*$^{-/-}$) mice showed enhancement of intracellular $O_2^{\bullet-}$ and various aging-like organ phenotypes, suggesting that cytoplasmic $O_2^{\bullet-}$-mediated oxidative damages primarily cause aging-like changes in various tissues [4]. Particularly, *Sod1* insufficiency resulted in both epidermal and dermal atrophies associated with downregulation of extracellular matrix-related genes including *Col1a1* and with upregulation of age-related genes including p53 [5, 6]. Therefore, *Sod1*$^{-/-}$ mouse is a suitable model for studying skin aging in older people.

Melinjo (Indonesian name; *Gnetum gnemon* Linn) is an arboreal dioecious plant that is widely cultivated in Southeast Asia. Its fruits and seeds are used as an ordinary vegetable in Indonesia. Melinjo seeds contain various stilbenoids including trans-resveratrol (3,5,4′-trihydroxy-trans-stilbene), its glucoside, resveratrol dimer (gnetin C), and resveratrol dimer glucoside (gnetin L, gnemonoside A, gnemonoside C, and gnemonoside D) [7]. Melinjo seed extract (MSE) revealed DPPH radical scavenging [7], lipase and α-amylase inhibitory [7], antimicrobial, immunostimulatory [7], angiogenesis inhibitory [8], tyrosinase inhibitory activities [9], promotion of melanin biosynthesis [9], and prevention of endothelial

senescence [10]. Recently, Tatefuji et al. also reported that acute and subchronic MSE administration showed no adverse effect in rat [11]. In human study, MSE administration decreases the serum uric acid levels by inhibiting the reabsorption of uric acid in the renal tubular epithelia as well as by increasing the HDL cholesterol levels by PPAR agonistic activity with no cause of the damage to health [12]. Furthermore, Tani et al. reported that single and repeated administration of MSE demonstrated no clinical noteworthy abnormalities [13]. MSE contains 1.2 mg/g (5.26 μmol/g) of RSV [13], while average content of RSV was 1.04 mg/L in red wine [14]. Therefore, MSE becomes a nutrient source of RSV with harmless long-term intake. RSV has been identified as a *Sirt1* activator that has been shown to protect various organs against aging [15, 16]. Furthermore, RSV possesses antioxidative activity and protective effect of ROS- and ultraviolet-induced cell death [17, 18]. In addition, *Sirt1* is a key modulator of cellular pathways involved in inherited dermatologic diseases and skin cancers [19], suggesting that *Sirt1* activation is a molecular target for dermatological therapy. In the present study, we investigated antiatrophic effects of MSE and RSV on age-related skin pathologies in *Sod1*$^{-/-}$ mice.

2. Materials and Methods

2.1. Reagents. MSE (Lot number YMP-M-110115) was provided by the Institute for Bee Products & Health Science, Yamada Bee Company, Inc. (Okayama, Japan). The MSE contains trans-resveratrol (RSV, 0.10% w/w), gnetin C (2.03% w/w), gnemonoside A (16.35% w/w), and gnemonoside D (3.97% w/w). Resveratrol (RSV) was obtained from Tokyo Chemical Industry Co. Ltd. (CAS 501-36-0, Tokyo, Japan). The purity of RSV is more than 98%.

2.2. Mice and Diets. *Sod1*$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Hrbor, ME, USA). The genotyping of *Sod1*$^{-/-}$ allele was performed using genomic PCR with genomic DNA isolated from the tail tip, as previously reported [20]. The animals were housed in a room temperature of 24 ± 1°C, a relative humidity of 55 ± 10%, and a 12 h light/dark cycle and were fed *ad libitum*. Experimental procedures were approved by the Animal Care and Use Committee of Chiba University. At 4 weeks of age, mice were randomly divided into four groups and fed respective experimental diets for 12 weeks: control MF diet (composition: 7.9% water, 21.1% protein, 5.1% lipid, 5.8% ash, 2.8% fiber, and 55.3% soluble nitrogen free extract, 359 kcal/100 g, Oriental Yeast Co., Ltd., Tokyo, Japan), control MF diet containing 0.04% (w/w) RSV as previously described [15], and control MF diets containing 0.1% or 0.5% (w/w) MSE (Lot number YMP-M-110115) according to the previous study [8].

2.3. Histology. For histological morphology, skin specimens from back tissues were dissected and fixed in a 20% formalin neutral buffer solution (Wako, Osaka, Japan) overnight. After dehydration and penetration, skin specimens were embedded in paraffin and sectioned on a microtome (ROM-380, Yamato Koki Kogyo Co. Ltd., Saitama, Japan) at 4 μm thickness by standard techniques. Hematoxylin and eosin staining for skin morphology and Sirius red staining for total collagen deposition were performed as described previously [21–23]. The thickness of the skin tissue was measured using Leica QWin V3 image software (Leica, Germany).

2.4. Measurement of Oxidative Stress Markers. In order to measure the 8-isoprostane content, blood was collected from the left ventricular space and centrifuged at 12,000 rpm for 5 min at room temperature. Plasma was separated from the clotted blood and added 100 μM indomethacin and 0.005% dibutylhydroxytoluene. The 8-isoprostane level was measured using the 8-isoprostane EIA Kit (Cayman Chemical Company) according to the manufacturer's instructions. The plasma was also assayed for the protein concentration using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA), and 8-isoprostane levels were normalized to the protein level.

For intracellular ROS measurement, bone marrow cells (5–10 × 10^5 cells/two tibias of a mouse) were collected by flushing tibias with phosphate-buffered saline using 26-G needles and stained with 10 μM of CM-H_2DCFDA (DCF, Life Technologies Corporation) for 30 min at 37°C. Primary dermal fibroblasts were incubated with 10 μM DCF for 30 min at 37°C. After incubation, cells were trypsinized and resuspended in PBS. Their fluorescence intensities were assessed using a flow cytometer (BD FACSCanto II, BD Biosciences).

2.5. Cell Culture. Skin tissues were dissected from *Sod1*$^{-/-}$ neonates at 5 days of age. The primary dermal fibroblasts were isolated by dissociation in 0.2% collagenase type 2 (Worthington Biochemical Corporation Lakewood, NJ, USA) at 37°C for 60 min. Cells were cultured in α-MEM (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS), 100 unit/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2 and 1% O_2. Cells were treated with 10 μM RSV at 72 h. We determined the concentration and duration of RSV treatment in this study according to our previous paper [6].

2.6. Outgrowth Assay. The back skin was sterilized with 70% ethanol, rinsed with PBS (Takara Bio Inc., Shiga, Japan), and punched out into discs measuring 5 mm in diameter using dermal punch (Nipro, Tokyo, Japan). The punched skin discs were placed into a 12-well culture plate (Falcon BD, Franklin Lakes, NJ) and cultured with or without 10 μM RSV in α-MEM containing 20% FBS, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin at 37°C in a humidified incubator with 5% CO_2 and 1% O_2. The number of outgrowth fibroblasts originating from the mouse skin disc was directly counted at 72 h after culture. The method of this experiment was performed as described previously [5].

2.7. Quantitative PCR. Total RNA was extracted from back skin using the Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using reverse transcriptase (ReverTra Ace qPCR RT Master Mix, Toyobo, Osaka, Japan). Real-time PCR was performed on

a MiniOpticon (Bio-Rad) with the SYBR Green PCR Master Mix (Bio-Rad) according to the manufacturer's instructions. All data were normalized to the level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The following primers were used for the analysis: *Gapdh*, forward, 5′-ATGTGTCCGTCGTGGATCTGA-3′, and reverse, 5′-TGCCTGCTTCACCACCTTCT-3′; *Col1a1*, forward, 5′-CATGTTCAGCTTTGTGGACCT-3′, and reverse, 5′-GCAGCTGACTTCAGGGATGT-3′; *p53*, forward, 5′-ACGCTTCTCCGAAGACTGG-3′, and reverse, 5′-AGGGAGCTCGAGGCTGATA-3′; *Sirt1*, forward, 5′-CAGTGAGAAAATGCTGGCCTA-3′, and reverse, 5′-TTGGTGGTACAAACAGGTATTGA-3′.

2.8. Statics. The statistical analyses were performed using Student's *t*-test for comparisons between two groups and Tukey's test for comparisons among three groups. Differences between the data were considered significant when the *P* values were less than 0.05. All data are expressed as the mean ± standard deviation (SD).

3. Results

3.1. MSE and RSV Attenuate the Skin Atrophy of Sod1$^{-/-}$ Mice. SOD1, one of the cellular antioxidant enzymes, plays a pivotal role in regulating oxidative and reductive balance. *Sod1*$^{-/-}$ mice showed age-related atrophic morphology in their skin accompanied by the degeneration of collagen [5]. Therefore, we have used *Sod1*$^{-/-}$ mice for skin aging research and for screening of antiatrophic compounds in skin thickness [24–26]. In this context, we investigated antiatrophic effects of MSE and RSV on the skin thickness of *Sod1*$^{-/-}$ mice.

In a preliminary experiment, MF diets containing 5% or 0.5% MSE were orally administrated to the *Sod1*$^{+/+}$ and *Sod1*$^{-/-}$ mice daily for 12 weeks beginning at 4 weeks of age. The results showed that both MSE diets improved the skin thickness of *Sod1*$^{-/-}$ mice and there were no adverse effects of skin pathologies of *Sod1*$^{+/+}$ mice (data not shown). Therefore, we selected the control diet containing 0.5% MSE to confirm antiatrophic effect on *Sod1*$^{-/-}$ skin. MSE and RSV were orally administered to the *Sod1*$^{+/+}$ and *Sod1*$^{-/-}$ mice under the same conditions. As shown in Figure 1(a), the skin of *Sod1*$^{-/-}$ mice was significantly thinner compared to that of *Sod1*$^{+/+}$ mice, confirming skin atrophy in *Sod1*$^{-/-}$ mice. The back skin of *Sod1*$^{-/-}$ that had been administrated with the MSE diets was significantly thicker compared to that of *Sod1*$^{-/-}$ mice treated with the control diet (Figures 1(b)–1(d)). RSV diet also improved skin atrophy of *Sod1*$^{-/-}$ mice compared to *Sod1*$^{-/-}$ mice treated with the control diet (Figures 1(b)–1(d)). To investigate the adverse effect of MSE and RSV diets, we similarly administered MSE and RSV to the *Sod1*$^{+/+}$ mice. No significant difference in skin thickness and morphology was observed in *Sod1*$^{+/+}$ mice treated with MSE and RSV (data not shown), indicating that MSE and RSV were safety food factors in skin during short-time treatment. In addition, Sirius red staining revealed that the skin of *Sod1*$^{-/-}$ mice was decreased in staining intensity compared to that observed in *Sod1*$^{+/+}$ mice (Figure 2(a)), confirming dermal collagen decline in *Sod1*$^{-/-}$ mice. Notably, both MSE and RSV diets increased the Sirius red intensity in *Sod1*$^{-/-}$ dermis (Figure 2(a)), implying enhancement of collagen level in *Sod1*$^{-/-}$ skin.

3.2. MSE and RSV Alter Gene Expression in Sod1$^{-/-}$ Skin. To investigate skin atrophy-preventing mechanism of MSE and RSV on skin atrophy in *Sod1*$^{-/-}$, we analyzed expression patterns of type I collagen and age-related genes in skin. In *Sod1*$^{-/-}$ skin, mRNA level of *Col1a1* was significantly downregulated compared to those of *Sod1*$^{+/+}$, indicating reduced collagen biosynthesis (Figure 2(b)). Moreover, p53, one of the major age-related genes, also significantly upregulated in *Sod1*$^{-/-}$ skin (Figure 2(c)). MSE and RSV treatment significantly normalized mRNA levels of *Col1a1* and *p53* in *Sod1*$^{-/-}$ skin (Figures 2(b) and 2(c)). Interestingly, we revealed that MSE and RSV treatment also significantly upregulated *Sirt1* expression, suggesting the molecular link between *Sirt1* expression and skin thinning in *Sod1*$^{-/-}$ mice (Figure 2(d)). These findings demonstrated that application of MSE and RSV diets improved the skin atrophy accompanied by normalization and activation of age-related genes in *Sod1*$^{-/-}$ mice.

3.3. MSE and RSV Significantly Attenuate Oxidative Damage in Sod1$^{-/-}$ Mice. *Sod1*$^{-/-}$ mice showed significant increase of several oxidative damage markers, including lipid peroxidation, in tissues [20, 24, 27, 28]. In order to evaluate oxidative damage, we measured the lipid peroxidation levels in the plasma. Regarding the 8-isoprostane levels, MSE and RSV containing diets significantly reduced the 8-isoprostane content in the plasma (Figure 3(a)). Furthermore, MSE and RSV containing diets decreased intracellular ROS level in cells from bone marrow (Figure 3(b)). These data indicate that MSE and RSV treatment mitigated oxidative damage in *Sod1*$^{-/-}$ mice.

3.4. MSE and RSV Significantly Restore Viability in Sod1$^{-/-}$ Fibroblasts. We investigated whether the RSV treatment attenuated intracellular ROS production and promoted the proliferation of *Sod1*$^{-/-}$ fibroblasts *in vitro*. Preliminary experiments revealed that RSV treatment for 24 h with various concentrations of 30 to 100 μM slightly suppressed cell viability of *Sod1*$^{+/+}$ fibroblasts. In contrast, 10 μM RSV treatment for 72 h showed no adverse effect of cell viability in *Sod1*$^{+/+}$ fibroblasts. Therefore, we determined dose and duration of the RSV experiment *in vitro*. Flow cytometer analysis indicated that RSV treatment significantly decreased intracellular ROS generation in *Sod1*$^{-/-}$ fibroblasts (Figure 3(c)). Moreover, the organ culture experiments using skin discs revealed that the *Sod1*$^{-/-}$ fibroblasts showed marked suppression of their outgrowth capacity compared to that observed in the *Sod1*$^{+/+}$ mice (Figure 4(a)). Treatment with 10 μM RSV significantly enhanced the fibroblasts outgrowth activity

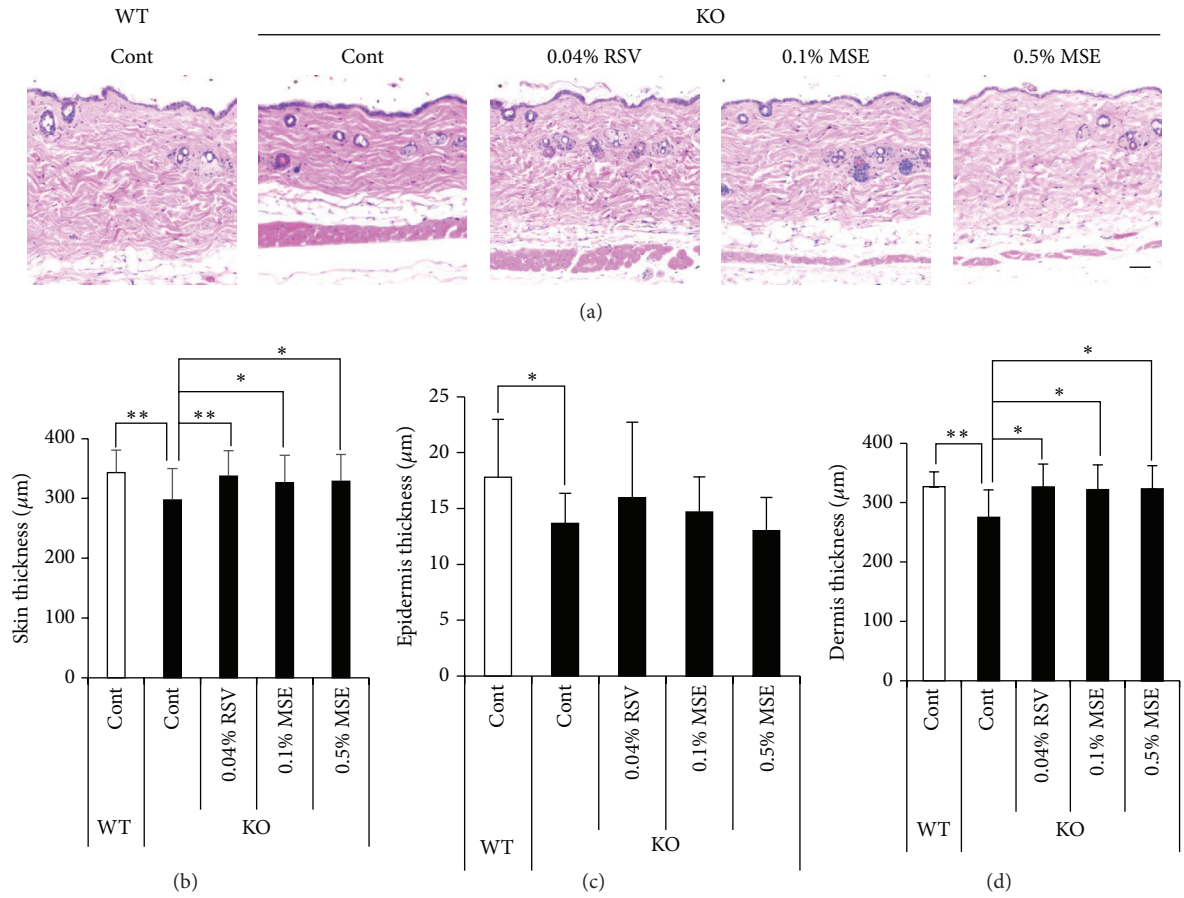

Figure 1: MSE and RSV attenuate skin atrophy in the *Sod1*$^{-/-}$ mice. (a) Hematoxylin and eosin staining of the back skin of *Sod1*$^{-/-}$ (KO) and *Sod1*$^{+/+}$ (WT) mice treated with the MSE or RSV. MSE and RSV containing diets were administrated for 12 weeks. The thickness of (b) total, (c) epidermis, and (d) dermis of the back skin of the *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice treated with MSE or RSV ($n = 10$–12). The statistical evaluations were performed using the Tukey's test. These data indicate the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$. The scale bar represents 100 μm.

from the *Sod1*$^{-/-}$ skin discs (Figure 4(b)). These findings collectively suggested that the RSV promoted the migration and proliferation of *Sod1*$^{-/-}$ fibroblasts *in vitro*.

4. Discussion

In the present study, we demonstrated that MSE and RSV significantly reversed skin thinning via reduction of oxidative damages in *Sod1*$^{-/-}$ mice (Figure 1). Recently, we have reported that *Sod1*$^{-/-}$ fibroblasts showed excessive ROS accumulation associated with mitochondrial dysfunction [6]. *In vitro* study also revealed that RSV treatment significantly reduced intracellular ROS generation and restored cell viability in *Sod1*$^{-/-}$ fibroblasts (Figures 3(c) and 4). Accumulating evidence revealed that RSV activates mitochondrial function and antioxidant defense leading to suppressing ROS generation [29]. Furthermore, SIRT1 also increases mitochondrial function and biogenesis and promotes cell proliferation and migration [15, 30, 31]. In a human study, treatment with a nutraceutical supplement containing resveratrol, procyanidin, and ellagic acid induced reduction of skin wrinkling, as well as reducing systemic and skin oxidative stress in a clinical setting [32]. These findings suggested that the *Sirt1*-mediated antioxidant activities of RSV contribute to attenuate skin damages in mammals. To rescue age-related changes in tissues of *Sod1*$^{-/-}$ mice, we have evaluated beneficial effects of several antioxidants *in vivo*. Ascorbic acid administration significantly attenuated bone loss and fragility of *Sod1*$^{-/-}$ mice [28]. Transdermal administration of ascorbic acid derivatives also normalized skin thinning of *Sod1*$^{-/-}$ mice [25, 26]. Furthermore, Iuchi et al. reported that oral N-acetylcysteine treatment mitigated hemolytic anemia of *Sod1*$^{-/-}$ mice by suppressed ROS generation in red blood cells [27]. Recently, Shibuya et al. showed that an SOD/catalase mimetic, PAPLAL, treatment attenuated skin atrophy [33]. These reports strongly supported that antioxidants, such as RSV, ascorbic acid, N-acetylcysteine, and PAPLAL, positively improved oxidative damage-induced organ pathologies.

As shown in Figure 2(c), *Sod1* deficiency showed upregulation of *p53* gene expression, which regulates cellular senescence and death, in skin (Figure 2(c)). We previously reported that *Sod1* loss induced $O_2^{\bullet-}$ generation and upregulated p53 protein level in skin fibroblasts [6]. Ascorbic acid derivatives significantly downregulated p53 expression and improved

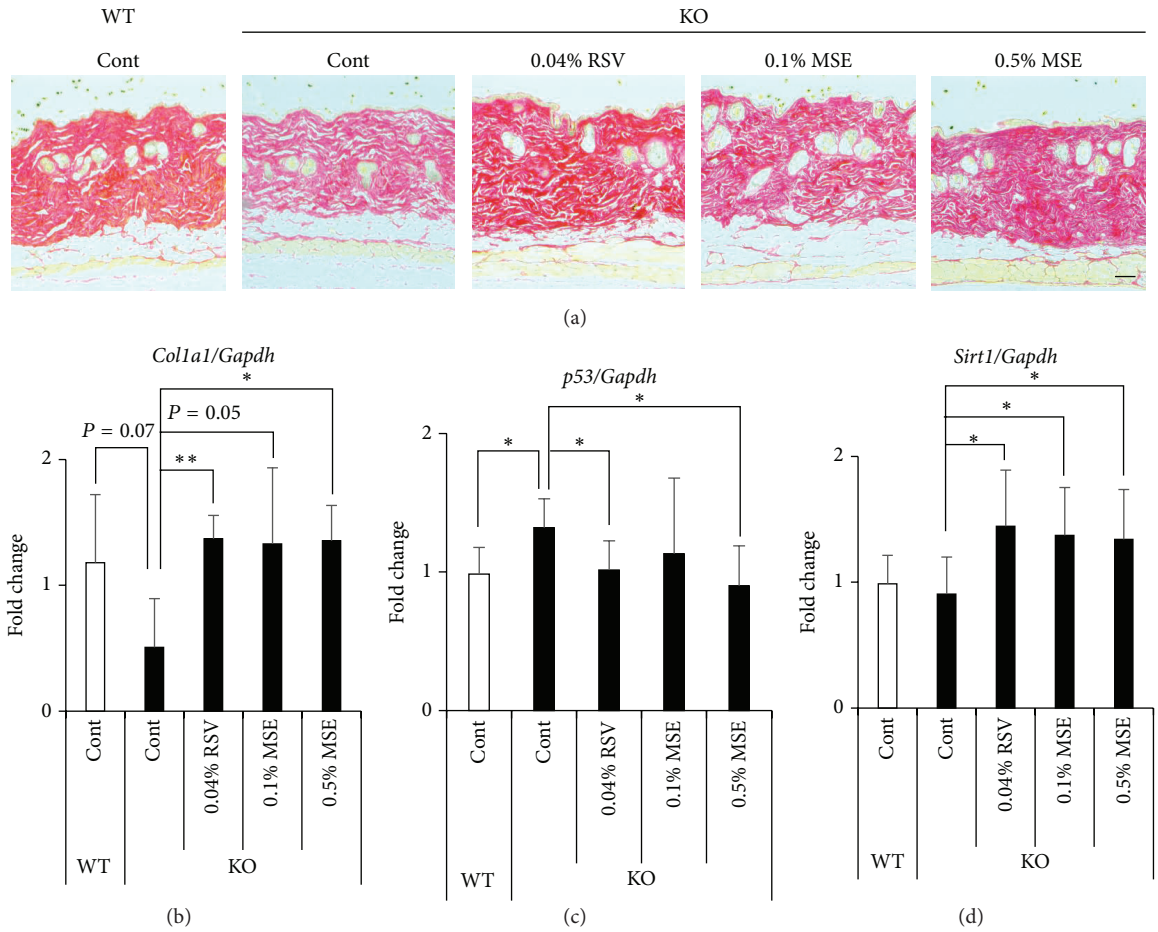

FIGURE 2: MSE and RSV attenuate collagen decline in skin tissues of *Sod1*$^{-/-}$ mice. (a) Sirius red staining of the back skin of *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice treated with the MSE or RSV. Relative mRNA expression of (b) *Sirt1*, (c) *Col1a1*, and (d) *p53*. Each of the mRNA expressions was determined by qRT-PCR (n = 8–12). The statistical evaluations were performed using the two-tailed Student's t-test for unpaired values. These data indicate the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$. The scale bar represents 100 μm.

cell viability in *Sod1*$^{-/-}$ fibroblasts [6, 25]. In a genetically modified model, p53 activation induced accelerated aging-like phenotypes, including skin atrophy, in p53 mutant mice [34]. Gannon et al. also reported that p53 activation by *Mdm2*-specific loss in keratinocytes induced epidermal stem cell senescence and atrophy in mice, suggesting that p53 activation in skin accelerated aging-like skin thinning in mice [35]. MSE and RSV treatment significantly downregulated mRNA level of *p53* in *Sod1*$^{-/-}$ skin (Figure 2(c)). These data indicated that MSE and RSV treatment may delay skin aging via reducing the p53 upregulation in skin.

RSV promotes the activity and expression of *Sirt1* [15]. MSE and RSV also normalized the gene expression of *Col1a1* and upregulated the gene expression of *Sirt1* in skin of *Sod1*$^{-/-}$ mice (Figures 2(b) and 2(d)). *Sirt1* upregulation by RSV may protect skin aging from oxidative damage in *Sod1*$^{-/-}$ mice. Actually, Lee et al. reported that RSV treatment or *Sirt1* overexpression significantly inhibited matrix metalloprotease-9 expression and appeared to protect collagen from degradation after ultraviolet radiation in human dermal fibroblasts and skin tissues [36]. Serravallo et al. reported that *Sirt1* plays a pivotal role in modulating skin diseases including psoriasis, autoimmune disease, cutaneous fungal infection, inherited dermatological diseases, and cancer [19]. These findings indicated that upregulation of *Sirt1* expression protected skin damages *in vivo*.

Recently, Konno et al. reported that RSV and MSE showed the agonistic activity for PPARα and PPARγ *in vitro* [12]. It is reported that a PPARα/γ dual agonist, MHY966, treatment significantly suppressed UVB-induced collagen digestion, lipid peroxidation, and inflammatory response via activating PPARα and PPARγ in mouse skin during photoaging [37]. Moreover, Mastrofrancesco et al. reported that PPARγ activation in skin normalized inflammatory response in IL-21-induced epithelial hyperplasia in mice [38]. These reports suggested that RSV and MSE may activate PPARα and PPARγ leading to attenuating the skin atrophy in *Sod1*$^{-/-}$ mice.

Finally, we, here, focused on RSV in MSE and antiatrophic effects of RSV in *Sod1*$^{-/-}$ skin. Since MSE also contains several RSV-derivatives such as gnetin C, gnemonoside A, and gnemonoside D, we cannot rule out antiatrophic effects of

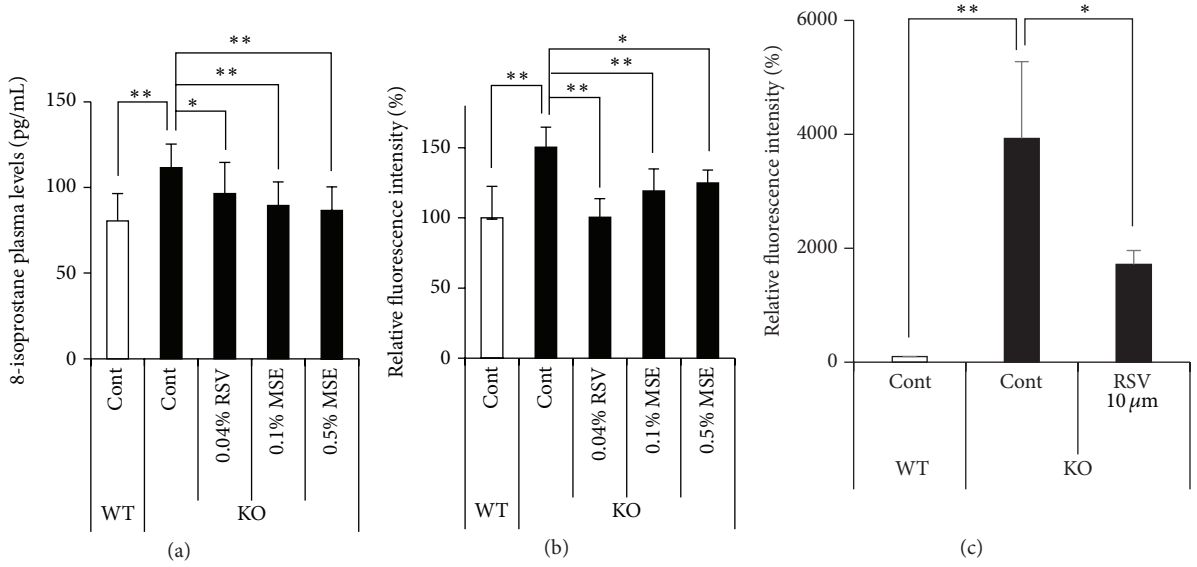

FIGURE 3: MSE and RSV decrease oxidative damage and ROS production. (a) 8-isoprostane content in plasma obtained from $Sod1^{-/-}$ and $Sod1^{+/+}$ mice treated with MSE and RSV (n = 10–12). (b) The intracellular ROS levels of bone marrow cells of $Sod1^{-/-}$ and $Sod1^{+/+}$ mice were measured using a DCF dye (n = 5-6). (c) The relative intracellular ROS level in $Sod1^{-/-}$ fibroblasts treated with 10 μM RSV for 72 h was measured by a DCF dye (n = 3). The statistical evaluations were performed using the Tukey's test. These data indicate the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$.

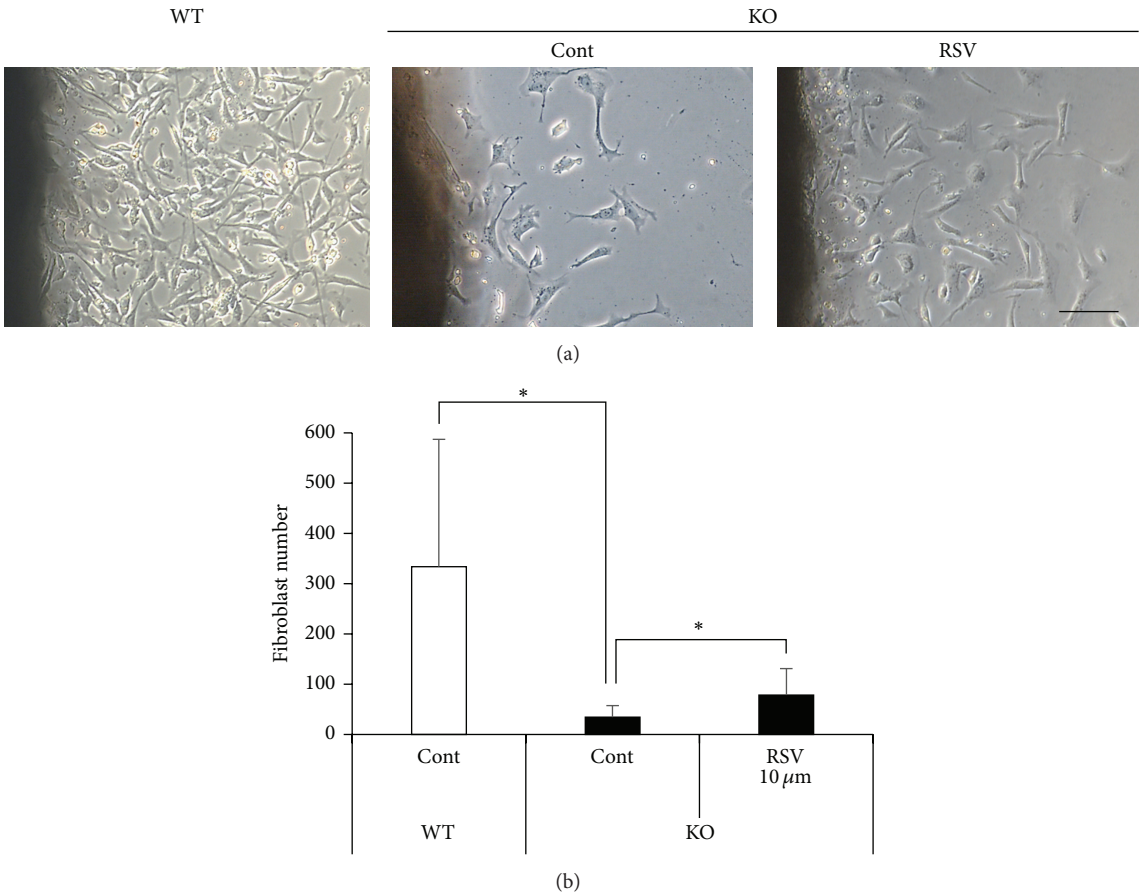

FIGURE 4: RSV promotes fibroblasts outgrowth from $Sod1^{-/-}$ skin. (a and b) Number of outgrowth fibroblasts of $Sod1^{+/+}$ and $Sod1^{-/-}$ mice in the skin disc culture treated with 10 μM RSV for 72 h (n = 8). Fibroblast number was counted on day 3. The statistical evaluations were performed using the two-tailed Student's t-test for unpaired values. These data indicate the mean ± SD; $^{*}P < 0.05$. The scale bar represents 100 μM.

the RSV derivatives in MSE. Further analysis should be needed to clarify the beneficial effect of other RSV derivatives in MSE on skin atrophy in *Sod1*$^{-/-}$ mice.

5. Conclusion

In the present study, we demonstrated that MSE and RSV treatment effectively attenuated aging-like skin pathologies accompanied by upregulation of *Sirt1* expression in *Sod1*$^{-/-}$ skin. MSE and RSV also exhibited less adverse effect on skin morphology. Consistent with our results, many interventions reported safety of MSE and RSV treatment in human. Therefore, MSE is useful for nutrient source of RSV as well as safety antioxidant for delaying skin aging in humans.

Conflict of Interests

This research was supported by the institute for Bee Products & Health Science, Yamada Bee Company, Inc.

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References

[1] B. Poljsak, R. G. Dahmane, and A. Godic, "Intrinsic skin aging: the role of oxidative stress," *Acta Dermatovenerol Alp Panonica Adriat*, vol. 21, no. 2, pp. 33–36, 2012.

[2] S. Shuster, M. M. Black, and E. McVitie, "The influence of age and sex on skin thickness, skin collagen and density," *British Journal of Dermatology*, vol. 93, no. 6, pp. 639–643, 1975.

[3] T. Finkel and N. J. Holbrook, "Oxidants, oxidative stress and the biology of ageing," *Nature*, vol. 408, no. 6809, pp. 239–247, 2000.

[4] T. Shimizu, H. Nojiri, S. Kawakami, S. Uchiyama, and T. Shirasawa, "Model mice for tissue-specific deletion of the manganese superoxide dismutase gene," *Geriatrics & Gerontology International*, vol. 10, supplement 1, pp. S70–S79, 2010.

[5] S. Shibuya, Y. Ozawa, T. Toda et al., "Collagen peptide and vitamin C additively attenuate age-related skin atrophy in Sod1-deficient mice," *Bioscience, Biotechnology, and Biochemistry*, vol. 78, no. 7, pp. 1212–1220, 2014.

[6] K. Watanabe, S. Shibuya, H. Koyama et al., "Sod1 loss induces intrinsic superoxide accumulation leading to p53-mediated growth arrest and apoptosis," *International Journal of Molecular Sciences*, vol. 14, no. 6, pp. 10998–11010, 2013.

[7] E. Kato, Y. Tokunaga, and F. Sakan, "Stilbenoids isolated from the seeds of melinjo (*Gnetum gnemon* L.) and their biological activity," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 6, pp. 2544–2549, 2009.

[8] K. Kunimasa, T. Ohta, H. Tani et al., "Resveratrol derivative-rich melinjo (*Gnetum gnemon* L.) seed extract suppresses multiple angiogenesis-related endothelial cell functions and tumor angiogenesis," *Molecular Nutrition and Food Research*, vol. 55, no. 11, pp. 1730–1734, 2011.

[9] M. Yanagihara, M. Yoshimatsu, A. Inoue, T. Kanno, T. Tatefuji, and K. Hashimoto, "Inhibitory effect of gnetin C, a resveratrol dimer from melinjo (*Gnetum gnemon*), on tyrosinase activity and melanin biosynthesis," *Biological and Pharmaceutical Bulletin*, vol. 35, no. 6, pp. 993–996, 2012.

[10] H. Ota, M. Akishita, H. Tani et al., "*trans*-resveratrol in *Gnetum gnemon* protects against oxidative-stress-induced endothelial senescence," *Journal of Natural Products*, vol. 76, no. 7, pp. 1242–1247, 2013.

[11] T. Tatefuji, M. Yanagihara, S. Fukushima, and K. Hashimoto, "Safety assessment of melinjo (*Gnetum gnemon* L.) seed extract: acute and subchronic toxicity studies," *Food and Chemical Toxicology*, vol. 67, pp. 230–235, 2014.

[12] H. Konno, Y. Kanai, M. Katagiri et al., "Melinjo (*Gnetum gnemon* L.) seed extract decreases serum uric acid levels in nonobese Japanese males: a randomized controlled study," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 589169, 9 pages, 2013.

[13] H. Tani, S. Hikami, S. Iizuna et al., "Pharmacokinetics and safety of resveratrol derivatives in humans after oral administration of melinjo (*Gnetum gnemon* L.) seed extract powder," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 8, pp. 1999–2007, 2014.

[14] M. Sato, Y. Suzuki, T. Okuda, and K. Yokotsuka, "Contents of resveratrol, piceid, and their isomers in commercially available wines made from grapes cultivated in Japan," *Bioscience, Biotechnology and Biochemistry*, vol. 61, no. 11, pp. 1800–1805, 1997.

[15] J. A. Baur and D. A. Sinclair, "Therapeutic potential of resveratrol: the *in vivo* evidence," *Nature Reviews Drug Discovery*, vol. 5, no. 6, pp. 493–506, 2006.

[16] B. P. Hubbard and D. A. Sinclair, "Small molecule SIRT1 activators for the treatment of aging and age-related diseases," *Trends in Pharmacological Sciences*, vol. 35, no. 3, pp. 146–154, 2014.

[17] J.-H. Jang and Y.-J. Surh, "Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells," *Mutation Research: Genetic Toxicology and Environmental Mutagenesis*, vol. 496, no. 1-2, pp. 181–190, 2001.

[18] V. M. Adhami, F. Afaq, and N. Ahmad, "Suppression of ultraviolet B exposure-mediated activation of NF-κB in normal human keratinocytes by resveratrol," *Neoplasia*, vol. 5, no. 1, pp. 74–82, 2003.

[19] M. Serravallo, J. Jagdeo, S. A. Glick, D. M. Siegel, and N. I. Brody, "Sirtuins in dermatology: applications for future research and therapeutics," *Archives of Dermatological Research*, vol. 305, no. 4, pp. 269–282, 2013.

[20] D. Morikawa, H. Nojiri, Y. Saita et al., "Cytoplasmic reactive oxygen species and SOD1 regulate bone mass during mechanical unloading," *Journal of Bone and Mineral Research*, vol. 28, no. 11, pp. 2368–2380, 2013.

[21] S. Uchiyama, T. Shimizu, and T. Shirasawa, "CuZn-SOD deficiency causes ApoB degradation and induces hepatic lipid accumulation by impaired lipoprotein secretion in mice," *The Journal of Biological Chemistry*, vol. 281, no. 42, pp. 31713–31719, 2006.

[22] H. Nojiri, T. Shimizu, M. Funakoshi et al., "Oxidative stress causes heart failure with impaired mitochondrial respiration," *Journal of Biological Chemistry*, vol. 281, no. 44, pp. 33789–33801, 2006.

[23] A. C. Nauta, M. Grova, D. T. Montoro et al., "Evidence that mast cells are not required for healing of splinted cutaneous

excisional wounds in mice," *PLoS ONE*, vol. 8, no. 3, Article ID e59167, 2013.

[24] K. Murakami, J. Inagaki, M. Saito et al., "Skin atrophy in cytoplasmic SOD-deficient mice and its complete recovery using a vitamin C derivative," *Biochemical and Biophysical Research Communications*, vol. 382, no. 2, pp. 457–461, 2009.

[25] S. Shibuya, K. Kinoshita, and T. Shimizu, "Protective effects of vitamin C derivatives on skin atrophy caused by Sod1 deficiency," in *Handbook of Diet, Nutrition and the Skin*, V. R. Preedy, Ed., pp. 351–364, Wageningen Academic, 2012.

[26] S. Shibuya, H. Nojiri, D. Morikawa, H. Koyama, and T. Shimizu, "Protective effects of vitamin C on age-related bone and skin phenotypes caused by intracellular reactive oxygen species," in *Oxidative Stress and Dietary Antioxidants*, V. R. Preedy, Ed., pp. 137–155, Academic Press, 2013.

[27] Y. Iuchi, F. Okada, K. Onuma et al., "Elevated oxidative stress in erythrocytes due to a SOD1 deficiency causes anaemia and triggers autoantibody production," *Biochemical Journal*, vol. 402, no. 2, pp. 219–227, 2007.

[28] H. Nojiri, Y. Saita, D. Morikawa et al., "Cytoplasmic superoxide causes bone fragility owing to low-turnover osteoporosis and impaired collagen cross-linking," *Journal of Bone and Mineral Research*, vol. 26, no. 11, pp. 2682–2694, 2011.

[29] Z. Ungvari, W. E. Sonntag, R. de Cabo, J. A. Baur, and A. Csiszar, "Mitochondrial protection by resveratrol," *Exercise and Sport Sciences Reviews*, vol. 39, no. 3, pp. 128–132, 2011.

[30] Y. Zhang, M. Zhang, H. Dong et al., "Deacetylation of cortactin by SIRT1 promotes cell migration," *Oncogene*, vol. 28, no. 3, pp. 445–460, 2009.

[31] N. L. Price, A. P. Gomes, A. J. Y. Ling et al., "SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function," *Cell Metabolism*, vol. 15, no. 5, pp. 675–690, 2012.

[32] D. Buonocore, A. Lazzeretti, P. Tocabens et al., "Resveratrol-procyanidin blend: nutraceutical and antiaging efficacy evaluated in a placebo-controlled, double-blind study," *Clinical, Cosmetic and Investigational Dermatology*, vol. 5, pp. 159–165, 2012.

[33] S. Shibuya, Y. Ozawa, K. Watanabe et al., "Palladium and platinum nanoparticles attenuate aging-like skin atrophy via antioxidant activity in mice," *PLoS ONE*, vol. 9, no. 10, Article ID e109288, 2014.

[34] S. D. Tyner, S. Venkatachalam, J. Choi et al., "p53 mutant mice that display early ageing-associated phenotypes," *Nature*, vol. 415, no. 6867, pp. 45–53, 2002.

[35] H. S. Gannon, L. A. Donehower, S. Lyle, and S. N. Jones, "Mdm2-p53 signaling regulates epidermal stem cell senescence and premature aging phenotypes in mouse skin," *Developmental Biology*, vol. 353, no. 1, pp. 1–9, 2011.

[36] J. S. Lee, K. Y. Park, H. G. Min et al., "Negative regulation of stress-induced matrix metalloproteinase-9 by Sirt1 in skin tissue," *Experimental Dermatology*, vol. 19, no. 12, pp. 1060–1066, 2010.

[37] M. H. Park, J. Y. Park, H. J. Lee et al., "The novel PPAR α/γ dual agonist MHY 966 modulates UVB-induced skin inflammation by inhibiting NF-κB activity," *PLoS ONE*, vol. 8, no. 10, Article ID e76820, 2013.

[38] A. Mastrofrancesco, D. Kovacs, M. Sarra et al., "Preclinical studies of a specific PPARγ modulator in the control of skin inflammation," *Journal of Investigative Dermatology*, vol. 134, no. 4, pp. 1001–1011, 2014.

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Research Article

Long Term Exposure to Polyphenols of Artichoke (*Cynara scolymus* L.) Exerts Induction of Senescence Driven Growth Arrest in the MDA-MB231 Human Breast Cancer Cell Line

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Polyphenolic extracts from the edible part of artichoke (*Cynara scolymus* L.) have been shown to be potential chemopreventive and anticancer dietary compounds. High doses of polyphenolic extracts (AEs) induce apoptosis and decrease the invasive potential of the human breast cancer cell line, MDA-MB231. However, the molecular mechanism underlying AEs antiproliferative effects is not completely understood. We demonstrate that chronic and low doses of AEs treatment at sublethal concentrations suppress human breast cancer cell growth via a caspases-independent mechanism. Furthermore, AEs exposure induces a significant increase of senescence-associated β-galactosidase (SA-β-gal) staining and upregulation of tumour suppressor genes, $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ in MDA-MB231 cells. AEs treatment leads to epigenetic alterations in cancer cells, modulating DNA hypomethylation and lysine acetylation levels in total proteins. Cell growth arrest correlates with increased reactive oxygen species (ROS) production in AEs treated breast cancer cells. Inhibition of ROS generation by N-acetylcysteine (NAC) attenuates the antiproliferative effect. These findings demonstrate that chronic AEs treatment inhibits breast cancer cell growth via the induction of premature senescence through epigenetic and ROS-mediated mechanisms. Our results suggest that artichoke polyphenols could be a promising dietary tool either in cancer chemoprevention or/and in cancer treatment as a nonconventional, adjuvant therapy.

1. Introduction

Cancer is one of the main causes of death worldwide, claiming over six million people each year [1]. According to current estimates, cancer is 30–40% preventable over time with appropriate nutrition, regular physical activity, and avoidance of obesity [2].

Chemoprevention is a promising strategy which uses natural dietary compounds and/or synthetic substances to block, inhibit, reverse, or delay the process of carcinogenesis. Important preventive mechanisms include suppression of cell proliferation and apoptosis and modulation of epigenetic processes [3–6].

Many epidemiological studies suggest that diets particularly rich in fruits and vegetables have cancer preventive properties [7–9]. The beneficial effect of diets is attributable, at least in part, to polyphenols which have antitumour activities both in animal models and in humans [8–14]. Furthermore, several clinical trials testing the efficacy of natural compounds are underway, http://www.clinicaltrials.gov/; some of them are already concluded and demonstrate the chemopreventive activity of dietary polyphenols [15–17].

The current growing interest for dietary plants has led to a renewed attention for artichoke because of its high polyphenolic content. In the edible part, it mainly consists of hydroxycinnamic derivatives, in particular chlorogenic and dicaffeoylquinic acids. Our studies of bioavailability demonstrate that ferulic acid is one of the metabolites present in human plasma after an artichoke meal [18]. Preclinical reports indicate that ferulic acid has antiproliferative and chemopreventive activities *in vitro* and *in vivo* [19–21] and proposed the potential use of ferulic acid as an adjuvant agent during chemo- and/or radiotherapy [22, 23].

Our previous findings indicate that polyphenolic artichoke extracts (AEs) protected hepatocytes from oxidative

stress and exhibited cancer chemopreventive properties, in part, by triggering apoptosis on human hepatoma cells Hep G2 [24] and on the human breast cancer cell line, MDA-MB231 [25].

Despite the growing scientific results regarding chemopreventive activities of natural dietary compounds [8], the cellular mechanisms underlying antitumour property of polyphenols are yet to be elucidated.

Cellular senescence, a state of cell cycle arrest, can be considered a relevant mechanism of tumour suppression [26–28]. Furthermore, emerging evidence has demonstrated that therapy-induced senescence is a critical mechanism through which many anticancer agents inhibit tumour progression [29–31]. Importantly, therapy-induced senescence can be achieved in administering agents at low doses. This approach can significantly reduce the side effects of conventional anticancer therapy, thus improving the quality of life for cancer patients [29, 30]. Innovative senescence therapies will be developed through improved knowledge of the molecular pathways controlling permanent growth arrest by specifically screening for senescence effectors.

Scientific evidence from *in vitro* studies indicates that the cancer prevention activity involved modulation of epigenetic processes. Epigenetics is defined as heritable changes in gene expression that are not accompanied by alterations in DNA sequence [32]. The main epigenetic processes are DNA methylation, histone modifications, and chromatin remodeling. Aberrant patterns of gene expression are key features of cancer and both genetic and epigenetic abnormalities are implicated in this molecular deregulation. In contrast to genetic modifications, epigenetic alterations are potentially reversible and strategies targeting the epigenome have been proposed for both cancer prevention and therapeutics [33].

Induction of premature senescence and modulation of epigenetic processes have been identified as relevant anticancer features of dietary polyphenolic compounds [34]. There are both *in vitro* and *in vivo* data showing that several bioactive food components can interfere with DNA methylation and histone modifications and affect the expression of genes involved in the carcinogenic process [35]. In particular, phenolic acids, including chlorogenic acid, have been shown to affect DNA methylation [36].

Since the induction of senescence requires moderate concentrations of anticancer agents and produces almost no side effects [37], we sought to investigate whether a low dose and chronic treatment of AEs could inhibit the growth of breast cancer cells through the induction of premature senescence. In the present study, we demonstrate that a moderate and chronic treatment of AEs causes a significant increase in senescence-associated β-galactosidase (SA-β-gal) detection and upregulation of $p21^{Cip1/Waf1}$ and $p16^{INK4a}$ protein expression in MDA-MB231 cells. Such a premature senescence is via ROS-mediated DNA damage and is associated with the modulation of epigenetic machinery. Altogether, these results highlight a significant contribution of senescence induction to AEs anticancer effects.

2. Materials and Methods

2.1. Artichoke Extract Preparation. The edible part (head) of fresh artichoke (*Cynara scolymus* L. cv Violetto di Provenza) buds was used for extract preparation and the analysis of polyphenols contained in the extracts was performed by HPLC as previously described [25].

2.2. Cell Lines and Cultured Conditions. Cell lines were maintained in a humidified incubator with 5% CO_2 and 95% air at 37°C. HCT 116 cells, human colon carcinoma cell line, MDA-MB231, oestrogen receptor-negative breast cancer cells, HEY cells, human ovarian cell line, and K-562 cells, human erythromyeloblastoid leukaemia cell line (kindly supplied by Dr. Maurizio Fanciulli, Dr. Paola Nisticò, and Dr. Maria Giulia Rizzo, Regina Elena National Cancer Institute Rome) were grown in RPMI medium (Invitrogen Life Technologies, Milan, Italy) supplemented with 10% FBS, 10 IU/mL penicillin, and 10 μg/mL streptomycin. GTL-16 cells, human gastric carcinoma cell line, DU 145 cells, human prostate carcinoma cell line, A549 cells, human lung carcinoma cell line, M14 cells, human melanoma cell line, U-373 MG cells, human astrocytic cell line, and Saos-2 cells, human osteosarcoma cell line (kindly supplied by Dr. Giovanni Blandino, Regina Elena National Cancer Institute, and Dr. Antonella Farsetti, CNR Rome, purchased from American Type Culture Collection, Rockville, MD, USA) were grown in D-MEM supplemented with 10% FBS, 10 IU/mL penicillin, and 10 μg/mL streptomycin.

2.3. Reagents. Artichoke extracts were dissolved in PBS and 0.1% dimethylsulfoxide (Me_2SO, Sigma-Aldrich, Milan, Italy). Paclitaxel (Ptx, Sigma-Aldrich) was dissolved in PBS. 5-Bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) was purchased from IBI Scientific (Peosta IA, USA) and used as previously described [38]. N-Acetyl-cysteine (NAC, Sigma-Aldrich) was dissolved in PBS. Dihydroethidium (DHE, Molecular Probes-Invitrogen UK) was dissolved in Me_2SO.

2.4. Cytotoxicity and Cell Proliferation Assays. Human cancer cells were seeded at density of 2.0×10^5 in 6-well plates (Sarstedt, Numbrecht, Germany) in triplicate and cultured for 24 h before adding either the vehicle (0.1% Me_2SO) or AEs. Cytotoxicity experiments were assessed in serum-free medium and cells were treated with AEs (200, 400, 600, and 800 μM) for 24 h.

For the cell proliferation experiment, MDA-MB231 were seeded at a density of 5×10^3 in 6-well plates in triplicate. After 24 h the cells were exposed to AEs (10 and 30 μM) for 10 days. To determine the role of ROS in AEs-mediated senescence, cells were pretreated with 10 mM NAC for 2 h and then exposed to AEs (10 and 30 μM) for 10 days.

Cells were harvested by trypsinization, stained with 0.4% trypan blue (Sigma-Aldrich), and counted using a haemocytometer.

2.5. Senescence-Associated β-Galactosidase (SA-β-gal) Assay. MDA-MB231 cells were seeded at a density of 5×10^3 in

6-well plates in triplicate and cultured for 24 h before adding either the vehicle or AEs (10 and 30 μM). After 10 days of a chronic treatment, with every 48 h renewal of medium and treatment, cultured cells were washed in PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde for 15 minutes at room temperature. Cells were then washed and incubated with fresh SA-β-gal staining solution containing 1 mg/mL X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$ for 16–18 h at 37°C. Blue-stained senescent cells were counted using a light microscopy (Olympus IX71-Olympus America, Center Valley, PA, USA) and the images were captured by digital camera (Olympus-Camedia C-5060).

2.6. Western-Blot Analysis. To prepare the whole-cell extract, cells were washed with PBS and suspended in ice cold RIPA lysis buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitors. After 30 minutes of mixing at 4°C, the mixture was centrifuged (10,000 ×g) for 10 minutes and the supernatants were collected as whole-cell extracts. The protein content was determined with a protein assay reagent (Bio-Rad, Milan, Italy) using bovine serum albumin as a standard. An equal protein content of total lysates (25 μg) from the control and from AEs treated samples was resolved on 10% SDS-PAGE with molecular weight markers (Bio-Rad). Proteins were then transferred to the PVDF membrane (EMD Millipore Billerica, MA, USA) and reacted with anti-$p16^{INK4a}$ and anti-$p21^{Cip1/Waf1}$ antibodies (Santa Cruz Biotechnology, CA, USA), acetylated-lysine polyclonal antibody (Cell Signalling Technology Inc., Danvers, MA, USA), and anti-actin antibody (Calbiochem-EMD Millipore Billerica, MA, USA) for protein normalization. The protein bands were revealed by chemiluminescence using an ECL detection kit (Amersham Bioscience, Cologno Monzese, Milan, Italy). Autoradiograms were quantified with ImageJ 1.43 software (National Institutes of Health, Bethesda, MD, USA).

2.7. Dot Blot Analysis of DNA 5-Methylcytosine and 5-mC Immunostaining. MDA-MB231 cells were seeded at a density of 2.5×10^3 in 6-well plates in triplicate and cultured for 24 h before adding either the vehicle or various concentrations of AEs (2.5, 5, 10, and 30 μM) for 10 days and then harvested. Genomic DNA was isolated using the Wizard DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, transferred onto positively charged Hybond N+ membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using dot filtration apparatus and fixed by baking the membrane for 30 minutes at 80°C. After baking, membranes were incubated with 5 μg/mL of 5-methylcytosine (5-mC) antibody (Calbiochem, San Diego, CA, USA) followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Membranes were then treated with chemiluminescence detection reagents and exposed to Kodak autoradiograph films. The intensity of each dot was quantified with ImageJ 1.43 software.

For the immunostaining analysis, cells (1×10^5) were cytospun using a Shandon Cytospin 3 (Thermo Scientific, Waltham, MA, USA) at 1000 rpm for 10 minutes and then processed for 5-mC immunostaining. Cells were permeabilized with 0.4% triton X-100 in PBS for 20 minutes. Cells were washed with PBS for 10 minutes and then blocked for 30 minutes with 3% preimmune goat serum in PBS. After 20 minutes of incubation with 3% H_2O_2 in order to quench endogenous peroxidase the cells were washed with PBS and incubated with 5-mC specific antibody (1 : 500 vol/vol) for 2 h. Cells were sequentially incubated with biotinylated prediluted antibody, horseradish peroxidase-conjugated streptavidin (UltraTek HRP, ScyTek Laboratories Inc, Logan, UT, USA) and AEC substrate (AEC substrate kit, ScyTek Laboratories Inc.) and counterstained with haematoxylin.

2.8. Intracellular ROS Detection. MDA-MB231 cells were treated with either the vehicle or AEs (10 and 30 μM). After 10 days, the oxidation-sensitive fluorescent probe DHE was used to assess the production of cytosolic superoxide anions. Briefly, at the end of exposure, cells were incubated with 5 μM DHE for 40 minutes at 37°C in the dark and then rinsed twice with PBS. The cell-permeant DHE entered the cells, was oxidized by superoxide anions to form ethidium (ETH) which binds to DNA, and produced the fluorescent ETH-DNA. The fluorescent signals were obtained exciting the cultured cells at λ_{ex} 300 nm and λ_{em} 610 nm. Cells were visualized and counted by fluorescence microscope (Olympus IX71-Olympus America).

The role of ROS on AEs-treated cell proliferation was evaluated using the antioxidant NAC. Cells were preincubated with 10 mM NAC for 2 h and then exposed to AEs. After 10 days, cells were harvested by trypsinization, stained with 0.4% trypan blue, and counted using a haemocytometer.

2.9. Statistical Analysis. Statistical analyses were performed by Student's t-test using GraphPad 5.1 software. For all statistical tests, a two-tailed p value <0.05 was considered significant. All data reported were verified at least in three independent experiments and expressed as mean ± SD.

3. Results

3.1. Phenolic Composition of Artichoke Extracts. The artichoke extracts were found to contain monocaffeoylquinic acids (MCQA), dicaffeoylquinic acids (DCQA), and small amounts of a luteolin and an apigenin glycoside. The main phenolic components of the AEs found were chlorogenic acid and two dicaffeoylquinic acids (3,5-DCQA and 1,5-DCQA) at a ratio of about 1 : 1 : 1. The concentrations of chlorogenic acid, 3,5-DCQA, and 1,5-DCQA, determined by HPLC, as previously described [25], were found to be 725 ± 70, 738 ± 58, and 632 ± 48 mgL^{-1}, respectively.

3.2. Effects of AEs on Human Cancer Cell Lines Viability. We have previously reported that AEs exhibited cancer chemopreventive activities on a human hepatoma cell line, Hep G2 [24], and on a human breast cancer cell line, MDA-MB231

Table 1: Cytotoxicity of AEs in human cancer cell lines.

	AEs μM									
	NT		200		400		600		800	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
HCT 116	84.5	3.7	77.2	6.1	84.0	2.7	79.0	4.0	77.0	4.5
GLT-16	93.5	1.3	89.2	0.9	89.5	4.5	83.2^{**}	2.7	69.7^{***}	3.6
MDA-MB231	90.0	2.6	83.7	1.5	81.7^{*}	1.0	43.2^{***}	1.0	16.0^{***}	1.4
HEY	91.7	2.2	91.0	3.6	90.0	2.1	73.5^{*}	4.9	27.7^{***}	1.7
DU 145	92.7	2.3	93.0	2.9	94.0	2.4	80.0^{**}	3.4	51.0^{***}	2.9
A549	86.5	5.9	86.2	5.5	82.5	11.2	70.0	2.0	28.3^{**}	3.5
M14	88.2	5.6	83.0	2.9	77.7	10.9	74.3	2.5	39.6^{**}	3.2
U-373 MG	93.7	3.7	95.0	1.4	90.2	6.3	91.5	1.7	16.5^{***}	2.0
Saos-2	89.8	2.3	86.5	6.5	85.3	4.6	37.3^{***}	6.9	18.3^{***}	1.5
K-562	89.7	4.3	15.2^{***}	4.3	2.7^{***}	1.2	3.7^{***}	1.5	1.5^{***}	0.6

Ten tumour cell lines derived from various human tissues are treated with increasing concentrations of AEs (from 200 to 800 μM) for 24 h. The results are the mean ± SD of at least three independent experiments. The statistical significance between groups was calculated using Student's t-test. Significant differences are indicated by asterisks.
GLT-16: $^{**}p = 0.0092$, $^{***}p = 0.0009$.
MDA-MB231: $^{*}p = 0.0129$, $^{***}p < 0.0001$.
HEY: $^{*}p = 0.0119$, $^{***}p < 0.0001$.
DU 145: $^{**}p = 0.0041$, $^{***}p < 0.0001$.
A549: $^{**}p = 0.0029$.
M14: $^{**}p = 0.0023$.
U-373 MG: $^{***}p = 0.0001$.
Saos-2: $^{***}p < 0.0002$.
K-562: $^{***}p < 0.0002$.

[25]. To investigate whether the antiproliferative activity of AEs could be extended to other tumours, we describe the effect of AEs on 10 cancer cell lines derived from various human tissues, as shown in Table 1. This panel provides a way of presenting the cellular sensitivity or resistance at three levels of effect. After 24 h, 800 μM AEs caused about 50% of death in prostate and melanoma cells; more than 50% of toxicity was detected in breast, ovary, lung, brain, bone, and leukaemia cells. Less than 50% of dead cells were observed in colon and gastric cancers. The more resistant cancer cells, such as gastric and colon, showed sensitivity to 1200 μM AEs (unpublished results). These data indicated that high doses of AEs are cytotoxic for all tested cancer cell lines without any effect on untreated counterparts.

3.3. Low Doses of AEs Inhibit Breast Cancer Cell Growth via a Caspases-Independent Mechanism. We have previously demonstrated [25] that high concentrations of AEs (from 200 to 800 μM for 24 h) are able to activate an apoptotic program in MDA-MB231 to halt tumour progression. Since bioactive compounds concentrations required to induce apoptosis in tumour cell lines might not be reachable in target tissues, we asked whether AEs chronically administered at low and sublethal doses can affect the growth of tumour cells as well. To this purpose, we focused on MDA-MB231, which provide the scientific rationale for testing AEs as an antitumour agent against invasive and hormone resistant breast cancer phenotype. After 10-day treatment (from 2.5 μM to 60 μM) direct cell count assay indicated that the cellular growth was significantly inhibited by chronic exposure to low doses of AEs (Figure 1(a)). Treatments up to 30 μM resulted in a relevant inhibition of cell proliferation in viable cells (about 90%); the highest concentration (60 μM) induced a dramatic growth arrest and was slightly cytotoxic (about 70% viability). Based on these data, we focused on two noncytotoxic concentrations of AEs, namely 10 and 30 μM, that modulate cell growth. Furthermore, we investigated whether these treatments caused a reduction in the number of living cells through the activation of caspases pathways. As shown in Figure 1(b), in these experimental conditions protein expression analysis revealed that caspase-9 was not activated. Conversely, high concentration of AEs (400 μM), used as positive control [25], triggers a significant activation/cleavage of caspase-9 after 24 h treatment. In addition, long term exposure to low concentrations of AEs did not activate caspase-8 (unpublished results). Altogether our results strongly suggested that low doses of AEs inhibit breast cancer cell growth via a caspases-independent mechanism.

3.4. AEs Induce Premature Senescence in Breast Cancer Cells. We investigated whether the cell growth arrest, in response to low doses of AEs, was caused by the induction of cellular senescence as demonstrated for chronic treatment of several polyphenols in cancer cells [39–42]. The detection of β-galactosidase positive cells reflects an increase in lysosomal mass in aging cells and it is widely regarded as a marker for senescence [43]. MDA-MB231 cells incubated without AEs showed no detectable SA-β-gal activity, whereas cells treated with 10 and 30 μM AEs revealed a marked X-gal staining in a dose-dependent manner after 10 days, as depicted in Figure 2. Most of the SA-β-gal positive cells showed an enlarged and flattened morphology with increased volume and granularity

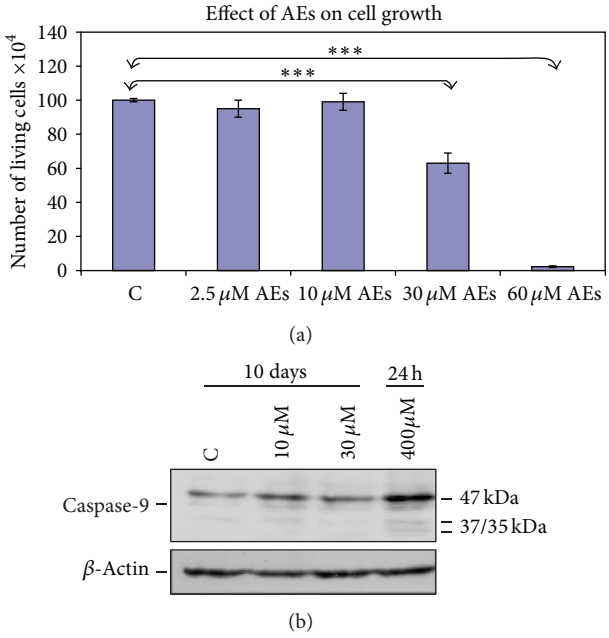


Figure 1: Low doses of AEs suppress breast cancer cell growth via a caspases-independent mechanism. (a) Cell growth inhibition by AEs. MDA-MB231 cells were treated with increasing concentrations of AEs (from 2.5 to 60 μM) for 10 days. The results are the mean ± SD of at least three independent experiments. Significant statistical differences are indicated by asterisks: $^{***}P < 0.0001$. (b) Effects of AEs on caspases pathway. The cells were treated with low doses of AEs (10 and 30 μM) for 10 days or with a high concentration of AEs (400 μM) for 24 h, as a positive control. Whole cell lysates (25 μg/lane) were tested for activation and cleavage of caspase-9. β-Actin was used as a protein loading control.

that were consistent with cellular senescence status, as shown in magnification area of Figure 2.

To further investigate this antiproliferative mechanism, we examined the expression levels of $p21^{Cip1/Waf1}$ and $p16^{INK4a}$, two pivotal cell cycle regulators involved in cellular senescence [44, 45]. Western blotting data demonstrated that the expression levels of $p21^{Cip1/Waf1}$ and $p16^{INK4a}$ were significantly increased in a dose-depending manner in AEs-treated cells (Figures 3(a) and 3(b)). Optical density measurements were performed using ImageJ software to obtain quantitative values for the protein expressions. Altogether, these findings suggested that low doses and chronic exposure to AEs induced senescence in MDA-MB231 breast cancer cells.

3.5. Effect of AEs on Global Methylation Level in Breast Cancer Cells. DNA hypermethylation is a major epigenetic modification that leads to silencing of tumour suppressor genes which may contribute to cancer development and progression [46]. Recent investigations suggest that some dietary phytochemicals may prevent cancer by modulating epigenetic processes [35, 47]. To examine whether chronic treatment has epigenetic effects on the DNA methylation level, cells were exposed to increasing concentrations of AEs, as shown in Figure 4. After 10 days, cells were harvested for immunocytostaining of DNA methylation using an antibody specific to 5-methylcytosine (5-mC). Treatment of AEs resulted in a reduced number of 5-mC-positive cells in a dose-dependent manner compared to untreated cells (Figures 4(a) and 4(b)).

To further verify the effect of AEs on DNA methylation, genomic DNA was isolated from cells and analysed by dot blot assay using anti-5mC antibody. As shown in Figure 4(c), AEs treatment of MDA-MB231 cells significantly decreased the DNA global methylation level as quantified by densitometric values. These results demonstrated that AEs have a relevant effect on regulation of DNA methylation machinery.

3.6. Effect of AEs on Acetylation of Total Proteins on Human Breast Cancer Cells. Protein acetylation of lysine residues is an important reversible modification controlling cellular protein expression [48]. We investigated the effect of AEs treatment on acetylation of total proteins in breast cancer cells. MDA-MB231 cells were cultured for 24 h before adding either the vehicle or various concentrations of AEs (10 and 30 μM) for 10 days and then harvested. As shown in Figure 5, the level of protein acetylation is markedly increased in treated cells as indicated by reported densitometric values. These results provide evidence that long term exposure to low concentrations of AEs is associated with increased level of lysines acetylation of total proteins.

3.7. AEs Treatment Increases ROS Production in Breast Cancer Cells. Since reactive oxygen species (ROS) are well-known inducers of cellular senescence, we tested whether AEs increase oxidative stress in breast cancer cells. To accomplish this, cells were incubated with DHE which is an indicator of the presence of superoxide anion, key radical in ROS generation. As shown in Figures 6(a) and 6(b) AEs-treated cells, according to the increased number of bright red fluorescent cells, showed enhanced level of superoxide anions compared to control cells.

To determine the role of ROS in AEs-induced growth arrest, we sought to examine whether inhibition of ROS production by antioxidant NAC has any impact on senescence in breast cancer cells. As shown in Figure 6(c), the presence of NAC significantly reduced the antiproliferative effect of 30 μM AEs for long term exposure. Furthermore, NAC treatment decreased percentage of SA-β-gal positive cells (unpublished results). According to data in literature regarding the natural products modulation of ROS expression in senescence [39, 41, 49], our findings strongly support the hypothesis that an oxidative pathway is involved in AEs-induced growth arrest in breast cancer cells.

4. Discussion

In this report, we provide evidence demonstrating that low doses and chronic AEs-treatments exert anticancer activity through induction of premature senescence in MDA-MB231, a triple negative and highly aggressive breast cancer cell line. Experimental data demonstrate that moderate doses of AEs inhibit the growth of breast cancer cells via a caspases-independent mechanism.

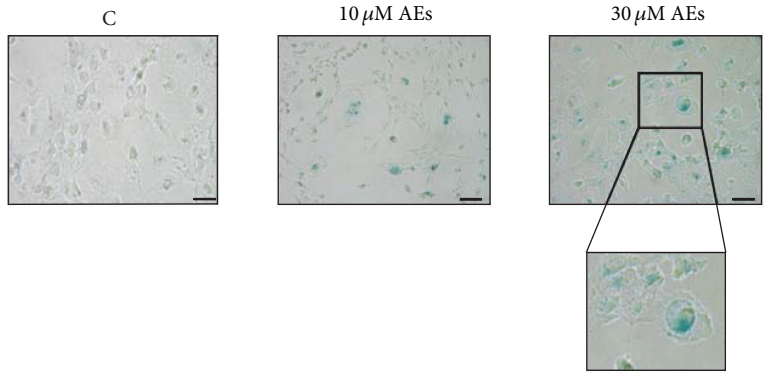


Figure 2: Chronic treatment of AEs induces cell senescence. MDA-MB231 cells were treated with low doses of AEs (10 and 30 μM) for 10 days and then analyzed for the SA-β-gal, senescence-associated β-galactosidase activity. The image shown is representative of at least three independent experiments. Scale bar: 50 μm. The senescent cells versus total cells were counted in random fields under an inverted microscope (20x) and the following data are the mean ± SD: C = 7.7 ± 0.5, 10 μM AEs = 31 ± 5.6, $p = 0.0044^{**}$, 30 μM AEs = 51 ± 6.6, $p = 0.0005^{***}$. Significant statistical differences are indicated by asterisks. Boxed area, regarding blue cells with a typical senescent flattened and enlarged morphology, is magnified (2x).

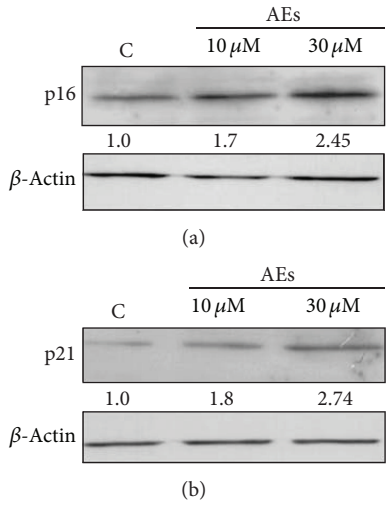


Figure 3: Chronic treatment of AEs induces increased $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ expression. MDA-MB231 cells were treated with low doses of AEs (10 and 30 μM) for 10 days. Whole cell lysates were tested for (a) $p16^{INK4a}$ and (b) $p21^{Cip1/Waf1}$ protein expression. Immunoblots are representative of at least three independent experiments. Intensities of electrophoretic bands relative to the immunoblot shown were quantified by densitometry and the values are reported.

Several reports point to a crucial physiological role for cellular senescence in fighting tumorigenesis. Cellular aging is a state of cell cycle arrest induced at the end of the cellular life span or in response to agents causing oxidative stress and DNA damage. Moreover, senescence is regarded as an important mechanism either for fighting premalignant tumours and/or for inducing tumour regression [2] since it limits the proliferative capacity of cells.

Increasing evidence has demonstrated that many phytochemicals exert anticancer and chemopreventive activities through the induction of senescence growth arrest [39–42].

Beside cell cycle inhibitors, $p21^{Cip1/Waf1}$ and $p16^{INK4a}$ are identified as senescence markers [44, 45].

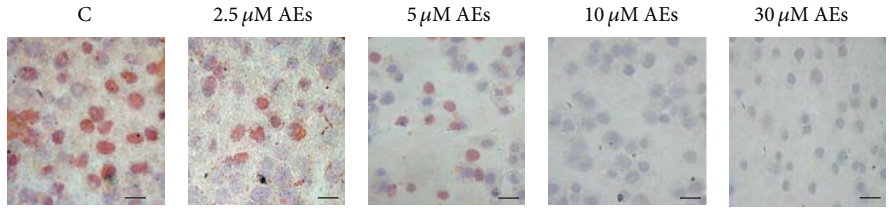
Both $p21^{Cip1/Waf1}$ and $p16^{INK4a}$ pathway induction can lead to the inhibition of pRb phosphorylation by inhibiting CDK2/cyclinE and CDK4/cyclinD complex, respectively. Furthermore, $p21^{Cip1/Waf1}$ and $p16^{INK4a}$ are likely to cooperate to keep pRb in a hypophosphorylated form during senescence [50]. According to data regarding the prosenescence property of a derivative of caffeic acid [51], we showed that low doses of AEs containing mono- and dicaffeoylquinic acids induce an increased number of SA-β-gal positive and flattened cells with enhanced expression of $p21^{Cip1/Waf1}$ and $p16^{INK4a}$.

High doses of AEs treatment induces cell death [25] in breast cancer as well as in other cell lines derived from various human cancers (Table 1) whereas AEs chronically administered on MDA-MB231 exert antiproliferative activity via induction of a caspases-independent mechanism.

This is a significant finding since the major challenge for anticancer therapeutic strategy leading to apoptosis *in vitro* is that effective concentrations of an antitumour agent should be too high to be reachable *in vivo* [52].

Data in literature are consistent with our results since dietary polyphenols [42, 49, 53, 54] activate apoptotic machinery when used at high doses whereas low level treatments induce senescence in cancer cells.

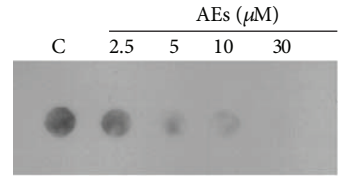
The senescence program involves epigenetic processes such as DNA methylation, histone modifications, and chromatin remodeling. DNA hypermethylation is a major epigenetic mechanism in the silencing of tumour suppressor genes. Protein lysine acetylation is a posttranslational change that has long been known to play a prominent role in the regulation of gene expression via modulation of chromatin structure. This epigenetic process involves modification of histone and nonhistone proteins through acetyltransferase (HATs) and deacetylase (HDACs) activities. Abnormal DNA methylation and dysregulated histone acetylation are implicated in numerous reported diseases,



(a)

	Mean	SD	p value
C	47.3	±2.5	
2.5 μM AEs	27.3	±2.1	0.0004***
5 μM AEs	24.3	±2.9	0.0005***
10 μM AEs	1.3	±0.8	<0.0001***
30 μM AEs	1.3	±0.8	<0.0001***

(b)



(c)

Figure 4: Effect of AEs on global DNA methylation levels in MDA-MB231 cells. Low doses of AEs (2.5–30 μM) for 10 days treatment decreased levels of 5-mC, 5-methylcytosine, in a dose-dependent manner. 5-mC specific antibody was used for (a) and (b) cytostaining and (c) for dot blot analysis on genomic DNA. (a) The image shown is representative of at least three independent experiments. Scale bar: 100 μm. (b) The 5-methylcytosine positive cells versus total cells were counted by using an inverted microscope (20x) and the results are reported as mean ± SD. (c) The intensity of individual DNA dots shown was quantified by densitometry.

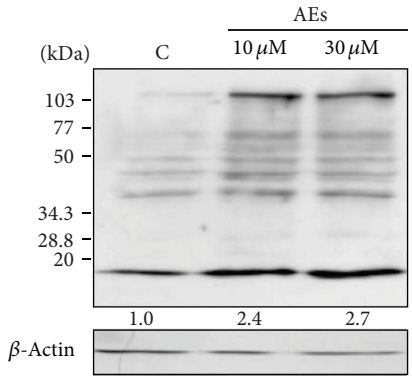


Figure 5: Effect of AEs on lysine acetylation of total proteins in MDA-MB231 cells. Cells were treated with low doses of AEs for 10 days. Cell lysates were analyzed for lysine acetylation of total proteins. Immunoblot is representative of at least three experiments. The intensities of electrophoretic bands relative to the immunoblot shown were quantified by densitometry.

including cancer [55]. In contrast to genetic modifications, epigenetic deregulation is potentially reversible and therapeutic strategies, targeting the epigenome, have been proposed for both cancer prevention and clinical treatment [56]. Many epigenetic modulators have been used in clinical trials (either completed or terminated) to treat human cancers (http://www.clinicaltrials.gov/). According to results of preclinical studies and clinical trials, epigenetic modulators, administered as monotherapy or in combination with conventional chemo- and radiotherapies, are potentially very useful [57].

Modulation of epigenetic processes has been identified as a relevant anticancer feature of many dietary polyphenolic compounds [33, 34, 58, 59]. There are both *in vitro* data and *in vivo* data showing that several bioactive food components can interfere with DNA methylation and histone modification affecting the expression of gene involved in the carcinogenic process [35]. Epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent of green tea, has been demonstrated to inhibit DNA methyl transferase in several cancer cell lines, including MDA-MB231 cells [60]. This activity was associated with promoter demethylation and reactivation of $p16^{INK4a}$. The positive clinical efficacy of EGCG in the treatment or prevention of chronic lymphocytic leukaemia [16] or prostate cancer [15] has been evaluated. A synergistic mixture of green tea plus capsicum has been demonstrated to have a chemopreventive effect versus different types of cancer in subjects testing positive for ENOX2, (ecto-nicotinamide adenine dinucleotide oxidase disulfide-thiol exchanger 2), which is ideally suited as a target for early diagnosis of cancer as well as for preventive intervention [17]. Curcumin has the potential to treat a wide variety of diseases including cancer [61]. Its epigenetic modulator activity has been a focus in clinical trials (terminated or completed). Phenol derivatives, including quercetin and resveratrol, have been shown to possess epigenetic properties through sirtuin activation [57]. Phenolic acids, including chlorogenic acid, the main component of AEs, have been shown to affect DNA methylation [36].

According to these data, we observed consistent DNA hypomethylation and increased total acetylation protein levels in AEs-treated MDA-MB231 cells.

It has been demonstrated that several anticancer polyphenolic compounds from fruit and vegetables induce tumour cellular growth arrest largely through the generation of ROS [62]. Low doses of resveratrol inhibit cell growth and enhance radiosensitization via the induction of ROS-mediated premature senescence in lung cancer cells [39, 49]. Sin et al. suggest that chronic treatment with 20(S)-ginsenoside Rg3, a

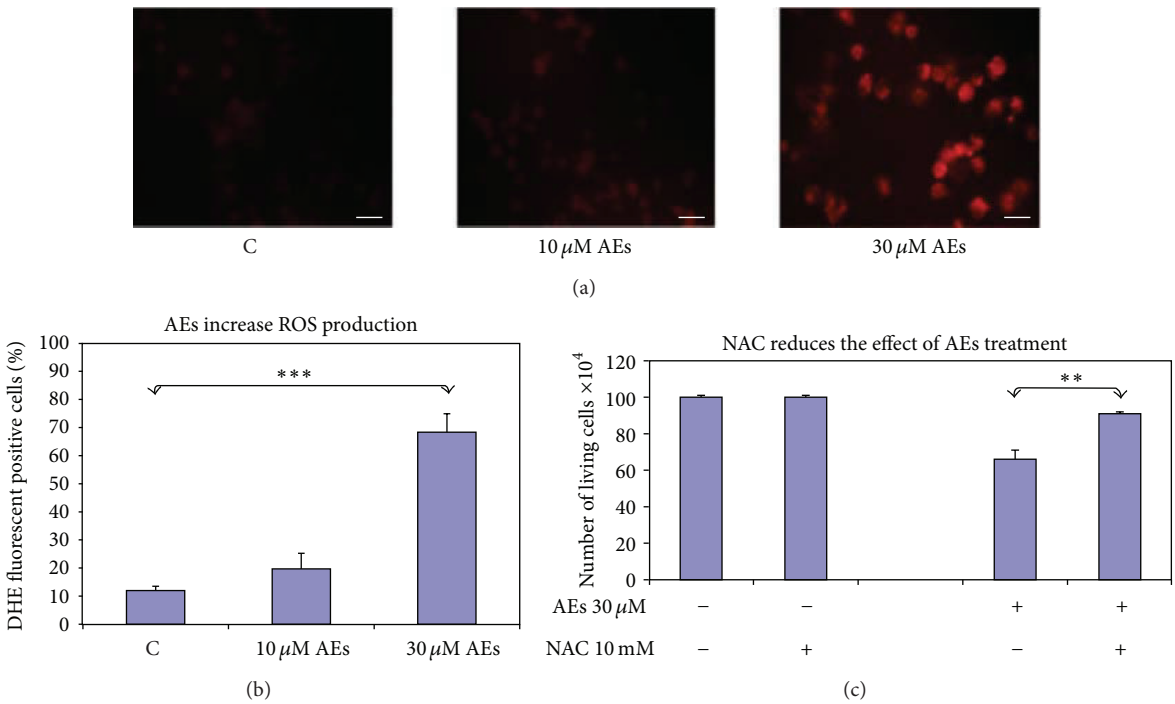


Figure 6: ROS production in MDA-MB231 cells treated with AEs. (a) The presence of ROS was detected by DHE fluorescent staining after 10 days treatment. Scale bar: 50 μm, original magnification 20x. (b) Red fluorescent-stained cells versus total cells were counted using an inverted fluorescence microscope. The results are the mean ± SD of at least three independent experiments. Significant statistical differences are indicated by asterisks: $^{**}P = 0.0012$. (c) NAC reduced the antiproliferative effect of AEs (30 μM for 10 days). The results are the mean ± SD of at least three independent experiments. Significant statistical differences are indicated by asterisks: $^{**}P = 0.0012$.

compound extracted from ginseng, at a subapoptotic concentration caused senescence-like growth arrest and increased ROS production in human glioma cells [54]. Moreover, bisdemethoxycurcumin, a natural derivative of curcumin, suppresses human breast cancer cell proliferation by inducing oxidative stress senescence [41]. A relevant role of ROS was also demonstrated for the phenethyl isothiocyanate induction of apoptosis and senescence in tumours [53]. Altogether, these findings suggest the crucial role of ROS as effectors of polyphenol-induced prooxidant damage in cancer cells. Accordingly, we show that AEs-induced growth arrest is associated with increased ROS production, suggesting that AEs may induce senescence via an oxidative-mediated damage in breast cancer cells. To confirm this important contribution of ROS, we demonstrate that antioxidant NAC attenuates AEs proliferative effect on MDA-MB231 cells.

It is important to highlight that we have previously shown that AEs have a prooxidant activity on breast cancer cells [25] and an antioxidant effect on normal hepatocyte [24]. Given that aberrant redox system is frequently observed in many tumour cells [63], we hypothesize that AEs may selectively inhibit the growth of tumour cells with little or no toxicity on normal cells based on their differential redox status.

Conventional cancer therapy is traditionally based on the efficacy of cytotoxic treatments even though severe side effects on patients are a clinical relevant problem. An alternative strategy is the induction of cytostatis, which disables the proliferative capacity of cells without inducing cancer cell death. A promising cytostatic approach is prosenescence therapy which may provide a relevant growth inhibitory effect in both early and late stage cancers [29, 64]. Targeted prosenescence therapies may be of remarkable clinical interest since it might minimize toxicity and improve quality of life for cancer patients [29, 30]. Over the last few years, it has been demonstrated that several prosenescence polyphenols and natural compounds could represent a promising novel therapeutic approach for cancer intervention [39, 41, 42, 54, 65]. The suppressive role of senescence in cancer progression has promoted the idea that induced premature cell aging could be an alternative or a complement to conventional anticancer treatments.

In line with this, our present study demonstrates for the first time that AEs may induce ROS accumulation in MDA-MB231 breast cancer cells and modulate the $p21^{Cip1/Waf1}$ and $p16^{INK4a}$ pathways to cause a senescence-mediated tumour suppression. Our study adds a novel aspect of the underlying mechanisms of the anticancer properties of AEs. However, in order to provide a solid basis for evaluating the efficacy in human clinical trials, further studies are required on animal models. In particular, deep pharmacokinetic and metabolic studies of AEs are needed.

In conclusion, our findings propose dietary artichoke polyphenols as a very promising tool either for the management of cancer prevention in healthy, high risk breast cancer women or for the design of innovative, nonconventional, adjuvant therapies in cancer treatment.

Conflict of Interests

The authors have declared no conflict of interests.

Authors' Contribution

Anna Maria Mileo, Stefania Miccadei, and Donato Di Venere conceived, designed, and performed the experiments. Claudia Abbruzzese analyzed the data. Stefania Miccadei and Anna Maria Mileo wrote the paper.

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References

[1] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[2] T. Norat, D. Aune, D. Chan, and D. Romaguera, "Fruits and vegetables: updating the epidemiologic evidence for the WCRF/AICR lifestyle recommendations for cancer prevention," *Cancer Treatment and Research*, vol. 159, pp. 35–50, 2014.

[3] L. Shu, K.-L. Cheung, T. O. Khor, C. Chen, and A.-N. Kong, "Phytochemicals: cancer chemoprevention and suppression of tumor onset and metastasis," *Cancer and Metastasis Reviews*, vol. 29, no. 3, pp. 483–502, 2010.

[4] A. C. Tan, I. Konczak, D. M.-Y. Sze, and I. Ramzan, "Molecular pathways for cancer chemoprevention by dietary phytochemicals," *Nutrition and Cancer*, vol. 63, no. 4, pp. 495–505, 2011.

[5] C. Gerhauser, "Cancer chemoprevention and nutri-epigenetics: state of the art and future challenges," *Topics in Current Chemistry*, vol. 329, pp. 73–132, 2013.

[6] V. S. Thakur, G. Deb, M. A. Babcook, and S. Gupta, "Plant phytochemicals as epigenetic modulators: role in cancer chemoprevention," *AAPS Journal*, vol. 16, no. 1, pp. 151–163, 2014.

[7] V. D. Longo and L. Fontana, "Calorie restriction and cancer prevention: metabolic and molecular mechanisms," *Trends in Pharmacological Sciences*, vol. 31, no. 2, pp. 89–98, 2010.

[8] M. González-Vallinas, M. González-Castejón, A. Rodríguez-Casado, and A. Ramírez de Molina, "Dietary phytochemicals in cancer prevention and therapy: a complementary approach with promising perspectives," *Nutrition Reviews*, vol. 71, no. 9, pp. 585–599, 2013.

[9] N. Namasivayam, "Chemoprevention in experimental animals," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 60–71, 2011.

[10] T. M. C. M. de Kok, P. de Waard, L. C. Wilms, and S. G. J. van Breda, "Antioxidative and antigenotoxic properties of vegetables and dietary phytochemicals: the value of genomics biomarkers in molecular epidemiology," *Molecular Nutrition and Food Research*, vol. 54, no. 2, pp. 208–217, 2010.

[11] S. M. Petiwala, A. G. Puthenveetil, and J. J. Johnson, "Polyphenols from the Mediterranean herb rosemary (*Rosmarinus officinalis*) for prostate cancer," *Frontiers in Pharmacology*, vol. 4, article 29, 2013.

[12] A. S. Darvesh and A. Bishayee, "Chemopreventive and therapeutic potential of tea polyphenols in hepatocellular cancer," *Nutrition and Cancer*, vol. 65, no. 3, pp. 329–344, 2013.

[13] P. Wang, J. V. Vadgama, J. W. Said et al., "Enhanced inhibition of prostate cancer xenograft tumor growth by combining quercetin and green tea," *The Journal of Nutritional Biochemistry*, vol. 25, no. 1, pp. 73–80, 2014.

[14] M. Iriti and E. M. Varoni, "Chemopreventive potential of flavonoids in oral squamous cell carcinoma in human studies," *Nutrients*, vol. 5, no. 7, pp. 2564–2576, 2013.

[15] J. McLarty, R. L. H. Bigelow, M. Smith, D. Elmajian, M. Ankem, and J. A. Cardelli, "Tea polyphenols decrease serum levels of prostate-specific antigen, hepatocyte growth factor, and vascular endothelial growth factor in prostate cancer patients and inhibit production of hepatocyte growth factor and vascular endothelial growth factor *in vitro*," *Cancer Prevention Research*, vol. 2, no. 7, pp. 673–682, 2009.

[16] T. D. Shanafelt, T. G. Call, C. S. Zent et al., "Phase 2 trial of daily, oral polyphenon e in patients with asymptomatic, Rai stage 0 to II chronic lymphocytic leukemia," *Cancer*, vol. 119, no. 2, pp. 363–370, 2013.

[17] C. Hanau, D. J. Morrè, and D. M. Morrè, "Cancer prevention trial of a synergistic mixture of green tea concentrate plus Capsicum (CAPSOL-T) in a random population of subjects ages 40–84," *Clinical Proteomics*, vol. 11, no. 1, pp. 1–11, 2014.

[18] E. Azzini, R. Bugianesi, F. Romano et al., "Absorption and metabolism of bioactive molecules after oral consumption of cooked edible heads of *Cynara scolymus* L. (cultivar Violetto di Provenza) in human subjects: A pilot study," *British Journal of Nutrition*, vol. 97, no. 5, pp. 963–969, 2007.

[19] C. Mancuso and R. Santangelo, "Ferulic acid: pharmacological and toxicological aspects," *Food and Chemical Toxicology*, vol. 65, pp. 185–195, 2014.

[20] B. Janicke, C. Hegardt, M. Krogh et al., "The antiproliferative effect of dietary fiber phenolic compounds ferulic acid and p-coumaric acid on the cell cycle of Caco-2 cells," *Nutrition and Cancer*, vol. 63, no. 4, pp. 611–622, 2011.

[21] N. Baskaran, S. Manoharan, S. Balakrishnan, and P. Pugalendhi, "Chemopreventive potential of ferulic acid in 7,12-dimethylbenz[a]anthracene-induced mammary carcinogenesis in Sprague-Dawley rats," *European Journal of Pharmacology*, vol. 637, no. 1–3, pp. 22–29, 2010.

[22] S. Hemaiswarya and M. Doble, "Combination of phenylpropanoids with 5-fluorouracil as anti-cancer agents against human cervical cancer (HeLa) cell line," *Phytomedicine*, vol. 20, no. 2, pp. 151–158, 2013.

[23] S. Karthikeyan, G. Kanimozhi, N. R. Prasad, and R. Mahalakshmi, "Radiosensitizing effect of ferulic acid on human cervical carcinoma cells in vitro," *Toxicology in Vitro*, vol. 25, no. 7, pp. 1366–1375, 2011.

[24] S. Miccadei, D. Di Venere, A. Cardinali et al., "Antioxidative and apoptotic properties of polyphenolic extracts from edible part of artichoke (*Cynara scolymus* L.) on cultured rat hepatocytes and on human hepatoma cells," *Nutrition and Cancer*, vol. 60, no. 2, pp. 276–283, 2008.

[25] A. M. Mileo, D. Di Venere, V. Linsalata, R. Fraioli, and S. Miccadei, "Artichoke polyphenols induce apoptosis and decrease

the invasive potential of the human breast cancer cell line MDA-MB231," *Journal of Cellular Physiology*, vol. 227, no. 9, pp. 3301–3309, 2012.

[26] M. Braig, S. Lee, C. Loddenkemper et al., "Oncogene-induced senescence as an initial barrier in lymphoma development," *Nature*, vol. 436, no. 7051, pp. 660–665, 2005.

[27] X. Guo, W. M. Keyes, C. Papazoglu et al., "TAp63 induces senescence and suppresses tumorigenesis *in vivo*," *Nature Cell Biology*, vol. 11, no. 12, pp. 1451–1457, 2009.

[28] M. Bennecke, L. Kriegl, M. Bajbouj et al., "Ink4a/Arf and oncogene-induced senescence prevent tumor progression during alternative colorectal tumorigenesis," *Cancer Cell*, vol. 18, no. 2, pp. 135–146, 2010.

[29] J. A. Ewald, J. A. Desotelle, G. Wilding, and D. F. Jarrard, "Therapy-induced senescence in cancer," *Journal of the National Cancer Institute*, vol. 102, no. 20, pp. 1536–1546, 2010.

[30] D. A. Gewirtz, S. E. Holt, and L. W. Elmore, "Accelerated senescence: an emerging role in tumor cell response to chemotherapy and radiation," *Biochemical Pharmacology*, vol. 76, no. 8, pp. 947–957, 2008.

[31] J. C. Acosta and J. Gil, "Senescence: a new weapon for cancer therapy," *Trends in Cell Biology*, vol. 22, no. 4, pp. 211–219, 2012.

[32] V. B. O. Ayissi, A. Ebrahimi, and H. Schluesenner, "Epigenetic effects of natural polyphenols: a focus on SIRT1-mediated mechanisms," *Molecular Nutrition & Food Research*, vol. 58, no. 1, pp. 22–32, 2014.

[33] P. Mummaneni and S. S. Shord, "Epigenetics and oncology," *Pharmacotherapy*, vol. 34, no. 5, pp. 495–505, 2014.

[34] T. P. Ong, F. S. Moreno, and S. A. Ross, "Targeting the epigenome with bioactive food components for cancer prevention," *Journal of Nutrigenetics and Nutrigenomics*, vol. 4, no. 5, pp. 275–292, 2012.

[35] S. Shankar, D. Kumar, and R. K. Srivastava, "Epigenetic modifications by dietary phytochemicals: Implications for personalized nutrition," *Pharmacology and Therapeutics*, vol. 138, no. 1, pp. 1–17, 2013.

[36] W. J. Lee and B. T. Zhu, "Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols," *Carcinogenesis*, vol. 27, no. 2, pp. 269–277, 2006.

[37] M. Lee and J.-S. Lee, "Exploiting tumor cell senescence in anticancer therapy," *BMB Reports*, vol. 47, no. 2, pp. 51–59, 2014.

[38] F. Debacq-Chainiaux, J. D. Erusalimsky, J. Campisi, and O. Toussaint, "Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and *in vivo*," *Nature Protocols*, vol. 4, no. 12, pp. 1798–1806, 2009.

[39] H. Luo, A. Yang, B. A. Schulte, M. J. Wargovich, and G. Y. Wang, "Resveratrol induces premature senescence in lung cancer cells via ROS-mediated DNA damage," *PLoS ONE*, vol. 8, no. 3, Article ID e60065, 2013.

[40] L. L. Zamin, E. C. Filippi-Chiela, P. Dillenburg-Pilla, F. Horn, C. Salbego, and G. Lenz, "Resveratrol and quercetin cooperate to induce senescence-like growth arrest in C6 rat glioma cells," *Cancer Science*, vol. 100, no. 9, pp. 1655–1662, 2009.

[41] Y.-B. Li, J.-L. Gao, Z.-F. Zhong, P.-M. Hoi, S. M.-Y. Lee, and Y.-T. Wang, "Bisdemethoxycurcumin suppresses MCF-7 cells proliferation by inducing ROS accumulation and modulating senescence-related pathways," *Pharmacological Reports*, vol. 65, no. 3, pp. 700–709, 2013.

[42] W. W. Quitschke, "Curcuminoid binding to embryonal Carcinoma cells: reductive metabolism, induction of apoptosis, senescence, and inhibition of cell proliferation," *PLoS ONE*, vol. 7, no. 6, Article ID e39568, 2012.

[43] G. P. Dimri, X. Lee, G. Basile et al., "A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 20, pp. 9363–9367, 1995.

[44] C. Papazoglu and A. A. Mills, "p53: at the crossroad between cancer and ageing," *The Journal of Pathology*, vol. 211, no. 2, pp. 124–133, 2007.

[45] W. Y. Kim and N. E. Sharpless, "The regulation of INK4/ARF in cancer and aging," *Cell*, vol. 127, no. 2, pp. 265–275, 2006.

[46] S. Kristiansen, D. Nielsen, and G. Sölétormos, "Methylated DNA for monitoring tumor growth and regression: how do we get there?" *Critical Reviews in Clinical Laboratory Sciences*, vol. 51, no. 3, pp. 149–159, 2014.

[47] S. M. Henning, P. Wang, C. L. Carpenter, and D. Heber, "Epigenetic effects of green tea polyphenols in cancer," *Epigenomics*, vol. 5, no. 6, pp. 729–741, 2013.

[48] K. E. Wellen and C. B. Thompson, "A two-way street: reciprocal regulation of metabolism and signalling," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 4, pp. 270–276, 2012.

[49] H. Luo, L. Wang, B. A. Schulte, A. Yang, S. Tang, and G. Y. Wang, "Resveratrol enhances ionizing radiation-induced premature senescence in lung cancer cells," *International Journal of Oncology*, vol. 43, no. 6, pp. 1999–2006, 2013.

[50] I. B. Roninson, "Tumor cell senescence in cancer treatment," *Cancer Research*, vol. 63, no. 11, pp. 2705–2715, 2003.

[51] A. Dong, Y. Fang, L. Zhang et al., "Caffeic acid 3,4-dihydroxyphenethyl ester induces cancer cell senescence by suppressing twist expression," *Journal of Pharmacology and Experimental Therapeutics*, vol. 339, no. 1, pp. 238–247, 2011.

[52] E. Scott, W. P. Steward, A. J. Gescher, and K. Brown, "Resveratrol in human cancer chemoprevention-choosing the "right" dose," *Molecular Nutrition and Food Research*, vol. 56, no. 1, pp. 7–13, 2012.

[53] N. Roy, I. Elangovan, D. Kopanja, S. Bagchi, and P. Raychaudhuri, "Tumor regression by phenethyl isothiocyanate involves DDB2," *Cancer Biology and Therapy*, vol. 14, no. 2, pp. 108–116, 2013.

[54] S. Sin, S. Y. Kim, and S. S. Kim, "Chronic treatment with ginsenoside Rg3 induces Akt-dependent senescence in human glioma cells," *International Journal of Oncology*, vol. 41, no. 5, pp. 1669–1674, 2012.

[55] J. D. Choi and J. S. Lee, "Interplay between epigenetics and genetics in cancer," *Genomics & Informatics*, vol. 11, no. 4, pp. 164–173, 2013.

[56] M. Conte and L. Altucci, "Molecular pathways: the complexity of the epigenome in cancer and recent clinical advances," *Clinical Cancer Research*, vol. 18, no. 20, pp. 5526–5534, 2012.

[57] A. Nebbioso, V. Carafa, R. Benedetti, and L. Altucci, "Trials with "epigenetic" drugs: an update," *Molecular Oncology*, vol. 6, no. 6, pp. 657–682, 2012.

[58] M. Schnekenburger, M. Dicato, and M. Diederich, "Plant-derived epigenetic modulators for cancer treatment and prevention," *Biotechnology Advances*, 2014.

[59] H. S. Lee and Z. Herceg, "The epigenome and cancer prevention: a complex story of dietary supplementation," *Cancer Letters*, vol. 342, no. 2, pp. 275–284, 2014.

[60] M. Fang, D. Chen, and C. S. Yang, "Dietary polyphenols may affect DNA methylation," *Journal of Nutrition*, vol. 137, no. 1, pp. 223S–228S, 2007.

[61] M.-H. Teiten, M. Dicato, and M. Diederich, "Curcumin as a regulator of epigenetic events," *Molecular Nutrition & Food Research*, vol. 57, no. 9, pp. 1619–1629, 2013.

[62] H. Y. Khan, H. Zubair, M. F. Ullah, A. Ahmad, and S. M. Hadi, "A prooxidant mechanism for the anticancer and chemopreventive properties of plant polyphenols," *Current Drug Targets*, vol. 13, no. 14, pp. 1738–1749, 2012.

[63] C. Gorrini, I. S. Harris, and T. W. Mak, "Modulation of oxidative stress as an anticancer strategy," *Nature Reviews Drug Discovery*, vol. 12, no. 12, pp. 931–947, 2013.

[64] M. E. LLeonart, A. Artero-Castro, and H. Kondoh, "Senescence induction; a possible cancer therapy," *Molecular Cancer*, vol. 8, article 3, 2009.

[65] Q. Yang, B. Wang, W. Zang et al., "Resveratrol inhibits the growth of gastric cancer by inducing G1 phase arrest and senescence in a Sirt1-dependent manner," *PLoS ONE*, vol. 8, no. 11, Article ID e70627, 2013.

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Review Article

Anticancer Properties of *Phyllanthus emblica* (Indian Gooseberry)

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There is a wealth of information emanating from both *in vitro* and *in vivo* studies indicating fruit extract of the *Phyllanthus emblica* tree, commonly referred to as Indian Gooseberries, has potent anticancer properties. The bioactivity in this extract is thought to be principally mediated by polyphenols, especially tannins and flavonoids. It remains unclear how polyphenols from *Phyllanthus emblica* can incorporate both cancer-preventative and antitumor properties. The antioxidant function of *Phyllanthus emblica* can account for some of the anticancer activity, but clearly other mechanisms are equally important. Herein, we provide a brief overview of the evidence supporting anticancer activity of Indian Gooseberry extracts, suggest possible mechanisms for these actions, and provide future directions that might be taken to translate these findings clinically.

1. Introduction

Cancer is a global epidemic with approximately fourteen million new cases being diagnosed each year, leading to an annual death toll of approximately eight million [1]. The societal burden resulting from such staggering numbers is almost immeasurable when one considers both the economic impact and quality of life consequences. However, a recent study puts the economic impact for individual cancers in the billions of dollars for Europe alone [2]. Obviously, more effective therapies are needed for many common cancers. But the holy grail of cancer research would be to identify either pharmacologic or lifestyle interventions that could prevent the onset of cancer. Towards this goal, research has shown that maintaining a healthy lifestyle decreases the risk of some cancers, and intake of some foods may also decrease risk [3–6]. Here, we will focus on the anticancer properties of the fruit-bearing tree *Phyllanthus emblica*.

Phyllanthus emblica is a tree indigenous to tropical regions of Southeast Asia. The tree produces a fruit commonly known as Indian Gooseberry or Amla. The *Phyllanthus emblica* fruit (also known as *Emblica officinalis*) or extract from these fruits has been used in traditional medicine for generations to treat symptoms ranging from constipation to the treatment of tumors [7]. Most commonly, the gooseberry was employed as a gentle laxative. However, the potential of *Phyllanthus emblica* extract to be utilized as an anticancer agent has been scrutinized using modern medical techniques over the past two decades. To date, there is substantial evidence that these extracts contain small molecules with both cancer-preventative and antitumor activity. Here, we will provide an overview of the literature supporting these concepts and attempt to lend insight into possible mechanisms whereby the anticancer properties are achieved.

2. Cancer Prevention

Each day we are exposed to agents in our environment and through our diet that can potentially compromise the integrity of our genome. Many xenobiotics and the reactive oxygen species generated during cell respiration are carcinogenic. Thus, reducing our exposure to harmful xenobiotics and increasing our capacity to "soak up" reactive oxygen species represented a potential means to reduce the likelihood of cancer. Using the classic measure of carcinogenicity, the Ames test, it was clearly demonstrated that *Emblica officinalis*

prevents mutagenesis *in vitro* [8]. Proving this concept *in vivo* is not as straightforward. Measuring cancer prevention in humans is a time consuming and complex endeavor, involving many confounding factors. Fortunately, chemopreventive properties of substances can be tested using rodent models of various cancers.

Taken prophylactically, *Emblica officinalis* extract reduced the genotoxic effects of heavy metals and the carcinogen benzopyrene in murine models [9, 10]. In a murine model of skin carcinogenesis, continuous administration of *Emblica officinalis* extract at 100 mg/kg reduced tumor incidence by ~60% [11]. Similarly, two independent studies showed the polyphenol or aqueous fractions of *Emblica officinalis* administered at 60–250 mg/kg prevented N-nitrosodiethylamine induced hepatocellular carcinoma by ~80–100% [12, 13]. However, such dramatic results were not reported when *Emblica officinalis* extract was examined for chemoprevention of liver tumors induced by initiation with diethylnitrosamine followed by promotion with 2-acetylaminofluorene [14]. This difference indicates *Emblica officinalis* has the capacity to prevent the onset of some, but not all cancers, depending on the initiator. This becomes fairly obvious when one considers carcinogenic compounds have differing modes of action; thus a single extract could not be expected to be universally chemopreventive. Hopefully, future studies will expand on these studies to examine the ability of *Emblica officinalis* to prevent tumors initiated by a wider variety of carcinogens at diverse tissue sites.

How are these extracts chemopreventive? There are four possibilities. First, *Phyllanthus emblica* has potent free radical scavenging activities that might prevent reactive oxygen species induced DNA damage and oncogenesis [15, 16]. However, in the animal models described above, it is unclear to what extent reactive oxygen species contribute to the underlying pathology, implying that there may be alternative mechanisms of action. Second, the extract has properties allowing it to reduce the levels of cytochrome enzymes in liver cells [17]. Cytochromes, such as Cyp 450, convert xenobiotics to potentially carcinogenic substances in an effort to clear them from the body. However, this concept is controversial as Amla extract was not found to decrease Cyp 450 levels in at least two other studies [18, 19]. Third, *Phyllanthus emblica* extracts have anti-inflammatory activities that might prevent inflammation related cancers [20]. Finally, as we will describe below, *Phyllanthus emblica* harbors potent antitumor activity [21, 22]. Even exposure to low levels of extract from these berries may be enough to impair tumor progression at early stages. It should be noted that there is concern regarding potential hepatotoxicity after long term Amla ingestion [23]. This matter may need to be resolved in the future by clinical and epidemiological studies before *Phyllanthus emblica* extract can be safely recommended for long term consumption for the prevention of cancer.

3. Cancer Therapy

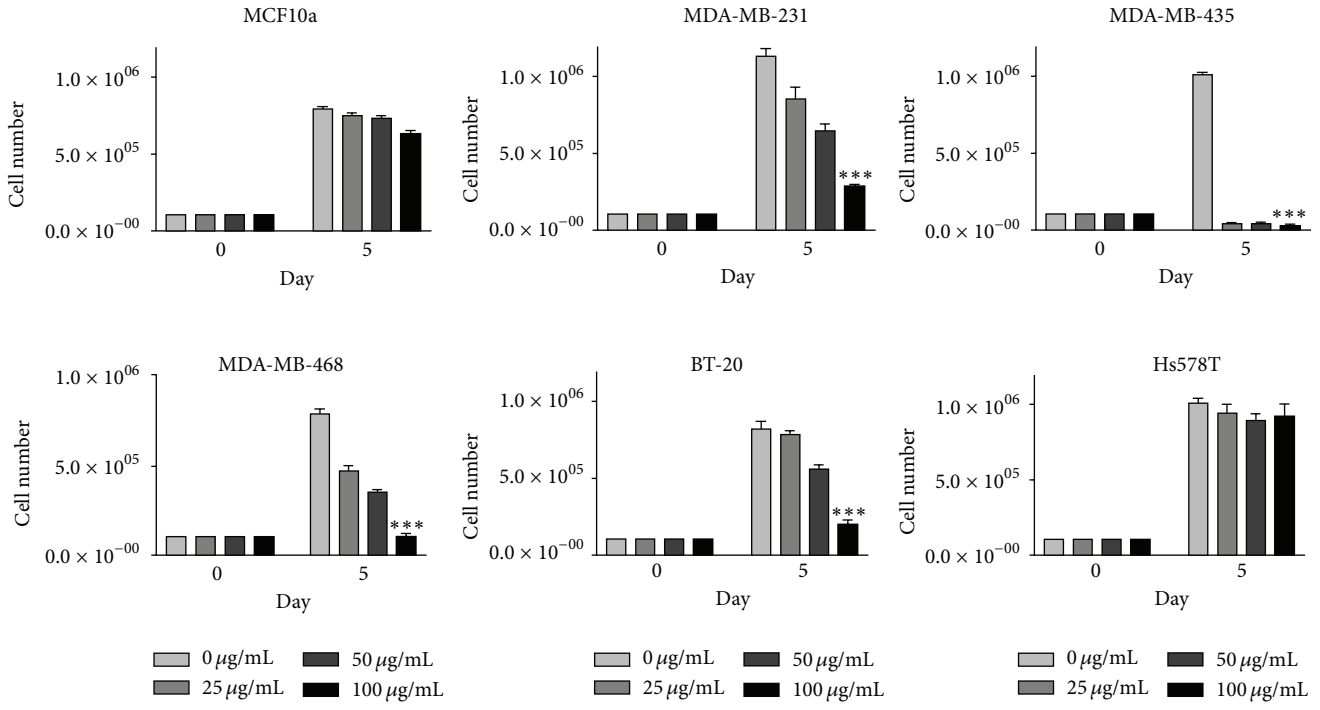
Phyllanthus emblica extracts have been demonstrated to have potent tumor repressive properties against a number of cancer types both *in vitro* and *in vivo*. Preclinical evidence using a diverse panel of cancer cell lines shows aqueous extract from *Phyllanthus emblica* berries induced apoptosis at concentrations ranging from 50 to 100 micrograms/mL [24]. In this study, normal fibroblasts were also included and showed 4-fold lower sensitivity to these extracts. This is in keeping with our own data comparing aqueous *Phyllanthus emblica* berry extracts (generous gift of Sabinsa Corporation) against triple-negative breast cancer cells (Figures 1(a) and 1(b)). We see exposure of these cells to doses of the soluble extract ranging from 25 to 100 micrograms/mL results in significant cytotoxicity (Figures 1(a) and 1(b)), but almost no effect is seen against normal breast epithelial cells (MCF10A). Other reports have shown extracts from blueberries and strawberries both limit the proliferation of triple-negative breast cancer cells *in vitro* and *in vivo* [25, 26]. However, neither of these extracts showed a considerable degree of antiproliferative activity at concentrations lower than 500 micrograms/mL, whereas soluble *Phyllanthus emblica* berry extract was potent even at 50 micrograms/mL in at least three (MDA-MB-231, MDA-MB-435, and MDA-MB-468) of the cell lines we tested. The sparsity of cells after exposure to the berry extract and appearance of debris indicate cells are undergoing apoptosis as opposed to cytostatic mechanisms of growth arrest. These data indicate *Phyllanthus emblica* extract or a constituent therein represents a potential treatment for breast cancer with low toxicity against nontransformed cells.

Phyllanthus emblica berry extract enriched for polyphenols or simple aqueous extracts have also shown cytotoxic activity against cervical and ovarian cancer cells [27, 28]. However, unlike other model systems tested, the reduced proliferation in ovarian cancer cells was attributed to the action of the autophagy pathway, independent of apoptosis [27]. Notably, in contrast to the studies outlined above where *Phyllanthus emblica* prevented liver carcinogenesis, *in vitro* studies using the human liver cancer cell line HepG2 show little evidence of cytotoxicity of aqueous extracts [29]. Overall, it appears that *Phyllanthus emblica* extract displays potent cytotoxic effects against most cell lines, but primary resistance mechanisms exist, as is seen with the Hs578T cell line (Figure 1(a)). Such resistant cells may prove to be a useful tool in determining the mechanism whereby these extracts carry out their cytotoxic effects.

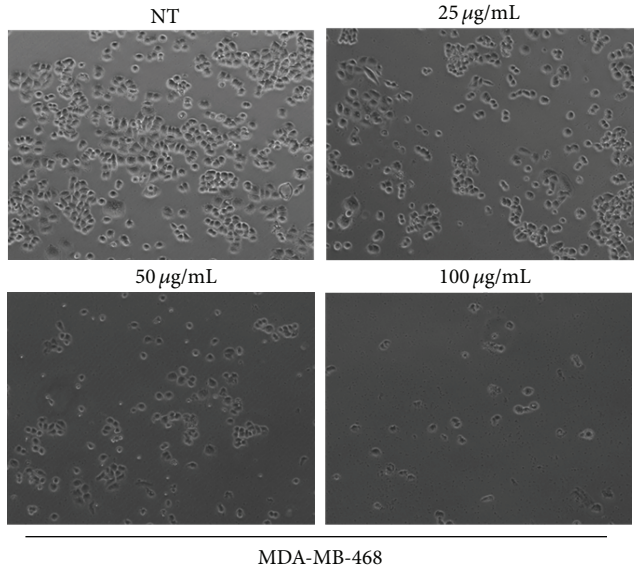
In contrast to the *in vitro* studies, there is sparse evidence regarding the tumor repressive activity of *Phyllanthus emblica* using *in vivo* models of cancer. Such preclinical models are necessary for the translation of these investigations into something clinically relevant. However, a striking study has been carried out employing aqueous extracts of the Indian Gooseberry administered orally against an ovarian cancer xenograft model [27]. In this report, the extracts were able to completely ablate the growth of ovarian xenografts, with little or no residual tumor being observed after treatment. Clearly, these remarkable data warrant further investigation into the *in vivo* anticancer properties of *Emblica officinalis* extracts.

4. So, What Is in There?

Extracts from *Phyllanthus emblica* tree have been documented to have a number of disparate properties including



(a)



(b)

Figure 1: Cytotoxic effects of *Phyllanthus emblica* (Indian Gooseberry) extract against triple-negative breast cancer cells. (a) MCF10A cells represent untransformed mammary epithelial cells. All other cell lines represent triple-negative breast cancer cell lines. Growth media for all cell lines were used according to ATCC recommendations. 10^4 cells were seeded in 24-well plates. 24 hours after plating, Indian Gooseberry extract (Saberry, Sabinsa Corporation) dissolved in PBS was added daily to fresh media at the indicated concentrations or PBS to control cells. Cell viability was measured using trypan blue exclusion with a hemocytometer after a five-day exposure period. Experiments were carried out multiple times in triplicate. Error bars represent SEM. *P* values for growth inhibition of MDA-MB-435, MDA-MB-468, MDA-MB-231, and BT20 cells exposed to 100 μg/mL *Phyllanthus emblica* compared to control cells were all <0.05 (denoted by $***$). MCF10A cells exposed to the same concentration showed no significant change. (b) Visualization of MDA-MB-468 cells after five-day exposure to *Phyllanthus emblica* extract at 10x magnification.

antioxidant, anti-inflammatory, proapoptotic, and proautophagy ones [21, 27, 30, 31]. Obviously these berry extracts are a complex mix of compounds [22, 32] and it is not trivial to decipher which molecules mediate these effects. It may be that combinations of molecules from these berries work in synergy with one another to achieve diverse biological activity. Highly purified fractions of low complexity are needed from *Phyllanthus emblica* to be interrogated for each biological property. That being said, some of the known molecules held within the Indian Gooseberryextract, identified by high pressure liquid chromatography (HPLC), can be matched to its anticancer activity [22, 32, 33]. This review will focus on molecules identified by HPLC from *Phyllanthus emblica* extract that have reproducibly been shown to have anticancer properties. Primarily, we will focus on the hydrolyzable tannin content of these extracts because of their high content within the Amla fruit [22] and importantly due to their known antioxidant, cancer-preventative, and cytotoxic activity *in vivo* [34, 35]. However, the flavonoid quercetin perhaps serves as the best paradigm for the future drug development from molecules within Amla extract having anticancer properties (described below), as this molecule has gone to clinical trial and novel analogues are being pursued. While there are other constituents of Amla extract such as vitamin C that have antioxidant activity, the only molecules having bona fide anticancer activity to date fall within the tannin or flavonoid categories.

The major constituents of *Phyllanthus emblica* that have been identified by HPLC and formally characterized as having anticancer activity are described below (and in Table 1). Overall, it seems reasonable that many of the anticancer properties of this fruit are derived from the tannin content. However, a notable exception to this concept is the flavonoid quercetin, which harbors potent antioxidant and anticancer properties as demonstrated utilizing multiple *in vivo* models of cancer including breast and leukemia [36–38].

Phyllanthus emblica is rich in polyphenols and hydrolysable tannin derived compounds that act as antioxidants [22, 32]. Well-studied examples include ellagic acid, gallic acid, and chebulagic acid [39–41]. These and other tannins from *Phyllanthus emblica* have been demonstrated to prevent mutagenesis and lipid peroxidation in response to carcinogens and reactive oxygen species [42]. It is likely that the combination of these compounds acting in synergy allows Amla extract to soak up free radicals with such efficiency and imparts the extracts with cancer-preventative properties. Alkylating carcinogens generate DNA mutations through carbon oxidation or conjugation reactions with nucleic acids [43]. It is likely that *Phyllanthus emblica* extract will reduce the oxidative damage induced mutations generated by such agents, but it remains to be seen whether conjugation reactions are also impaired.

There are also similarities between the proapoptotic and autophagy effects of *Phyllanthus emblica* extract and purified components. Most *in vitro* data indicate the antiproliferative effect of these extracts is mediated via activation of an apoptotic cascade. Interestingly, either crude *Emblica officinalis* extracts or purified components are capable of NF-kappaB inhibition [44–47]. Tannins from *Phyllanthus emblica* impairing NF-kappaB inhibition include chebulagic acid, ellagic acid, and corilagin. All of them have been shown to have antiproliferative and proapoptotic properties against cancer cells [48–50]. The same is true for gallic acid, a product of tannin hydrolysis, and major constituent of Amla extract [22]. NF-kappaB is an important survival factor in many cancers, and its inhibition leads to pervasive apoptosis [51, 52]. Thus, many of the anticancer properties of these extracts may be brought about through inhibition of this transcription factor binding with its cognate DNA binding elements. Further, NF-kappaB activation is also a potent inducer of inflammation [53] and again it is reasonable to think that the anti-inflammatory properties of the Amla extract are mediated by NF-kappaB inhibition. However, it should be noted that a least one of the *Phyllanthus emblica* components having potent *in vivo* antitumor activity, pyrogallol, is known to activate reactive oxygen species and NF-kappaB binding [54, 55]. NF-kappaB independent tumor suppression has been described for gallotannin, indicating alternative mechanisms for growth inhibition may be relevant depending on the tissue being targeted [34].

But what about the induction of autophagy? Cell death in response to chebulagic acid and the flavonoid quercetin has been linked to autophagy [27, 56]. Considering apoptosis is tightly linked to the autophagosome, it is likely that many of the tannins present in *Phyllanthus emblica* extract may activate the autophagy and apoptotic response simultaneously [57].

Similar to the tannins outlined in Table 1, quercetin also induces apoptosis and autophagy. However, distinct from tannins, quercetin exhibits a well-characterized inhibition of growth factor signaling pathways. This includes oncogenic

Table 1: Molecules from *Phyllanthus emblica* fruit extract having proven anticancer properties.

Phenolic compounds from *Phyllanthus emblica* extract identified by HPLC having anticancer properties	Cancer model utilized to identify antiproliferative and antitumor properties
Ellagic acid (tannin)	Colon, prostate cell lines, and breast and prostate xenografts
Corilagin (tannin)	Ovarian cancer cells, liver cancer cells, and hepatocarcinoma xenografts
Pyrogallol (tannin)	Lung cancer cells, gastric cancer cells, and lung adenocarcinoma xenografts
Chebulagic acid (tannin)	Retinoblastoma colon cancer, breast cancer, prostate cancer, and leukemia cancer cell lines
Gallic acid (tannin)	Breast and lung cancer cell lines, some activity against lung cancer xenograft
Quercetin (flavonoid)	Numerous cancer cell lines from multiple tissue types, transgenic murine model of breast cancer, leukemia xenograft, and phase I clinical trial

signaling pathways such as the EGFR [58, 59]. Quercetin accomplishes this feat through inhibition of PI3K signaling, which plays a pivotal role in relaying oncogenic growth signals from the EGFR. Structural studies of the quercetin analog LY-29004 reveal its activity as a competitive inhibitor of ATP binding to the PI3K catalytic domain [60]. Of course, as is true for any inhibitor, Ly-29004 also has off-target effects, such as CKII inhibition [61], that may play a role in its repression of proliferation.

To become relevant to humans, preclinical data using mouse models of cancer are necessary to interrogate the *in vivo* potential of hydrolyzable tannins. In fact, a number of the components of *Phyllanthus emblica* extract have been demonstrated to have striking anticancer activity against currently incurable cancer using xenograft models. Pyrogallol ablates the growth of lung adenocarcinoma xenografts at only 75 μg/kg [54]. Gallotannin has shown significant tumor response against triple-negative breast cancers and cholangiocarcinoma [34, 62]. Xenograft models of pancreatic and triple-negative breast cancers showed considerable response to ellagic acid [48, 63]. Recent evidence also indicates ellagic acid may act as a prophylactic, protecting against the onset of prostate or breast cancer in animal models [64, 65]. Corilagin demonstrates high antitumor activity against hepatocellular carcinoma xenograft models at 15 mg/kg [66]. Gallic acid shows antitumor qualities against lung and osteosarcoma, but the impact may be not substantial enough to warrant future studies [67, 68].

The flavonoid quercetin has been demonstrated to attenuate tumor growth in multiple animal models. This includes xenograft model of leukemia and pancreatic cancer [38, 69]. Based on the well-defined mechanism of action and encouraging data using murine models, quercetin was tested in a phase I clinical trial where it showed antityrosine kinase activity *in vivo* [70]. Analogues of quercetin designed as more specific PI3K inhibitors also demonstrate potent antiproliferative activities. Such analogues of other small molecules found with the Amla extract may facilitate further development into clinical relevant drugs.

Overall, the data published to date highlights the enormous potential of naturally occurring molecules from *Phyllanthus emblica* as pharmacological agents for the treatment of cancer.

5. Future Perspective

For *Phyllanthus emblica* to become relevant clinically, it is imperative that the molecules mediating the antitumor effects of the plant be identified and even more potent, patentable derivatives synthesized. Without the possibility of patents, the pharmaceutical industry will undoubtedly not invest the enormous amount of money required to carry out clinical trials using these putative chemotherapeutics. Such evidenced-based trials will eventually be necessary to prove the worth of these extracts in preventing and treating human cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit et al., "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012," *International Journal of Cancer*, vol. 136, no. 5, pp. E359–E386, 2015.

[2] P. Hanly, I. Soerjomataram, and L. Sharp, "Measuring the societal burden of cancer: the cost of lost productivity due to premature cancer-related mortality in Europe," *International Journal of Cancer*, vol. 136, no. 4, pp. E136–E45, 2015.

[3] G. A. Colditz and K. Bohlke, "Priorities for the primary prevention of breast cancer," *CA Cancer Journal for Clinicians*, vol. 64, no. 3, pp. 186–194, 2014.

[4] C. C. Huang, W. T. Lee, S. T. Tsai et al., "Tea consumption and risk of head and neck cancer," *PLoS ONE*, vol. 9, no. 5, Article ID e96507, 2014.

[5] L. Wang, X. Zhang, J. Liu, L. Shen, and Z. Li, "Tea consumption and lung cancer risk: a meta-analysis of case-control and cohort studies," *Nutrition*, vol. 30, no. 10, pp. 1122–1127, 2014.

[6] A. H. Eliassen, S. J. Hendrickson, L. A. Brinton et al., "Circulating carotenoids and risk of breast cancer: pooled analysis of eight prospective studies," *Journal of the National Cancer Institute*, vol. 104, no. 24, pp. 1905–1916, 2012.

[7] D. W. Unander, G. L. Webster, and B. S. Blumberg, "Records of usage or assays in *Phyllanthus* (Euphorbiaceae) I. Subgenera *Isocladus*, *Kirganelia*, *Cicca* and *Emblica*," *Journal of Ethnopharmacology*, vol. 30, no. 3, pp. 233–264, 1990.

[8] N. Sharma, P. Trikha, M. Athar, and S. Raisuddin, "In vitro inhibition of carcinogen-induced mutagenicity by *Cassia occidentalis* and **Emblica officinalis**," *Drug and Chemical Toxicology*, vol. 23, no. 3, pp. 477–484, 2000.

[9] P. Nandi, G. Talukder, and A. Sharma, "Dietary chemoprevention of clastogenic effects of 3,4-benzo(a)pyrene by *Emblica officinalis* Gaertn. fruit extract," *British Journal of Cancer*, vol. 76, no. 10, pp. 1279–1283, 1997.

[10] H. Dhir, A. K. Roy, A. Sharma, and G. Talukder, "Modification of clastogenicity of lead and aluminium in mouse bone marrow cells by dietary ingestion of Phyllanthus emblica fruit extract," *Mutation Research*, vol. 241, no. 3, pp. 305–312, 1990.

[11] G. Sancheti, A. Jindal, R. Kumari, and P. K. Goyal, "Chemopreventive action of *Emblica officinalis* on skin carcinogenesis in mice," *Asian Pacific Journal of Cancer Prevention*, vol. 6, no. 2, pp. 197–201, 2005.

[12] N. V. Rajeshkumar, M. R. Pillai, and R. Kuttan, "Induction of apoptosis in mouse and human carcinoma cell lines by *Emblica officinalis* polyphenols and its effect on chemical carcinogenesis," *Journal of Experimental and Clinical Cancer Research*, vol. 22, no. 2, pp. 201–212, 2003.

[13] K. J. Jeena, K. L. Joy, and R. Kuttan, "Effect of *Emblica officinalis*, *Phyllanthus amarus* and *Picrorrhiza kurroa* on *N*-nitrosodiethylamine induced hepatocarcinogenesis," *Cancer Letters*, vol. 136, no. 1, pp. 11–16, 1999.

[14] S. Sultana, S. Ahmed, and T. Jahangir, "*Emblica officinalis* and hepatocarcinogenesis: a chemopreventive study in Wistar rats," *Journal of Ethnopharmacology*, vol. 118, no. 1, pp. 1–6, 2008.

[15] B. Hazra, R. Sarkar, S. Biswas, and N. Mandal, "Comparative study of the antioxidant and reactive oxygen species scavenging properties in the extracts of the fruits of *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis*," *BMC Complementary and Alternative Medicine*, vol. 10, article 20, 2010.

[16] M. Majeed, B. Bhat, A. N. Jadhav, J. S. Srivastava, and K. Nagabhushanam, "Ascorbic acid and tannins from *Emblica*

officinalis Gaertn. Fruits—a revisit," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 1, pp. 220–225, 2009.

[17] S. M. Banu, K. Selvendiran, J. P. V. Singh, and D. Sakthisekaran, "Protective effect of *Emblica officinalis* ethanolic extract against 7,12-dimethylbenz(a)anthracene (DMBA) induced genotoxicity in Swiss albino mice," *Human and Experimental Toxicology*, vol. 23, no. 11, pp. 527–531, 2004.

[18] K.-H. Chen, B.-R. Lin, C.-T. Chien, and C.-H. Ho, "*Emblica officinalis* gaertn. attentuates *N*-nitrosodiethylamine-induced apoptosis, autophagy, and inflammation in rat livers," *Journal of Medicinal Food*, vol. 14, no. 7-8, pp. 746–755, 2011.

[19] S. A. Tasduq, D. M. Mondhe, D. K. Gupta, M. Baleshwar, and R. K. Johri, "Reversal of fibrogenic events in liver by *Emblica officinalis* (fruit), an Indian natural drug," *Biological and Pharmaceutical Bulletin*, vol. 28, no. 7, pp. 1304–1306, 2005.

[20] M. Golechha, V. Sarangal, S. Ojha, J. Bhatia, and D. S. Arya, "Anti-inflammatory effect of *Emblica officinalis* in rodent models of acute and chronic inflammation: involvement of possible mechanisms," *International Journal of Inflammation*, vol. 2014, Article ID 178408, 6 pages, 2014.

[21] M. S. Baliga and J. J. Dsouza, "Amla (*Emblica officinalis* Gaertn), a wonder berry in the treatment and prevention of cancer," *European Journal of Cancer Prevention*, vol. 20, no. 3, pp. 225–239, 2011.

[22] B. Yang and P. Liu, "Composition and biological activities of hydrolyzable tannins of fruits of phyllanthus emblica," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 3, pp. 529–541, 2014.

[23] C. Wiart, "Note on the relevance of *Emblica officinalis* Gaertn. for the treatment and prevention of cancer," *European Journal of Cancer Prevention*, vol. 22, no. 2, article 198, 2013.

[24] C. Ngamkitidechakul, K. Jaijoy, P. Hansakul, N. Soonthornchareonnon, and S. Sireeratawong, "Antitumour effects of *Phyllanthus emblica* L.: induction of cancer cell apoptosis and inhibition of in vivo tumour promotion and in vitro invasion of human cancer cells," *Phytotherapy Research*, vol. 24, no. 9, pp. 1405–1413, 2010.

[25] L. S. Adams, S. Phung, N. Yee, N. P. Seeram, L. Li, and S. Chen, "Blueberry phytochemicals inhibit growth and metastatic potential of MDA-MB-231 breast cancer cells through modulation of the phosphatidylinositol 3-kinase pathway," *Cancer Research*, vol. 70, no. 9, pp. 3594–3605, 2010.

[26] R. R. Somasagara, M. Hegde, K. K. Chiruvella, A. Musini, B. Choudhary, and S. C. Raghavan, "Extracts of strawberry fruits induce intrinsic pathway of apoptosis in breast cancer cells and inhibits tumor progression in mice," *PLoS ONE*, vol. 7, no. 10, Article ID e47021, 2012.

[27] A. De, C. Papasian, S. Hentges, S. Banerjee, I. Haque, and S. K. Banerjee, "*Emblica officinalis* extract induces autophagy and inhibits human ovarian cancer cell proliferation, angiogenesis, growth of mouse xenograft tumors," *PLoS ONE*, vol. 8, no. 8, Article ID e72748, 2013.

[28] X. Zhu, J. Wang, Y. Ou, W. Han, and H. Li, "Polyphenol extract of *Phyllanthus emblica* (PEEP) induces inhibition of cell proliferation and triggers apoptosis in cervical cancer cells," *European Journal of Medical Research*, vol. 18, no. 1, article 46, 2013.

[29] M. M. Shivananjappa and M. K. Joshi, "Influence of *Emblica officinalis* aqueous extract on growth and antioxidant defense system of human hepatoma cell line (HepG2)," *Pharmaceutical Biology*, vol. 50, no. 4, pp. 497–505, 2012.

[30] S. Rawal, P. Singh, A. Gupta, and S. Mohanty, "Dietary intake of *Curcuma longa* and *Emblica officinalis* increases life span in *Drosophila melanogaster*," *BioMed Research International*, vol. 2014, Article ID 910290, 7 pages, 2014.

[31] S. Mahata, A. Pandey, S. Shukla et al., "Anticancer activity of *Phyllanthus emblica* Linn. (Indian Gooseberry): inhibition of transcription factor ap-1 and HPV gene expression in cervical cancer cells," *Nutrition and Cancer*, vol. 65, supplement 1, pp. 88–97, 2013.

[32] E. A. Poltanov, A. N. Shikov, H. J. D. Dorman et al., "Chemical and antioxidant evaluation of Indian gooseberry (*Emblica officinalis* Gaertn., syn. *Phyllanthus emblica* L.) supplements," *Phytotherapy Research*, vol. 23, no. 9, pp. 1309–1315, 2009.

[33] B. Yang, M. Kortesniemi, P. Liu, M. Karonen, and J.-P. Salminen, "Analysis of hydrolyzable tannins and other phenolic compounds in emblic leafflower (*Phyllanthus emblica* L.) fruits by high performance liquid chromatography-electrospray ionization mass spectrometry," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 35, pp. 8672–8683, 2012.

[34] T. Zhao, Q. Sun, S. V. Del Rincon, A. Lovato, M. Marques, and M. Witcher, "Gallotannin imposes S phase arrest in breast cancer cells and suppresses the growth of triple-negative tumors in vivo," *PLoS ONE*, vol. 9, no. 3, Article ID e92853, 2014.

[35] Y. Chai, H.-J. Lee, A. A. Shaik et al., "Penta-O-galloyl-β-D-glucose induces G1 arrest and DNA replicative S-phase arrest independently of P21 cyclin-dependent kinase inhibitor 1A, P27 cyclin-dependent kinase inhibitor 1B and P53 in human breast cancer cells and is orally active against triple-negative xenograft growth," *Breast Cancer Research*, vol. 12, no. 5, article R67, 2010.

[36] C. Spagnuolo, M. Russo, S. Bilotto, I. Tedesco, B. Laratta, and G. L. Russo, "Dietary polyphenols in cancer prevention: the example of the flavonoid quercetin in leukemia," *Annals of the New York Academy of Sciences*, vol. 1259, no. 1, pp. 95–103, 2012.

[37] J. L. Steiner, J. M. Davis, J. L. McClellan et al., "Dose-dependent benefits of quercetin on tumorigenesis in the C3(1)/SV40Tag transgenic mouse model of breast cancer," *Cancer Biology & Therapy*, vol. 15, no. 11, pp. 1456–1467, 2014.

[38] S. Cheng, N. Gao, Z. Zhang et al., "Quercetin induces tumor-selective apoptosis through downregulation of Mcl-1 and activation of Bax," *Clinical Cancer Research*, vol. 16, no. 23, pp. 5679–5691, 2010.

[39] A. Galano, M. Francisco Marquez, and A. Pérez-González, "Ellagic acid: an unusually versatile protector against oxidative stress," *Chemical Research in Toxicology*, vol. 27, no. 5, pp. 904–918, 2014.

[40] M. Singh, A. Jha, A. Kumar, N. Hettiarachchy, A. K. Rai, and D. Sharma, "Influence of the solvents on the extraction of major phenolic compounds (punicalagin, ellagic acid and gallic acid) and their antioxidant activities in pomegranate aril," *Journal of Food Science and Technology*, vol. 51, no. 9, pp. 2070–2077, 2014.

[41] A. Manosroi, P. Jantrawut, H. Akazawa, T. Akihisa, and J. Manosroi, "Biological activities of phenolic compounds isolated from galls of *Terminalia chebula* Retz. (Combretaceae)," *Natural Product Research*, vol. 24, no. 20, pp. 1915–1926, 2010.

[42] P. S. Makena and K.-T. Chung, "Effects of various plant polyphenols on bladder carcinogen benzidine-induced mutagenicity," *Food and Chemical Toxicology*, vol. 45, no. 10, pp. 1899–1909, 2007.

[43] A. Dipple, "DNA adducts of chemical carcinogens," *Carcinogenesis*, vol. 16, no. 3, pp. 437–441, 1995.

[44] R. Piva, L. Penolazzi, M. Borgatti et al., "Apoptosis of human primary osteoclasts treated with molecules targeting nuclear

factor-kappaB," *Annals of the New York Academy of Sciences*, vol. 1171, pp. 448–456, 2009.

[45] H.-H. Ho, C.-S. Chang, W.-C. Ho, S.-Y. Liao, C.-H. Wu, and C.-J. Wang, "Anti-metastasis effects of gallic acid on gastric cancer cells involves inhibition of NF-κB activity and downregulation of PI3K/AKT/small GTPase signals," *Food and Chemical Toxicology*, vol. 48, no. 8-9, pp. 2508–2516, 2010.

[46] H. H. Ho, C.-S. Chang, W.-C. Ho, S.-Y. Liao, W.-L. Lin, and C.-J. Wang, "Gallic acid inhibits gastric cancer cells metastasis and invasive growth via increased expression of RhoB, downregulation of AKT/small GTPase signals and inhibition of NF-kappaB activity," *Toxicology and Applied Pharmacology*, vol. 266, no. 1, pp. 76–85, 2013.

[47] L. Penolazzi, I. Lampronti, M. Borgatti et al., "Induction of apoptosis of human primary osteoclasts treated with extracts from the medicinal plant *Emblica officinalis*," *BMC Complementary and Alternative Medicine*, vol. 8, article 59, 2008.

[48] M. Edderkaoui, I. Odinokova, I. Ohno et al., "Ellagic acid induces apoptosis through inhibition of nuclear factor κB in pancreatic cancer cells," *World Journal of Gastroenterology*, vol. 14, no. 23, pp. 3672–3680, 2008.

[49] P. Anitha, R. V. Priyadarsini, K. Kavitha, P. Thiyagarajan, and S. Nagini, "Ellagic acid coordinately attenuates Wnt/beta-catenin and NF-kappaB signaling pathways to induce intrinsic apoptosis in an animal model of oral oncogenesis," *European Journal of Nutrition*, vol. 52, no. 1, pp. 75–84, 2013.

[50] L. Jia, H. Jin, J. Zhou et al., "A potential anti-tumor herbal medicine, Corilagin, inhibits ovarian cancer cell growth through blocking the TGF-β signaling pathways," *BMC Complementary and Alternative Medicine*, vol. 13, article 33, 2013.

[51] C. Gasparini, C. Celeghini, L. Monasta, and G. Zauli, "NF-kappaB pathways in hematological malignancies," *Cellular and Molecular Life Sciences*, vol. 71, no. 11, pp. 2083–2102, 2014.

[52] H.-M. Shen and V. Tergaonkar, "NFkappaB signaling in carcinogenesis and as a potential molecular target for cancer therapy," *Apoptosis*, vol. 14, no. 4, pp. 348–363, 2009.

[53] B. Hoesel and J. A. Schmid, "The complexity of NF-κB signaling in inflammation and cancer," *Molecular Cancer*, vol. 12, no. 1, article 86, 2013.

[54] C.-J. Yang, C.-S. Wang, J.-Y. Hung et al., "Pyrogallol induces G2-M arrest in human lung cancer cells and inhibits tumor growth in an animal model," *Lung Cancer*, vol. 66, no. 2, pp. 162–168, 2009.

[55] I. M. Adcock, C. R. Brown, O. Kwon, and P. J. Barnes, "Oxidative stress induces NFκB DNA binding and inducible NOS mRNA in human epithelial cells," *Biochemical and Biophysical Research Communications*, vol. 199, no. 3, pp. 1518–1524, 1994.

[56] H. J. Kim, J. Kim, K. S. Kang, K. T. Lee, and H. O. Yang, "Neuroprotective effect of chebulagic acid via autophagy induction in SH-SY5Y cells," *Biomolecules & Therapeutics*, vol. 22, no. 4, pp. 275–281, 2014.

[57] D. M. Benbrook and A. Long, "Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis," *Experimental Oncology*, vol. 34, no. 3, pp. 286–297, 2012.

[58] L. T. Lee, Y. T. Huang, J. J. Hwang et al., "Blockade of the epidermal growth factor receptor tyrosine kinase activity by quercetin and luteolin leads to growth inhibition and apoptosis of pancreatic tumor cells," *Anticancer Research*, vol. 22, no. 3, pp. 1615–1627, 2002.

[59] F. A. Bhat, G. Sharmila, S. Balakrishnan et al., "Quercetin reverses EGF-induced epithelial to mesenchymal transition and invasiveness in prostate cancer (PC-3) cell line via EGFR/PI3K/Akt pathway," *The Journal of Nutritional Biochemistry*, vol. 25, no. 11, pp. 1132–1139, 2014.

[60] E. H. Walker, M. E. Pacold, O. Perisic et al., "Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine," *Molecular Cell*, vol. 6, no. 4, pp. 909–919, 2000.

[61] S. P. Davies, H. Reddy, M. Caivano, and P. Cohen, "Specificity and mechanism of action of some commonly used protein kinase inhibitors," *Biochemical Journal*, vol. 351, no. 1, pp. 95–105, 2000.

[62] C. Marienfeld, L. Tadlock, Y. Yamagiwa, and T. Patel, "Inhibition of cholangiocarcinoma growth by tannic acid," *Hepatology*, vol. 37, no. 5, pp. 1097–1104, 2003.

[63] N. Wang, Z.-Y. Wang, S.-L. Mo et al., "Ellagic acid, a phenolic compound, exerts anti-angiogenesis effects via VEGFR-2 signaling pathway in breast cancer," *Breast Cancer Research and Treatment*, vol. 134, no. 3, pp. 943–955, 2012.

[64] M. V. Vadhanam, S. Ravoori, F. Aqil, and R. C. Gupta, "Chemoprevention of mammary carcinogenesis by sustained systemic delivery of ellagic acid," *European Journal of Cancer Prevention*, vol. 20, no. 6, pp. 484–491, 2011.

[65] A. Naiki-Ito, T. Chewonarin, M. Tang et al., "Ellagic acid, a component of pomegranate fruit juice, suppresses androgen-dependent prostate carcinogenesis via induction of apoptosis," *The Prostate*, vol. 75, no. 2, pp. 151–160, 2015.

[66] D. K.-P. Hau, G.-Y. Zhu, A. K.-M. Leung et al., "*In vivo* anti-tumour activity of corilagin on Hep3B hepatocellular carcinoma," *Phytomedicine*, vol. 18, no. 1, pp. 11–15, 2010.

[67] B. C. Ji, W. H. Hsu, J. S. Yang, and et al, "Gallic acid induces apoptosis via caspase-3 and mitochondrion-dependent pathways in vitro and suppresses lung xenograft tumor growth in vivo," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 16, pp. 7596–7604, 2009.

[68] C.-Z. Liang, X. Zhang, H. Li et al., "Gallic acid induces the apoptosis of human osteosarcoma cells in vitro and in vivo via the regulation of mitogen-activated protein kinase pathways," *Cancer Biotherapy and Radiopharmaceuticals*, vol. 27, no. 10, pp. 701–710, 2012.

[69] E. Angst, J. L. Park, A. Moro et al., "The flavonoid quercetin inhibits pancreatic cancer growth in vitro and in vivo," *Pancreas*, vol. 42, no. 2, pp. 223–229, 2013.

[70] D. R. Ferry, A. Smith, J. Malkhandi et al., "Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition," *Clinical Cancer Research*, vol. 2, no. 4, pp. 659–668, 1996.

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Review Article

Psychiatric Disorders and Polyphenols: Can They Be Helpful in Therapy?

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The prevalence of psychiatric disorders permanently increases. Polyphenolic compounds can be involved in modulation of mental health including brain plasticity, behaviour, mood, depression, and cognition. In addition to their antioxidant ability other biomodulating properties have been observed. In the pathogenesis of depression disturbance in neurotransmitters, increased inflammatory processes, defects in neurogenesis and synaptic plasticity, mitochondrial dysfunction, and redox imbalance are observed. *Ginkgo biloba*, green tea, and *Quercus robur* extracts and curcumin can affect neuronal system in depressive patients. ADHD patients treated with antipsychotic drugs, especially stimulants, report significant adverse effects; therefore, an alternative treatment is searched for. An extract from *Ginkgo biloba* and from *Pinus pinaster* bark, Pycnogenol, could become promising complementary supplements in ADHD treatment. Schizophrenia is a devastating mental disorder, with oxidative stress involved in its pathophysiology. The direct interference of polyphenols with schizophrenia pathophysiology has not been reported yet. However, increased oxidative stress caused by haloperidol was inhibited *ex vivo* by different polyphenols. Curcumin, extract from green tea and from *Ginkgo biloba,* may have benefits on serious side effects associated with administration of neuroleptics to patients suffering from schizophrenia. Polyphenols in the diet have the potential to become medicaments in the field of mental health after a thorough study of their mechanism of action.

1. Introduction

The prevalence of psychiatric disorders permanently increases. In the WHO European Region nearly 4 out of 15 people suffer from major depression and anxiety. Neuropsychiatric disorders are the second cause of disability in Europe and account for 19% in comparison to cardiovascular disorders with only 4%. In 28 countries of EU with a population of 466 million, at least 21 million people were affected by depression, out of which almost 80% are men. The treatment of psychiatric disorders is very expensive. The total annual cost of depression in Europe was estimated at Euro 118 billion in 2004, which corresponds to a cost of Euro 253 per inhabitant. The cost of depression corresponds to 1% of the total economy of Europe. These reasons provide support for the importance of increased research efforts in this field, better detection, prevention based on improvement of lifestyle factors including diet, and effectiveness of treatment [1].

Results of many animal and human studies support the role of different natural polyphenolic compounds in modulation of mental health including brain plasticity, behaviour, mood through anxiolytic, antidepressant-like properties, and cognition. Research demonstrates that dietary factors and exercise can affect the maintenance and development of neurons and protect the brain from insult associated with neurological illnesses or injuries [2–7].

2. Polyphenolic Compounds

Polyphenols (known as *polyhydroxyphenols*) are predominantly secondary metabolites of plants. They belong to

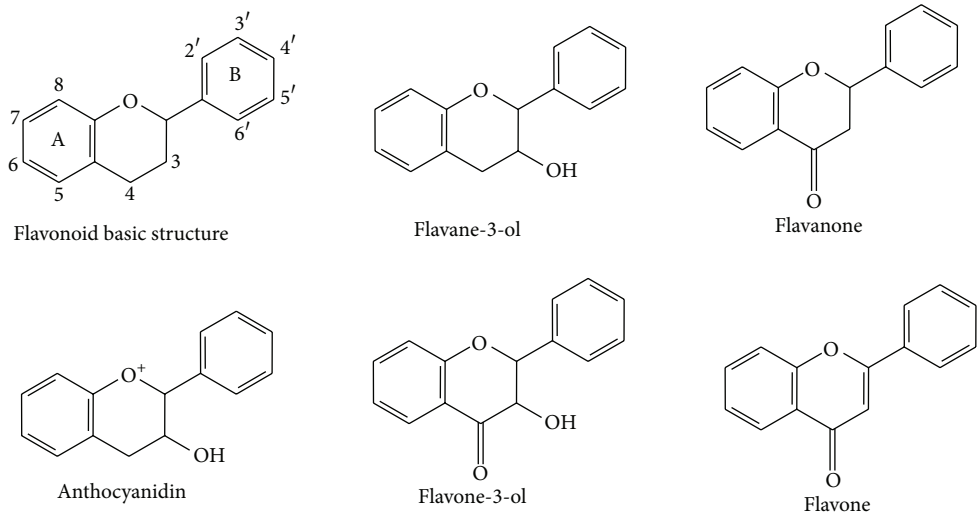

Figure 1: Basic flavonoid structures.

structural class of organic compounds characterized by the presence of large multiples of phenol structural units. These phenol structures underlie the unique physical, chemical, and biological (metabolic, toxic, therapeutic, etc.) properties of particular members of the class. The name derives from the ancient Greek word πολύς (polus, meaning "many, much") and the word phenol which refers to a chemical structure formed by attaching a hydroxyl (–OH) group to an aromatic phenyl ring. They are divided into three groups according to their hydrolytic cleavage products: (i) tannins: derivatives of catechin or gallic acid with mostly antioxidant properties [8], (ii) phenylpropane derivatives (lignans, ellagitannins, cinnamic acid derivatives, and others); for example, higher dietary intake of lignans is associated with better cognitive functions in postmenopausal women [9] or extract of ellagitannins from oak wood reduced many of key symptoms of chronic fatigue [10], and (iii) flavonoids: phenolic compounds spread in the plant kingdom. They include more than 4000 different derivatives and their list constantly increases. Formation of so many derivatives is possible due to the substitution of hydrogen atoms by hydroxyl, methoxyl, and other groups at different sites of the basic structures. The basic flavonoid structures include the following: *flavan-3-ols* (epicatechin and gallocatechin), *flavanones* (naringenin and hesperidin), *flavones* (apigenin and luteolin), *flavone-3-ol* (quercetin and myricetin), *anthocyanidins* (cyanidin and pelargonidin), and *isoflavones* (genistein and daidzein) [11] (Figure 1).

Polyphenols occur in food (vegetables and fruits) either as free monomers (quercetin and catechin) or oligomers (procyanidins). They are bound to saccharides as glycosides or occasionally they are found as free aglycones. After ingestion, flavonoids can undergo biotransformation to their metabolites which can be detected in plasma reaching concentration of about 1 μmol·L^{-1} [12, 13].

Consumption of polyphenol-rich foods is associated with a lower incidence of coronary heart disease, myocardial infarction [14], cancer [15], neurodegenerative diseases [16], psychiatric disorders (like ADHD) [17], and other chronic diseases [18]. Since in the pathology of these diseases, in addition to other factors, oxidative stress has been assumed to play a role, dietary flavonoids have been suggested to exert health benefits through antioxidant mechanisms. In experiments *in vitro*, flavonoids exert a significant antioxidant [8, 19] and redox modulating [20] ability. Polyphenols act as strong antioxidants *in vitro* through the numerous mechanisms, such as radical scavenging, metal ions (Fe, Cu, and others) chelation, and the modulation of antioxidant enzyme activities [8, 21]. In the scavenging ability the position and the number of phenolic –OH groups play a role through donation of a hydrogen atom from their hydroxyl groups to radicals, resulting in radical moiety elimination. During this reaction, phenoxyl radical is formed that can form stable compound and terminates radical reaction via reaction with another radical [20].

Upon consumption of food, polyphenols are available in the form of esters, glycosides, or polymers that cannot be absorbed in the intestine. The original molecules of polyphenolic compounds are hydrolyzed by microbial enzymes in colon and transformed via methylation, sulfation, and glucuronidation to derivatives of original molecules followed by their absorption in the colon and travelling through blood to various tissues and organs such as the brain. However, due to the diverse susceptibilities of phenolic compounds to colon enzyme metabolism, their bioavailability can vary from very low to very high [22, 23]. The low bioavailability and transformation of polyphenolic compounds *in vivo* to different derivatives lead to their low direct antioxidant activity in comparison to other low-molecular weight antioxidants, such as vitamins C and E and uric acid [24].

However, in addition to antioxidant activities polyphenols at low concentration can exert also other biological effects *in vivo*. Polyphenols can participate in modulation of different signaling pathways, thus influencing the fate of cells [25] including nerve cells via influencing the neuronal survival, regeneration, development, or death [26].

Polyphenolic compounds possess also antimutagenic ability [27], vasodilating [28], antithrombic [29], antiapoptotic [30], and anti-inflammatory [31] effects.

Anti-inflammatory effects of polyphenols in cerebral tissue can be realised via binding to various receptors. Flavonoid-induced receptor stimulation can modulate active state of different kinases, such as the mitogen-activated protein (MAP) kinase (naringenin), phosphoinositide-3-(PI3) kinase (curcumin), nuclear factor-kappaB (resveratrol and epigallocatechin gallate), and protein kinase C (PKC) pathways (resveratrol). Polyphenols can thus influence differentiation and apoptosis, cell survival (inhibition of apoptosis), inflammatory response, learning and memory, and reduction of amyloid plaque formation. Polyphenols can affect the activation of glial cells in brain, which are the residence of macrophages, via inhibiting the inflammation mediated by macrophages through the reduction of proinflammatory cytokines (IL-1 beta, TNF-alpha) formation [32].

The neuroprotection of polyphenols can be assigned to the improvement of cerebral blood flow via stimulation of NO formation in the endothelium and inhibition of platelet aggregation [32].

The principle question is how and in which form and amount can polyphenols reach the brain and modulate its function? This problem is not completely solved. Results from animal experiments indicate that diet supplementation with some polyphenolic extracts (e.g., from grapes, blueberries, and blackberries) results in deposition and bioavailability of polyphenols and their metabolites in the brain of animals where they can directly exert their protective effects. However, we can only assume that a diet rich in vegetables and fruits will result in increased cerebral deposition of these beneficial compounds. Polyphenols might modify brain function at three locations: (i) outside the CNS, by improving cerebral blood flow or by altering signaling pathways from peripheral organs to the brain, (ii) at the blood-brain barrier, by altering multi-drug-resistant protein-development influx/exflux mechanisms of different biomolecules, and (iii) inside the CNS, by modifying the activity of neurons and glial cells [33].

On the other hand, flavonoids could exert also their prooxidative properties *in vitro*, for example, in the presence of high concentration of Cu^{2+} ions (25–100 μmol·L^{-1}) and oxygen. The issue whether prooxidative effects of flavonoids can be exerted also *in vivo* has not been addressed yet and the answer to this question requires further studies [34, 35].

3. Psychiatric Disorders

Psychiatric disorders, including major depression, attention deficit hyperactivity disease (ADHD), and schizophrenia, contribute largely to mental problems of children, adolescents, and adults.

According to the PubMed, under the terms "polyphenols" and "mental health," the number of studies dealing with polyphenols and mental health is much lower (21 papers) compared with cardiovascular diseases (924 papers) or neurodegenerative diseases (291 papers). For this reason, any new paper dealing with this topic is very important.

It is believed that in pathology of psychiatric disorders also oxidative stress plays a role (Figure 2). Oxidative stress is defined as the disbalance between production of free radicals and/or reactive oxygen species/reactive nitrogen species (ROS/RNS) and antioxidant defence in favour of ROS/RNS leading to oxidative damage to lipids, proteins, and DNA and thus to the dysfunction of cells and organs [25]. Although the brain forms less than 2% of the body weight, it consumes about 20% of the oxygen available through respiration. The brain is also a lipid-rich organ, which can contribute to its susceptibility to oxidative damage [36]. The brain has a large potential oxidative capacity but a limited ability to counteract oxidative stress. Cells in the central nervous system are more sensitive to toxic effects of ROS, than cells in other organs of the body. Moreover, in the brain there is a low activity of catalase, insufficient activities of glutathione peroxidase (both enzymes decompose hydrogen peroxide or organic peroxides) and superoxide dismutase (decomposes superoxide anion radical to hydrogen peroxide and oxygen), and higher level of iron ions and ascorbic acid (together they form optimal conditions for formation of very toxic hydroxyl radical) in comparison to other cells. These facts increase the susceptibility of brain to oxidative and peroxidative damages to biomolecules [25, 32].

In addition to antioxidant properties of polyphenols, research has shown that polyphenols can exert their neuroprotective properties through modulation of specific cellular signaling pathways involved in cognitive processes such as synaptic plasticity, notably, pathways with CREB (cAMP-response element-binding protein) signalling. CREB is a transcription factor linked with genes that express brain-derived neurotrophic factor (BDNF). The importance of CREB in brain function is emphasized by studies that demonstrate impairments in memory formation induced by the disruption of CREB activity and, similarly, accelerations in memory formation stimulated by increased CREB activity [37]. Polyphenols can directly modulate these signaling pathways by induction of CREB and subsequently by BDNF activation [23].

4. Major Depression

Major depression is a psychiatric disorder which represents the fourth leading cause of disability worldwide and is expected to become the second most prevalent disease after ischemic heart disease by 2020. Depression is also one of the most costly disorders in western countries, and antidepressants account for 20% of total CNS drug sales [38].

Depression has a multifactorial aetiology arising from genetic, environmental, psychological, and biological factors. These factors are mainly applied jointly in aetiology of depression, and their impact on the status and severity of disease are mutually intertwined (Figure 2).

Firstly, the causal relation is assumed between disturbance in monoamine (neurotransmitters) metabolism, especially serotonin, abnormalities in its receptor, and depression [39], but also dopamine, adrenaline, and glutamate are involved [40, 41]. Metabolism of neurotransmitters is

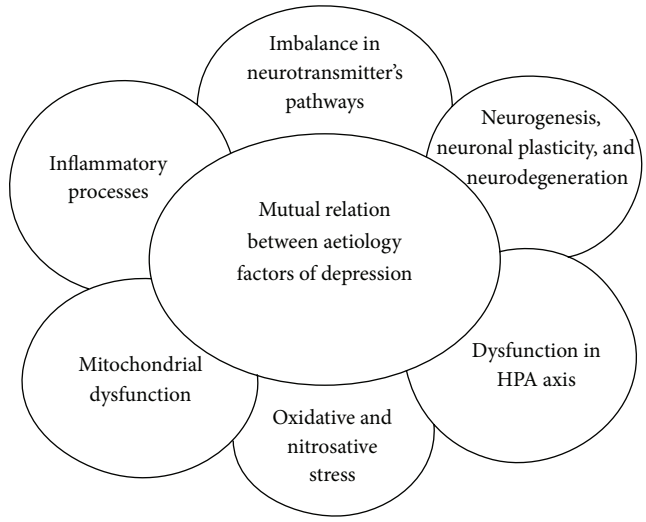

Figure 2: Mutual relations between aetiology factors of depression. Explanation of individual factors is given in the text. HPA: hypothalamic-pituitary-adrenal.

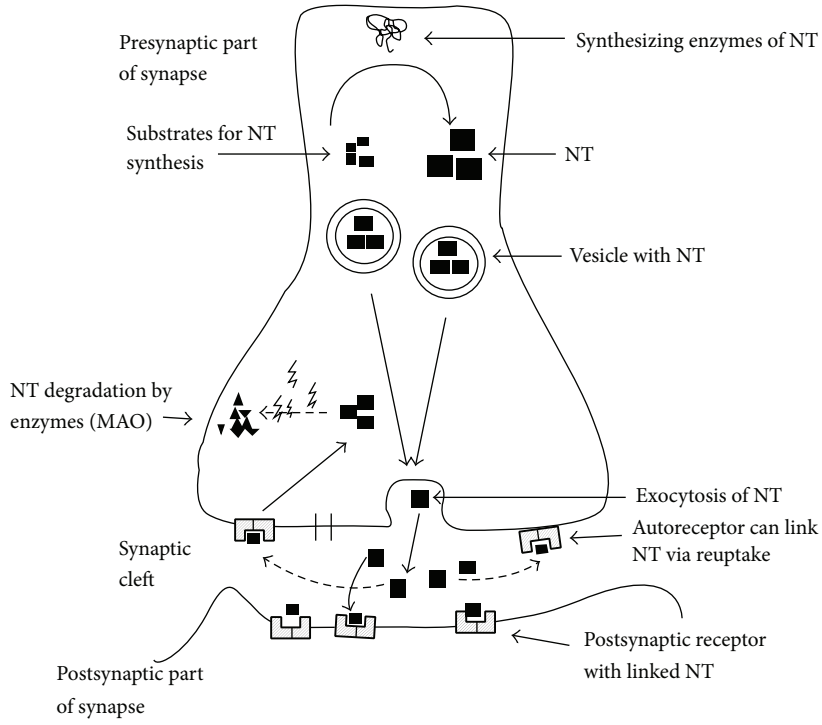

Figure 3: Neurotransmitters in synapse. Molecules of NT are synthesized from their substrates by enzymes. NT are stored in vesicles. Vesicles after action potential fuse with presynaptic membrane and NT are released into synapse cleft. Released NT are linked to postsynaptic receptors and signal is transferred (→) to postsynapse. NT can be reuptaken by autoreceptor and neurotransmission is inhibited (⇢). Reuptaken NT can be enzymatically degraded (MAO). NT: neurotransmitter; MAO: monoaminooxidase.

influenced by enzymes involved in their degradation, like monoamine oxidase (MAO), and in synthesis of their amino acid precursor tryptophan by enzyme tryptophan hydroxylase [42]. The causal association between neurotransmitters and depression is also based on successful treatment of depression with selective serotonin reuptake inhibitors (SSRIs) into the presynaptic cells increasing the level of serotonin in the synaptic cleft available to bind to the postsynaptic receptor (Figure 3). The process of neurotransmission is explained in the text to the figure.

Secondly, increased inflammatory processes are also involved in the pathology of depression which was first reported by Maes et al. [43]. Increased proinflammatory cytokines can induce various clinical characteristics such as

disturbed serotonin metabolic pathway and neurovegetative symptoms [44]. Increased level of proinflammatory cytokines (IL-6, tumor necrosis factor (TNF-alpha)) and C-reactive protein (CRP) in blood are recognized as good markers of increased inflammation in depressive patients. These reliable markers of nonspecific inflammation showed positive correlation with the severity of depressive symptoms and some comorbidities like impaired sleep, cognitive dysfunction, and fatigue [38, 45, 46].

Thirdly, neurogenesis and synaptic plasticity in the context of adult hippocampal neurogenesis (AHN) are compromised in patients with depression with subsequent neurodegeneration [47]. This results in stress-induced alteration in the number and shape of neurons and glial cells in brain regions of depressed patients and decreased proliferation of neural stern cells [48]. The most abundant neurotrophin in the central nervous system involved in neuronal survival, growth, and proliferation is the brain-derived neurotrophic factor (BDNF). In patients with depression the levels of BDNF are very low [49, 50].

Fourthly, dysfunction in hypothalamic-pituitary-adrenal (HPA) axis is characteristic for patients suffering from depression [51]. This gives rise to the failure in the secretion of cortisol and glucocorticoids depending on the type of depression and to the hypersecretion of corticotropin-releasing hormone (CRH). Treatment with antidepressants regulates levels of these hormones [52–54].

Fifthly, depression is associated with mitochondrial dysfunction related to lower activities of respiratory chain enzymes, ATP production, and damage to mitochondrial DNA [55–58].

Sixthly, it is assumed that redox imbalance (increased oxidative and nitrosative stress) also plays a role in the pathology of depression. The antioxidant defence systems are decreased and the level of low molecular-weight antioxidants, such as vitamin C, vitamin E, and coenzyme Q as well as the activity of antioxidant enzyme glutathione peroxidase are reduced [59–62]. Deficiencies in antioxidant defence systems impair protection of cells and organs against free radicals and reactive oxygen and nitrogen species leading to the damage to proteins, DNA, and lipids through oxidation of fatty acids in phospholipids of lipoproteins and membranes [8].

Increased oxidative stress is manifested by increased markers of oxidative stress in depressed patients, such as increased level of malondialdehyde and isoprostanes, products of lipoperoxidation [63, 64], peroxides in plasma [65], xanthine oxidase activity [66], and oxidative damage to DNA represented by increased level of 8-hydroxy-2-deoxyguanosine [67, 68].

4.1. Can Polyphenols Influence Aetiology Factors of Depression? Several *in vitro* and *in vivo* studies indicate that polyphenols can affect neuronal system and processes [3]. It should be kept in mind that the effects of polyphenols *in vivo* may be different from the effects *in vitro*, as *in vivo* rather metabolites of polyphenols are active than original substances. For example, curcumin, a nonflavonoid phenolic compound present in *Curcuma longa*, known and used in Indian traditional medicine, after administration significantly decreased depression-like behaviour in rats probably through improvement of the BDNF level [69]. Curcumin coadministered with alkaloid piperine inhibited MAO activity and increased serotonin and dopamine level in mice [70]. Sanmukhani et al. [71] provides clinical evidence that curcumin (1000 mg/day) administered for 6 weeks to 60 patients with major depression in randomized and placebo controlled trial may be used as an effective and safe modality for treatment of depressive patients without concurrent suicidal ideation. On the contrary to results of Sanmukhani et al., Bergman et al. [72] did not observe significant differences between the groups of patients with administered curcumin (500 mg) and placebo for 5 weeks in randomized, double-blind, and placebo controlled clinical study, probably due to low daily doses used. However, the patients in the curcumin group demonstrated a trend to a more rapid relief of depressive symptoms in comparison to those in the placebo group.

Curcumin is a lipophilic compound that can easily cross the blood-brain barrier and directly induce neuroprotection probably through its antioxidant ability to inhibit lipid peroxidation and neutralize ROS and RNS [73]. In addition, curcumin can affect number of cellular pathways on molecular level and via anti-inflammatory properties it can inhibit cyclooxygenase 1 and cyclooxygenase 2 and influence many other signalling pathways leading to cell protection and enhancement of cell survival [74].

Flavonoid derived from catechin, epigallocatechin gallate (EGCG), present in green tea, was used in traditional Chinese medicine for at least 4000 years. At present EGCG is known for its powerful antioxidant properties and for its ability to attenuate stress and depression. In experimental study on mice increased level of BDNF was found after a long-term administration of green tea polyphenols [75] or reduced serum corticosterone and adrenocorticotropic hormone levels after forced swimming test [76]. *In vitro* experiments with cultured hippocampal neurons confirmed the specific modulation of the GABA-A receptor benzodiazepine site by application of EGCG [77]. In a double-blind, randomized, and placebo controlled human study with seventy-four subjects who were administered green tea or placebo for 5 weeks, subjects with the long-term green tea extract supplementation increased the reward learning and prevented the depressive symptoms [78]. Also an extract of polyphenols from *Ginkgo biloba,* consisting, namely, of flavonol quercetin and kaemferol, has been shown to have antidepressant-like effects in mice probably through increasing BDNF level, neuronal survival and plasticity, and inhibition of MAO towards serotonin [79]. MAO is also inhibited *in vitro* by anthocyanins from berries, flavone apigenin from celery, and stilbene *trans*-resveratrol from red wine [80]. The flavonoids from cocoa showed also antidepressant-like effects in the animal model using the forced swimming test in rats [81] and reduced symptoms of chronic fatigue in ten subjects enrolled in double-blind, randomized, clinical pilot crossover study [82]. Polyphenolic extract from the wood of *Quercus robur* (*Robuvit*, Horphag Res. Ltd.) is a proprietary extract with concentrated water-soluble components of the wood

(ellagitannins) also found in wine resting in oak barrels. Robuvit contains roburins (A, B, C, D, and E) and grandinin. These components belong to the group of hydrolysable tannins (ellagitannins). Clinical investigation in healthy volunteers and patients with primary lymphedema has shown an increased antioxidant capacity of blood and a decrease in peripheral edema after supplementation with Robuvit [83, 84].

In the study of Natella et al. [83], Robuvit actions were studied on modulation of gene expression. Robuvit affects ribosomes, cell cycle, and spliceosome pathway. The effects of Robuvit on stimulation of ribosomal activity and protein translation are suggested to be involved in relieving fatigue in healthy volunteers and chronic fatigue syndrome in patients [85]. In addition, Robuvit was shown to accelerate healing in patients with temporary hepatic damage [86]. In another study, intake of 300 mg/day of Robuvit was associated with improving effect on energy, tiredness, and tension subscales evaluating fatigue in 20 volunteers with lower baseline of feelings scoring [87]. In the same volunteers, the decrease of markers of oxidative stress and increase of activity of antioxidant enzymes, Cu/Zn superoxide dismutase, catalase, and total antioxidant capacity of plasma *in vivo* were observed [88].

5. Attention Deficit Hyperactivity Disorders (ADHD)

Attention deficit hyperactivity disorder is the most prevalent childhood disorder, estimated to affect 2–18% of children depending largely on diagnostic criteria [89]. The economic consequences of ADHD persisting into adulthood are significant with one US analysis finding an average of 35 days of annual lost work performance, representing 120 million days of annual lost work in the labor force, equivalent to 19.5 billion USD lost human capital [90].

ADHD is a complex polygenic disorder with high levels of heterogeneity, influenced by the interaction of multiple aetiological factors [91]. Twin, family, and adoption studies of ADHD have supported a strong genetic contribution to the disorder, with heritability ranging from 60 to 90%. A plausible genetic hypothesis for ADHD is a mixture of dominant and recessive major genes that act with complex polygenic transmission patterns. Molecular genetic studies have implicated a number of possible genes (DRD4, DRD5, DAT1, DRD1, and Taq1). However, each of these genes only increases relative risk of ADHD slightly. Pre-, peri-, and postnatal environmental factors play an important role in the pathogenesis of ADHD. Prenatal factors are associated with maternal lifestyle during pregnancy. For example, prenatal alcohol exposure is known to induce brain structural anomalies, especially in the cerebellum. Maternal smoking produces a 2.7-fold increased risk for ADHD. Perinatal factors have also been implicated, with a twofold increase in ADHD in very low-birthweight children and an increased rate of pregnancy with birth complications. Among postnatal factors, a role for malnutrition and dietary deficiency in ADHD has been proposed. An imbalance of essential fatty acid (omega-3 and omega-6) intake has been suggested to be potentially involved in the development of ADHD. Iron deficiency has been implicated in some cases. Early deprivation of social environment during the postnatal period may also have significant effects [92].

Studies have identified various structural and functional abnormalities in frontostriatal network. This network involves the lateral prefrontal cortex, the dorsal anterior cingulate cortex, and the caudate nucleus and putamen. In ADHD patients, reductions in volume have been observed in total cerebral, the prefrontal cortex, the basal ganglia (striatum), the dorsal anterior cingulate cortex, the corpus callosum, and the cerebellum. A developmental trajectories study in ADHD patients showed a delay in cortical maturation. The delay was most prominent in prefrontal regions important in the control of cognitive processes including attention and motor planning. Compensatory networks including basal ganglia, insula, and cerebellum have been implicated for relative lower cognitive load tasks in ADHD patients.

Genetic influences predispose a child to catecholaminergic dysregulation (deficits in dopamine, noradrenaline, and serotonin transmission) and abnormalities in their metabolism [93, 94]. There is also persuasive relation between ADHD and suboptimal level of catecholamines and the composition of consumed essential fatty acids [95] as well as consumption of certain additives or food preservatives [96].

For diagnosis clear evidence of clinically significant impairment in social, academic, or occupational functioning is required. The essential feature of ADHD is a persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development. Inattention manifests behaviorally in ADHD as wandering off task, lacking persistence, having difficulty sustaining focus, and being disorganized which is not due to defiance or lack of comprehension. Hyperactivity refers to excessive motor activity (such as a child running around) when it is not appropriate, or excessive fidgeting, tapping, or talkativeness. In adults, hyperactivity may manifest as extreme restlessness or wearing others out with their activity. Impulsivity refers to hasty actions that occur in the moment without forethought and that have high potential for harm to the individual (e.g., darting into the street without looking) (DSM V, APA, 2013).

Comorbidity is common in ADHD, with strong links to oppositional defiance disorder, learning disorders in children, major depressive disorder, anxiety disorders, social dysfunction, and substance abuse in adults. Academic issues surrounding ADHD in childhood are linked to a higher drop-out rate from secondary (high) school with fewer than 5% completing a university degree [97].

Conventional treatment options usually include, either in isolation or in combination, a pharmaceutical component, a behavioural component, and a psychosocial component. Pharmacotherapies. which inhibit the reuptake of noradrenaline and dopamine such as the psychostimulants methylphenidate and dextroamphetamine, and nonstimulating prefrontal cortex noradrenaline reuptake inhibitor atomoxetine, are the standard Western treatments for ADHD. Selective serotonin reuptake inhibitors (SSRIs) and other antidepressants are also used with varying degrees of success.

A third of ADHD patients who take stimulants for ADHD report significant adverse effects including anorexia, weight loss, abdominal pain, sleep disturbances, headaches, irritability, depressed mood, and appetite, with some reports of stimulant induced psychosis. Increasing apprehension regarding stimulant medication and the ramifications of its use in children has led to the investigation and acknowledgment of alternative therapeutic medications [94].

While more than 900 results can be found for the term "depression" in Pubmed, only 47 studies including just one systematic review can be found for the terms "oxidative stress" and ADHD [98]. When compared to oxidative stress, results from six studies with total 231 ADHD patients and 207 controls indicate that the association between ADHD and antioxidant status was not significant. However, results with markers of oxidative stress are controversial. Malondialdehyde (MDA), the marker of lipoperoxidation, was found increased in 20 adult patients and correlated with the score of hyperactivity [99], but in 30 children with ADHD, this parameter was not different from healthy controls [100]. In contrast to results of Oztop et al. [100], Essawy et al. [101] found higher level of MDA and decreased level of antioxidant element, zink, in children with ADHD.

Selek et al. [102] found increased level of NO which at low concentration exhibits important physiological functions in neurotransmitters release, memory, and learning [103], but at high concentration NO with superoxide can form very damaging oxidant, peroxynitrite $ONOO^{-}$. At low SOD activity, which was found by Selek et al. [102], a redox imbalance and oxidative stress can be observed in adults with ADHD. However, in children, decreased activity of SOD was not observed [104]. Authors found altered activities also of other antioxidant enzymes, glutathione peroxidase, and non-significantly changed catalase in comparison to the controls. Our results found in 61 children with ADHD investigated in double-blind, randomized, and placebo controlled study suggest increased level of the marker of oxidative damage to DNA, 8-oxo-7,8-dihydroxyquanine (8-oxo-G) and decreased total antioxidant status in comparison to the controls [24]. We also investigated levels of neurotransmitters in urine. In ADHD children, adrenaline and noradrenaline concentrations positively correlated with plasma levels of oxidized glutathione and noradrenaline positively correlated with the degree of hyperactivity [105].

However, the determination of only one or two markers of oxidative stress cannot reflect the real redox state in the organism. Therefore, the evaluation of total oxidative status (TOS) and total antioxidant status (TAS) and their ratio as oxidative stress index (OSI) could be useful for identification of redox imbalance [106–108].

However, a small number of studies and their variety do not allow drawing definitive conclusions concerning involvement of oxidative stress in pathophysiology of ADHD.

5.1. Polyphenols in ADHD Treatment. In experimental conditions, *Ginkgo biloba* extract (EGb 761) was tested on synaptosomal fraction prepared from mice cerebral cortex. EGb 761 significantly increased uptake of serotonin. Similar effect was observed, when synaptosomes were prepared from the cortex of mice treated orally with EGb 761. These observations were found in an area of suspected deficit in people with ADHD [109]. In ADHD patients several polyphenolic compounds were tested for treatment [110]. The extract from *Ginkgo biloba* at daily dose 80–120 mg administered during 6 weeks to fifty children treated with methylphenidate had no benefits in double blind, randomized, and placebo controlled study [111]. However, in another study increased dosage with the maximum of 240 mg/day was administered to 20 children with ADHD in an open clinical pilot study over 3 to 5 weeks. Improvement of ADHD symptoms, as well as brain-electrical activity was observed [112].

St. John's wort from *Hypericum perforatum* (900 mg/day) was used for treatment of ADHD symptoms in a double blind, randomized, and placebo controlled study with 54 children. Positive results were observed after 8 weeks of treatment [113]. The effect of traditional Chinese medicine compound (*Ningdong*, NDG) at daily dose of 5 mg/kg was studied in 72 children with ADHD and compared with effects of methylphenidate (1 mg/kg) in a randomized double-blind trial. After 8 weeks of treatment NDG significantly reduced ADHD symptoms. The level of dopamin was not changed but serum level of homovanillic acid (a degrading product of catecholamine catabolism) increased [114]. *Oroxylin A* is an O-methylated flavone, a chemical compound that can be found in the medicinal plant *Scutellaria baicalensis* and the *Oroxylum indicum* tree. It has demonstrated a dopamine but not noradrenaline, reuptake inhibitor activity. Its analogue, 5,7-dihydroxy-6-methoxy-4′-phenoxyflavone, showed the most remarkable inhibition of dopamine reuptake comparable to methylphenidate, but not modulation of GABA pathway in spontaneously hypertensive rat model of attention-deficit hyperactivity disorder [115, 116].

Pycnogenol (Horphag, Ltd), a standardized extract of French maritime pine bark *Pinus pinaster*, was also studied in relation to mental health, especially to ADHD. Pycnogenol is a defined mixture of polyphenols, mainly procyanidins, catechin, taxifolin, and a small amount of phenolic acids [117]. It exhibits a number of biological activities, especially antioxidant properties *in vitro* and many different biomodulating activities *in vivo* [118]. The exact mechanism by which Pycnogenol improves brain functions and mental health is not entirely clear yet. Several works on different levels (cell cultures, experimental animal models, and human studies) deal with effects of Pycnogenol on brain functions or mental health. The first condition for the positive effect of substances in the brain is the ability to cross the blood-brain barrier. Pycnogenol is able to cross blood brain barrier [119] as well as other cell membranes. Kurlbaum et al. [120] analysed the binding of constituents and the metabolite M1 (delta (3,4-dihydroxyphenyl)-gamma-valerolactone) of Pycnogenol that had been previously detected in plasma samples of human Pycnogenol consumers, to human erythrocytes. Authors found a transporter-mediated accumulation of the flavonoid metabolite, probably via GLUT-1 transporter. It was also found that Pycnogenol significantly increased the membrane fluidity predominantly at the membrane surface. Pycnogenol efficacy to modify effectively some membrane

dependent processes is related not only to the chemical action of Pycnogenol but also to its ability to interact directly with cell membranes and/or penetrate the membrane thus inducing modification of the lipid bilayer and lipid-protein interactions [121]. The ability to modify membrane fluidity can be related to the pathology of psychiatric disorders through modification of adrenergic receptors [122]. Pycnogenol protected cultured SH-SY5Y neuroblastoma cells against acrolein-induced oxidative stress toxicity probably through its antioxidant properties and increased level of GSH [123]. The same cells were used in another experiment, in which Pycnogenol and extract from *Hypericum perforatum* (St. John's wort) were used as alternatives to the classical ADHD drugs. Pycnogenol exerted no significant effect on ATP level but increased cell survival at the concentrations 32.25 and 250 ng/mL [124].

Also results obtained from animal models support the positive effects of Pycnogenol on mental health. Increased oxidative stress is implicated in the pathogenesis of Parkinson disease in which dopaminergic neurons are intrinsically susceptible to oxidative stress. In Parkinson disease model mice treated with Pycnogenol (20 mg/kg) for 15 days decreased number of dopaminergic D2 receptors and increased levels of dopamin and its metabolites were observed [125]. Neuroprotective effect of Pycnogenol was observed by Scheff et al. [126] in a rat model after traumatic brain injury following increased oxidative stress, increased level of proinflammatory cytokines in cortex and hippocampus. In treated animals ameliorated level of protein carbonyls, lipid peroxides, protein nitrations, and proinflammatory cytokines were observed. In mentioned rat model the same group of authors also observed decreased level of thiobarbituric acid reactive substances (TBARS) in brain and injury-related declines in pre- and postsynaptic proteins after Pycnogenol treatment (1–10 mg/kg) [127].

Influence of Pycnogenol on cognitive functions and enhancement of "normal" mental performance was studied in 53 students in evaluation study. After 8 weeks of supplementation, attention, memory, executive functions, and mood rating were improved [128]. Influence of Pycnogenol on cognitive functions, attention, mental performance, and specific professional skills together with oxidative stress in healthy professionals was studied in 30 subjects and results were compared with comparable control group. After 12 weeks of Pycnogenol supplementation at the dose of 150 mg/day improved cognitive functions and oxidative stress parameters compared to the control group [129].

First case reports about positive effects following supplementation of ADHD; children with Pycnogenol were collected by Passwater [130]. Heimann [131] reported that coadministration of Pycnogenol and dextroamphetamine clearly improved symptoms of ADHD of a 10-year-old boy. Withdrawal of Pycnogenol while continuing dextroamphetamine treatment caused a relapse; reinstated Pycnogenol caused again the significant improvement. Positive experience with Pycnogenol was also reported by Hanley in her book "Attention Deficit Disorder" [132]. Masao published in Japan a success rate of 70% when treating 40 children with 1 mg/kg Pycnogenol [133]. An attempt to demonstrate reduction of ADHD symptoms in adults failed in a double-blind, placebo controlled, comparative study with 24 adults [134]. No significant differences were found between placebo, methylphenidate, and Pycnogenol groups. As the study could not show a difference between the active drug, methylphenidate, and placebo, the relevance of these results is questionable.

One randomized, double blind, and placebo controlled study examined the role of Pycnogenol in alleviating ADHD symptoms. 61 children with ICD-10 diagnoses of ADHD were enrolled to either Pycnogenol or placebo groups. Children in Pycnogenol group were administered Pycnogenol at the dose of 1 mg/kg/day for one month followed by 1 wash-out month. No serious side effects were reported. A significant reduction of symptoms was noted in the intervention group of the teacher-rated Child Attention Problems for hyperactivity and inattention, with symptoms returning to pretreatment levels after the wash-out period. Reduction of these symptoms was not observed in the placebo group. When rated by parents and teachers on Conners' rating scale, symptoms decreased slightly compared to the baseline and placebo but did not reach significance. Also positive effects were detected on visual-motor coordination and concentration tasks in intervention but not in the placebo group. The relatively small number of 44 patients treated with Pycnogenol and the short duration of the study limits the generalization of our findings [17]. In this study, also levels of catecholamines in urine were investigated. Patients suffering from ADHD had significantly higher levels of adrenaline and noradrenaline at the baseline compared to healthy age-matched controls. The concentration of noradrenaline in urine of patients with ADHD positively correlated with the score for inattention. Treatment with Pycnogenol resulted in significantly decreased dopamine levels, while adrenaline and noradrenaline showed only a trend toward reduced levels [105]. Parallelly, improvement of GSH/GSSG ratio was determined [135] as well as an increase of total antioxidant status and decrease of oxidative damage to DNA [24]. These results indicate that Pycnogenol can inhibit oxidative stress by normalizing catecholamine levels in children with ADHD, which may, in turn, reduce hyperactivity and increase attention [110]. After completion of the study, parents asked that their ADHD children continue the additional treatment with Pycnogenol. Mentioned studies indicate that Pycnogenol could become a promising additive and complementary supplement in ADHD treatment; however, more studies are needed to confirm this conclusion [136].

6. Schizophrenia

Schizophrenia is a devastating mental disorder, expressed in the form of abnormal mental functions and disturbed behaviour. It has a life-time prevalence of approximately 1% of the world's population [137]. Genetic and early environmental factors, as well as psychological and social processes, appear to be important contributory factors. Many possible combinations of symptoms have triggered debate about whether the diagnosis represents a single disorder or a number of separate syndromes.

Symptoms begin typically in young adulthood, and about 0.3–0.7% of people are affected during their lifetime. The disorder is thought to mainly affect the ability to think, but it also usually contributes to chronic problems with behavior and emotions. People with schizophrenia are likely to have additional comorbidity, including major depression and anxiety disorders. Social problems, such as long-term unemployment, poverty, and homelessness, are common. The average life expectancy of people with the disorder is 12 to 15 years less than those without schizophrenia. This is the result of increased physical health problems and a higher suicide rate (about 5%). The mainstay of treatment is antipsychotic medication, which primarily suppresses dopamine receptor activity. Some recreational and prescription drugs appear to cause or worsen symptoms.

It is assumed that increased oxidative stress may be relevant to the pathophysiology of schizophrenia [138]. Molecular mechanisms contributing to oxidative stress are very complex and not fully understood yet. Although oxidative stress may not be the main cause, oxidative damage to important biomolecules has been suggested to be a common pathogenic process contributing to deteriorating course and poor outcome [139, 140]. Brain has a high rate of oxidative metabolic activity (see chapter, Psychiatric disorders). Moreover, neurotransmitters (dopamine, adrenaline, and noradrenaline) present in excess in the brain can be autooxidized to form relatively large amount of hydrogene peroxide. Additionally, neuronal mitochondria can form excess of superoxide anion radical. Due to insufficient activity of Mn-superoxide dismutase (MnSOD) and low concentration of major free radical scavenger in brain, glutathione (GSH), mitochondria become damaged and dysfunctioned [141]. Glutathione and redox regulation have a critical role in myelination processes and white matter maturation in the prefrontal cortex of rodent and human, a mechanism potentially disrupted in schizophrenia [142]. However, data for the brain redox status are limited and contradictory in human. The majority of information for oxidative stress in schizophrenia is received predominantly from determination of markers in plasma/serum, blood cells, or urine, respectively. Reduced level of GSH was observed in plasma of patients with schizophrenia [143]. The lower level of another endogenous low-molecular weight antioxidant, uric acid, was found in plasma of schizophrenic patients [144]. The presence of this antioxidant in the CNS is limited by the blood-brain-barrier and is about ten times lower than in blood [145]. Concerning activities of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, or catalase, controversial results in their activities (decreased, increased, and unchanged in comparison to healthy subjects) were observed in schizophrenic patients [146].

Similarly, contrasting results were observed in markers of lipid peroxidation (malondialdehyde, thiobarbituric acid reactive substances (TBARS), 4-hydroxynonenal, and isoprostanes) in patients with schizophrenia [147]. Meta-analysis of studies on MDA levels in schizophrenic patients showed very large heterogeneity of the results [148]. More accepted and more sensitive marker of nonenzymatic lipid peroxidation is F2-isoprostane, the product of peroxidation of arachidonic acid liberated from phospholipids [149]. This marker was found to be increased in patients with schizophrenia [150]. Also some other markers were investigated in schizophrenic patients. There were monitored markers of oxidative damage to proteins (protein carbonyls or 3-nitrotyrosine) [150], DNA such as 8-oxo-7,8-dihydro-2-deoxyguanosine, which was increased by 20% in 40 schizophrenic patients when compared to the controls [151], or leukocyte telomere length in 53 schizophrenic patients which was found to be gender dependent but not different from controls [152].

Oxidative stress is also related to apoptotic hypothesis of schizophrenia. Apoptosis (a programmed cell death) is a mechanism of cell death that operates in normal neurodevelopment and is increasingly recognized for its role in diverse neuropathological conditions. Activation of apoptosis can lead to rapid and complete elimination of neurons and glial cells in the CNS. In certain conditions, proapoptotic triggers can lead to sublethal and localized apoptotic activity that produces neuritic and synaptic loss without causing cell death. Neuropathology of schizophrenia includes reduced neuropil (especially synaptic elements) and limited and often layer-specific reduction of neurons suggesting progressive loss of cortical gray matter in first episode of psychosis, when antioxidant activity is low [153, 154]. Apoptotic mechanism that can influence synaptic connectivity and neuronal complexity seems to support the apoptotic hypothesis of schizophrenia connected also with oxidative stress [155].

Oxidative stress markers could be used to indicate the degree of severity of the disease in untreated schizophrenic patients and may be associated with the subtype of disorder [156].

6.1. Polyphenols in Schizophrenia. There are no studies yet, reporting the direct interference of polyphenols with pathophysiology or pathobiochemistry of schizophrenia in human. Understanding of the molecular foundations of schizophrenia pathophysiology would allow a targeted application of pharmacotherapy. However, this cannot be studied in human trials. Therefore, especially in chronic neurodegenerative and psychiatric disorders, the use of animal experiments is necessary. Conclusions of these experiments may then be more or less used for application in human biomedical field.

Preclinical studies suggest that the green tea extract with the main polyphenol *epigallocatechin-3-gallate (EGCG)* may possibly benefit patients with schizophrenia. Loftis et al. [157] were interested in whether EGCG at doses of 600 mg per day is a useful adjunct for maintenance treatment with antipsychotic medication in 34 patients in the double-blind and placebo controlled study. Authors have not found therapeutic effects of EGCG on psychotic symptoms in comparison to placebo. In schizophrenic patients only few works investigated the influence of polyphenols on side effects following antipsychotic treatment. Tardive dyskinesia (TD) is a serious adverse effect associated with the long-term administration of neuroleptics. The pathophysiology of antipsychotic treatment-induced TD is still unclear, although several reports assumed that free radicals may be involved

[158]. Involvement of oxidative stress in the development of haloperidol-induced orofacial TD was confirmed by Bishnoi et al. [159]. Authors found that chronic administration of haloperidol increased vacuous chewing movements, tongue protrusions, facial jerking, and also oxidative damage in all major regions of rat brain. These changes were dose-dependently inhibited by curcumin. Authors point to curcumin as a possible therapeutic option to treat this hyperkinetic movement. Similarly, in experimental conditions flavonoid quercetin (3,5,7,3′,4′-pentahydroxyflavone) reverses haloperidol-induced extrapyramidal side effects, catalepsy, usually associated with catatonic schizophrenia. It is a physical condition, characterized by suspension of sensation, muscular rigidity, fixity of posture, and often loss of contact with surroundings [160]. Besides this, quercetin and also resveratrol (3′,4′,5′-trihydroxystilbene) reduced lipid peroxidation in human plasma caused by a first-generation antipsychotics, haloperidol in *ex vivo* experiments. The amisulpride, the second-generation of antipsychotic drugs did not influence the level of lipid peroxidation biomarker TBARS in comparison to the controls [161].

Flavonoid epicatechin, present as a major component in green tea, inhibits lipid peroxidation in human plasma caused by haloperidol in experiment *ex vivo* [162].

Plasma lipid peroxidation induced by atypical antipsychotic drug ziprasidone was also inhibited by polyphenols from berries isolated from *Aronia melanocarpa* in *ex vivo* experiments [163]. However, results of experiment *ex vivo* should be read and interpreted with caution, because polyphenols passing through the GIT are metabolized to derivatives and, therefore, the effect on lipid peroxidation *ex vivo* may not be identical to the effect of *in vivo*.

Extract from *Ginkgo biloba (EGb-761)*, which components are mostly lipophilic, crosses the blood-brain barrier and protects the brain against damaging effect of oxidative stress. In the study by Zhang et al. [164], 157 patients suffering from schizophrenia were included in the double-blind and placebo controlled study. Patients in EGb-761 group were administered daily dose of 240 mg EGb-761 for 12 weeks. Significant improvement of TD symptoms in schizophrenic patients was observed in EGb-761 group in comparison to placebo. The improvement may be mediated through the well-known antioxidant activities of this extract.

Genistein, a polyphenol belonging to phytoestrogens together with amino acid leucine, is able to potentiate the haloperidol-induced catalepsy in rats compared with the haloperidol treated group and reduced the number of fights and increased latency to fights in foot shock-induced aggression [165].

Since not all polyphenols are able to pass through the blood-brain barrier, it is necessary to look for new therapeutic approaches. One of the new approaches is the use of exosomes. Exosomes are small (30–150 nm) extracellular cell membrane-derived vesicles that are present in many and perhaps all biological fluids, including blood and urine. Exosomes are either released from the cells when multivesical bodies fuse with the plasma membrane or released directly from the plasma membrane. It is becoming increasingly clear that exosomes have specialized functions and play a key role in, for example, coagulation, intercellular signaling, and waste management. Exosomes' simple structure and abilities to be incorporated into plasma membrane and to cross the blood-brain barrier allow them to be utilized as drug delivery vehicles (in our case polyphenols) or genetic elements in the treatment of immune, psychiatric, and neurologic disorders [166].

Several questions remain open for the role of oxidative stress in schizophrenia. Antipsychotic drugs have been suspected to generate increased ROS resulting in increased oxidative stress. What kind of antipsychotic drugs is involved in oxidative stress? What are the symptom domains associated with the oxidative stress? Is the oxidative stress an attribute of early or chronic stages of the disease? What is the role of current treatment on oxidative stress? The answer to these questions and explanation of the participation of oxidative stress in pathology of schizophrenia need further validation [167].

7. Conclusions

A large number of studies have focused on investigation of effects of natural polyphenols in mental disorders, but their use in clinical practice is still a long way off [168]. There might be several reasons for such a slow and ineffective research.

(1) There are no sufficient sophisticated analytical methods for determination of levels of polyphenolic compounds and their metabolites in brain, (2) it is very difficult to find a suitable animal model that would mimic the exact status of human mental disorder, (3) isolated studies of interorgan actions and reactions between brain and peripheral organs cannot give the complex view, (4) application of information obtained from *in vitro* or *ex vivo* experiments into *in vivo* conditions of the complex nervous system is complicated by the biotransformation of original polyphenols to entirely different metabolites, and (5) antipsychotic effects of polyphenols have not been sufficiently validated in clinical practice yet.

Due to the enormous complexity of the human brain, the exact pathophysiology of psychiatric disorders is not known yet and the understanding of these complex relations needs to collect huge amount of data on all levels of research, experimental and human.

Identification of the exact mechanism of pathological components of mental disorders on molecular level can lead to the development of effective treatments. Polyphenols in the diet have the potential to become medicaments in the field of mental health after a thorough study of their mechanism of action. Members of the International Society for Nutritional Psychiatry Research advocated recognition of diet and nutrition as central determinants of both, physical and mental health [169].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] P. Sobocki, B. Jönsson, J. Angst, and C. Rehnberg, "Cost of depression in Europe," *Journal of Mental Health Policy and Economics*, vol. 9, no. 2, pp. 87–98, 2006.

[2] R. Meeusen, "Exercise, nutrition and the brain," *Sports Medicine*, vol. 44, supplement 1, pp. S47–S56, 2014.

[3] G. P. Dias, N. Cavegn, A. Nix et al., "The role of dietary polyphenols on adult hippocampal neurogenesis: molecular mechanisms and behavioural effects on depression and anxiety," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 541971, 18 pages, 2012.

[4] H. Mo, Y. Chen, L. Huang, H. Zhang, J. Li, and W. Zhou, "Neuroprotective effect of tea polyphenols on oxyhemoglobin induced subarachnoid hemorrhage in mice," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 743938, 7 pages, 2013.

[5] B. Wright, "Forging a modern generation of polyphenol-based therapeutics," *British Journal of Pharmacology*, vol. 169, no. 4, pp. 844–847, 2013.

[6] U. Gundimeda, T. H. McNeill, J. E. Schiffman, D. R. Hinton, and R. Gopalakrishna, "Green tea polyphenols potentiate the action of nerve growth factor to induce neuritogenesis: possible role of reactive oxygen species," *Journal of Neuroscience Research*, vol. 88, no. 16, pp. 3644–3655, 2010.

[7] M. Ashafaq, S. S. Raza, M. M. Khan et al., "Catechin hydrate ameliorates redox imbalance and limits inflammatory response in focal cerebral ischemia," *Neurochemical Research*, vol. 37, no. 8, pp. 1747–1760, 2012.

[8] Z. Ďuračková, "Free radicals and antioxidants for non-experts," in *Systems Biology of Free Radicals and Antioxidants*, I. Laher, Ed., Springer, Berlin, Germany, 2014.

[9] M. Franco, "New option for chronic fatigue syndrome," *Life Extension Magazine*, p. 18, 2014.

[10] G. Belcaro, U. Cornelli, R. Luzzi et al., "QR (Quercus Robur Extract, Robuvit) supplementation in subjects with chronic fatigue syndrome (CFS) and increased oxidative stress. A pilot registry ," *Journal of Neurosurgical Sciences*. In press.

[11] J. A. Ross and C. M. Kasum, "Dietary flavonoids: bioavailability, metabolic effects, and safety," *Annual Review of Nutrition*, vol. 22, pp. 19–34, 2002.

[12] C. Manach and J. L. Donovan, "Pharmacokinetics and metabolism of dietary flavonoids in humans," *Free Radical Research*, vol. 38, no. 8, pp. 771–785, 2004.

[13] T. Grimm, R. Skrabala, Z. Chovanová et al., "Single and multiple dose pharmacokinetics of maritime pine bark extract (Pycnogenol) after oral administration to healthy volunteers," *BMC Clinical Pharmacology*, vol. 6, article 4, 2006.

[14] Y. Curin, M. F. Ritz, and R. Andriantsitohaina, "Cellular mechanisms of the protective effect of polyphenols on the neurovascular unit in strokes," *Cardiovascular & Hematological Agents in Medicinal Chemistry*, vol. 4, no. 4, pp. 277–288, 2006.

[15] M. Valko, C. J. Rhodes, J. Moncol, M. Izakovic, and M. Mazur, "Free radicals, metals and antioxidants in oxidative stress-induced cancer," *Chemico-Biological Interactions*, vol. 160, no. 1, pp. 1–40, 2006.

[16] C. Ramassamy, "Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets," *European Journal of Pharmacology*, vol. 545, no. 1, pp. 51–64, 2006.

[17] J. Trebatická, S. Kopasová, Z. Hradečná et al., "Treatment of ADHD with French maritime pine bark extract, Pycnogenol," *European Child and Adolescent Psychiatry*, vol. 15, no. 6, pp. 329–335, 2006.

[18] P. Knekt, J. Kumpulainen, R. Järvinen et al., "Flavonoid intake and risk of chronic diseases," *The American Journal of Clinical Nutrition*, vol. 76, no. 3, pp. 560–568, 2002.

[19] R. J. Williams, J. P. E. Spencer, and C. Rice-Evans, "Flavonoids: antioxidants or signalling molecules?" *Free Radical Biology and Medicine*, vol. 36, no. 7, pp. 838–849, 2004.

[20] S. B. Lotito and B. Frei, "Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon?" *Free Radical Biology and Medicine*, vol. 41, no. 12, pp. 1727–1746, 2006.

[21] M. Koláček, J. Muchová, S. Vranková et al., "Effect of natural polyphenols, pycnogenol on superoxide dismutase and nitric oxide synthase in diabetic rats," *Prague Medical Report*, vol. 111, no. 4, pp. 279–288, 2010.

[22] B. Halliwell, J. Rafter, and A. Jenner, "Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not?" *The American Journal of Clinical Nutrition*, vol. 81, supplement 1, pp. 268S–276S, 2005.

[23] F. Gomez-Pinilla and T. T. J. Nguyen, "Natural mood foods: the actions of polyphenols against psychiatric and cognitive disorders," *Nutritional Neuroscience*, vol. 15, no. 3, pp. 127–133, 2012.

[24] Z. Chovanová, J. Muchová, M. Sivoňová et al., "Effect of polyphenolic extract, Pycnogenol, on the level of 8-oxoguanine in children suffering from attention deficit/hyperactivity disorder," *Free Radical Research*, vol. 40, no. 9, pp. 1003–1010, 2006.

[25] Z. Ďuračková, "Some current insights into oxidative stress," *Physiological Research*, vol. 59, no. 4, pp. 459–469, 2010.

[26] J. P. E. Spencer, "Interactions of flavonoids and their metabolites with cell signaling cascades," in *Nutrigenomics*, G. Rimbach, J. Fuchs, and L. Packer, Eds., Taylor & Francis, Boca Raton, Fla, USA, 2005.

[27] L. Križková, Z. Chovanová, Z. Ďuračková, and J. Krajčovič, "Antimutagenic in vitro activity of plant polyphenols: Pycnogenol® and Ginkgo biloba extract (EGb 761)," *Phytotherapy Research*, vol. 22, no. 3, pp. 384–388, 2008.

[28] Z. Ďuračková, B. Trebatický, V. Novotný, I. Žitňanová, and J. Breza, "Lipid metabolism and erectile function improvement by Pycnogenol, extract from the bark of *Pinus pinaster* in patients suffering from erectile dysfunction—a pilot study," *Nutrition Research*, vol. 23, no. 9, pp. 1189–1198, 2003.

[29] J. Golański, J. Muchova, R. Golański, Z. Durackova, L. Markuszewski, and C. Watała, "Does pycnogenol intensify the efficacy of acetylsalicylic acid in the inhibition of platelet function? In vitro experience," *Postepy Higieny I Medycyny Doswiadczalnej*, vol. 60, pp. 316–321, 2006.

[30] G. Nie, C. Jin, Y. Cao, S. Shen, and B. Zhao, "Distinct effects of tea catechins on 6-hydroxydopamine-induced apoptosis in PC12 cells," *Archives of Biochemistry and Biophysics*, vol. 397, no. 1, pp. 84–90, 2002.

[31] A. Schäfer, Z. Chovanová, J. Muchová et al., "Inhibition of COX-1 and COX-2 activity by plasma of human volunteers after ingestion of French maritime pine bark extract (Pycnogenol)," *Biomedicine and Pharmacotherapy*, vol. 60, no. 1, pp. 5–9, 2006.

[32] D. Nowak, "Antioxidant plant polyphenols and cognitive disorders," in *Studies on Psychiatric Disorders*, A. Dietrich-Muszalska, V. Chauhan, and S. Grignon, Eds., Humana Press, New York, NY, USA, 2015.

[33] S. Schaffer and B. Halliwell, "Do polyphenols enter the brain and does it matter? Some theoretical and practical considerations," *Genes and Nutrition*, vol. 7, no. 2, pp. 99–109, 2012.

[34] J. Labuda, M. Bučková, L. Heilerová, S. Šilhár, and I. Štepánek, "Evaluation of the redox properties and anti/pro-oxidant effects of selected flavonoids by means of a DNA-based electrochemical biosensor," *Analytical and Bioanalytical Chemistry*, vol. 376, no. 2, pp. 168–173, 2003.

[35] D. Procházková, I. Boušová, and N. Wilhelmová, "Antioxidant and prooxidant properties of flavonoids," *Fitoterapia*, vol. 82, no. 4, pp. 513–523, 2011.

[36] G. P. Kumar and F. Khanum, "Neuroprotective potential of phytochemicals," *Pharmacognosy Reviews*, vol. 6, no. 12, pp. 81–90, 2012.

[37] C. Finsterwald, H. Fiumelli, J.-R. Cardinaux, and J.-L. Martin, "Regulation of dendritic development by BDNF requires activation of CRTC1 by glutamate," *The Journal of Biological Chemistry*, vol. 285, no. 37, pp. 28587–28595, 2010.

[38] G. Scapagnini, S. Davinelli, F. Drago, A. de Lorenzo, and G. Oriani, "Antioxidants as antidepressants: fact or fiction?" *CNS Drugs*, vol. 26, no. 6, pp. 477–490, 2012.

[39] G. V. Carr and I. Lucki, "The role of serotonin receptor subtypes in treating depression: a review of animal studies," *Psychopharmacology (Berl.)*, vol. 213, no. 2-3, pp. 265–287, 2011.

[40] M. J. Owens, "Selectivity of antidepressants: from the monoamine hypothesis of depression to the SSRI revolution and beyond," *Journal of Clinical Psychiatry*, vol. 65, no. 4, pp. 5–10, 2004.

[41] V. Maletic, M. Robinson, T. Oakes, S. Iyengar, S. G. Ball, and J. Russell, "Neurobiology of depression: an integrated view of key findings," *International Journal of Clinical Practice*, vol. 61, no. 12, pp. 2030–2040, 2007.

[42] S. Matthes, V. Mosienko, S. Bashammakh, N. Alenina, and M. Bader, "Tryptophan hydroxylase as novel target for the treatment of depressive disorders," *Pharmacology*, vol. 85, no. 2, pp. 95–109, 2010.

[43] M. Maes, E. Bosmans, E. Suy, C. Vandervorst, C. De Jonckheere, and J. Raus, "Immune disturbances during major depression: upregulated expression of interleukin-2 receptors," *Neuropsychobiology*, vol. 24, no. 3, pp. 115–120, 1990.

[44] M. Maes, "The monocyte-T-lymphocyte hypothesis of major depression," *Psychoneuroendocrinology*, vol. 20, no. 2, pp. 111–116, 1995.

[45] C. A. Meyers, M. Albitar, and E. Estey, "Cognitive impairment, fatigue, and cytokine levels in patients with acute myelogenous leukemia or myelodysplastic syndrome," *Cancer*, vol. 104, no. 4, pp. 788–793, 2005.

[46] S. J. Motivala, A. Sarfatti, L. Olmos, and M. R. Irwin, "Inflammatory markers and sleep disturbance in major depression," *Psychosomatic Medicine*, vol. 67, no. 2, pp. 187–194, 2005.

[47] B.-H. Lee and Y.-K. Kim, "The roles of BDNF in the pathophysiology of major depression and in antidepressant treatment," *Psychiatry Investigation*, vol. 7, no. 4, pp. 231–235, 2010.

[48] H. Eyre and B. T. Baune, "Neuroplastic changes in depression: a role for the immune system," *Psychoneuroendocrinology*, vol. 37, no. 9, pp. 1397–1416, 2012.

[49] R. S. Duman, "Neuronal damage and protection in the pathophysiology and treatment of psychiatric illness: stress and depression," *Dialogues in Clinical Neuroscience*, vol. 11, no. 3, pp. 239–255, 2009.

[50] A. L. Lopresti, S. D. Hood, and P. D. Drummond, "A review of lifestyle factors that contribute to important pathways associated with major depression: diet, sleep and exercise," *Journal of Affective Disorders*, vol. 148, no. 1, pp. 12–27, 2013.

[51] C. M. Pariante and S. L. Lightman, "The HPA axis in major depression: classical theories and new developments," *Trends in Neurosciences*, vol. 31, no. 9, pp. 464–468, 2008.

[52] P. W. Gold and G. P. Chrousos, "Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states," *Molecular Psychiatry*, vol. 7, no. 3, pp. 254–275, 2002.

[53] C. Anacker, P. A. Zunszain, L. A. Carvalho, and C. M. Pariante, "The glucocorticoid receptor: pivot of depression and of antidepressant treatment?" *Psychoneuroendocrinology*, vol. 36, no. 3, pp. 415–425, 2011.

[54] C. Anacker, P. A. Zunszain, A. Cattaneo et al., "Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor," *Molecular Psychiatry*, vol. 16, no. 7, pp. 738–750, 2011.

[55] S. Koene, T. L. Kozicz, R. J. T. Rodenburg et al., "Major depression in adolescent children consecutively diagnosed with mitochondrial disorder," *Journal of Affective Disorders*, vol. 114, no. 1–3, pp. 327–332, 2009.

[56] A. Gardner, A. Johansson, R. Wibom et al., "Alterations of mitochondrial function and correlations with personality traits in selected major depressive disorder patients," *Journal of Affective Disorders*, vol. 76, no. 1–3, pp. 55–68, 2003.

[57] A. Gardner and R. G. Boles, "Mitochondrial energy depletion in depression with somatization," *Psychotherapy and Psychosomatics*, vol. 77, no. 2, pp. 127–129, 2008.

[58] A. Gardner and R. G. Boles, "Beyond the serotonin hypothesis: mitochondria, inflammation and neurodegeneration in major depression and affective spectrum disorders," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 35, no. 3, pp. 730–743, 2011.

[59] S. D. Khanzode, G. N. Dakhale, S. S. Khanzode, A. Saoji, and R. Palasodkar, "Oxidative damage and major depression: the potential antioxidant action of selective serotonin-re-uptake inhibitors," *Redox Report*, vol. 8, no. 6, pp. 365–370, 2003.

[60] A. J. Owen, M. J. Batterham, Y. C. Probst, B. F. S. Grenyer, and L. C. Tapsell, "Low plasma vitamin E levels in major depression: diet or disease?" *European Journal of Clinical Nutrition*, vol. 59, no. 2, pp. 304–306, 2005.

[61] M. Maes, I. Mihaylova, M. Kubera, M. Uytterhoeven, N. Vrydags, and E. Bosmans, "Lower plasma Coenzyme Q10 in depression: a marker for treatment resistance and chronic fatigue in depression and a risk factor to cardiovascular disorder in that illness," *Neuroendocrinology Letters*, vol. 30, no. 4, pp. 462–469, 2009.

[62] M. Maes, I. Mihaylova, M. Kubera, M. Uytterhoeven, N. Vrydags, and E. Bosmans, "Lower whole blood glutathione peroxidase (GPX) activity in depression, but not in myalgic encephalomyelitis/chronic fatigue syndrome: another pathway that may be associated with coronary artery disease and

neuroprogression in depression," *Neuroendocrinology Letters*, vol. 32, no. 2, pp. 133–140, 2011.

[63] M. E. Ozcan, M. Gulec, E. Ozerol, R. Polat, and O. Akyol, "Antioxidant enzyme activities and oxidative stress in affective disorders," *International Clinical Psychopharmacology*, vol. 19, no. 2, pp. 89–95, 2004.

[64] Y.-C. Wei, F.-L. Zhou, D.-L. He et al., "The level of oxidative stress and the expression of genes involved in DNA-damage signaling pathways in depressive patients with colorectal carcinoma," *Journal of Psychosomatic Research*, vol. 66, no. 3, pp. 259–266, 2009.

[65] H. Herken, A. Gurel, S. Selek et al., "Adenosine deaminase, nitric oxide, superoxide dismutase, and xanthine oxidase in patients with major depression: impact of antidepressant treatment," *Archives of Medical Research*, vol. 38, no. 2, pp. 247–252, 2007.

[66] M. Maes, I. Mihaylova, M. Kubera, M. Uytterhoeven, N. Vrydags, and E. Bosmans, "Increased plasma peroxides and serum oxidized low density lipoprotein antibodies in major depression: markers that further explain the higher incidence of neurodegeneration and coronary artery disease," *Journal of Affective Disorders*, vol. 125, no. 1–3, pp. 287–294, 2010.

[67] M. J. Forlenza and G. E. Miller, "Increased serum levels of 8-hydroxy-2′-deoxyguanosine in clinical depression," *Psychosomatic Medicine*, vol. 68, no. 1, pp. 1–7, 2006.

[68] M. Maes, I. Mihaylova, M. Kubera, M. Uytterhoeven, N. Vrydags, and E. Bosmans, "Increased 8-hydroxy-deoxyguanosine, a marker of oxidative damage to DNA, in major depression and myalgic encephalomyelitis/chronic fatigue syndrome," *Neuroendocrinology Letters*, vol. 30, no. 6, pp. 715–722, 2009.

[69] Z. Huang, X.-M. Zhong, Z.-Y. Li, C.-R. Feng, A.-J. Pan, and Q.-Q. Mao, "Curcumin reverses corticosterone-induced depressive-like behavior and decrease in brain BDNF levels in rats," *Neuroscience Letters*, vol. 493, no. 3, pp. 145–148, 2011.

[70] S. K. Kulkarni, M. K. Bhutani, and M. Bishnoi, "Antidepressant activity of curcumin: Involvement of serotonin and dopamine system," *Psychopharmacology*, vol. 201, no. 3, pp. 435–442, 2008.

[71] J. Sanmukhani, V. Satodia, J. Trivedi et al., "Efficacy and safety of curcumin in major depressive disorder: a randomized controlled trial," *Phytotherapy Research*, vol. 28, no. 4, pp. 579–585, 2014.

[72] J. Bergman, C. Miodownik, Y. Bersudsky et al., "Curcumin as an add-on to antidepressive treatment: a randomized, double-blind, placebo-controlled, pilot clinical study," *Clinical Neuropharmacology*, vol. 36, no. 3, pp. 73–77, 2013.

[73] Sreejayan and M. N. A. Rao, "Nitric oxide scavenging by curcuminoids," *Journal of Pharmacy and Pharmacology*, vol. 49, no. 1, pp. 105–107, 1997.

[74] G. Scapagnini, S. Vasto, N. G. Abraham, C. Caruso, D. Zella, and G. Fabio, "Modulation of Nrf2/ARE pathway by food polyphenols: a nutritional neuroprotective strategy for cognitive and neurodegenerative disorders," *Molecular Neurobiology*, vol. 44, no. 2, pp. 192–201, 2011.

[75] W.-Q. Chen, X.-L. Zhao, Y. Hou et al., "Protective effects of green tea polyphenols on cognitive impairments induced by psychological stress in rats," *Behavioural Brain Research*, vol. 202, no. 1, pp. 71–76, 2009.

[76] W. L. Zhu, H. S. Shi, Y. M. Wei et al., "Green tea polyphenols produce antidepressant-like effects in adult mice," *Pharmacological Research*, vol. 65, no. 1, pp. 74–80, 2012.

[77] M. Vignes, T. Maurice, F. Lanté et al., "Anxiolytic properties of green tea polyphenol (-)-epigallocatechin gallate (EGCG)," *Brain Research*, vol. 1110, no. 1, pp. 102–115, 2006.

[78] Q. Zhang, H. Yang, J. Wang et al., "Effect of green tea on reward learning in healthy individuals: a randomized, double-blind, placebo-controlled pilot study," *Nutrition Journal*, vol. 12, no. 1, article 84, 7 pages, 2013.

[79] Y. Hou, M. A. Aboukhatwa, D.-L. Lei, K. Manaye, I. Khan, and Y. Luo, "Anti-depressant natural flavonols modulate BDNF and beta amyloid in neurons and hippocampus of double TgAD mice," *Neuropharmacology*, vol. 58, no. 6, pp. 911–920, 2010.

[80] A. Dreiseitel, G. Korte, P. Schreier et al., "Berry anthocyanins and their aglycons inhibit monoamine oxidases A and B," *Pharmacological Research*, vol. 59, no. 5, pp. 306–311, 2009.

[81] M. Messaoudi, J.-F. Bisson, A. Nejdi, P. Rozan, and H. Javelot, "Antidepressant-like effects of a cocoa polyphenolic extract in Wistar-Unilever rats," *Nutritional Neuroscience*, vol. 11, no. 6, pp. 269–276, 2008.

[82] T. Sathyapalan, S. Beckett, A. S. Rigby, D. D. Mellor, and S. L. Atkin, "High cocoa polyphenol rich chocolate may reduce the burden of the symptoms in chronic fatigue syndrome," *Nutrition Journal*, vol. 9, no. 1, article 55, 2010.

[83] F. Natella, G. Leoni, M. Maldini et al., "Absorption, metabolism, and effects at transcriptome level of a standardized french oak wood extract, Robuvit, in healthy volunteers: Pilot Study," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 2, pp. 443–453, 2014.

[84] G. Belcaro, M. Dugall, S. Hu, A. Ledda, and E. Ippolito, "French oak wood (*Quercus robur*) extract (Robuvit) in primary lymphedema: a supplement, pilot, registry evaluation," *International Journal of Angiology*, 2014.

[85] G. Belcaro, U. Cornelli, R. Luzzi et al., "Improved management of primary chronic fatigue syndrome with the supplement French oak wood extract (Robuvit): a pilot, registry evaluation," *Panminerva Medica*, vol. 56, no. 1, pp. 63–72, 2014.

[86] G. Belcaro, G. Gizzi, S. Hu et al., "Robuvit (French oak wood extract) in the management of functional, temporary hepatic damage. A registry, pilot study," *Minerva Medica*, vol. 105, no. 1, pp. 41–50, 2014.

[87] Z. Országhová, I. Waczulíková, C. Burki, P. Rohdewald, and Z. Ďuračková, "An effect of oak-wood extract (Robuvit) on energy levels in healthy adults—a pilot study," Submitted to *Phytotherapy Research*.

[88] M. Horváthová, Z. Országhová, L. Laubertová et al., "Effect of the French oak wood extract Robuvit on markers of oxidative stress and activity of antioxidant enzymes in healthy volunteers: a pilot study," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 639868, 6 pages, 2014.

[89] N. Sinn, "Nutritional and dietary influences on attention deficit hyperactivity disorder," *Nutrition Reviews*, vol. 66, no. 10, pp. 558–568, 2008.

[90] R. C. Kessler, L. Adler, M. Ames et al., "The prevalence and effects of adult attention deficit/hyperactivity disorder on work performance in a nationally representative sample of workers," *Journal of Occupational and Environmental Medicine*, vol. 47, no. 6, pp. 565–572, 2005.

[91] D. Coghill, S. Bonnar, S. Duke, J. Graham, and S. Seth, *Child and Adolescent Psychiatry*, Oxford University Press, New York, NY, USA, 2009.

[92] P. Curatolo, E. D'Agati, and R. Moavero, "The neurobiological basis of ADHD," *Italian Journal of Pediatrics*, vol. 36, article 79, 2010.

[93] L. T. Curtis and K. Patel, "Nutritional and environmental approaches to preventing and treating autism and attention

deficit hyperactivity disorder (ADHD): a review," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 79–85, 2008.

[94] J. Sarris, J. Kean, I. Schweitzer, and J. Lake, "Complementary medicines (herbal and nutritional products) in the treatment of Attention Deficit Hyperactivity Disorder (ADHD): a systematic review of the evidence," *Complementary Therapies in Medicine*, vol. 19, no. 4, pp. 216–227, 2011.

[95] N. Sinn and J. Bryan, "Effect of supplementation with polyunsaturated fatty acids and micronutrients on learning and behavior problems associated with child ADHD," *Journal of Developmental and Behavioral Pediatrics*, vol. 28, no. 2, pp. 82–91, 2007.

[96] D. McCann, A. Barrett, A. Cooper et al., "Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial," *The Lancet*, vol. 370, no. 9598, pp. 1560–1567, 2007.

[97] R. E. Cimera, *Making ADHD a Gift: Teaching Superman How to Fly*, Scarecrow Education, Rowman & Littlefield, Lanham, Md, USA, 2002.

[98] N. Joseph, Y. Zhang-James, A. Perl, and S. V. Faraone, "Oxidative stress and ADHD: a meta-analysis," *Journal of Attention Disorders*, 2013.

[99] M. Bulut, S. Selek, H. S. Gergerlioglu et al., "Malondialdehyde levels in adult attention-deficit hyperactivity disorder," *Journal of Psychiatry and Neuroscience*, vol. 32, no. 6, pp. 435–438, 2007.

[100] D. Oztop, H. Altun, G. Baskol, and S. Ozsoy, "Oxidative stress in children with attention deficit hyperactivity disorder," *Clinical Biochemistry*, vol. 45, no. 10-11, pp. 745–748, 2012.

[101] H. Essawy, I. El-Ghohary, A. El-Missiry, O. Kahla, A. Soliman, and O. El-Rashidi, "Oxidative stress in attention deficit hyperactivity disorder patients," *Current Psychiatry*, vol. 16, no. 1, p. 5669, 2009.

[102] S. Selek, H. A. Savas, H. S. Gergerlioglu, M. Bulut, and H. R. Yilmaz, "Oxidative imbalance in adult attention deficit/hyperactivity disorder," *Biological Psychology*, vol. 79, no. 2, pp. 256–259, 2008.

[103] O. Akyol, S. Zoroglu, F. Armutcu, S. Sahin, and A. Gurel, "Nitric oxide as a physiopathological factor in neuropsychiatric disorders," *In Vivo*, vol. 18, no. 3, pp. 377–390, 2004.

[104] M. Ceylan, S. Sener, A. C. Bayraktar, and M. Kavutcu, "Oxidative imbalance in child and adolescent patients with attention-deficit/hyperactivity disorder," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 34, no. 8, pp. 1491–1494, 2010.

[105] M. Dvořáková, D. Ježová, P. Blažíček et al., "Urinary catecholamines in children with attention deficit hyperactivity disorder (ADHD): modulation by a polyphenolic extract from pine bark (Pycnogenol)," *Nutritional Neuroscience*, vol. 10, no. 3-4, pp. 151–157, 2007.

[106] O. Erel, "A novel automated method to measure total antioxidant response against potent free radical reactions," *Clinical Biochemistry*, vol. 37, no. 2, pp. 112–119, 2004.

[107] O. Erel, "A new automated colorimetric method for measuring total oxidant status," *Clinical Biochemistry*, vol. 38, no. 12, pp. 1103–1111, 2005.

[108] S. Selek and M. F. Ceylan, "A relationship between oxidative status and attention deficit hyperactivity disorder," in *Studies on Psychiatric Disorders*, A. Dietrich-Muszalska, V. Chauhan, and S. Grignon, Eds., Humana Press, New York, NY, USA, 2015.

[109] C. Ramassamy, Y. Christen, F. Clostre, and J. Costentin, "The Ginkgo biloba extract, EGb761, increases synaptosomal uptake of 5-hydroxytryptamine: in-vitro and ex-vivo studies," *Journal of Pharmacy and Pharmacology*, vol. 44, no. 11, pp. 943–945, 1992.

[110] J. J. Rucklidge, J. Johnstone, and B. J. Kaplan, "Nutrient supplementation approaches in the treatment of ADHD," *Expert Review of Neurotherapeutics*, vol. 9, no. 4, pp. 461–476, 2009.

[111] B. Salehi, R. Imani, M. R. Mohammadi et al., "Ginkgo biloba for attention-deficit/hyperactivity disorder in children and adolescents: a double blind, randomized controlled trial," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 34, no. 1, pp. 76–80, 2010.

[112] H. Uebel-von Sandersleben, A. Rothenberger, B. Albrecht, L. G. Rothenberger, S. Klement, and N. Bock, "Ginkgo biloba extract EGb 761 in children with ADHD," *Zeitschrift für Kinder- und Jugendpsychiatrie und Psychotherapie*, vol. 42, no. 5, pp. 337–347, 2014.

[113] W. Weber, A. Vander Stoep, R. L. McCarty, N. S. Weiss, J. Biederman, and J. McClellan, "Hypericum perforatum (St John's Wort) for attention-deficit/hyperactivity disorder in children and adolescents: a randomized controlled trial," *The Journal of the American Medical Association*, vol. 299, no. 22, pp. 2633–2641, 2008.

[114] J.-J. Li, Z.-W. Li, S.-Z. Wang et al., "Ningdong granule: a complementary and alternative therapy in the treatment of attention deficit/hyperactivity disorder," *Psychopharmacology*, vol. 216, no. 4, pp. 501–509, 2011.

[115] I. C. Dela Peña, S. Y. Yoon, Y. Kim et al., "5,7-Dihydroxy-6-methoxy-4′-phenoxyflavone, a derivative of oroxylin A improves attention-deficit/hyperactivity disorder (ADHD)-like behaviors in spontaneously hypertensive rats," *European Journal of Pharmacology*, vol. 715, no. 1–3, pp. 337–344, 2013.

[116] S. Y. Yoon, I. D. Peña, S. M. Kim et al., "Oroxylin A improves attention deficit hyperactivity disorder-like behaviors in the spontaneously hypertensive rat and inhibits reuptake of dopamine in vitro," *Archives of Pharmacal Research*, vol. 36, no. 1, pp. 134–140, 2013.

[117] P. Rohdewald, "A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology," *International Journal of Clinical Pharmacology and Therapeutics*, vol. 40, no. 4, pp. 158–168, 2002.

[118] P. Rohdewald, "Letter to the editor: pycnogenol protects DNA against oxidative damage in vivo," *Phytotherapy Research*, vol. 19, no. 3, p. 262, 2005.

[119] S. A. Baldwin, I. Fugaccia, D. R. Brown, L. V. Brown, and S. W. Scheff, "Blood-brain barrier breach following cortical contusion in the rat," *Journal of Neurosurgery*, vol. 85, no. 3, pp. 476–481, 1996.

[120] M. Kurlbaum, M. Mülek, and P. Högger, "Facilitated uptake of a bioactive metabolite of maritime pine bark extract (pycnogenol) into human erythrocytes," *PLoS ONE*, vol. 8, no. 4, Article ID e63197, 2013.

[121] M. Sivoňová, I. Waczulíková, E. Kilanczyk et al., "The effect of Pycnogenol on the erythrocyte membrane fluidity," *General Physiology and Biophysics*, vol. 23, no. 1, pp. 39–51, 2004.

[122] J. E. Piletz, M. Sarasua, M. Chotani, A. Saran, and A. Halaris, "Relationship between membrane fluidity and adrenoceptor binding in depression," *Psychiatry Research*, vol. 38, no. 1, pp. 1–12, 1991.

[123] M. A. Ansari, J. N. Keller, and S. W. Scheff, "Protective effect of Pycnogenol in human neuroblastoma SH-SY5Y cells

following acrolein-induced cytotoxicity," *Free Radical Biology and Medicine*, vol. 45, no. 11, pp. 1510–1519, 2008.

[124] A. J. Schmidt, J.-C. Krieg, U. M. Hemmeter et al., "Impact of plant extracts tested in attention-deficit/hyperactivity disorder treatment on cell survival and energy metabolism in human neuroblastoma SH-SY5Y cells," *Phytotherapy Research*, vol. 24, no. 10, pp. 1549–1553, 2010.

[125] M. M. Khan, M. N. Hoda, T. Ishrat et al., "Amelioration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced behavioural dysfunction and oxidative stress by Pycnogenol in mouse model of Parkinson's disease," *Behavioural Pharmacology*, vol. 21, no. 5-6, pp. 563–571, 2010.

[126] S. W. Scheff, M. A. Ansari, and K. N. Roberts, "Neuroprotective effect of Pycnogenol following traumatic brain injury," *Experimental Neurology*, vol. 239, no. 1, pp. 183–191, 2013.

[127] M. A. Ansari, K. N. Roberts, and S. W. Scheff, "Dose- and time-dependent neuroprotective effects of Pycnogenol following traumatic brain injury," *Journal of Neurotrauma*, vol. 30, no. 17, pp. 1542–1549, 2013.

[128] R. Luzzi, G. Belcaro, C. Zulli et al., "Pycnogenol supplementation improves cognitive function, attention and mental performance in students," *Panminerva Medica*, vol. 53, supplement 1, no. 3, pp. 75–82, 2011.

[129] G. Belcaro, R. Luzzi, M. Dugall, E. Ippolito, and A. Saggino, "Pycnogenol improves cognitive function, attention, mental performance and specific professional skills in healthy professionals age 35–55," *Journal of Neurosurgical Sciences*, vol. 58, no. 4, pp. 239–248, 2014.

[130] R. A. Passwater, *All about Pycnogenol*, Avery Publishing Group, New York, NY, USA, 1998.

[131] S. W. Heimann, "Pycnogenol for ADHD?" *Journal of the American Academy of Child & Adolescent Psychiatry*, vol. 38, no. 4, pp. 357–358, 1999.

[132] J. L. Hanley, *Attention Deficit Disorder*, Impact Communications, Green Bay, Wis, USA, 1999.

[133] H. Masao, "Pycnogenol's therapeutic effect in improving ADHD symptoms in children confirmed," *Mainichi Shimbun*, vol. 10, 2000.

[134] S. Tenenbaum, J. C. Paull, E. P. Sparrow, D. K. Dodd, and L. Green, "An experimental comparison of Pycnogenol and methylphenidate in adults with Attention-Deficit/Hyperactivity Disorder (ADHD)," *Journal of Attention Disorders*, vol. 6, no. 2, pp. 49–60, 2002.

[135] M. Dvořáková, M. Sivoňová, J. Trebatická et al., "The effect of polyphenolic extract from pine bark, Pycnogenol, on the level of glutathione in children suffering from attention deficit hyperactivity disorder (ADHD)," *Redox Report*, vol. 11, no. 4, pp. 163–172, 2006.

[136] A. Schoonees, J. Visser, A. Musekiwa, and J. Volmink, "Pycnogenol (extract of French maritime pine bark) for the treatment of chronic disorders," *Cochrane Database of Systematic Reviews*, vol. 4, Article ID CD008294, 2012.

[137] M. Bošković, T. Vovk, B. K. Plesničar, and I. Grabnar, "Oxidative stress in schizophrenia," *Current Neuropharmacology*, vol. 9, no. 2, pp. 301–312, 2011.

[138] K. Q. Do, "Schizophrenia: genes, environment and neurodevelopment," *Revue Médicale de la Suisse Romande*, vol. 9, no. 398, pp. 1672–1677, 2013 (French).

[139] S. P. Mahadik and S. Mukherjee, "Free radical pathology and antioxidant defense in schizophrenia: a review," *Schizophrenia Research*, vol. 19, no. 1, pp. 1–17, 1996.

[140] I. Pérez-Neri, J. Ramírez-Bermúdez, S. Montes, and C. Ríos, "Possible mechanisms of neurodegeneration in schizophrenia," *Neurochemical Research*, vol. 31, no. 10, pp. 1279–1294, 2006.

[141] V. Tang and J.-F. Wang, "Mitochondrial dysfunction and oxidative stress in bipolar disorder," in *Systems Biology of Free Radicals and Antioxidants*, I. Laher, Ed., Springer, Berlin, Germany, 2014.

[142] A. Monin, P. S. Baumann, A. Griffa et al., "Glutathione deficit impairs myelin maturation: relevance for white matter integrity in schizophrenia patients," *Molecular Psychiatry*, 2014.

[143] A. Dietrich-Muszalska and B. Olas, "Isoprostenes as indicators of oxidative stress in schizophrenia," *The World Journal of Biological Psychiatry*, vol. 10, no. 1, pp. 27–33, 2009.

[144] R. Reddy, M. Keshavan, and J. K. Yao, "Reduced plasma antioxidants in first-episode patients with schizophrenia," *Schizophrenia Research*, vol. 62, no. 3, pp. 205–212, 2003.

[145] G. L. Bowman, J. Shannon, B. Frei, J. A. Kaye, and J. F. Quinn, "Uric acid as a CNS antioxidant," *Journal of Alzheimer's Disease*, vol. 19, no. 4, pp. 1331–1336, 2010.

[146] T. M. Michel, J. Thome, D. Martin et al., "Cu, Zn- and Mn-superoxide dismutase levels in brains of patients with schizophrenic psychosis," *Journal of Neural Transmission*, vol. 111, no. 9, pp. 1191–1201, 2004.

[147] A. Ciobica, M. Padurariu, I. Dobrin, C. Stefanescu, and R. Dobrin, "Oxidative stress in schizophrenia—focusing on the main markers," *Psychiatria Danubina*, vol. 23, no. 3, pp. 237–245, 2011.

[148] S. Grignon and J. M. Chianetta, "Assessment of malondialdehyde levels in schizophrenia: a meta-analysis and some methodological considerations," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 31, no. 2, pp. 365–369, 2007.

[149] J. D. Morrow, "The isoprostanes—unique products of arachidonate peroxidation: their role as mediators of oxidant stress," *Current Pharmaceutical Design*, vol. 12, no. 8, pp. 895–902, 2006.

[150] A. Dietrich-Muszalska, B. Olas, R. Głowacki, and E. Bald, "Oxidative/nitrative modifications of plasma proteins and thiols from patients with schizophrenia," *Neuropsychobiology*, vol. 59, no. 1, pp. 1–7, 2009.

[151] A. Jorgensen, K. Broedbaek, A. Fink-Jensen et al., "Increased systemic oxidatively generated DNA and RNA damage in schizophrenia," *Psychiatry Research*, vol. 209, no. 3, pp. 417–423, 2013.

[152] D. Malaspina, R. Dracxler, J. Walsh-Messinger et al., "Telomere length, family history, and paternal age in schizophrenia," *Molecular Genetics & Genomic Medicine*, vol. 2, no. 4, pp. 326–331, 2014.

[153] L. F. Jarskog, L. A. Glantz, J. H. Gilmore, and J. A. Lieberman, "Apoptotic mechanisms in the pathophysiology of schizophrenia," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 29, no. 5, pp. 846–858, 2005.

[154] L. A. Glantz, J. H. Gilmore, J. A. Lieberman, and L. F. Jarskog, "Apoptotic mechanisms and the synaptic pathology of schizophrenia," *Schizophrenia Research*, vol. 81, no. 1, pp. 47–63, 2006.

[155] A. Dietrich-Muszalska, "Oxidative stress in schizophrenia," in *Studies on Psychiatric Disorders*, A. Dietrich-Muszalska, V. Chauhan, and S. Grignon, Eds., Humana Press, NewYork, NY, USA, 2015.

[156] O. Pazvantoglu, S. Selek, I. T. Okay et al., "Oxidative mechanisms in schizophrenia and their relationship with illness subtype and symptom profile," *Psychiatry and Clinical Neurosciences*, vol. 63, no. 5, pp. 693–700, 2009.

[157] J. M. Loftis, C. J. Wilhelm, and M. Huckans, "Effect of epigallocatechin gallate supplementation in schizophrenia and bipolar disorder: an 8-week, randomized, double-blind, placebo-controlled study," *Therapeutic Advances in Psychopharmacology*, vol. 3, no. 1, pp. 21–27, 2013.

[158] G. Tsai, D. C. Goff, R. W. Chang, J. Flood, L. Baer, and J. T. Coyle, "Markers of glutamatergic neurotransmission and oxidative stress associated with tardive dyskinesia," *The American Journal of Psychiatry*, vol. 155, no. 9, pp. 1207–1213, 1998.

[159] M. Bishnoi, K. Chopra, and S. K. Kulkarni, "Protective effect of Curcumin, the active principle of turmeric (*Curcuma longa*) in haloperidol-induced orofacial dyskinesia and associated behavioural, biochemical and neurochemical changes in rat brain," *Pharmacology Biochemistry and Behavior*, vol. 88, no. 4, pp. 511–522, 2008.

[160] P. S. Naidu and S. K. Kulkarni, "Quercefin, a bioflavonoid, reverses haloperidol-induced catalepsy," *Methods and Findings in Experimental and Clinical Pharmacology*, vol. 26, no. 5, pp. 323–326, 2004.

[161] A. Dietrich-Muszalska, B. Olas, B. Kontek, and J. Rabe-Jabłońska, "Beta-glucan from Saccharomyces cerevisiae reduces plasma lipid peroxidation induced by haloperidol," *International Journal of Biological Macromolecules*, vol. 49, no. 1, pp. 113–116, 2011.

[162] A. Dietrich-Muszalska, B. Kontek, B. Olas, and J. Rabe-Jablońska, "Epicatechin inhibits human plasma lipid peroxidation caused by haloperidol in vitro," *Neurochemical Research*, vol. 37, no. 3, pp. 557–562, 2012.

[163] A. Dietrich-Muszalska, J. Kopka, and B. Kontek, "Polyphenols from berries of *Aronia melanocarpa* reduce the plasma lipid peroxidation induced by Ziprasidone," *Schizophrenia Research and Treatment*, vol. 2014, Article ID 602390, 7 pages, 2014.

[164] W.-F. Zhang, Y.-L. Tan, X.-Y. Zhang, R. C. K. Chan, H.-R. Wu, and D.-F. Zhou, "Extract of *Ginkgo biloba* treatment for tardive dyskinesia in schizophrenia: a randomized, double-blind, placebo-controlled trial," *Journal of Clinical Psychiatry*, vol. 72, no. 5, pp. 615–621, 2011.

[165] P. Suresh and A. B. Raju, "Antidopaminergic effects of leucine and genistein on shizophrenic rat models," *Neurosciences*, vol. 18, no. 3, pp. 235–241, 2013.

[166] I. Tsilioni, S. Panagiotidou, and T. C. Theoharides, "Exosomes in Neurologic and Psychiatric Disorders," *Clinical Therapeutics*, 2014.

[167] P. O'Donnell, K. Q. Do, and C. Arango, "Oxidative/nitrosative stress in psychiatric disorders: are we there yet?" *Schizophrenia Bulletin*, vol. 40, no. 5, pp. 960–962, 2014.

[168] L. Pathak, Y. Agrawal, and A. Dhir, "Natural polyphenols in the management of major depression," *Expert Opinion on Investigational Drugs*, vol. 22, no. 7, pp. 863–880, 2013.

[169] J. Sarris, A. C. Logan, T. N. Akbaraly et al., "Nutritional medicine as mainstream in psychiatry," *The Lancet Psychiatry*, 2015.

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Research Article

Cardiac and Vascular Synergic Protective Effect of *Olea europea* L. Leaves and *Hibiscus sabdariffa* L. Flower Extracts

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This study was aimed at investigating the cardiovascular effects of an *Olea europea* L. leaf extract (OEE), of a *Hibiscus sabdariffa* L. flower extract (HSE), and of their 13 : 2 w/w mixture in order to assess their cardiac and vascular activity. Both extracts were fully characterized in their bioactive compounds by HPLC-MS/MS analysis. The study was performed using primary vascular endothelial cells (HUVECs) to investigate the antioxidant and cytoprotective effect of the extracts and their mixture and isolated guinea-pig left and right atria and aorta to evaluate the inotropic and chronotropic activities and vasorelaxant properties. In cultured HUVECs, OEE and HSE reduced intracellular reactive oxygen species formation and improved cell viability, following oxidative stress in dose-dependent manner. OEE and HSE exerted negative inotropic and vasorelaxant effects without any chronotropic property. Interestingly, the mixture exerted higher cytoprotective effects and antioxidant activities. Moreover, the mixture exerted an inotropic effect similar to each single extract, while it revealed an intrinsic negative chronotropic activity different from the single extract; its relaxant activity was higher than that of each single extract. In conclusion OEE and HSE mixture has a good potential for pharmaceutical and nutraceutical application, thanks to the synergistic effects of the single phytochemicals.

1. Introduction

Hypertension is a chronical medical condition which represents a major risk factor for myocardial infarction, heart failure, stroke, peripheral arterial disease, and aortic aneurysm and is a cause of chronic kidney disease. This pathology often occurs along with metabolic syndrome and it is associated with a decreased life expectancy [1–3].

The central role in the pathogenesis belongs to the vascular endothelium. Vascular biology assumes a pivotal role in the initiation and perpetuation of hypertension and cardiovascular organ damage. Oxidative stress (ROS and RNS), inflammation, increased expression of redox-sensitive proinflammatory genes, cell adhesion molecules, and recruitment migration vascular dysfunction (T cells and B cells) are the primary pathophysiologic and functional mechanisms that induce vascular disease [4]. All these are closely interrelated and establish a deadly combination that leads to endothelial dysfunction (ED), vascular smooth muscle and cardiac dysfunction, hypertension, vascular disease, atherosclerosis, and cardiovascular diseases (CVD) [5]. Conventional pharmacological treatment for hypertension includes diuretics, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blocker, β-receptors blockers, L-Type calcium channel blockers, and central α-receptors agonists.

International medical societies have long since introduced nonpharmacological recommendations in their treatment guidelines [6], due to the several limitation of the pharmacological treatments: possible side effects, poor adherence to therapy (half of the patients stop taking their medication

at one year from starting) [7], or also the very slow actual development of new drugs for hypertension.

The use of nonpharmacological treatments, including, among others, the administration of nutraceutical supplements based on botanicals, has been growing in the recent years. Several plants, such as *Eucommia ulmoides* Oliv. [8], *Allium sativum* L. [9], and *Nigella sativa* L. [10], exert antihypertensive effects through different mechanisms and have been investigated for their clinical efficacy [11–13]. The main class of natural compounds responsible for the vascular effects is represented by polyphenols. Some grape juices and grape skin extracts rich in polyphenols exert endothelium-dependent relaxations in aortic rings [14]. Furthermore, polyphenol-rich sources, including extracts from red wines and green and black tea, determine endothelium-dependent relaxations in large arteries, arterioles, and veins that are prevented by competitive inhibitors of eNOS and guanylyl cyclase [14, 15]. In addition, red wine polyphenols have been shown to induce endothelium-dependent relaxation in porcine coronary artery rings [16]. Other studies demonstrated that polyphenol-rich red wine extracts suppress the angiotensin II-stimulated upregulation of several NADPH oxidase subunits including NOX1 and p22phox and the associated oxidative stress and hypertension [17]. Kane et al. [18] demonstrated that red wine polyphenols decrease angiotensin II-induced vascular expression of COX and the increased endothelium-derived contracting factors.

In addition, a chestnut wood extract, rich in ellagitannins, has been shown to exhibit an antioxidant activity and to produce a cardioprotective effect [19]. Polyphenols from terrestrial and marine plants exert several biological activities which result not only in a reduction of the blood pressure, but also in a protection from a wild variety of chronic diseases, including those affecting cardiovascular system [20, 21].

In this study we focused our attention on *Olea europaea* L. leaf extract (OEE) and on *Hibiscus sabdariffa* L. calyces extract (HSE). Phenolic compounds found in olive plant (*Olea europaea* L.), including hydroxytyrosol, oleuropein, flavonoids, chalcones, and tannins have been shown to exert many beneficial effects towards cardiovascular system [22]. Several studies have demonstrated that olive leaves exert antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic, and hypocholesterolemic effects [23]. In addition, olive leaf extract, or its main component oleuropein, shows protective effects in atherosclerosis [24], diabetes [25], hypertension [26], cardiotoxicity [27, 28], neurotoxicity [29], gastric lesions [30], and cancers [31].

Olive leaf extract exerts the hypotensive action through a direct activity on vascular smooth muscle where it determines a calcium antagonistic effect [32] and on endothelium [33]. A clinical trial confirmed the efficacy and safety of an olive leaf extract, named EFLA 943, in lowering systolic and diastolic blood pressures in subjects with stage-1 hypertension [34].

Hibiscus sabdariffa L. calices extracts have been reported to contain several polyphenols including flavonoids such as cyanidin 3-rutinoside, delphinidin 3-sambubioside, cyanidin 3-sambubioside, cyaniding-3-glucoside, delphinidin 3-glucoside, and hibiscus acid [35]. All these compounds of *Hibiscus sabdariffa* L. extract have been shown to attenuate atherosclerosis through several mechanisms such as the antioxidative activity [36], inhibition of LDL oxidation [37], and smooth muscle cell proliferation [38]. The hypotensive action of *Hibiscus sabdariffa* L. extract occurs, at least in part, through a diuretic activity, due to the modulation of the aldosterone action [39]. The latter action is mainly exerted by anthocyanins such as delphinidin-3-sambubioside and cyanidin-3-sambubioside, by phenylpropanoids such as chlorogenic acid, and, to a lesser extent, by flavonoids such as quercetin and rutin [39]. In addition, the anthocyanins delphinidin- and cyanidin-3-O-sambubiosides from *Hibiscus sabdariffa* L. are ACE competitive inhibitors [40]. HSE relaxes, in a concentration-dependent manner, aortic rings precontracted with KCl high concentration (80 mM) and phenylephrine (PE), thus it probably exerts a vasorelaxant activity through a mechanism involving voltage and receptor operated Ca^{2+} calcium channels (VOCC and ROCC, resp.) [41]. In addition, HSE exerts a direct activity towards vascular endothelium, producing a vasorelaxant effect via the activation of endothelium-derived nitric oxide/cGMP-relaxant pathway and the increased synthesis/release of endothelium-derived nitric oxide [41]. Several clinical trials confirmed hypotensive and antihypertensive effectiveness and safety of *Hibiscus sabdariffa* L. extract [42–44].

This study is aimed at investigating the cardiovascular effects of an *Olea europea* L. leaf extract (EFLA 943), of an *Hibiscus sabdariffa* L. calyces extract, and of a mixture of the two extracts, containing 86.67% of EFLA 943 and 13.33% of *Hibiscus sabdariffa* L. flower powder extract (13 : 2 w/w) in order to assess their cardiac and vascular synergistic or antagonistic activity. This EFLA 943/HSE ratio is commercially available as an adjuvant food supplement in the treatment of hypertension.

In this study, we have fully characterized both EFLA 943 and HSE extracts in their polyphenol composition, and we have evaluated the antioxidant and cytoprotective activity of both single extracts and their mixture in human umbilical vein endothelial cells (HUVECs). Moreover, the effect on cardiovascular system has been investigated by the evaluation of inotropic and chronotropic effects on guinea pig isolated left and right atria and of vasorelaxant effect in guinea pig isolated aortic strips. Comparison with ileum longitudinal smooth muscle was performed to discriminate the potential relaxant effect in vascular and nonvascular smooth muscle tissue.

2. Materials and Methods

2.1. Materials. *Olea europea* L. leaves extract (Benolea (EFLA 943)) (OEE) was supplied by Frutarom (Switzerland Ltd.). The extract, manufactured from the dried leaves of *Olea europaea* L., was obtained applying an ethanol (80% m/m) extraction procedure as previously described [45] and the product was purified by a patented procedure (US Patent 6024998) to remove undesired contaminants and residues. The solvent was subsequently removed resulting in a free flowing powder, containing 18–26% w/w oleuropein by HPLC analysis.

The *Hibiscus sabdariffa* L. flower powder extract (HSE) was supplied by Nutraceutica S.r.l. (via Idice 270/1 40050 Monterenzio, Bologna, Italy). Briefly dried calyces of *Hibiscus sabdariffa* L. were subjected to extraction with distilled water for 48 hrs. The extract was filtered and concentrated under reduced pressure and completely evaporated in a vacuum oven at a temperature not exceeding 40°C. The aqueous extract was dried using Freeze Dryer system. For deep information please visit Nutraceutica website (http://www.nutraceutica.it/).

The mixture of two extracts, containing 86.67% of EFLA 943 and 13.33% of *Hibiscus sabdariffa* L. flower powder extract, represents the active ingredient of a food supplement proposed as coadjuvant in the treatment of hypertension.

The extracts and the mixture were dissolved in water shortly before use.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) was from VWR (Milano, Italy), and formic acid (98%–100%) was from Merck (Darmstadt, Germany). Deionized water was obtained from an Elix 10 water purification system from Millipore (Bedford, MA, USA).

2.2. Phytochemical Analysis

2.2.1. Extraction of Phenolic Compounds. OEE and HSE (0.1 g) were subjected to extraction using 2 mL of a methanol/water solution (50 : 50, v/v) in a 15-mL centrifuge tube. The mixture was blended (Ultra-Turrax, IKA, Staufen, Germany) for 5 min and then centrifuged for 5 min at 2500 g. The hydroalcoholic extract was collected and the powders were reextracted with 2 mL of methanol/water solution. Finally, the hydroalcoholic fractions were combined, diluted up to 5 mL and filtered through 0.2 μm regenerated cellulose filters (Schleicher & Schuell, Dassel, Germany).

2.2.2. Liquid Chromatography Analysis of OEE and HSE. The analysis of OEE and HSE to detect phytochemicals was performed by a 1290 Infinity series liquid chromatography instrument (HPLC) equipped with a quaternary pump (Agilent Technologies, Waldbronn, Germany) coupled online with a UV-Vis detector. The separation of phenolic compounds was carried out on a reverse phase C18 100 Å Kinetex column (2.6 μm, 100 × 3.00 mm I.D., Phenomenex, Torrance, CA, USA). Gradient elution was carried out with a solvent system of water/formic acid (100 : 0.5 v/v) as mobile phase A and acetonitrile as mobile phase B; the total run-time was 30 minutes and the gradient elution was as follows: from 0 to 5 min solvent B increased from 5% to 15%, at 10 min solvent B reached 25%, at 23 min solvent B reached 50%, and finally at 28 min solvent B was 100%; at 30 min 5% solvent B was restored. The column was thermostated at 30°C and equilibrated for 5 min prior to each analysis. An injection volume of 2.5 μL and a flow rate of 0.7 mL min^{-1} were used. The chromatograms were monitored at three wavelengths (280, 320, and 345 nm) characteristic for a high number for aromatic compounds. Each wavelength was suitable for each group of compounds: 280 nm was used for secoiridoids, 320 nm for hydroxycinnamic acids, and 345 nm for flavones and flavonols.

For the structural elucidation and the detection of other compounds such as hibiscus acid, a typical compound present in HSE, the HPLC system was coupled online to a triple quadrupole mass spectrometer detector (QqQ, 6420 Triple Quad/LC MS, Agilent Technologies) equipped with a TurboIonSpray source operating in negative-ion mode. The declustering potential (DP), collision energy (CE), and focusing potential (FP) were optimized for oleuropein and vanillic acid. TurboIonSpray source of QqQ setting was as follows: capillary voltage −3500 V; nebulizer gas (N2) 50 (arbitrary units); curtain gas (N2) 12 (arbitrary units); collision gas (N2) 4 (arbitrary units); focusing potential −200 V; entrance potential 10 V; drying gas (N2) heated to 250°C and introduced to a flow rate of 12 mL min^{-1}. Full-scan data acquisition was performed scanning from m/z 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 μm and a pause between each scan of 0.002 s; dwell time was set at 0.016 s.

In order to quantify through UV-Vis detector the amount of phenolic compounds in the hydroalcoholic OE extract, calibration curves were prepared with the available standards: oleuropein, verbascoside, quercetin-3-*O*-rhamnosyl-glucoside (rutin), and apigenin. The other compounds, for which no commercially standards were available, were tentatively quantified on basis of the other compounds bearing similar structures.

A calibration curve ($r^2 > 0.99$) of vanillic acid was also obtained to quantify hibiscus acid by ESI-QqQ-MS detector.

2.3. Animals. Guinea-pigs (males and females, 300–400 g) obtained from Charles River (Calco, Como, Italy) were housed in a controlled environment with a 12:12-h light-dark cycle at 22°C and provided with chow diet and water *ad libitum*. All animals used in this study were housed and treated according to the directives on the protection of animals used for scientific purposes (Directive 2010/63/EU of the European Parliament and of the Council) and the WMA Statement on Animal Use in Biomedical Research. All procedures followed the guidelines of animal care and were approved by the Ethics Committee of the University of Bologna (Bologna, Italy). The animals were sacrificed by cervical dislocation; the organs were immediately removed and used as below described.

2.3.1. Atrial Preparations. The removed heart was washed by perfusion through the aorta with oxygenated Tyrode solution containing (mM): NaCl 136.9; KCl 5.4; $CaCl_2$ 2.5; $MgCl_2$ 1.0; $NaH_2PO_4xH_2O$ 0.4; $NaHCO_3$ 11.9; and glucose 5.5. The physiological salt solution (PSS) was buffered at pH 7.4 by saturation with 95% O_2–5% CO_2 gas, and the temperature was maintained at 35°C. The following isolated guinea-pig heart preparations were used: spontaneously beating right atria and left atria driven at 1 Hz. For each preparation, the entire left and right atria were dissected from the ventricles,

cleaned of excess tissue, and hung vertically in a 15 mL organ bath containing PSS continuously bubbled with 95% O_2–5% CO_2 at 35°C, pH 7.4. The contractile activity was recorded isometrically by means of force transducer (FT 0.3, Grass Instruments Corporation, Quincy, MA, USA) using Power Lab software (ADInstruments Pty Ltd., Castle Hill, Australia). The left atria were stimulated by rectangular pulses of 0.6–0.8 ms duration and about 50% threshold voltage through two platinum contact electrodes in the lower holding clamp (Grass S88 Stimulator). The right atria were in spontaneous activity. After the tissues were beating for several min, a length-tension curve was determined, and the muscle length was maintained at the value eliciting 90% of maximum contractile force observed at the optimal length. A stabilization period of 45–60 min was allowed before the atria were challenged by various agents. During the equilibration period, the bathing solution was changed every 15 min and the threshold voltage was ascertained for the left atria. Atrial muscle preparations were used to examine the inotropic and chronotropic activity of the extracts and mixture (0.01–10 mg/mL), dissolved in PSS. During the generation of cumulative concentration-response curves, the next higher concentration of different extracts or mixture was added only after the preparation reached a steady state. All data are reported as means ± SEM. The EC_{50} values were calculated from concentration-response curves [46].

2.3.2. Aortic Strips and Ileum Longitudinal Smooth Muscle (GPLSM) Preparations. The thoracic aorta and ileum were placed in Tyrode solution containing (mM): NaCl, 118; KCl 4.75; $CaCl_2$ 2.54; $MgSO_4$ 1.20; KH_2PO_4 1.19; $NaHCO_3$ 25; and glucose 11, equilibrated with 95% O_2–5% CO_2 at pH 7.4. The vessel was cleaned of extraneous connective tissue. Two helicoidal strips (10 mm × 1 mm) were cut from each aorta beginning from the end most proximal to the heart. Vascular strips were then tied with surgical thread (6–0) and suspended in a jacketed tissue bath (15 mL) containing aerated PSS at 35°C. Aortic strips were secured at one end to plexiglass hooks and connected via the surgical thread to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction. Aortic strips were subjected to a resting force of 1 g. The intestine was removed above the ileocaecal junction. GPILSM segments of 2 cm length were mounted under a resting tension of 300–400 mg. Strips were secured at one end to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction and washed every 20 min with fresh PSS for 1 h. After the equilibration period, guinea-pig aortic and GPLSM strips were contracted by washing in PSS containing 80 mM KCl (equimolar substitution of K^+ for Na^+). When the contraction reached a plateau (about 45 min) different concentrations of the extracts and mixture (0.01–10 mg/mL) were added cumulatively to the bath allowing for any relaxation to obtain an equilibrated level of force. All data are reported as means ± S.E.M. The IC_{50} were calculated from concentration-response curves [46].

2.4. Antioxidant and Cytoprotective Activities

2.4.1. Cell Culture and Treatments. HUVECs were cultured as previously reported [47]. Briefly, cells were plated on gelatin-coated multiwell plates and maintained in complete medium M200 containing 10% FBS and growth factors at 37°C with 5% CO_2. Cells from passages 3 to 6 were used. Cells at 80% confluence were treated for 24 h with different concentrations (0.05–100 μg/mL) of OEE and HSE or a 13 : 2 w/w mix of the two and used for further analysis.

2.4.2. Determination of Cell Viability. Viability of control and treated cells was measured using the MTT assay as previously reported [48]. For the flow cytometry analysis the cells were double labelled with Annexin V conjugated to Phycoerythrin (Annexin V-PE) and 7-amino-actinomycin D (7 AAD) and immediately analysed on a GuavaEasyCyte flow cytometer (Guava Technologies, Hayward, CA) in accordance with the manufacturer's instructions as reported in [49]. The percentage of viable cells was reported with respect to the total number of cells.

2.4.3. Detection of Intracellular Reactive Oxygen Species. The formation of reactive oxygen species (ROS) was evaluated using a fluorescent probe, DCFH-DA, as previously reported [50]. Briefly, controls and treated cells were washed with PBS and then incubated with 5 μM DCFH-DA in PBS for 30 min. After DCFH-DA removal, the cells were incubated with 100 μM H_2O_2 for 30 min. Cell fluorescence from each well was measured using a microplate spectrofluorometer (λ_{ex} = 485 nm and λ_{em} = 535 nm). Intracellular antioxidant activity was expressed as the percentage of inhibition of intracellular ROS produced by H_2O_2 exposure.

2.4.4. Determination of Cytoprotective Effect. Cytoprotection against H_2O_2 induced cell damage was assessed using the MTT assay as previously reported [19]. Control and treated cells were exposed to 150 μM H_2O_2 in PBS for 3 h after which cells were changed to a fresh culture medium. MTT was added to the medium at the final concentration of 0.5 mg/mL and incubated for 4 h at 37°C. DMSO was added to dissolve the formazan crystals and the absorbance was measured at 595 nm using a microplate reader VICTOR3 V Multilabel Counter. Data were expressed as percentage of viable cells with respect to controls times. For the flow cytometry analysis the cells were double labelled with Annexin V conjugated to Annexin V-PE and 7 AAD and analysed on a GuavaEasy-Cyte flow cytometer in accordance with the manufacturer's instruction.

2.5. Statistical Analysis. Data on atria and on vascular and nonvascular smooth muscle were analyzed by the Student's *t*-test and presented as means ± S.E.M. [46]. *P* value less than 0.05 has been considered significant. The potency of different extract and its mixture, defined as EC_{50}, EC_{30}, and IC_{50} were calculated from concentration-response curves (Probit analysis using Litchfield and Wilcoxon [46] or GraphPad Prism [51, 52] software).

Table 1: UV absorption bands, mass spectrometry, and quantitative analysis of hibiscus acid and phenolic compounds in HSE and OEE.

Compound	λ_{max} (nm)	Mass spectrometry data			Quantitative data (mg g^{-1})	
		MW	$[M-H]^-$	Main fragments	OEE	HSE
Hibiscus acid[1]	—	190	189		—	139.2 ± 0.47
Secoiridoids						
Hydroxyoleuropein[2]	280	556	555	539, 377, 197	8.96 ± 0.42	—
Elenolic acid glucoside[2]	240	404	—	—	23.65 ± 0.79	—
Oleuropein[2]	280	540	539	377, 197	215.1 ± 1.64	—
Oleuropein isomer[2]	280	540	523	377, 197	51.09 ± 0.35	—
Ligstroside[2]	280	524	523	361, 191	11.03 ± 0.04	—
Hydroxycinnamic acids						
Verbascoside[3]	320	624	623	461, 315, 135	4.67 ± 0.44	—
Flavonols						
Rutin[4]	345	610	609	301, 179	0.69 ± 0.06	—
Flavones						
Luteolin-7-*O*-rutinoside[5]	345	594	593	447, 285	0.74 ± 0.04	—
Luteolin-7-*O*-glucoside[5]	345	448	447	285	5.83 ± 0.24	—
Luteolin-4-*O*-glucoside[5]	345	448	447	285	4.20 ± 0.12	—

Hibiscus acid and phenolic compounds quantified as: [1]vanillic acid mg/g; [2]oleuropein mg/g; [3]verbascoside mg/g; [4]rutin mg/g; [5]apigenin mg/g. Values are means ± S.D. ($n = 3$).

Data from HUVEC cultures are means ± S.D. and were analyzed by one-way analysis of variance

(ANOVA) followed by Dunnett's test, and $P < 0.05$ has been considered significant.

3. Results

3.1. Characterization of Olea europea L. Leaf and Hibiscus sabdariffa L. Flower Extracts. A complete characterization of OEE and HSE was realized by HPLC coupled to a UV-Vis and QqQ-Ms detector. When reference standard was not available, a tentative identification of compounds was made on the basis of spectroscopic properties, molecular weight, and the search of the main [M-H]-ion together with the interpretation of its fragments (Table 1).

Hibiscus acid was detected using mass spectrometry by matching the information of molecular ion at *m/z* 189 in negative mode (Figure 1). Elenolic acid glucoside was tentatively identified by UV spectra at 240 nm but not corroborated by mass spectra. The identification of hydroxy oleuropein and oleuropein isomer was corroborated by detection of the molecular ion (at *m/z* 555 and 539, resp.) and their aglycone fragment at *m/z* 377. Verbascoside was identified by molecular ion at *m/z* 623 and various fragments (*m/z* 461, 315) that are in accordance with the fragmentation pathway. Luteolin-4-O-rutinoside, luteolin-7-*O*-glucoside, and luteolin-4-*O*-glucoside were detected by an intense molecular ion at *m/z* 447 and the diagnostic fragment at *m/z* 285 of luteolin derivatives.

To quantify phenolic compounds, calibration curves were obtained for each standard with high linearity ($r^2 = 0.99$) by plotting the standard concentration as a function of the peak area obtained from HPLC-UV. Hibiscus acid was expressed as equivalents of vanillic acid, not present in the extract, but used as standard for the quantification of this compound. Only hibiscus acid was identified and quantified in HSE, probably due to the extraction process used by the producer that washed or degraded the phenolic acids and flavonoids commonly reported in other hibiscus extracts. Phenolic compounds characterized in OEE include oleuropein and its isomers, luteolin-4-O- and luteolin-7-O-glucoside, luteolin-4-O-rutinoside, ligstroside, elenolic acid glucoside, verbascoside, and rutin (Figure 1).

The four major compounds found in olive leaf powder extract were oleuropein and oleuropein isomer and two glucoside isomers of luteolin (215, 51, and 5.8 and 4.2 mg g^{-1}, resp.). Other minor compounds, detected in less quantity were hydroxy oleuropein, ligstroside, verbascoside, and rutin as reported in Table 1. All the compounds reported are typically present in olive leaf as previously reported [53, 54].

3.2. Effects of OEE, HSE, and Their Mixture on Guinea-Pig Isolated Cardiac and Smooth Muscle Tissues. The effects of single extracts and mixture were derived on guinea-pig isolated left and right atria to evaluate their inotropic and chronotropic effects, respectively, and on K^+-depolarized (80 mM) guinea-pig vascular (aorta) and nonvascular ileum longitudinal smooth muscle (GPILSM) strips to assess calcium antagonist activity. Tested samples were checked at increasing doses to evaluate the percent decrease of developed tension on isolated left atrium driven at 1 Hz (negative inotropic activity), the percent decrease in atrial rate on spontaneously beating right atrium (negative chronotropic activity), and the percent inhibition of calcium-induced contraction on K^+-depolarized aortic strips and GPILSM (vascular and nonvascular relaxant activity, resp.).

3.2.1. Guinea-Pig Left and Right Atria. Data relative to inotropic and chronotropic activities of OEE, HSE, and their mixture were reported in Table 2. Both extracts (1 mg/mL)

Table 2: Inotropic and chronotropic effects of OEE, HSE, and their mixture.

Extract	Left atrium Negative inotropy			Right atrium Negative chronotropy		
	Activity[a] (M ± SEM)	EC_{50} [b] (mg/mL)	95% conf. lim.	Activity[c] (M ± SEM)	EC_{30} [b] (mg/mL)	95% conf. lim.
OEE	68 ± 2.4	0.14	0.10–0.18	37 ± 2.4		
HSE	76 ± 0.9	0.27	0.21–0.35	46 ± 0.7[d]		
Mixture	60 ± 1.4	0.16	0.12–0.20	84 ± 2.0	1.21	1.10–1.33

[a]Decrease in developed tension on isolated guinea-pig left atrium at 1 mg/mL concentration, expressed as percent changes from the control (n = 5-6). The left atria were driven at 1 Hz. The 1 mg/mL concentration gave the maximum effect for most compounds. [b]Calculated from concentration-response curves (Probit analysis by Litchfield and Wilcoxon [46] with n = 6-7). When the maximum effect was <50%, the EC_{50} ino., EC_{30} chrono., values were not calculated. [c]Decrease in atrial rate on guinea-pig spontaneously beating isolated right atrium 10 mg/mL concentration, expressed as percent changes from the control (n = 7-8). The 10 mg/mL concentration gave the maximum effect for most compounds. Pretreatment heart rate ranged from 165 to 190 beats/min. [d]At 1 mg/mL concentration.

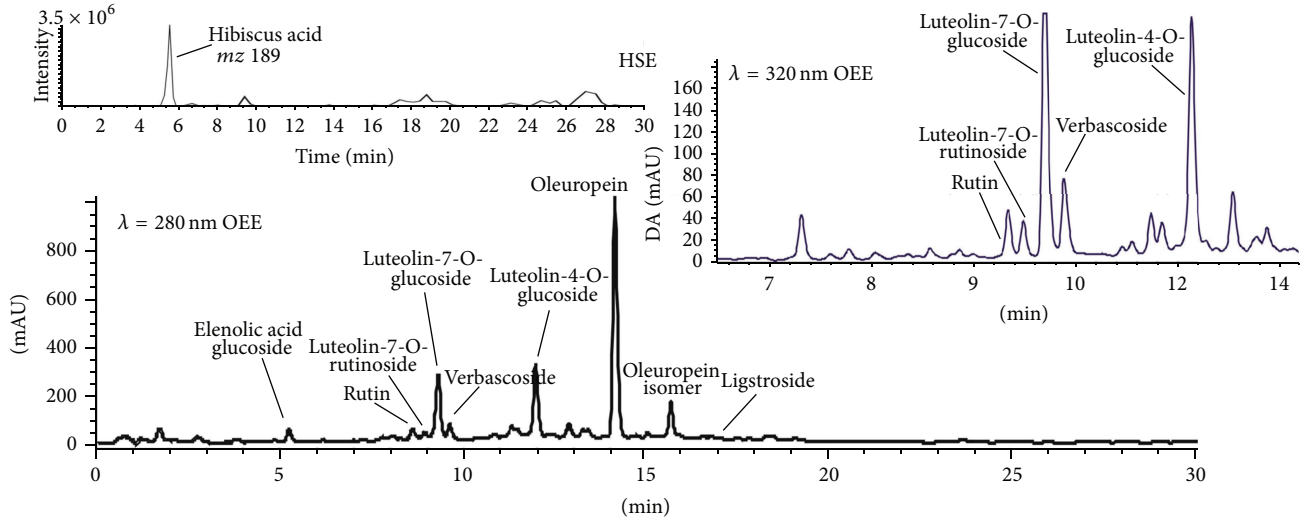

Figure 1: Phenolic profile of OEE at λ = 280 and 320 nm and extracted ion chromatography of hibiscus acid. The analysis of phenolic compounds was performed by liquid chromatography coupled online with a UV-Vis detection and a triple quadrupole mass spectrometer as reported in Methods section.

produced negative inotropic effect in left atria driven at 1 Hz (68 ± 2.4% and 76 ± 0.9%, resp.). On the contrary, OEE potency was 1.9 times higher than that of HSE extract (EC_{50} = 0.14 mg/mL (c.l. 0.10–0.18) and EC_{50} = 0.27 mg/mL (c.l. 0.21–0.35), resp.). The mixture exerts an intrinsic activity, slightly lower than that exerted by the single extracts; however its potency was not significantly different from the potency of OEE (EC_{50} = 0.16 mg/mL (c.l. 0.12–0.020) and EC_{50} = 0.14 mg/mL (c.l. 0.10–0.18), resp.) but it was 1.7 times higher than that of HSE (EC_{50} = 0.16 mg/mL (c.l. 0.12–0.20) and EC_{50} = 0.27 mg/mL (c.l. 0.21–0.35) resp.). Both extracts did not show negative chronotropic activity. As shown in Table 2 intrinsic activity was less than 50% (37 ± 2.4% and 46 ± 0.7%, resp.) at the maximal dose tested (10 mg/mL for OEE and 1 mg/mL for HSE, resp.). On the contrary, the mixture (1 mg/mL) revealed a negative chronotropic intrinsic activity (84 ± 2.0% at 10 mg/mL) with a potency 7.6 time higher than the inotropic negative potency (EC_{30} = 1.21 mg/mL (c.l. 1.10–1.33) and EC_{50} = 0.16 mg/mL (c.l. 0.12–0.20), resp.).

3.2.2. Guinea Pig Smooth Muscle. Both extracts reduced in a dose-dependent manner the contraction induced by 80 mM K^+ on the vascular smooth muscle (Table 3). Both OEE and HSE had similar intrinsic activity at the same maximal concentration (10 mg/mL) analyzed. On the contrary, the vasorelaxant potency of OEE was 6.7 times higher than that of HSE (IC_{50} = 5.15 mg/mL (c.l. 4.68–5.59) and IC_{50} = 6.63 mg/mL (c.l. 6.34–6.92), resp.). The mixture revealed an intrinsic activity lower than that of the single extracts at the same maximal concentration tested. In agreement with data on negative inotropic effect, the potency of the mixture was not significantly different from those of OEE (IC_{50} = 5.89 mg/mL (c.l. 5.56–6.25) and IC_{50} = 5.15 mg/mL (c.l. 4.68–5.59), resp.). Moreover, the vasorelaxant effect of mixture is about 1.1 times higher than that of the HSE (IC_{50} = 5.89 mg/mL (c.l. 5.56–6.25) and IC_{50} = 6.63 mg/mL (c.l. 6.34–6.92), resp.). On the contrary, only OEE demonstrated intrinsic activity in nonvascular smooth muscle (Table 3). The OEE was 6.7 times more potent in the nonvascular (GPLSM) than in the vascular smooth muscle (aorta) (IC_{50} = 0.77 mg/mL (c.l. 0.34–1.01) and IC_{50} = 5.15 mg/mL (c.l. 4.68–5.59), resp.) evidencing its selectively. In GPILSM, the mixture revealed an intrinsic activity not significantly different from these of OEE (Table 2) with a potency 1.8 times lower

Table 3: Relaxant activity of OEE, HSE, and their mixture on K^+-depolarized guinea pig smooth muscle.

Extract	Aorta			GPILSM		
	Activity[a] (M ± SEM)	IC_{50}[b] (mg/mL)	95% conf. lim.	Activity[a] (M ± SEM)	IC_{50}[b] (mg/mL)	95% conf. lim.
OEE	90 ± 1.1	5.15	4.68–5.59	90 ± 1.7	0.77	0.34–1.01
HSE	93 ± 1.4	6.63	6.34–6.92	21 ± 0.7[c]		
Mixture	70 ± 3.6	5.89	5.56–6.25	90 ± 1.2	1.39	1.13–1.54

[a]Percent inhibition of calcium-induced contraction on K^+-depolarized (80 mM) guinea-pig aortic strips at 10 mg/mL concentration. The 10 mg/mL concentration gave the maximum effect for most compounds, respectively. [b]Calculated from concentration-response curves (Probit analysis by Litchfield and Wilcoxon [46] with n = 6-7). When the maximum effect was <50%, the IC_{50} values were not calculated. [c]At 5 mg/mL concentration.

than that of OEE alone (IC_{50} = 1.39 mg/mL (c.l. 1.13–1.54) and IC_{50} = 0.77 mg/mL (c.l. 0.34–1.01), resp.) suggesting that the addition of a little amount of HSE to OEE is able to reduce the selectivity toward the nonvascular tissue.

3.3. Antioxidant and Cytoprotective Effects in Cultured HUVECs. Since olive leaves and hibiscus flower extracts are rich in phenolic compounds, we have investigated the ability of the extracts to protect cultured HUVECs from oxidative stress.

Figure 2 shows, by both MTT (Figures 2(a), 2(b), and 2(c)) and flow cytometry analysis (Figures 2(d), 2(e), and 2(f)), that OEE, HSE, and their mixture did not exert any toxic effect on cultured HUVECs on a wide range of concentrations (0.05–100 μg/mL).

ROS level measurement revealed that ROS production was significantly reduced in extract treated cells after 24 h in a dose-dependent manner. Figure 3(a) shows significant decrease in ROS production, as detected by DCFH-DA assay that was observed in both OEE and HSE treated HUVECs following exposure to H_2O_2 (Figures 3(a) and 3(b)), with OEE exhibiting the highest effect, with a significant decrease in ROS production at 1 μg/mL, while HSE evidenced a significant effect only at 5 μg/mL.

The mix revealed a significant antioxidant activity at 1 μg/mL (Figure 3(c)). The calculated IC_{50} values were 17.34 μg/mL and 25.04 μg/mL for OEE and HSE, respectively. Interestingly, the IC_{50} value of the mixture (17.00 μg/mL) was lower, although not significantly, than the OEE one.

Incubation of HUVECs with 150 μM H_2O_2 for 3 hours caused a significant decrease in cell viability (Figure 4), as detected by MTT reduction assay. OEE revealed a significant cytoprotective effect at 10 μg/mL (Figure 4(a)) while HSE at 50 μg/mL (Figure 4(b)). The mixture was able to significantly protect cells against H_2O_2 induced damage at 5 μg/mL (Figure 4(c)), suggesting a synergistic effect in cytoprotection. Flow cytometry analysis confirmed MTT data (Figures 4(d), 4(e), and 4(f)).

4. Discussion

OEE and HSE are rich in phytochemicals, the effects of which have been widely investigated. They have been used in the human diet as an extract, a herbal tea, and a powder. Their bioactive compounds have been demonstrated to have antioxidant, antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic, and hypocholesterolemic properties [23, 55].

In the present paper, we have focused the attention on the potential application of OEE and HSE in the prevention/counteraction of hypertension, a pathological condition affecting a large number of populations. Hypertension management is a primary factor in the prevention of pathological events as heart attacks, cerebrovascular diseases, and renal failure.

Conventional approach relies on drugs with different biological targets. Oral antihypertensive drugs associated to exercise, lifestyle, and dietary modification are essential approaches for hypertension therapy [1]. However, hypertension control is not always satisfactory [56], and nowadays some patients have turned to natural substances [57], which, as complementary therapy, appear to be promising in reducing blood pressure and relieving signs and symptoms in hypertensive patients [58].

Olea europea L. and *Hibiscus sabdariffa* L. extracts are known to exert antihypertensive effects [23, 55]. In this paper using both cultured HUVECs and guinea-pig left and right atria and vascular smooth muscle we have demonstrated their antioxidant and cytoprotective effects and their ability to modulate cardiac inotropy and chronotropy together with their relaxant activity on vascular smooth muscle.

Olea europea L. is an evergreen tree typical of the Mediterranean region. Not only olive oil, but also olive leaves revealed antihypertensive properties mainly attributed to the presence of polyphenols (as oleuropein and analogues), due to their L-type calcium channels blocking ability [26, 32]. Oleuropein is therefore a functional analogue of well-known L-Type calcium channel entry blockers such as nifedipine.

Interesting studies on oleuropein and olive polyphenols metabolism and bioavailability have been recently conducted. Suárez et al. identified the presence of oleuropein metabolites in a simulated in vitro model of gastrointestinal digestion [59]. Data on oleuropein bioavailability have been reported by García-Villalba et al., who demonstrated that many phenolic metabolites are detectable in urine sample of women supplemented with 250 mg of an oleuropein-rich olive leaves extract. Moreover the authors showed that some metabolites such as hydroxytyrosol glucuronide are present in urine at micromolar concentration [60]. Absorption of biologically active compounds has been demonstrated after chronic administration of olive leaves extract to rats and mice [61].

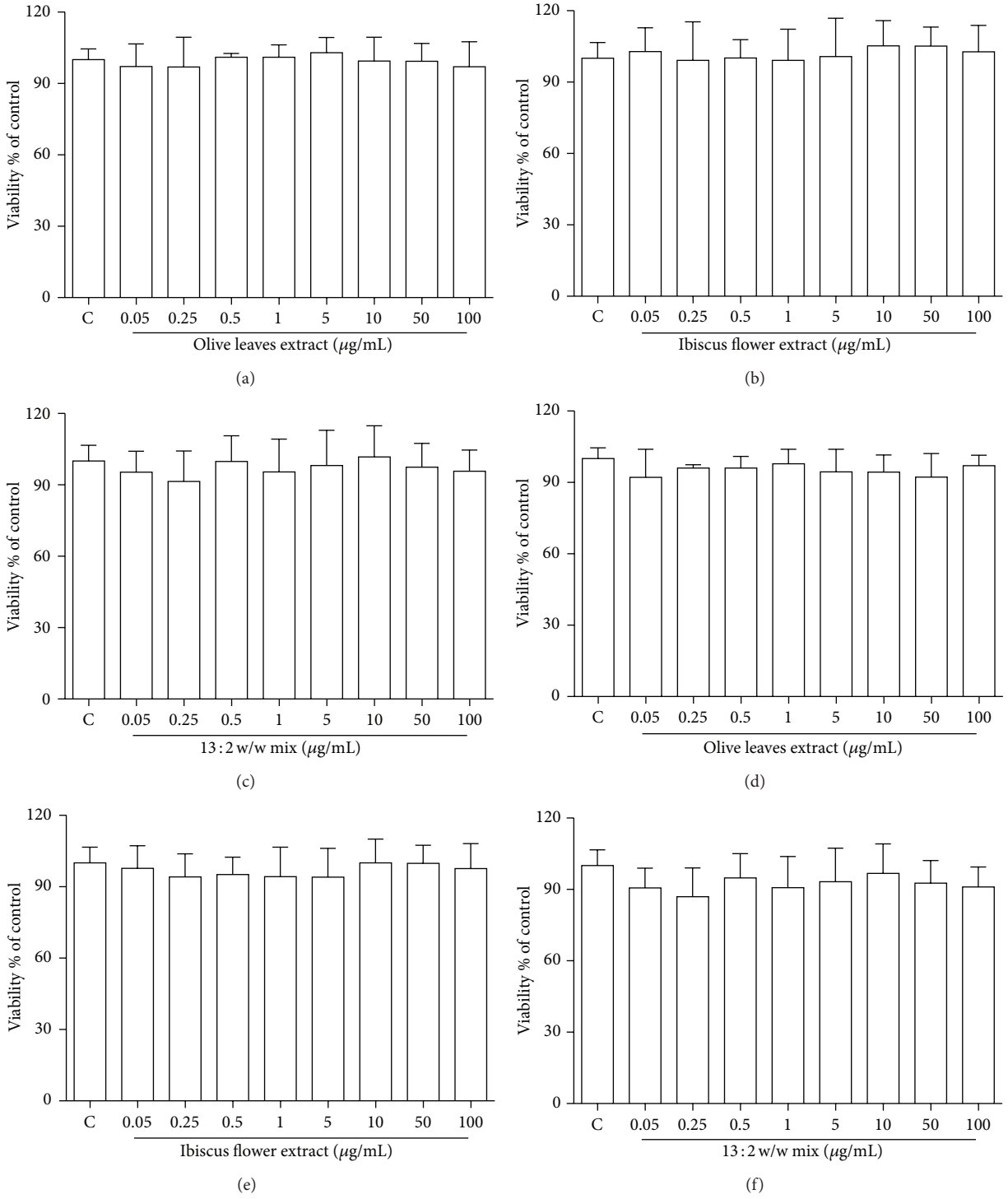

Figure 2: Cell viability of HUVECs treated with OEE, HSE, and their mixture. HUVECs were treated as described in Methods section. Cell viability was analysed by the MTT test as reported in the Methods section ((a), (b), and (c)). Cell viability was analysed by flow cytometry. Cells were double-labelled with Annexin V-PE 7 AAD and analyzed by a Guava EasyCyte flow cytometer ((d), (e), and (f)). Data are reported as means ± S.D. of four independent experiments.

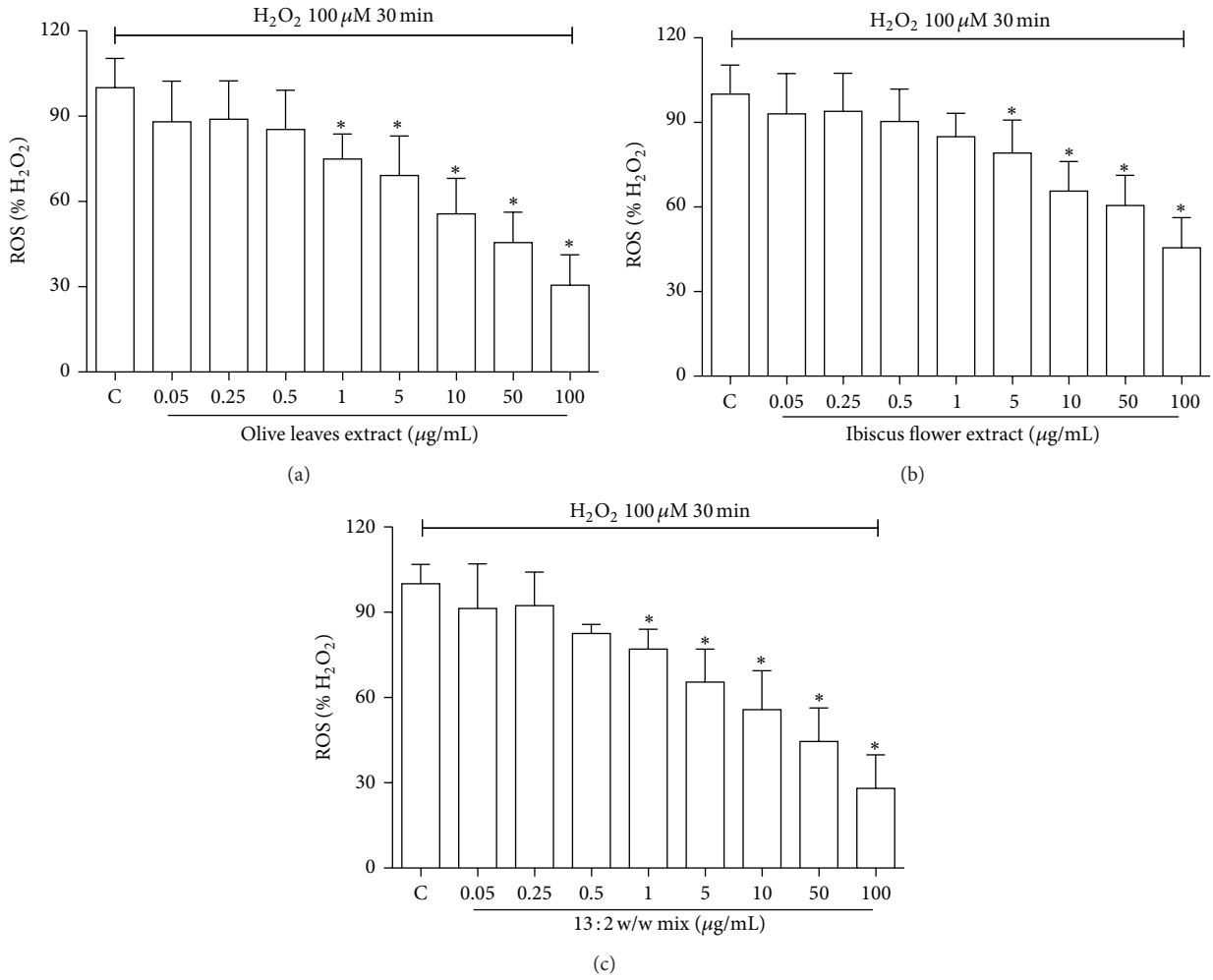

Figure 3: Effect of OEE, HSE, and their mixture on intracellular ROS production. HUVECs were treated with OEE (a), HSE (b), and their mixture (c) (0.05–100 μg/mL) for 24 h; oxidative damage was then induced exposing the cells to 100 μM H_2O_2 for 30 min and intracellular ROS were determined using the peroxide-sensitive fluorescent probe DCFH-DA as described in Methods section. Data are expressed as percent of control cells treated with H_2O_2. Values represent means ± S.D. of four independent experiments. $^*P < 0.05$ with respect to H_2O_2-treated cells.

de Bock et al. published data showing that oleuropein is bioavailable in the parental form after oral ingestion of an olive leaves extract with a peak plasma concentration in the order of ng/mL [62]. Possible mechanisms have been proposed for oleuropein and oleuropein metabolites absorption; Manna et al. proposed that oleuropein-glycoside may diffuse through the lipid bilayer of the epithelial cell membrane and be absorbed via a glucose transporter. Moreover additional mechanisms for oleuropein-glycoside absorption are potential via the paracellular or transcellular passive diffusion [63].

OEE concentrations used in "in vitro" assays on HUVEC cells are representative of oleuropein and oleuropein metabolite concentrations found in plasma after ingestion of olive leaf extracts.

Data obtained on cardiac guinea pig isolated tissues show that OEE exerted negative inotropic effect on left atrium driven at 1 Hz, without negative chronotropic effect on spontaneous beating right atrium in contrast with nifedipine, which present both negative inotropic and chronotropic effects [64]. Differently from nifedipine, OEE is presumably selective for the Cav1.2 subunit, known as cardiac isoform, widely expressed in the cardiovascular system where it regulates the vascular tone and cardiac inotropy, without effect on Cav1.3 subunit predominantly expressed in neurons and in cardiac pacemaker cells responsible for the chronotropic effect [65]. Similarly to nifedipine, OEE reduced the potassium (80 mM) induced contraction on guinea-pig aorta strips. As far as the vascular and not vascular smooth muscle relaxant activity, OEE is similar to nifedipine. Nifedipine selectivity for vascular smooth muscle over the cardiac parameters has so far allowed its use and that of subsequent 1,4-DHP's generations in the treatment of hypertension [66, 67]. Differently from nifedipine, in OEE, the inotropic effect prevails over the effects on smooth muscle.

Olive (*Olea europaea*) leaf extract, at the dosage regimen of 500 mg twice daily, was similarly effective in lowering

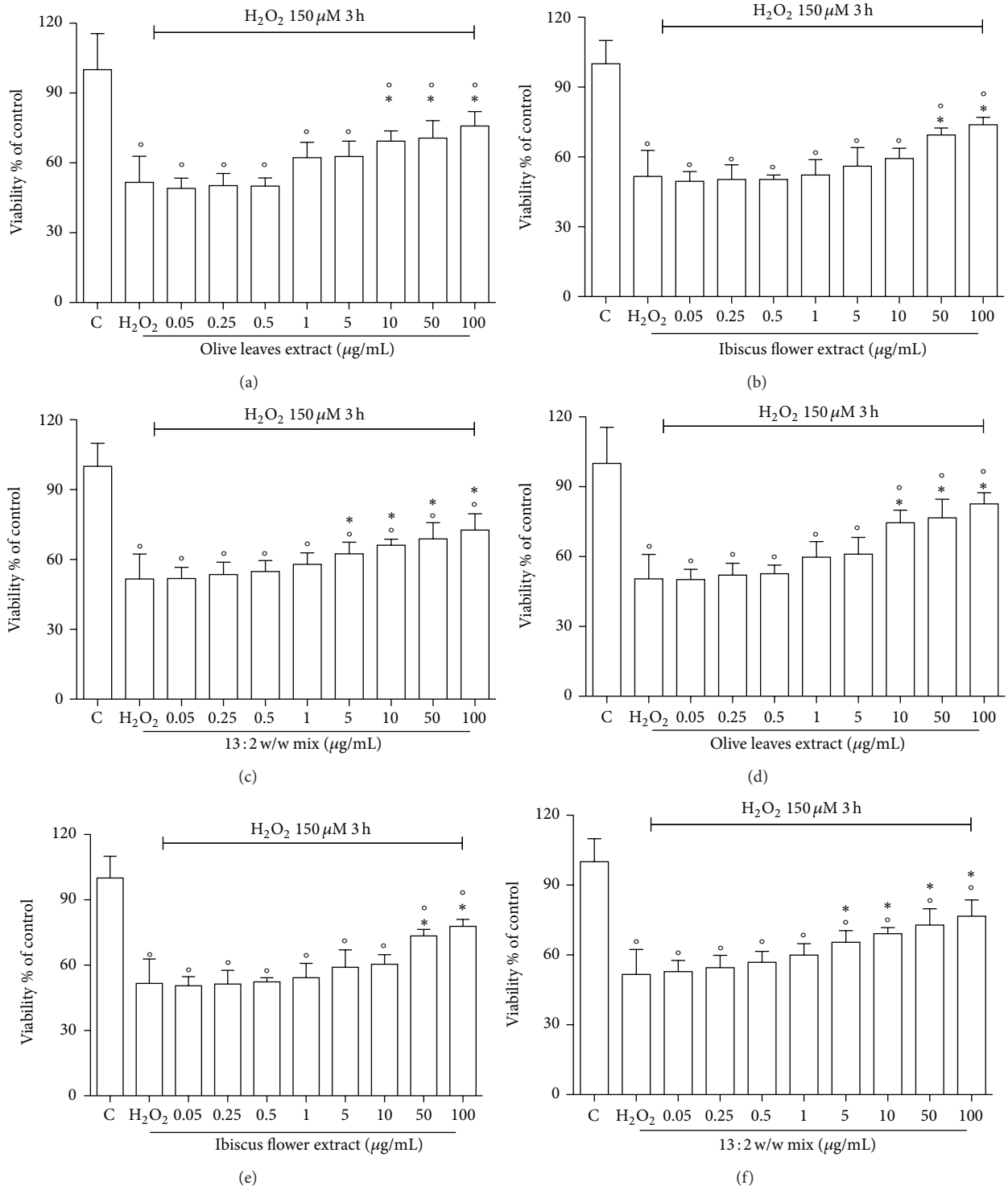

Figure 4: Effect of OEE, HSE, and their mixture on cell viability in HUVECs exposed to H_2O_2. HUVECs were treated with OEE, HSE, and their mixture (0.05–100 μg/mL) for 24 h; oxidative damage was then induced exposing the cells to 150 μM H_2O_2 for 3 h and cellular damage was assessed by both MTT assay ((a), (b), and (c)) and flow-cytometry analysis ((d), (e), and (f)). Data are reported as percent cell viability in comparison to control cells. Each bar represents the mean ± S.D. of four independent experiments. $^*P < 0.05$ with respect to H_2O_2-treated cells, $^\circ P < 0.05$ with respect to control cells.

systolic and diastolic blood pressures in subjects with stage-1 hypertension as Captopril, given at its effective dose of 12.5–25 mg twice daily [34].

The OEE concentrations used in "ex vivo" assays on isolated tissue are in line with doses reported in human intervention studies with oleuropein enriched nutritional supplements [34, 68]

Hibiscus sabdariffa L. is a native shrub of tropical Africa. Dried flowers are used to prepare an infusion with soothing and refreshing properties [69]. The hypotensive activity is partly due to its vascular smooth muscle vasorelaxant properties, as shown in Table 3. HSE share common inotropic and chronotropic effects with OEE, but with less potency. HSE, similarly to OEE, shows relaxant effects on the vascular but not on the nonvascular smooth muscle. It has been recently reported that diuresis and inhibition of the angiotensin I-converting enzyme are less important mechanisms to explain the beneficial actions than those related to the antioxidant, anti-inflammatory, and endothelium-dependent effects [70].

These effects could be ascribed to hibiscus acid, the main phytochemical present in hibiscus calyces [71]; moreover hibiscus flowers are rich in anthocyanosides [72], known to increase microvessels resistance and reduce the permeability and damage [73, 74].

Recently Fernández-Arroyo et al. published data regarding bioavailability and metabolism of *Hibiscus sabdariffa* L. organic acids and polyphenols after oral ingestion in rats. Through HPLC-ESI-TOF-MS analysis, the authors demonstrated that phenolic acids were detectable in plasma without any structural modification; most flavonols were found as quercetin or kaempferol glucuronide conjugates. After oral administration, hibiscus acid, hibiscus acid hydroxyethyl ester, and the metabolite hydroxycitric acid reached high concentrations in plasma, contributing to micromole amounts of organic acids in plasma [75].

To demonstrate that a multifaceted and likely synergistic mechanism accounts for the hypotensive action of OEE and HSE mixture, in vitro study on HUVECs has been performed to evaluate the antioxidant and cytoprotective effects of the two extracts and mixture towards endothelial cells which exert a central role in the regulation of blood pressure.

OEE and HSE revealed cytoprotective and antioxidant properties and OEE extract showed the highest effect. Several authors demonstrated the synergistic effect of many plant extracts administered in polyherbal formulations [75–78]. In this study we have demonstrated that the mixture of the two extracts exerts higher cytoprotective and antioxidant activities than each single extract. Both of these functions are critically involved in cardiovascular protection and hypertension control as clearly reported in the literature. Oxidative stress is also markedly increased in hypertensive patients. If oxidative stress is indeed a cause of hypertension, then antioxidants should have beneficial effects on hypertension control and reduction of oxidative damage should result in a reduction in blood pressure [79].

Another key factor in evaluating the effect of OEE and HSE on the endothelium is their capacity to react to different injuries [80]. Tissue damage at the vascular wall and inflammation leads to stress in the endothelium, so studies on the cytoprotective effect at the endothelium level are of great importance. Some factors have been identified which impair vascular endothelium function, both by their direct effects on the vascular vasomotor capacity, or by influencing cellular regulators, such as inflammatory mediators (ICAM, VCAM). What we currently know about the etiopathogeny of atherosclerosis is that it is a chronic oxidative stress-related inflammatory disease. This study performed on the antioxidant and cytoprotective effects of OEE and HSE throws light on this important point, delineating the cellular mechanism in vascular health protection.

The mixture showed an intrinsic inotropic activity (left atrium) lower than each single extracts, while it exerted negative chronotropic effect; globally the inotropic effect is lower than chronotropic effect, in contrast to nifedipine, characterized by a higher chronotropic than inotropic effect [64]. Despite the low percentage in the mixture, HSE was able to strengthen the effect of OEE, revealing a synergistic cardiac and vascular protective effect. However, even if the amounts of the other phytochemicals are very low, the percentage of HSE is sufficient to reduce the effects of OEE on nonvascular tissue, directing the mixture selectivity towards the vascular tissue.

On the whole, these findings show that the combined treatment of cultured cells, atria, and vascular tissue with the mixture revealed high cardiac and vascular protective activities that, in our specific experimental conditions, were higher than that observed for the most powerful component (OEE).

In conclusion OEE and HSE mixture has a huge potential for pharmaceutical and nutraceutical application, thanks to the synergistic effects of the single phytochemicals.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Matteo Micucci and Marco Malaguti contributed equally to the paper.

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References

[1] A. V. Chobanian, G. L. Bakris, H. R. Black et al., "Seventh report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure," *Hypertension*, vol. 42, no. 6, pp. 1206–1252, 2003.

[2] S. Behradmanesh and P. Nasri, "Serum cholesterol and LDL-C in association with level of diastolic blood pressure in type 2

diabetic patients," *Journal of Renal Injury Prevention*, vol. 1, pp. 23–26, 2012.

[3] S. Behradmanesh and H. Nasri, "Association of serum calcium with level of blood pressure in type 2 diabetic patients," *Journal of Nephropathology*, vol. 2, pp. 254–257, 2013.

[4] Y. Yin, J. L. Pastrana, X. Li et al., "Inflammasomes: sensors of metabolic stresses for vascular inflammation," *Frontiers in Bioscience*, vol. 18, no. 2, pp. 638–649, 2013.

[5] M. Houston, "The role of nutrition and nutraceutical supplements in the treatment of hypertension," *World Journal of Cardiology*, vol. 6, no. 2, pp. 38–66, 2014.

[6] L. A. Al-Ansary, A. C. Tricco, Y. Adi et al., "A systematic review of recent clinical practice guidelines on the diagnosis, assessment and management of hypertension," *PLoS ONE*, vol. 8, no. 1, Article ID e53744, 2013.

[7] B. Vrijens, G. Vincze, P. Kristanto, J. Urquhart, and M. Burnier, "Adherence to prescribed antihypertensive drug treatments: longitudinal study of electronically compiled dosing histories," *British Medical Journal*, vol. 336, no. 7653, pp. 1114–1117, 2008.

[8] L. F. Luo, W. H. Wu, Y. J. Zhou, J. Yan, G. P. Yang, and D. S. Ouyang, "Antihypertensive effect of Eucommia ulmoides Oliv. extracts in spontaneously hypertensive rats," *Journal of Ethnopharmacology*, vol. 129, no. 2, pp. 238–243, 2010.

[9] K. Ried, O. R. Frank, N. P. Stocks, P. Fakler, and T. Sullivan, "Effect of garlic on blood pressure: a systematic review and meta-analysis," *BMC Cardiovascular Disorders*, vol. 8, article 13, 2008.

[10] F. R. Dehkordi and A. F. Kamkhah, "Antihypertensive effect of *Nigella sativa* seed extract in patients with mild hypertension," *Fundamental and Clinical Pharmacology*, vol. 22, no. 4, pp. 447–452, 2008.

[11] F. Greenway, Z. Liu, Y. Yu, and A. Gupta, "A clinical trial testing the safety and efficacy of a standardized *Eucommia ulmoides* oliver bark extract to treat hypertension," *Alternative Medicine Review*, vol. 16, no. 4, pp. 338–347, 2011.

[12] K. Ried, O. R. Frank, and N. P. Stocks, "Aged garlic extract reduces blood pressure in hypertensives: a dose-response trial," *European Journal of Clinical Nutrition*, vol. 67, no. 1, pp. 64–70, 2013.

[13] H. F. Huseini, M. Amini, R. Mohtashami et al., "Blood pressure lowering effect of *Nigella sativa* l. seed oil in healthy Volunteers: a randomized, double-blind, placebo-controlled clinical trial," *Phytotherapy Research*, vol. 27, no. 12, pp. 1849–1853, 2013.

[14] D. F. Fitzpatrick, S. L. Hirschfield, and R. G. Coffey, "Endothelium-dependent vasorelaxing activity of wine and other grape products," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 265, no. 2, pp. H774–H778, 1993.

[15] V. B. Schini-Kerth, C. Auger, J.-H. Kim, N. Étienne-Selloum, and T. Chataigneau, "Nutritional improvement of the endothelial control of vascular tone by polyphenols: role of NO and EDHF," *Pflugers Archiv*, vol. 459, no. 6, pp. 853–862, 2010.

[16] M. Ndiaye, M. Chataigneau, I. Lobysheva, T. Chataigneau, and V. B. Schini-Kerth, "Red wine polyphenol-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery," *The FASEB Journal*, vol. 19, no. 3, pp. 455–457, 2005.

[17] M. Sarr, M. Chataigneau, S. Martins et al., "Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase," *Cardiovascular Research*, vol. 71, no. 4, pp. 794–802, 2006.

[18] M. O. Kane, N. Etienne-Selloum, S. V. F. Madeira et al., "Endothelium-derived contracting factors mediate the Ang II-induced endothelial dysfunction in the rat aorta: preventive effect of red wine polyphenols," *Pflugers Archiv*, vol. 459, no. 5, pp. 671–679, 2010.

[19] A. Chiarini, M. Micucci, M. Malaguti et al., "Sweet chestnut (*Castanea sativa* Mill.) bark extract: cardiovascular activity and myocyte protection against oxidative damage," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 471790, 10 pages, 2013.

[20] C. Carpéné, S. Gomez-Zorita, S. Deleruyelle, and M. A. Carpéné, "Novel strategies for preventing diabetes and obesity complications with natural polyphenols," *Current Medicinal Chemistry*, vol. 22, no. 1, pp. 150–164, 2014.

[21] T. H. Kim, S. K. Ku, T. Lee, and J.-S. Bae, "Vascular barrier protective effects of phlorotannins on HMGB1-mediated proinflammatory responses in vitro and in vivo," *Food and Chemical Toxicology*, vol. 50, no. 6, pp. 2188–2195, 2012.

[22] I. Fistonić, M. Šitum, V. Bulat, M. Harapin, N. Fistonić, and D. Verbanac, "Olive oil biophenols and women's health," *Medicinski Glasnik (Zenica)*, vol. 9, no. 1, pp. 1–9, 2012.

[23] S. N. El and S. Karakaya, "Olive tree (*Olea europaea*) leaves: potential beneficial effects on human health," *Nutrition Reviews*, vol. 67, no. 11, pp. 632–638, 2009.

[24] L. Wang, C. Geng, L. Jiang et al., "The anti-atherosclerotic effect of olive leaf extract is related to suppressed inflammatory response in rabbits with experimental atherosclerosis," *European Journal of Nutrition*, vol. 47, no. 5, pp. 235–243, 2008.

[25] H. F. Al-Azzawie and M.-S. S. Alhamdani, "Hypoglycemic and antioxidant effect of oleuropein in alloxan-diabetic rabbits," *Life Sciences*, vol. 78, no. 12, pp. 1371–1377, 2006.

[26] L. I. Somova, F. O. Shode, P. Ramnanan, and A. Nadar, "Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from Olea europaea, subspecies africana leaves," *Journal of Ethnopharmacology*, vol. 84, no. 2-3, pp. 299–305, 2003.

[27] I. Andreadou, E. K. Iliodromitis, E. Mikros et al., "The olive constituent oleuropein exhibits anti-ischemic, antioxidative, and hypolipidemic effects in anesthetized rabbits," *Journal of Nutrition*, vol. 136, no. 8, pp. 2213–2219, 2006.

[28] I. Andreadou, F. Sigala, E. K. Iliodromitis et al., "Acute doxorubicin cardiotoxicity is successfully treated with the phytochemical oleuropein through suppression of oxidative and nitrosative stress," *Journal of Molecular and Cellular Cardiology*, vol. 42, no. 3, pp. 549–558, 2007.

[29] L. Seddik, T. M. Bah, A. Aoues, M. Slimani, and M. Benderdour, "Elucidation of mechanisms underlying the protective effects of olive leaf extract against lead-induced neurotoxicity in Wistar rats," *Journal of Toxicological Sciences*, vol. 36, no. 6, pp. 797–809, 2011.

[30] D. Dekanski, S. Janićijević-Hudomal, S. Ristić et al., "Attenuation of cold restraint stress-induced gastric lesions by an olive leaf extract," *General Physiology and Biophysics*, vol. 28, pp. 135–142, 2009.

[31] H. K. Hamdi and R. Castellon, "Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor," *Biochemical and Biophysical Research Communications*, vol. 334, no. 3, pp. 769–778, 2005.

[32] A. Scheffler, H. W. Rauwald, B. Kampa, U. Mann, F. W. Mohr, and S. Dhein, "*Olea europaea* leaf extract exerts L-type Ca^{2+} channel antagonistic effects," *Journal of Ethnopharmacology*, vol. 120, no. 2, pp. 233–240, 2008.

[33] M. T. Khayyal, M. A. El-Ghazaly, D. M. Abdallah, N. N. Nassar, S. N. Okpanyi, and M.-H. Kreuter, "Blood pressure lowering effect of an olive leaf extract (*Olea europaea*) in L-NAME induced hypertension in rats," *Arzneimittel-Forschung/Drug Research*, vol. 52, no. 11, pp. 797–802, 2002.

[34] E. Susalit, N. Agus, I. Effendi et al., "Olive (*Olea europaea*) leaf extract effective in patients with stage-1 hypertension: comparison with Captopril," *Phytomedicine*, vol. 18, no. 4, pp. 251–258, 2011.

[35] H. A. Sindi, L. J. Marshall, and M. R. A. Morgan, "Comparative chemical and biochemical analysis of extracts of *Hibiscus sabdariffa*," *Food Chemistry*, vol. 164, pp. 23–29, 2014.

[36] T.-H. Tseng, E.-S. Kao, C.-Y. Chu, F.-P. Chou, H.-W. L. Wu, and C.-J. Wang, "Protective effects of dried flower extracts of *Hibiscus sabdariffa* L. against oxidative stress in rat primary hepatocytes," *Food and Chemical Toxicology*, vol. 35, no. 12, pp. 1159–1164, 1997.

[37] J.-H. Chen, C.-J. Wang, C.-P. Wang, J.-Y. Sheu, C.-L. Lin, and H.-H. Lin, "Hibiscus sabdariffa leaf polyphenolic extract inhibits LDL oxidation and foam cell formation involving up-regulation of LXRα/ABCA1 pathway," *Food Chemistry*, vol. 141, no. 1, pp. 397–406, 2013.

[38] C.-N. Huang, K.-C. Chan, W.-T. Lin, S.-L. Su, C.-J. Wang, and C.-H. Peng, "*Hibiscus sabdariffa* inhibits vascular smooth muscle cell proliferation and migration induced by high glucoses—a mechanism involves connective tissue growth factor signals," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 8, pp. 3073–3079, 2009.

[39] E. Jiménez-Ferrer, J. Alarcón-Alonso, A. Aguilar-Rojas et al., "Diuretic effect of compounds from hibiscus sabdariffa by modulation of the aldosterone activity," *Planta Medica*, vol. 78, no. 18, pp. 1893–1898, 2012.

[40] D. Ojeda, E. Jiménez-Ferrer, A. Zamilpa, A. Herrera-Arellano, J. Tortoriello, and L. Alvarez, "Inhibition of angiotensin convertin enzyme (ACE) activity by the anthocyanins delphinidin- and cyanidin-3-*O*-sambubiosides from *Hibiscus sabdariffa*," *Journal of Ethnopharmacology*, vol. 127, no. 1, pp. 7–10, 2010.

[41] M. Ajay, H. J. Chai, A. M. Mustafa, A. H. Gilani, and M. R. Mustafa, "Mechanisms of the anti-hypertensive effect of *Hibiscus sabdariffa* L. calyces," *Journal of Ethnopharmacology*, vol. 109, no. 3, pp. 388–393, 2007.

[42] A. Herrera-Arellano, S. Flores-Romero, M. A. Chávez-Soto, and J. Tortoriello, "Effectiveness and tolerability of a standardized extract from *Hibiscus sabdariffa* in patients with mild to moderate hypertension: a controlled and randomized clinical trial," *Phytomedicine*, vol. 11, no. 5, pp. 375–382, 2004.

[43] A. Herrera-Arellano, J. Miranda-Sánchez, P. Ávila-Castro et al., "Clinical effects produced by a standardized herbal medicinal product of *Hibiscus sabdariffa* on patients with hypertension. A randomized, double-blind, lisinopril-controlled clinical trial," *Planta Medica*, vol. 73, no. 1, pp. 6–12, 2007.

[44] D. L. McKay, C.-Y. O. Chen, E. Saltzman, and J. B. Blumberg, "*Hibiscus sabdariffa* L. tea (tisane) lowers blood pressure in prehypertensive and mildly hypertensive adults," *Journal of Nutrition*, vol. 140, no. 2, pp. 298–303, 2010.

[45] T. Perrinjaquet-Moccetti, A. Busjahn, C. Schmidlin, A. Schmidt, B. Bradl, and C. Aydogan, "Food supplementation with an olive (*Olea europaea* L.) leaf extract reduces blood pressure in borderline hypertensive monozygotic twins," *Phytotherapy Research*, vol. 22, no. 9, pp. 1239–1242, 2008.

[46] R. J. Tallarida and R. B. Murray, *Manual of Pharmacologic Calculations with Computer Programs*, Springer, New York, NY, USA, 2nd edition, 1987.

[47] C. Caliceti, G. Aquila, M. Pannella et al., "17β-estradiol enhances signalling mediated by VEGF-A-delta-like ligand 4-notch1 axis in human endothelial cells," *PLoS ONE*, vol. 8, no. 8, Article ID e71440, 2013.

[48] C. Angeloni, E. Leoncini, M. Malaguti, S. Angelini, P. Hrelia, and S. Hrelia, "Role of quercetin in modulating rat cardiomyocyte gene expression profile," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 294, no. 3, pp. H1233–H1243, 2008.

[49] C. Angeloni, E. Motori, D. Fabbri et al., "H_2O_2 preconditioning modulates phase II enzymes through p38 MAPK and PI3K/Akt activation," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 6, pp. H2196–H2205, 2011.

[50] C. Angeloni, J. P. E. Spencer, E. Leoncini, P. L. Biagi, and S. Hrelia, "Role of quercetin and its in vivo metabolites in protecting H9c2 cells against oxidative stress," *Biochimie*, vol. 89, no. 1, pp. 73–82, 2007.

[51] H. Motulsky and A. Christopoulos, *Fitting Models to Biological Data Using Linear and Non Linear Regression*, 2003, http://www.graphpad.com/.

[52] H. J. Motulsky, *Prism 5 Statistics Guide*, GraphPad Software Inc., San Diego, Calif, USA, 2007, http://www.graphpad.com.

[53] S. Silva, L. Gomes, F. Leitão, A. V. Coelho, and L. V. Boas, "Phenolic compounds and antioxidant activity of *Olea europaea* L. Fruits and leaves," *Food Science and Technology International*, vol. 12, no. 5, pp. 385–396, 2006.

[54] R. Quirantes-Piné, J. Lozano-Sánchez, M. Herrero, E. Ibáñez, A. Segura-Carretero, and A. Fernández-Gutiérrez, "HPLC-ESI-QTOF-MS as a powerful analytical tool for characterising phenolic compounds in olive-leaf extracts," *Phytochemical Analysis*, vol. 24, no. 3, pp. 213–223, 2013.

[55] A. L. Hopkins, M. G. Lamm, J. L. Funk, and C. Ritenbaugh, "*Hibiscus sabdariffa* L. in the treatment of hypertension and hyperlipidemia: a comprehensive review of animal and human studies," *Fitoterapia*, vol. 85, no. 1, pp. 84–94, 2013.

[56] H. Redwood, "Hypertension, society, and public policy," *European Heart Journal*, vol. 9, Supplement B, pp. B13–B18, 2007.

[57] E. Ernst, "Complementary/alternative medicine for hypertension: a mini-review," *Wiener Medizinische Wochenschrift*, vol. 155, no. 17-18, pp. 386–391, 2005.

[58] J. Wang and X. Xiong, "Current situation and perspectives of clinical study in integrative medicine in China," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 268542, 11 pages, 2012.

[59] M. Suárez, M.-P. Romero, and M.-J. Motilva, "Development of a phenol-enriched olive oil with phenolic compounds from olive cake," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 19, pp. 10396–10403, 2010.

[60] R. García-Villalba, M. Larrosa, S. Possemiers, F. A. Tomás-Barberán, and J. C. Espín, "Bioavailability of phenolics from an oleuropein-rich olive (*Olea europaea*) leaf extract and its acute effect on plasma antioxidant status: comparison between pre- and postmenopausal women," *European Journal of Nutrition*, vol. 53, pp. 1015–1027, 2014.

[61] P. L. Huang, P. L. Huang, and S. Lee-Huang, "Oleuropein and related compounds reduce atherosclerosis," *The Open Conference Proceedings Journal*, vol. 1, no. 1, pp. 81–86, 2010.

[62] M. de Bock, E. B. Thorstensen, J. G. B. Derraik, H. V. Henderson, P. L. Hofman, and W. S. Cutfield, "Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (*Olea europaea* L.) leaf extract," *Molecular Nutrition & Food Research*, vol. 57, no. 11, pp. 2079–2085, 2013.

[63] C. Manna, P. Galletti, G. Maisto, V. Cucciolla, S. D'Angelo, and V. Zappia, "Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells," *FEBS Letters*, vol. 470, no. 3, pp. 341–344, 2000.

[64] A. Locatelli, S. Cosconati, M. Micucci et al., "Ligand based approach to L-type calcium channel by imidazo[2,1-b]thiazole-1,4-dihydropyridines: from heart activity to brain affinity," *Journal of Medicinal Chemistry*, vol. 56, no. 10, pp. 3866–3877, 2013.

[65] A. Zuccotti, S. Clementi, T. Reinbothe, A. Torrente, D. H. Vandael, and A. Pirone, "Structural and functional differences between L-type calcium channels: crucial issues for future selective targeting," *Trends in Pharmacological Sciences*, vol. 32, no. 6, pp. 366–375, 2011.

[66] P. Ioan, E. Carosati, M. Micucci et al., "1,4-dihydropyridine scaffold in medicinal chemistry, the story so far and perspectives (Part 1): action in ion channels and GPCRs," *Current Medicinal Chemistry*, vol. 18, no. 32, pp. 4901–4922, 2011.

[67] E. Carosati, P. Ioan, M. Micucci et al., "1,4-Dihydropyridine scaffold in medicinal chemistry, the story so far and perspectives (part 2): action in other targets and antitargets," *Current Medicinal Chemistry*, vol. 19, no. 25, pp. 4306–4323, 2012.

[68] T. Perrinjaquet-Moccetti, A. Busjahn, C. Schmidlin, A. Schmidt, B. Bradl, and C. Aydogan, "Food supplementation with an olive (*Olea europaea* L.) leaf extract reduces blood pressure in borderline hypertensive monozygotic twins," *Phytotherapy Research*, vol. 22, no. 9, pp. 1239–1242, 2008.

[69] I. Da-Costa-Rocha, B. Bonnlaender, H. Sievers, I. Pischel, and M. Heinrich, "*Hibiscus sabdariffa* L.—a phytochemical and pharmacological review," *Food Chemistry*, vol. 165, pp. 424–443, 2014.

[70] J. Joven, I. March, E. Espinel et al., "*Hibiscus sabdariffa* extract lowers blood pressure and improves endothelial function," *Molecular Nutrition & Food Research*, vol. 58, no. 6, pp. 1374–1378, 2014.

[71] M. M. Ramirez-Rodrigues, M. L. Plaza, A. Azeredo, M. O. Balaban, and M. R. Marshall, "Physicochemical and phytochemical properties of cold and hot water extraction from *Hibiscus sabdariffa*," *Journal of Food Science*, vol. 76, no. 3, pp. C428–C435, 2011.

[72] G. A. Camelo-Méndez, J. A. Ragazzo-Sánchez, A. R. Jiménez-Aparicio, P. E. Vanegas-Espinoza, O. Paredes-López, and A. A. del Villar-Martínez, "Comparative study of anthocyanin and volatile compounds content of four varieties of Mexican roselle (*Hibiscus sabdariffa* L.) by multivariable analysis," *Plant Foods for Human Nutrition*, vol. 68, no. 3, pp. 229–234, 2013.

[73] E. Mian, S. B. Curri, A. Lietti, and E. Bombardelli, "Anthocyanosides and microvessels wall: new findings on the mechanism of action of their protective effect in syndromes due to abnormal capillary fragility," *Minerva Medica*, vol. 68, no. 52, pp. 3565–3581, 1977.

[74] C. Ghiringhelli, L. Gregoratti, and F. Marastoni, "Capillarotropic action of anthocyanosides in high dosage in phlebopathic statis," *Minerva Cardioangiologica*, vol. 26, pp. 255–276, 1978.

[75] S. Fernández-Arroyo, M. Herranz-López, R. Beltrán-Debón et al., "Bioavailability study of a polyphenol-enriched extract from *Hibiscus sabdariffa* in rats and associated antioxidant status," *Molecular Nutrition and Food Research*, vol. 56, no. 10, pp. 1590–1595, 2012.

[76] R. R. Petchi, C. Vijaya, and S. Parasuraman, "Antidiabetic activity of polyherbal formulation in streptozotocin—nicotinamide induced diabetic wistar rats," *Journal of Traditional and Complementary Medicine*, vol. 4, no. 2, pp. 108–117, 2014.

[77] N. Aziz, M. H. Mehmood, S. R. Mandukhal, S. Bashir, S. Raoof, and A. H. Gilani, "Antihypertensive, antioxidant, antidyslipidemic and endothelial modulating activities of a polyherbal formulation (POL-10)," *Vascular Pharmacology*, vol. 50, no. 1-2, pp. 57–64, 2009.

[78] J. L. Gao, T. C. He, Y. B. Li, and Y. T. Wang, "A traditional chinese medicine formulation consisting of *Rhizoma corydalis* and *Rhizoma curcumae* exerts synergistic anti-tumor activity," *Oncology Reports*, vol. 22, no. 5, pp. 1077–1083, 2009.

[79] A. Baradaran, H. Nasri, and M. Rafieian-Kopaei, "Oxidative stress and hypertension: possibility of hypertension therapy with antioxidants," *Journal of Research in Medical Sciences*, vol. 19, no. 4, pp. 358–367, 2014.

[80] J. Delgado-Lista, P. Perez-Martinez, A. Garcia-Rios, A. I. Perez-Caballero, F. Perez-Jimenez, and J. Lopez-Miranda, "Mediterranean diet and cardiovascular risk: beyond traditional risk factors," *Critical Reviews in Food Science and Nutrition*, 2014.

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Research Article

Impact of Procyanidins from Different Berries on Caspase 8 Activation in Colon Cancer

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Scope. The aim of this work is to identify which proapoptotic pathway is induced in human colon cancer cell lines, in contact with proanthocyanidins extracted from various berries. *Methods and Results*. Proanthocyanidins (Pcys) extracted from 11 berry species are monitored for proapoptotic activities on two related human colon cancer cell lines: SW480-TRAIL-sensitive and SW620-TRAIL-resistant. Apoptosis induction is monitored by cell surface phosphatidylserine (PS) detection. Lowbush blueberry extract triggers the strongest activity. When tested on the human monocytic cell line THP-1, blueberry Pcys are less effective for PS externalisation and DNA fragmentation is absent, highlighting a specificity of apoptosis induction in gut cells. In Pcys-treated gut cell lines, caspase 8 (apoptosis extrinsic pathway) but not caspase 9 (apoptosis intrinsic pathway) is activated after 3 hours through P38 phosphorylation (90 min), emphasizing the potency of lowbush blueberry Pcys to eradicate gut TRAIL-resistant cancer cells. *Conclusion*. We highlight here that berries Pcys, especially lowbush blueberry Pcys, are of putative interest for nutritional chemoprevention of colorectal cancer in view of their apoptosis induction in a human colorectal cancer cell lines.

1. Introduction

Colorectal cancer is presently the 3rd more diagnosed cancer in the world (1 million cases), after lung (1,35 million cases) and breast cancers (1,15 million cases) [1]. Concerning the worldwide incidence, colorectal cancer is placed at the 4th rank for men and at the 3rd rank for women. Epidemiologic studies indicate that physical inactivity and excess body weight as abdominal fat are risk factors to develop a colorectal cancer, in particular as a result of an unbalanced diet (high in saturated fats, low in vegetables and fruits) [2]. It is currently admitted that 60 to 80% of colorectal cancers could be avoided by a diet modification [3–5]. Colon carcinogenesis is a process which takes place on 10 to 20 years [6–8], representing a rather long period of time on a human life scale and accounting for the late average diagnosis age. This is the reason why chemoprevention is a particularly relevant strategy in the context of colorectal cancer. Chemoprevention, first described by Sporn in 1976, consists in the administration of natural, synthetic, or biochemical compounds able to prevent cancer apparition or suppress or even reverse its progression and extension [9, 10]. In the case of colorectal cancer, primary chemoprevention concerns the whole population with an average risk to develop this kind of cancer. Secondary chemoprevention is much aimed for a population with a high risk to develop a colorectal cancer [6, 11].

Nutritional cancer chemoprevention consists in *per os* administration of bioactive dietary compounds [12], presenting anticancer activities via different and complementary mechanisms of action [13, 14]. Bioactive dietary phytoconstituents are thus able to exert their activities on precancerous

and cancerous colorectal cells at low but regular doses, such as a metronomic chemotherapy approach [15]. Strategies consisting in preventing and even treating cancer with natural dietary compounds able to induce apoptosis of cancerous cells are currently largely admitted and studied [16–21].

Proanthocyanidins (Pcys), also known as condensed tannins, are the most widely represented products of plants secondary metabolism throughout nature, after lignins [22, 23]. They are constituted by the assembly of flavan-3-ol monomer units, giving rise to Pcy oligomers (2 to 10 monomer units) and Pcy polymers (>10 units, up to 200) [24, 25]. These monomer units are most frequently epicatechin, epiafzelechin, and epigallocatechin, forming procyanidins, propelargonidins, and prodelphinidins, respectively [22]. Flavan-3-ol units can be linked by 2 types of bounds [22, 23, 25]: type B link, mostly C4 → C8, or less frequent type A link consisting in a double bounding, for example, C4 → C8 and C2 → O → C7. The more widespread Pcys are procyanidins and heterogeneous combinations of different monomer units [22]. Pcys protect plants against external aggressions like UV, bacteria, fungi, insects, and herbivores [26, 27], as they are present in certain fruits, nuts, spices, and beverages [28, 29]. Pcys represent a large part of phytoconstituents in a balanced diet [30], and thus they can exert a wide variety of beneficial biological effects [28, 31]. While their *in vivo* antioxidant [32], anti-inflammatory [21], and vasculoprotective [33] activities have already been demonstrated, they are also currently studied for their beneficial effects against cancer, at different stages of its evolution [34].

Earlier, we demonstrated the *in vitro* and *in vivo* colon chemopreventive activities of apple Pcys [35–37]. We postulate here that Pcys from other natural edible sources may exert beneficial anticancer effects as well. Anticancer properties of lowbush blueberry (*Vaccinium myrtillus*) in colorectal cancer remain focused on their anthocyanins effective *in vitro* [38, 39] and *in vivo* [40]. Despite the growing interest on anticancer activities of Pcys [34], there is nowadays no study on proapoptotic activities on colorectal cell lines of lowbush blueberry Pcys. Therefore, we screened for proapoptotic activities of different Pcy-rich fractions obtained from various local fruits. Proapoptotic activities on a validated cellular model of colon cancer progression from a primary tumor were tested on SW480 a TRAIL-sensitive cell line [41] and its corresponding metastatic TRAIL-resistant SW620 sister cell line [42]. We finally focused on *Vaccinium myrtillus* berries, whose Pcys were found to be the most active, trying to clarify elements of their proapoptotic mechanism of action.

2. Materials and Methods

2.1. Fruit Extraction and Proanthocyanidin Enrichment. The following berries were obtained from a local organic producer "Les Fruits d'Altitude" (Fresse-sur-Moselle, France): wild lowbush blueberry (*Vaccinium myrtillus*), highbush blueberry (*Vaccinium corymbosum*), lingonberry (*Vaccinium vitis-idaea*), raspberry (*Rubus idaeus*), wild blackberry (*Rubus fruticosus*), thornfree blackberry (*Rubus fruticosus* "Thornfree"), redcurrant (*Ribes rubrum*), gooseberry (*Ribes uva-crispa*), blackcurrant (*Ribes nigrum*), and jostaberry (*Ribes nidigrolaria*). Cranberry (*Vaccinium macrocarpon*) was purchased from Ocean Spray (Canada).

Fresh fruits were extracted with acetone/water 6 : 4 (1 L/kg) for 3 × 24 h stirring, away from light. The crude extract was then dried and then taken up in water and extracted successively with cyclohexane and ethyl acetate. The aqueous extract was centrifuged to remove insoluble polymers of high MW and then fractionated on Sephadex LH-20 with 100% water followed by addition of 10% methanol with 10% (100 mL) and then washed with acetone/6/4 water (500 mL). Fractions were grouped according to their chromatographic profile (proanthocyanidins and degree of polymerization of greater than 3) and proapoptotic activity. Dosage of proanthocyanidins by vanillin showed that the enriched blueberry extract contains 101 ± 1 g catechin equivalents per 100 g of extract. DMAC proanthocyanidins dosage showed that enriched blackberry extract contains 32.5 g ± 5.1 g of procyanidin A2 equivalent to 100 g of extract. Pcy-rich extracts were obtained by mixing several of the above-cited fractions.

2.2. Proanthocyanidin Determination. DMAC (*p*-dimethylaminocinnamaldehyde) Pcy dosage generates more stable and reproducible results than vanillin [43]. In acidic conditions, DMAC specifically reacts with *meta*-diphenols to form a green carbonium ion detected at 640 nm. The DMAC dosage is as highly specific to Pcys as it does not react with other flavonoids like anthocyanins [44]. However, the colour development depends on the procyanidin structural conformation, and although it has not been demonstrated yet, several authors have suggested that the DMAC reagent could react with only one flavan-3-ol monomer inside a Pcys [45, 46] leading us to an underestimation of Pcy contents, especially for polymers. Procyanidin A2 was our internal standard, as recently validated by several laboratories for cranberry Pcy dosage [47]. Nevertheless, as apple (which we used as our internal standard) contains only type B Pcys, we checked the similarity to procyanidin B2 an internal standard recently published [48]. We observed similar results with both procyanidins A2 and B2 as internal standards.

2.3. Cells. SW480 is a cell line derived from a grade B primary colon carcinoma (Duke's classification) of a 50-year-old patient. SW620 cell line is derived from a metastasis located in a lymphatic node of the same patient, which is removed 6 months later. Both cell lines are obtained through European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They are cultured in Alpha modified Eagle's medium supplemented by 10% heat-inactivated (56°C/30 min) fetal calf serum, 1% penicillin/streptomycin (10 000 U and 20 mg/mL), and 1% L-glutamine (PAN Biotech GmbH, Aidenbach, Germany). Incubations were carried out at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 48 h. Cells were detached by 5 mL trypsin/EDTA (0.05%/0.02% in PBS) (PAN Biotech GmbH, Aidenbach, Germany). All experiments were carried out during exponential phase cell growth. THP-1 (TIB-202) and BxPC-3 (CRL-1687)

cells were grown in RPMI 1640 medium with 2 mM L-alanyl-L-glutamine additionally supplemented with 10% (v/v) fetal bovine serum and 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma-Aldrich). HepG2 (HB-8065) cell line was maintained in MEM media supplemented with 10% (v/v) fetal bovine serum, 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma-Aldrich), and 2 mM glutamine. Cells were grown in humidified atmosphere with 5% CO_2 at 37°C in 25 cm^2 and 75 cm^2 flasks up to 70–80% confluency prior to treatment.

Cells were incubated with the different compounds (Pcy extracts and/or TRAIL) while seeding and incubated for 24 h (SW620) or 48 h (SW480). For control, ethanol used to dissolve Pcy samples was added to the cells at a final concentration of 0.25% (v/v). Apple procyanidins (apple Pcy) used as a Pcy internal control are obtained from Applephenon extract (Maypro Industries, NY, USA) [35].

2.4. Apoptosis. Early and late apoptosis are monitored by flow cytometry (Guava PCA-96 Merck/Millipore, Molsheim, France). Late apoptotic cells are double labeled by Annexin V and 7-AAD (Guava Nexin Reagent kit Merck/Millipore). Apoptotic positive control for each experiment is obtained by celastrol (50 μM) dissolved in DMSO [49, 50]. Celastrol, a gift from Pr. A. C. Allison (Alavita Pharmaceuticals Inc., CA USA), validated each experimental 96-well plate with a 90 to 100% observed rate of apoptosis.

Recombinant human TRAIL was purchased from R&D Systems Europe (Abingdon, UK). TRAIL was dissolved in DPBS supplemented by 1% fetal calf serum and tested at concentrations ranging from 0 to 100 ng/mL.

2.5. Cell Cycle Phase Distribution Analysis and Quantitation of Hypodiploid Sub-G1 Cell Population. Cells were cultured in 25 or 75 cm^2 culture flasks at a density of 10^5 up to 10^6 cells/mL depending on the cell line in accordance with ATCC recommendations. After seeding for 24 h cells were exposed to the extracts for different time periods. Then cells were washed with phosphate-buffered saline (PBS), resuspended in ethanol 70%, and placed for 24 h at −20°C. After centrifugation at 400 g for 5 min, cells were washed twice with PBS buffer. Cells were then resuspended in 500 μL PBS, incubated in FxCycle PI/RNase Staining Solution (Life technologies, Thermo Fisher Scientific Inc., USA), and kept in the dark at room temperature for 30 min. Cellular DNA content was then assessed by flow cytometry in a Guava EasyCyte Plus HP system (EMD Millipore Corporation, Billerica, MA, USA). A minimum of 10,000 cells were acquired per sample and analyzed on the InCyte software. The percentage of cells in G0/G1, S, G2/M, and sub-G1 was determined from DNA content histograms.

2.6. Death Receptors. TRAIL-R1 (DR4/CD261), TRAIL-R2 (DR5/CD262), and Fas (CD95/APO1) specific fluorescent antibodies were used to monitor their expressions onto the cell surface. TRAIL-R1 was labeled by an anti-human mouse monoclonal anti-TRAIL-R1 antibody coupled to Alexa Fluor 488 (AbD Serotec, Düsseldorf, Germany). TRAIL-R2 was revealed by an anti-human mouse monoclonal anti-TRAIL-R2 antibody coupled to phycoerythrin (PE) from CliniSciences (Montrouge, France). Fas was labeled by an anti-human mouse monoclonal anti-Fas antibody coupled to phycoerythrin-Cy5 (PE-Cy5) as well from CliniSciences (Montrouge, France).

After 24 h (SW620 cell line) or 48 h (SW480 cell line) incubation with Pcys, cells were centrifuged 5 min at 200 g and 4°C. Used medium was replaced by culture medium supplemented with antibody solutions. Cells were then incubated 3-4 h at 0°C protected from light. After incubation, cells were centrifuged again, and medium was replaced by fresh culture medium. Cells were then analysed by flow cytometry, on a Guava EasyCyte Plus device for TRAIL-R1 and TRAIL-R2 detection or on a Guava PCA-96 device for Fas detection. The excitation laser wavelength of the Guava EasyCyte Plus is 488 nm, whereas it is 525 nm for the Guava PCA-96.

2.7. Caspases 8 and 9. Caspases 8 and 9 activation was assessed by marking cells with Guava Caspase kit (Merck/Millipore, Molsheim, France). After 24 h (SW620 cell line) or 48 h (SW480 cell line) incubation with Pcys, cells were washed with PBS. Caspases were marked with FLICA reagents covalently marked with a fluorescent probe: FAM (6-fluorescein amidite) for caspase 8 and SR for caspase 9. Cells were incubated for 1 h at 37°C. They were then washed twice with PBS, marked with 7-AAD, and analyzed by flow cytometry on the Guava EasyCyte 8HT device. The Guava EasyCyte 8HT is equipped with two lasers whose excitation wavelengths are 488 and 640 nm.

2.8. ATF2 and P38. Measuring the activity of cell signaling pathways (ATF2 and P38) by flow cytometry was done with the FlowCellect p38 Stress Pathway Activation Detection Kit (Merck/Millipore, Molsheim, France). The anti-pP38 (Thr180/Tyr182) antibody is Alexa Fluor 488 labeled and the anti-TF2 (Thr69/71) antibody is tagged with Alexa Fluor 647. Cell staining protocol was done according to the manufactured recommendations and was analyzed on the Guava EasyCyte 8HT device.

2.9. Statistics. Data are reported as mean ± standard deviation of the mean (SD). Statistical analyses were evaluated using Student's t-test. For the preliminary screening on the thirteen fruits Pcy-rich fractions, values were corrected by Bonferroni's multiple comparison (threshold = $7.6 \cdot 10^{-4}$).

Generally, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

EC_{50} (effective concentration, 50%) determinations with sigmoidal dose-response were computed using GraphPad Prism version 5.0f for OSX (GraphPad Software, San Diego, California USA, http://www.graphpad.com/).

3. Results

3.1. Fruit Extraction and Proanthocyanidin Enrichment Yield. Twelve locally grown fruits were extracted and then fractioned as described in order to obtain several Pcy-rich fractions per fruit (Table 1).

Table 1: Procyanidin A2 equivalents of apple Pcys, lowbush blueberry, and lingonberry Pcy-rich extracts obtained by the BL-DMAC dosage as described under M&M.

Proanthocyanidin-rich sample	Procyanidin A2 equivalents in mg/100 g fresh fruit
Apple	72 ± 10
Lingonberry	48 ± 7
Lowbush blueberry	33 ± 5

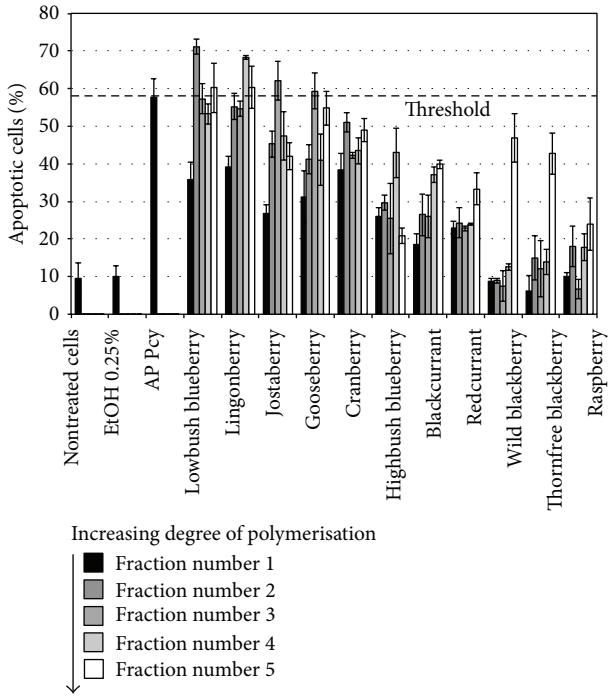

Figure 1: Proapoptotic activities. The 55 proanthocyanidin-rich fractions obtained from 11 fruits (5 fractions per fruit) were tested at 50 μg/mL (final concentration) on SW620 cells for their apoptosis induction properties after 24 h of incubation. Apoptosis yield was evaluated in cytometry by PS cell surface expression as described under Materials and Methods.

3.2. Screening of Proapoptotic Activities of Pcy-Rich Fractions from Various Berries. The obtained fractions were then evaluated for proapoptotic activities on SW620 cells (Figure 1) and compared to apple procyanidins (apple Pcy), a standard well described [35–37].

Pcys from two locally grown fruits showed stronger proapoptotic activities than apple Pcy so that lowbush blueberry and lingonberry were chosen for further investigations on their proapoptotic activities on both SW620 and SW480 cell lines.

3.3. Proapoptotic Activities of Pcys from Lowbush Blueberry and Lingonberry on SW480 and SW620 Cell Lines. Pcy-rich extracts from lowbush blueberry and lingonberry were obtained by combining Pcy-rich fractions no. 2 to no. 5 from each fruit, respectively. Their Pcy richness was assessed by BL-DMAC dosage that estimates procyanidin A2 equivalents contents and then compared to apple Pcy (Table 1).

Table 2: EC_{50} and maximum proapoptotic activity, expressed in percentage of apoptotic cells. Results were observed after 24 h (SW620) or 48 h (SW480) treatment with apple Pcys, lowbush blueberry, and lingonberry Pcy-rich extracts.

Fruit	Human colon cancer cell line			
	SW620	SW480	SW620	SW480
	EC_{50} in μg/ml		% of apoptotic cells at 75 μg/ml	
Lowbush blueberry	24.7 ± 0.1	25.2 ± 4.5	95 ± 1%	92 ± 6%
Lingonberry	24.3 ± 0.2	24.7 ± 0.1	37 ± 2%	41 ± 5%
Apple	46.0 ± 4.5	34.6 ± 0.3	64 ± 9%	55 ± 9%

These extracts were tested for their proapoptotic activities on SW480 and SW620 cell lines at several concentrations. Dose-response curves were computed to obtain EC_{50} values (Figure 2) with noteworthy differences between cell lines or Pcy origins. One should notice that only lowbush blueberry Pcys were able to induce more than 90% of apoptosis on both cell lines (92–95%), followed by apple Pcys (55–64%) and finally by lingonberry Pcys (37–41%) as shown in Table 2.

No clear correlation between activity and procyanidin A2 equivalents content could be observed.

Lowbush blueberry Pcys were selected as most active to carry out a more fine mechanistic approach.

3.4. Lowbush Blueberry Pcys Activities on the Human THP-1 Monocytic Cell Line. At around 100 μg/mL Pcys, 50% of the cells exhibited PS on the cell surface (early apoptosis) when at the same time (Figure 3) celastrol (50 μM) induced 97% of cells in late apoptosis. At 100 μg/mL of fruit Pcys, Chacón et al. reported a percentage of viability >97% when monitoring LDH [51]. In the same manner after 24H of treatment, no increase in number of hypodiploid sub-G1 cells could be observed when monocytic cells THP-1 were exposed to 50 μg/mL of lowbush blueberry Pcys (Table 3) suggesting no apoptotic activity in these cells. Taken together these data suggest an important cell surface disturbant activity of Pcys on THP-1 inflammatory cells without leading to an apoptotic cell death.

Fruit Pcys were reported to possess multiple biological activities including anti-inflammatory [52]. Fruit Pcys decrease production of inflammatory cytokines (at 100 μg/mL), tumor necrosis factor-alpha, and interleukin-6, in cultured human monocytic THP-1 cells, in response to lipopolysaccharide [53]. It appears that, for THP-1 cells, Pcys exhibit a protective action on DNA degradation with mainly anti-inflammatory actions.

3.5. Lowbush Blueberry Pcys Are Not Able to Sensitize SW480 and SW620 Cell Lines to TRAIL-Induced Apoptosis. We described previously that apple Pcys were able to sensitize SW620 and SW480 cell lines to TRAIL-induced apoptosis [54]; we wanted to investigate whether lowbush blueberry Pcys could as well potentiate TRAIL-induced apoptosis of

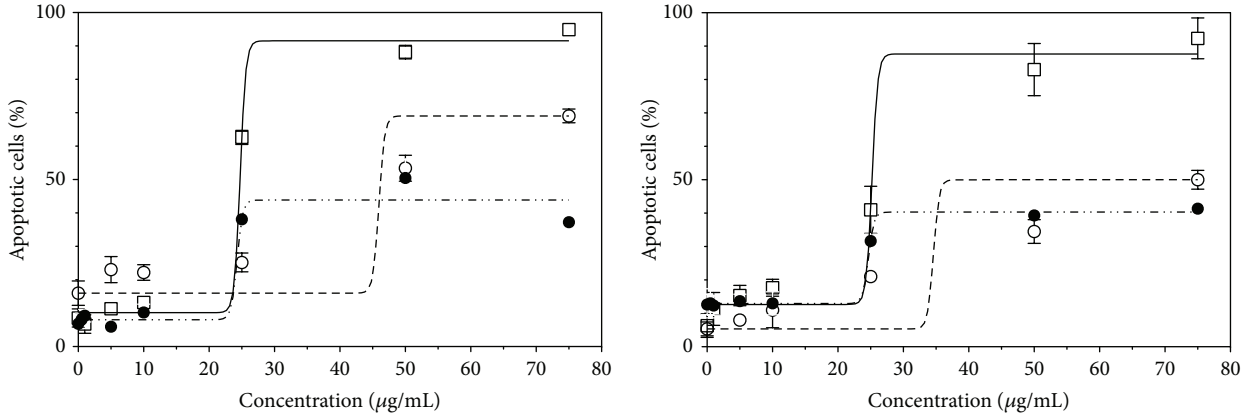

FIGURE 2: EC_{50} of proapoptotic activities. Dose effect curves were plotted for apple Pcy (- - -), proanthocyanidin-rich extracts obtained from lowbush blueberry (—), and lingonberry (·····) when tested on SW620 (a) or SW480 (b) cell lines.

TABLE 3: Cell cycle analyses on THP-1 monocytic cells. Results were observed after 6 and 24 h treatment with lowbush blueberry Pcy-rich extract (50 μg/ml).

Time	Pcys	Sub G0/G1	G0/G1	S	G2/M	% ± SD
6 h	—	0.4 ± 0.2	68.8 ± 5.0	13.0 ± 2.0	17.7 ± 4.3	(n = 12)
	Lowbush blueberry	0.4 ± 0.2	66.3 ± 6.5	13.7 ± 1.7	19.5 ± 6.6	(n = 6)
24 h	—	0.7 ± 0.8	66.1 ± 6.1	17.4 ± 3.9	15.8 ± 8.3	(n = 14)
	Lowbush blueberry	0.6 ± 0.2	69.4 ± 3.4	13.1 ± 5.0	16.8 ± 5.8	(n = 6)

SW480 cell line and sensitize TRAIL-induced apoptosis on SW620 cell line.

A combination of increasing TRAIL and lowbush blueberry Pcy concentrations was tested on both cell lines with no proapoptotic synergistic effect (Figure 3) but lowbush blueberry Pcys are not able to sensitize neither SW480 nor SW620 cell lines to TRAIL-induced apoptosis.

3.6. TRAIL-R1, TRAIL-R2, and Fas Death Receptors Cell Surface Expression Are Not Modified by Lowbush Blueberry Pcys Treatment on SW480 Nor SW620 Cell Lines. According to the membrane disturbance activities observed on human monocytic cells and according to the fact that apple Pcys trigger an increase of TRAIL-R1 and TRAIL-R2 at the cell surface on both cell lines [54], we here investigated whether lowbush blueberry Pcys could induce as well an increase of the three studied death receptors, that is, TRAIL-R1, TRAIL-R2, and Fas receptors, at the cell surface of SW620 and SW480 cells.

Lowbush blueberry Pcys did not modify TRAIL-R1 or TRAIL-R2 (Figure 4) nor Fas receptor expression (Figure 5). Only a slight decrease could be noticed for TRAIL-R2 and Fas in SW480 cell line.

3.7. Lowbush Blueberry Pcys Induce Caspase 8 at 3 and 6 Hours but Not Caspase 9. After 24 hours, apple Pcys were shown to trigger activation of caspase 8 in both cell lines, but only caspase 9 in SW620 cells [54]. After 48 hours, the two caspases were fully activated. Our results here emphasize the fact that lowbush blueberry Pcys are more potent as they significantly activate both caspases 8 and 9 in SW620 (a) and SW480 (b) cell lines (Figure 6) after 24 and 48 hours. As for apple Pcys, shorter incubation times (i.e., 3 and 6 hours) resulted in caspase 8 activation only, highlighting the importance of the extrinsic apoptosis pathway.

3.8. Lowbush Blueberry Pcys Induce P38 Phosphorylation in SW620 Cells. P38 MAK is involved in regulation of Hsp27 and MAPKAP-2 and several transcription factors including ATF2, STAT1, MEF-2, and ELK-1 [55]. Monitoring the activity of cell signaling pathways (P38 and ATF2) underlined the fast activation of cell signals when apoptosis is induced by lowbush blueberry Pcys. IC_{50} value (15 μg/mL) obtained for P38 phosphorylation was equivalent to the value assessed for caspase 8 activation after 3 hours (8 μg/mL) showing a nice correlation between the two events.

4. Discussion

Pcy chemopreventive effects on CRC remain less studied as those induced by other polyphenols [56, 57]. One explanation could be that Pcy extraction is not an easy task [58, 59]. Hence, the extractable Pcys do not exactly reflect qualitatively nor quantitatively the total Pcys of the studied vegetal material. The activities of the extracted Pcys may not exactly reflect the whole fruit activity. The method we use here allows us to enrich our extracts in Pcy oligomers and polymers.

When we screened for proapoptotic activities, the Pcy-enriched fractions from various berries on the TRAIL-resistant SW620 cell line, two berries showed more activity:

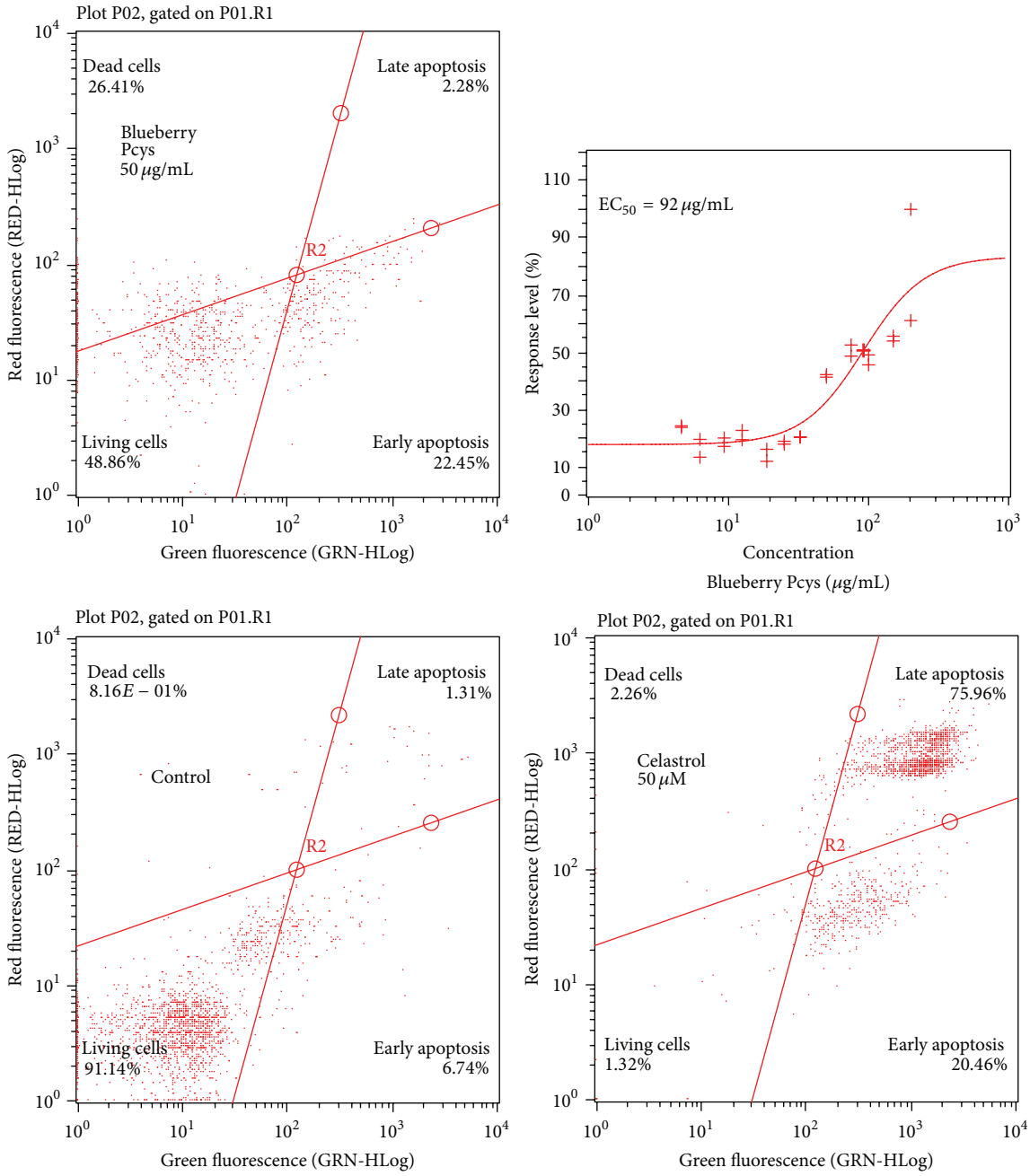

Figure 3: PS exposure on THP-1 monocytic cells induced by lowbush blueberry Pcys. Dose-response curve and EC_{50} value for lowbush blueberry Pcys incubated in raising concentrations were determined on THP-1 cells (upper right graph) after 24 h of incubation. Cells were stained using Annexin V-/PI apoptotic assay as described in Section 2.3 ($n = 3$ independent experiments; 2,000 events per sample were analyzed). Cytograms presented here illustrate the fact that Pcys on early apoptosis could be observed on the contrary of the data obtained for 50 μM celastrol (lower right cytogram).

lingonberry and lowbush blueberry. It looks like the Pcy richness of an extract is not directly related to proapoptotic activity, but two criteria seem relevant: polymer concentration and polymerization degree (PD). Even if lingonberry contains more Pcys in terms of procyanidin A2 equivalents, lowbush blueberry, with more polymerized Pcys, shows stronger proapoptotic activities. In a same sense, raspberry and blackberry fractions, containing very few polymers, were less active on our cellular models. Indeed, small polymer percentages over total Pcys (0% for raspberry and 5,6% for blackberry), with low mPDs (2,1 for raspberry and 2,3 for blackberry), were already reported [28]. Such a Pcy polymers richness of lowbush blueberry was described before by several authors [28, 29].

The proapoptotic dose-response curves of lowbush blueberry and lingonberry Pcy-enriched extracts on SW480 and

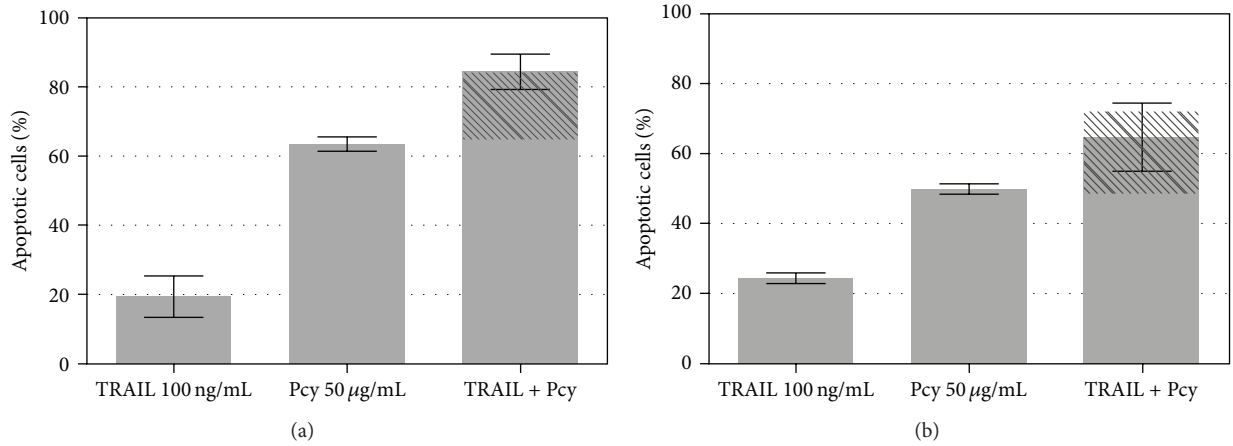

Figure 4: Effect of lowbush blueberry Pcy on TRAIL-mediated apoptosis in SW480 and SW620 cells. The nonsynergistical effects of TRAIL (100 ng/mL) and lowbush blueberry proanthocyanidins (Pcys) (50 μg/mL) combination on apoptosis of SW620 (a) and SW480 (b) cell lines are illustrated by the dashed area reporting TRAIL alone activation and overlaid on the data obtained for TRAIL + Pcy.

SW620 cell lines fit with a higher maximum proapoptotic activity for lowbush blueberry Pcy-enriched extract when compared to lingonberry and this is for both cell lines. Thus, the importance of the Pcy structures has to be emphasized in relation to their proapoptotic activities. The importance of their PD on antiproliferative activities was described earlier for grape seed and pine bark [60], apple [35], and American wild blueberry (*Vaccinium angustifolium*) [61]. If, on one hand, the strength of an extract *in vitro* proapoptotic activity could be linked to its polymer proportion and to their mPD, on the other hand, their chemical structures probably play, as well, an important role in their proapoptotic activities, especially

(i) proportions of type A and type B boundings and their respective positions inside the Pcy molecule;

(ii) proportions of the different possible flavan-3-ol units (e.g., (epi)catechin, (epi)gallocatechin, and (epi)afzelechin) and respective positions inside the Pcy molecule; lingonberry (*Vaccinium vitis-idaea*) contains types A and B procyanidins (catechin/epicatechin) whereas lowbush blueberry (*Vaccinium myrtillus*) contains as well type A and B prodelphinidins (gallocatechin/epigallocatechin) [28–30].

All these parameters will influence the Pcy tridimensional structure and therefore their interaction with cellular elements (receptors, membranes), conditioning biological activities [62–64]. This could be one of the reasons why the most active Pcy extract is the one obtained from lowbush blueberry which, among all tested fruits, presents the higher polymer rate.

TRAIL and related signalization pathways via its receptors TRAIL-R1 and TRAIL-R2 were monitored in view of the capacity of lowbush blueberry Pcy-enriched extract to induce a very strong apoptosis (92–95% at 75 μg/mL) on both cell lines, which notably differ on their TRAIL sensitivity. The mechanism of action could involve TRAIL, inducing suppression of TRAIL resistance in the SW620 cells. The Fas receptor, representative of the TNF superfamily death receptors, was monitored in parallel, as some phytoconstituents were previously described as being able to induce cells apoptosis via extrinsic pathway activation and increase of death receptors number at the cell surface, whether it was for TRAIL-R1 and R2, with sensitization to TRAIL-induced apoptosis [37, 65, 66] or Fas [67–69].

In our hands, lowbush blueberry Pcys were not capable of sensitizing neither SW480 nor SW620 cells to commit TRAIL-induced apoptosis. Indeed, lowbush blueberry Pcys treatment did not modify the expression of TRAIL-R1 and TRAIL-R2 as well as Fas death receptors at the cell surface, and this is for both cell lines.

Unaffected death receptor's numbers at the cell surface as well as nonsensitization to TRAIL by lowbush blueberry Pcys can be explained in two different ways. Lowbush blueberry Pcy-induced apoptosis is mediated

(i) by TRAIL-R1, TRAIL-R2, and/or Fas receptors with an increase of their number at the cell surface but highly polymerized lowbush blueberry Pcys, by forming a coating around the cell, prevented their detection by specific antibodies,

(ii) by other TNF superfamily death receptors inducing caspases 8 and 9 activation, for example, TNF-R1, DR3, or DR6; further experimentations to elucidate these issues were considered, that is, using fluorescent labeled Pcys to visualize any cell surface/receptor coating; however, any chemical grafting of a fluorescent entity will induce changes in the chemophysical properties of the labeled Pcys; fluorescent entities are always presenting high ring density of π electrons generating the fluorescence; thus, such grafted Pcys will compromise their interaction with cellular membrane not permitting to decide which explanation is the most realistic, even with confocal image based investigations.

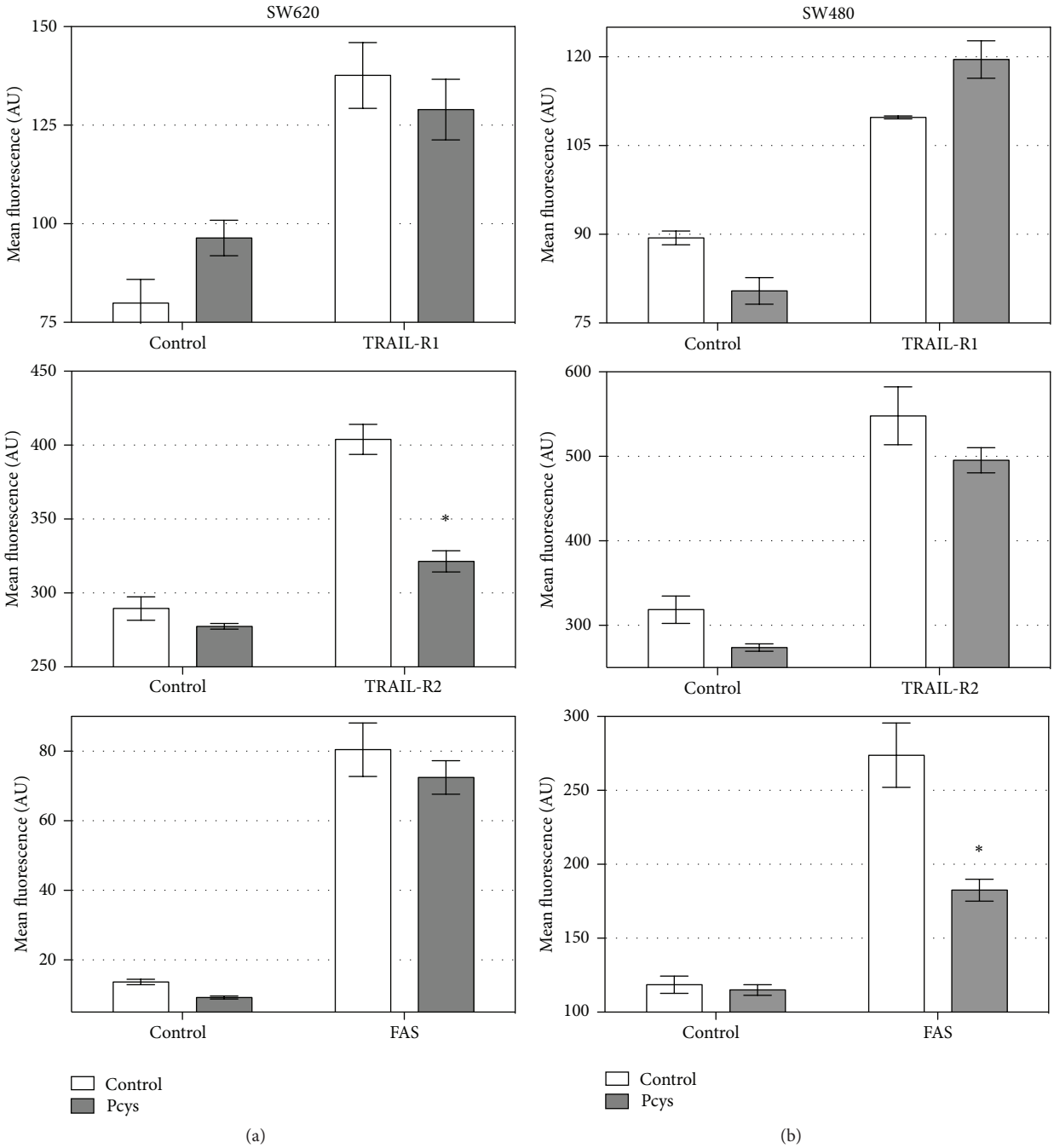

Figure 5: Effect of lowbush blueberry proanthocyanidins (Pcys) (50 μg/mL) on the density of death receptors present at the cell surface of SW620 (a) and SW480 (b) cells. TRAIL-R1 receptor is specifically recognized by a monoclonal antibody Ac1 coupled to Alexa 488 (which emits at 525 nm) whereas TRAIL-R2 receptor is specifically recognized by a monoclonal antibody Ac2 coupled to phycoerythrin (which emits at 575 nm). Fas receptor is specifically recognized by a monoclonal antibody Ac3 coupled to phycoerythrin-Cy5 (PE-Cy5) (which emits at 670 nm). TRAIL-R1 receptor presence is materialized by the mean green fluorescence emitted by Ac1 antibody on SW620 or SW480 cell lines, while TRAIL-R2 receptor is materialized by the mean yellow fluorescence emitted by Ac2 antibody and Fas receptor is materialized by the red green fluorescence emitted by Ac3 antibody. Statistical significant differences (n = 3 independent experiments) based on mean fluorescence (AU) of the cell population between labeled cells in absence of proanthocyanidins and cells labeled and treated with Pcys are represented by the symbol "$*$."

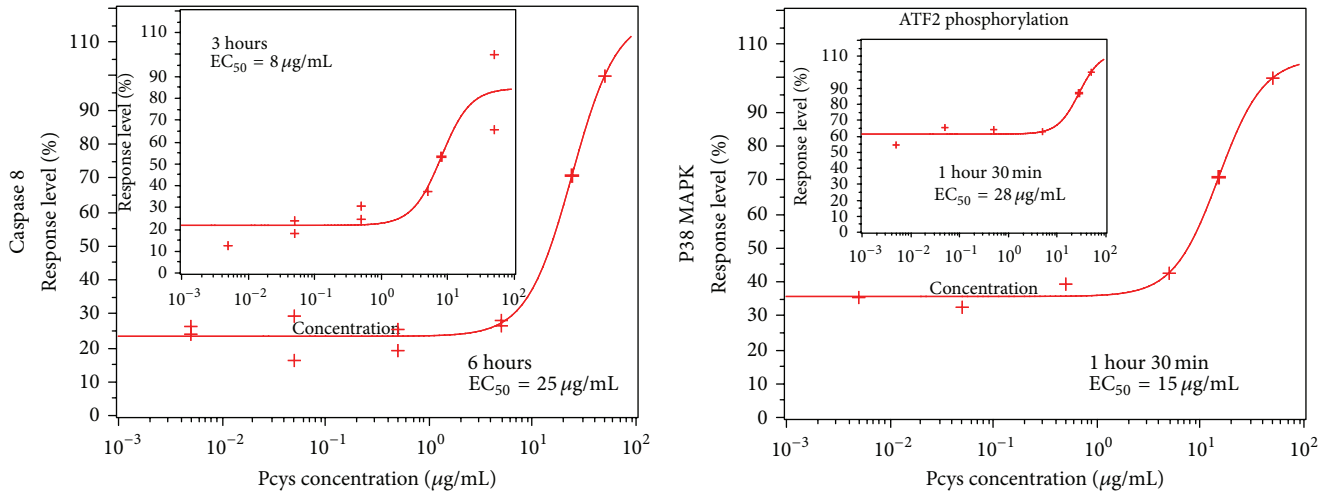

Figure 6: Caspase 8 and P38 MAK pathways activation by lowbush blueberry proanthocyanidins in SW620 cells. (a) Caspase 8 activation curve responses were obtained with two fluorochrome-conjugated inhibitors of caspases consisting of a fluorophore (sulforhodamine for caspase 8 and carboxyfluorescein for caspase 9), a peptide specific for the active site of a particular caspase or many caspases, and a reactive functional group (fluoromethylketone or FMK). These inhibitors are cell permeable and noncytotoxic. Once inside the cell, the caspase inhibitors bind specifically through the peptide moiety to caspases that have been activated in apoptosis, and the FMK moiety covalently links the inhibitor to the caspase. The resulting signal is proportional to the number of active caspase enzymes that are present in the cell. (b) Directly conjugated phosphospecific antibodies were used to monitor the activation of P38 MAK and ATF2 pathways. All signals were monitored by capillary flow cytometry as described under M&M.

Nevertheless, lowbush blueberry Pcys trigger apoptosis via the extrinsic pathway, and this is for both colonic cell lines. It is only after 48 hours that caspases 8 and 9 are similarly greatly activated in the two cell lines (70–80%). We know that extrinsic (caspase 8) and intrinsic (caspase 9) pathways are linked by protein Bid: activated caspase 8 splits Bid, which then later activates caspase 9. Both caspases can then be activated simultaneously only when both extrinsic and intrinsic apoptosis pathways are activated. The whole process is thus triggered by the extrinsic pathway, that is to say, from the cell membrane, consistent with the fact that Pcys (starting from trimers) are unable to enter the cell [63]. In view of the early P38 MAK pathway activation one could make a parallel to apoptosis induced by ROS entities following an oxidative stress. Then lowbush blueberry Pcys are not commonly accepted as passing passively the cellular membrane, unless one takes into account the phenomenon of "sliding through the membrane" recently stated by the group of P. Trouillas [70, 71]. These authors were interested particularly in polyphenols and other π-conjugated compounds. Their molecular dynamics simulations give a very good estimate of the ability of these compounds to insert into membranes. Positions and "exact" directions compounds can thus be obtained *in silico*. Such an insert into the membrane could easily activate the P38 MAK pathways and caspase 8.

The degree of polymerization of proanthocyanidins has a major impact on their fate in the body [28]. Studies have shown that the proanthocyanidins are not degraded in the stomach. If conditions are in any case not quite drastic, they are also protected by the buffering effect of the bolus [34, 72]. The proanthocyanidol polymers appear to be 10 to 100 times less well absorbed than monomers [27, 28]. Thus, the polymers are much less well absorbed through the digestive track portion, monomers, dimers, and trimers [73], due to their lower cellular absorption and their complexation with protein and luminal mucosa [27, 72, 74]. During passage through the small intestine, the proanthocyanidol polymers form complexes with proteins, starch, and digestive enzymes; these complexes are less easily digested, explaining why proanthocyanidol polymers thus reach the colon unchanged [73]. Dimers and trimers intact and undamaged trimers were detected at low levels in urine and plasma of rats after ingestion of a procyanidin-rich extract from grape seeds [75, 76], proving their limit to the systemic circulation; gastrointestinal absorption of procyanidins from the tetramer is suggested to be low or zero. In addition, human plasma levels of procyanidins are very low (nanomolar) after ingestion of cocoa [77] and grape seed [78]. Proanthocyanidins with a polymerization degree greater than or equal to 2 are not depolymerized bioavailable monomers during their passage through the gastrointestinal tract [28, 72, 73, 76].

Authors previously described over a physiologically relevant dose range (up to 50 μg/mL gallic acid equivalents) that digested and fermented berry extracts demonstrated significant activities on colonocytes [79] indicating that phenolic compounds from berries, even after their passage through the gastrointestinal tract, retain biological activity and can modulate cellular processes associated with colon cancer. So once procyanidins have reached the colon, they can do their job. The enriched extract of blueberry procyanidins contains large procyanidins (trimers and beyond) that cannot enter the cells, as already demonstrated [80, 81], but may have effects

on cell membranes [80, 82]. Some authors showed that the polymers are not absorbed across a monolayer of Caco-2 cells and partially adhere to the cell surface [83]. Furthermore, Maldonado-Celis showed that apple procyanidin activates the extrinsic pathway of apoptosis via membrane receptors [37].

In conclusion, Pcys beyond trimers, the major dietary Pcys, are not absorbed throughout the digestive tract, particularly due to their huge molecular weights [27, 74]. Thus, they reach the colon practically intact, where they are able to locally exert their anticancer activities on colorectal precancerous and cancerous cells [72]. Although their metabolism by the colon microflora still remains unclear [74], it is believed to play a major role in Pcys varied biological activities *in vivo* [28, 75, 84]. We estimated that a human of 60–75 kg bodyweight should ingest 40 ± 5 g/day of fresh blueberries to have a quantity of procyanidins reaching the colon equivalent to the one tested in our *in vitro* experiments. This amount seems reasonably attainable through diet.

Our present work emphasizes the potential of berries in chemoprevention, especially CRC chemoprevention, due in part to their polyphenol content and notably to their neglected Pcys. To our knowledge, this is the first report on proapoptotic activities of lowbush blueberry and lingonberry Pcys on human colorectal cell lines.

Abbreviations

DMAC: *p*-Dimethylaminocinnamaldehyde
Pcy: Proanthocyanidin
PD: Polymerization degree
TRAIL: TNF-related apoptosis-inducing ligand.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] D. M. Parkin, F. Bray, J. Ferlay, and P. Pisani, "Global cancer statistics, 2002," *CA: A Cancer Journal for Clinicians*, vol. 55, no. 2, pp. 74–108, 2005.

[2] M. Wiseman, "The second World Cancer Research Fund/American Institute for Cancer Research expert report. Food, nutrition, physical activity, and the prevention of cancer: a global perspective," *Proceedings of the Nutrition Society*, vol. 67, no. 3, pp. 253–256, 2008.

[3] I. T. Johnson, "New approaches to the role of diet in the prevention of cancers of the alimentary tract," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 551, no. 1-2, pp. 9–28, 2004.

[4] T. Norat, S. Bingham, P. Ferrari et al., "Meat, fish, and colorectal cancer risk: the European prospective investigation into cancer and nutrition," *Journal of the National Cancer Institute*, vol. 97, no. 12, pp. 906–916, 2005.

[5] F. J. B. Van Duijnhoven, H. B. Bueno-De-Mesquita, P. Ferrari et al., "Fruit, vegetables, and colorectal cancer risk: the European Prospective Investigation into Cancer and Nutrition," *The American Journal of Clinical Nutrition*, vol. 89, no. 5, pp. 1441–1452, 2009.

[6] E. T. Hawk, A. Umar, and J. L. Viner, "Colorectal cancer chemoprevention—an overview of the science," *Gastroenterology*, vol. 126, no. 5, pp. 1423–1447, 2004.

[7] A. Jemal, R. C. Tiwari, T. Murray et al., "Cancer statistics, 2004," *CA: A Cancer Journal for Clinicians*, vol. 54, no. 1, pp. 8–29, 2004.

[8] I. Dove-Edwin and H. J. Thomas, "Review article: the prevention of colorectal cancer," *Alimentary Pharmacology & Therapeutics*, vol. 15, no. 3, pp. 323–336, 2001.

[9] M. B. Sporn, N. M. Dunlop, D. L. Newton, and J. M. Smith, "Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids)," *Federation Proceedings*, vol. 35, no. 6, pp. 1332–1338, 1976.

[10] R. G. Mehta, G. Murillo, R. Naithani, and X. Peng, "Cancer chemoprevention by natural products: how far have we come?" *Pharmaceutical Research*, vol. 27, no. 6, pp. 950–961, 2010.

[11] D. M. Gustin and D. E. Brenner, "Chemoprevention of colon cancer: Current status and future prospects," *Cancer and Metastasis Reviews*, vol. 21, no. 3-4, pp. 323–348, 2002.

[12] Y.-J. Surh, "Cancer chemoprevention with dietary phytochemicals," *Nature Reviews Cancer*, vol. 3, no. 10, pp. 768–780, 2003.

[13] M. M. Manson, "Cancer prevention—the potential for diet to modulate molecular signalling," *Trends in Molecular Medicine*, vol. 9, no. 1, pp. 11–18, 2003.

[14] B. B. Aggarwal and S. Shishodia, "Molecular targets of dietary agents for prevention and therapy of cancer," *Biochemical Pharmacology*, vol. 71, no. 10, pp. 1397–1421, 2006.

[15] R. S. Kerbel and B. A. Kamen, "The anti-angiogenic basis of metronomic chemotherapy," *Nature Reviews Cancer*, vol. 4, no. 6, pp. 423–436, 2004.

[16] S. Elmore, "Apoptosis: a review of programmed cell death," *Toxicologic Pathology*, vol. 35, no. 4, pp. 495–516, 2007.

[17] S. Mondal, S. Bandyopadhyay, M. K. Ghosh, S. Mukhopadhyay, S. Roy, and C. Mandal, "Natural products: promising resources for cancer drug discovery," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 12, no. 1, pp. 49–75, 2012.

[18] S. Ramos, "Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention," *Journal of Nutritional Biochemistry*, vol. 18, no. 7, pp. 427–442, 2007.

[19] N. Khan, V. M. Adhami, and H. Mukhtar, "Apoptosis by dietary agents for prevention and treatment of prostate cancer," *Endocrine-Related Cancer*, vol. 17, no. 1, pp. R39–R52, 2010.

[20] M.-H. Pan, G. Ghai, and C.-T. Ho, "Food bioactives, apoptosis, and cancer," *Molecular Nutrition and Food Research*, vol. 52, no. 1, pp. 43–52, 2008.

[21] Y. Yoshioka, H. Akiyama, M. Nakano et al., "Orally administered apple procyanidins protect against experimental inflammatory bowel disease in mice," *International Immunopharmacology*, vol. 8, no. 13-14, pp. 1802–1807, 2008.

[22] L. Gu, M. A. Kelm, J. F. Hammerstone et al., "Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 25, pp. 7513–7521, 2003.

[23] T. Shoji, M. Mutsuga, T. Nakamura, T. Kanda, H. Akiyama, and Y. Goda, "Isolation and structural elucidation of some procyanidins from apple by low-temperature nuclear magnetic resonance," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 13, pp. 3806–3813, 2003.

[24] L. Gu, M. Kelm, J. F. Hammerstone et al., "Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 17, pp. 4852–4860, 2002.

[25] M. A. Kelm, J. C. Johnson, R. J. Robbins, J. F. Hammerstone, and H. H. Schmitz, "High-performance liquid chromatography separation and purification of cacao (*Theobroma cacao* L.) procyanidins according to degree of polymerization using a diol stationary phase," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 5, pp. 1571–1576, 2006.

[26] S. González-Manzano, C. Santos-Buelga, J. J. Pérez-Alonso, J. C. Rivas-Gonzalo, and M. T. Escribano-Bailón, "Characterization of the mean degree of polymerization of proanthocyanidins in red wines using Liquid Chromatography-Mass Spectrometry (LC-MS)," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 12, pp. 4326–4332, 2006.

[27] P. M. Aron and J. A. Kennedy, "Flavan-3-ols: nature, occurrence and biological activity," *Molecular Nutrition & Food Research*, vol. 52, no. 1, pp. 79–104, 2008.

[28] R. L. Prior and L. Gu, "Occurrence and biological significance of proanthocyanidins in the American diet," *Phytochemistry*, vol. 66, no. 18, pp. 2264–2280, 2005.

[29] J. K. Hellstrom, A. R. Torronen, and P. H. Mattila, "Proanthocyanidins in common food products of plant origin," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 17, pp. 7899–7906, 2009.

[30] L. Gu, M. A. Kelm, J. F. Hammerstone et al., "Concentrations of proanthocyanidins in common foods and estimations of normal consumption," *Journal of Nutrition*, vol. 134, no. 3, pp. 613–617, 2004.

[31] R. de La Iglesia, F. I. Milagro, J. Campión, N. Boqué, and J. A. Martínez, "Healthy properties of proanthocyanidins," *BioFactors*, vol. 36, no. 3, pp. 159–168, 2010.

[32] T. Koga, K. Moro, K. Nakamori et al., "Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 5, pp. 1892–1897, 1999.

[33] J. Balzer, T. Rassaf, C. Heiss et al., "Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients a double-masked, randomized, controlled trial," *Journal of the American College of Cardiology*, vol. 51, no. 22, pp. 2141–2149, 2008.

[34] V. Nandakumar, T. Singh, and S. K. Katiyar, "Multi-targeted prevention and therapy of cancer by proanthocyanidins," *Cancer Letters*, vol. 269, no. 2, pp. 378–387, 2008.

[35] F. Gossé, S. Guyot, S. Roussi et al., "Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis," *Carcinogenesis*, vol. 26, no. 7, pp. 1291–1295, 2005.

[36] M. E. Maldonado-Celis, S. Roussi, C. Foltzer-Jourdainne et al., "Modulation by polyamines of apoptotic pathways triggered by procyanidins in human metastatic SW620 cells," *Cellular and Molecular Life Sciences*, vol. 65, no. 9, pp. 1425–1434, 2008.

[37] M. E. Maldonado-Celis, S. Bousserouel, F. Gossé, A. Lobstein, and F. Raul, "Apple procyanidins activate apoptotic signaling pathway in human colon adenocarcinoma cells by a lipid-raft independent mechanism," *Biochemical and Biophysical Research Communications*, vol. 388, no. 2, pp. 372–376, 2009.

[38] N. Katsube, K. Iwashita, T. Tsushida, K. Yamaki, and M. Kobori, "Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 1, pp. 68–75, 2003.

[39] C. Zhao, M. M. Giusti, M. Malik, M. P. Moyer, and B. A. Magnuson, "Effects of commercial anthocyanin-rich on colonic cancer and nontumorigenic colonic cell growth," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 20, pp. 6122–6128, 2004.

[40] G. Lala, M. Malik, C. Zhao et al., "Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats," *Nutrition and Cancer*, vol. 54, no. 1, pp. 84–93, 2006.

[41] A. Leibovitz, J. C. Stinson, W. B. McCombs III, and et al, "Classification of human colorectal adenocarcinoma cell lines," *Cancer Research*, vol. 36, no. 12, pp. 4562–4569, 1976.

[42] R. E. Hewitt, A. McMarlin, D. Kleiner et al., "Validation of a model of colon cancer progression," *The Journal of Pathology*, vol. 192, no. 4, pp. 446–454, 2000.

[43] R. L. Prior, E. Fan, H. Ji et al., "Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders," *Journal of the Science of Food and Agriculture*, vol. 90, no. 9, pp. 1473–1478, 2010.

[44] S. Y. Ong, A. Wain, L. Groombridge, and E. Grimes, "Forensic identification of urine using the DMAC test: a method validation study," *Science and Justice*, vol. 52, no. 2, pp. 90–95, 2012.

[45] I. McMurrough and J. McDowell, "Chromatographic separation and automated analysis of flavanols," *Analytical Biochemistry*, vol. 91, no. 1, pp. 92–100, 1978.

[46] C. W. Nagel and Y. Glories, "Use of a modified dimethylaminocinnamaldehyde reagent for analysis of flavanols," *The American Journal of Enology and Viticulture*, vol. 42, no. 4, pp. 364–366, 1991.

[47] R. L. Prior, X. Wu, L. Gu et al., "Purified berry anthocyanins but not whole berries normalize lipid parameters in mice fed an obesogenic high fat diet," *Molecular Nutrition and Food Research*, vol. 53, no. 11, pp. 1406–1418, 2009.

[48] M. J. Payne, W. J. Hurst, D. A. Stuart et al., "Determination of total procyanidins in selected chocolate and confectionery products using DMAC," *Journal of AOAC International*, vol. 93, no. 1, pp. 89–96, 2010.

[49] R. Kannaiyan, K. A. Manu, L. Chen et al., "Celastrol inhibits tumor cell proliferation and promotes apoptosis through the activation of c-Jun N-terminal kinase and suppression of PI3 K/Akt signaling pathways," *Apoptosis*, vol. 16, no. 10, pp. 1028–1041, 2011.

[50] B. Sung, B. Park, V. R. Yadav, and B. B. Aggarwal, "Celastrol, a triterpene, enhances TRAIL-induced apoptosis through the down-regulation of cell survival proteins and up-regulation of death receptors," *Journal of Biological Chemistry*, vol. 285, no. 15, pp. 11498–11507, 2010.

[51] M. R. Chacón, V. Ceperuelo-Mallafré, E. Maymó-Masip et al., "Grape-seed procyanidins modulate inflammation on human

differentiated adipocytes *in vitro*," *Cytokine*, vol. 47, no. 2, pp. 137–142, 2009.

[52] D. Bagchi, M. Bagchi, S. J. Stohs, S. D. Ray, C. K. Sen, and H. G. Preuss, "Cellular protection with proanthocyanidins derived from grape seeds," *Annals of the New York Academy of Sciences*, vol. 957, pp. 260–270, 2002.

[53] T. Tatsuno, M. Jinno, Y. Arima et al., "Anti-inflammatory and anti-melanogenic proanthocyanidin oligomers from peanut skin," *Biological and Pharmaceutical Bulletin*, vol. 35, no. 6, pp. 909–916, 2012.

[54] M.-E. Maldonado-Celis, S. Bousserouel, F. Gossé, C. Minker, A. Lobstein, and F. Raul, "Differential induction of apoptosis by apple procyanidins in TRAIL-sensitive human colon tumor cells and derived TRAIL-resistant metastatic cells," *Journal of Cancer Molecules*, vol. 5, no. 1, pp. 21–30, 2009.

[55] M. Löwenberg, M. P. Peppelenbosch, and D. W. Hommes, "Therapeutic modulation of signal transduction pathways," *Inflammatory Bowel Diseases*, vol. 10, no. 1, pp. S52–S57, 2004.

[56] A. Yang, H. Li, W. Y. Zhang, Y. T. Chung, J. Liao, and G.-Y. Yang, "Chemoprevention of chronic inflammatory bowel disease-induced carcinogenesis in Rodent Models by Berries," in *Berries and Cancer Prevention*, N. P. Seeram and G. D. Stoner, Eds., pp. 227–243, Springer, New York, NY, USA, 2011.

[57] J. R. Araújo, P. Gonçalves, and F. Martel, "Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines," *Nutrition Research*, vol. 31, no. 2, pp. 77–87, 2011.

[58] S. Arranz, F. Saura-Calixto, S. Shaha, and P. A. Kroon, "High contents of nonextractable polyphenols in fruits suggest that polyphenol contents of plant foods have been underestimated," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 16, pp. 7298–7303, 2009.

[59] J. Pérez-Jiménez, S. Arranz, and F. Saura-Calixto, "Proanthocyanidin content in foods is largely underestimated in the literature data: an approach to quantification of the missing proanthocyanidins," *Food Research International*, vol. 42, no. 10, pp. 1381–1388, 2009.

[60] D. Lizarraga, C. Lozano, J. J. Briedé et al., "The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts," *FEBS Journal*, vol. 274, no. 18, pp. 4802–4811, 2007.

[61] B. M. Schmidt, J. W. Erdman Jr., and M. A. Lila, "Differential effects of blueberry proanthocyanidins on androgen sensitive and insensitive human prostate cancer cell lines," *Cancer Letters*, vol. 231, no. 2, pp. 240–246, 2006.

[62] R. Maestre, V. Micol, L. Funes, and I. Medina, "Incorporation and interaction of grape seed extract in membranes and relation with efficacy in muscle foods," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 14, pp. 8365–8374, 2010.

[63] Z. Dong and Y. J. Surh, *Dietary Modulation of Cell Signaling Pathways*, Taylor & Francis, 2008.

[64] S. V. Verstraeten, J. F. Hammerstone, C. L. Keen, C. G. Fraga, and P. I. Oteiza, "Antioxidant and membrane effects of procyanidin dimers and trimers isolated from peanut and cocoa," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 12, pp. 5041–5048, 2005.

[65] I. A. Siddiqui, A. Malik, V. M. Adhami et al., "Green tea polyphenol EGCG sensitizes human prostate carcinoma LNCaP cells to TRAIL-mediated apoptosis and synergistically inhibits biomarkers associated with angiogenesis and metastasis," *Oncogene*, vol. 27, no. 14, pp. 2055–2063, 2008.

[66] S. Shankar, S. Ganapathy, Q. Chen, and R. K. Srivastava, "Curcumin sensitizes TRAIL-resistant xenografts: molecular mechanisms of apoptosis, metastasis and angiogenesis," *Molecular Cancer*, vol. 7, article 16, 2008.

[67] C.-H. Liang, L.-Y. Shiu, L.-C. Chang, H.-M. Sheu, E.-M. Tsai, and K.-W. Kuo, "Solamargine enhances HER2 expression and increases the susceptibility of human lung cancer H661 and H69 cells to trastuzumab and epirubicin," *Chemical Research in Toxicology*, vol. 21, no. 2, pp. 393–399, 2008.

[68] M. A. Shammas, P. Neri, H. Koley et al., "Specific killing of multiple myeloma cells by (-)-epigallocatechin-3- gallate extracted from green tea: biologic activity and therapeutic implications," *Blood*, vol. 108, no. 8, pp. 2804–2810, 2006.

[69] M. Saleem, M. H. Kweon, J. M. Yun et al., "A novel dietary triterpene lupeol induces fas-mediated apoptotic death of androgen-sensitive prostate cancer cells and inhibits tumor growth in a xenograft model," *Cancer Research*, vol. 65, no. 23, pp. 11203–11213, 2005.

[70] J. Vacek, M. Zatloukalová, T. Desmier et al., "Antioxidant, metal-binding and DNA-damaging properties of flavonolignans: a joint experimental and computational highlight based on 7-*O*-galloylsilybin," *Chemico-Biological Interactions*, vol. 205, no. 3, pp. 173–180, 2013.

[71] P. Podloucká, K. Berka, G. Fabre et al., "Lipid bilayer membrane affinity rationalizes inhibition of lipid peroxidation by a natural lignan antioxidant," *The Journal of Physical Chemistry B*, vol. 117, no. 17, pp. 5043–5049, 2013.

[72] S. E. Rasmussen, H. Frederiksen, K. S. Krogholm, and L. Poulsen, "Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease," *Molecular Nutrition and Food Research*, vol. 49, no. 2, pp. 159–174, 2005.

[73] J. Serrano, R. Puupponen-Pimiä, A. Dauer, A.-M. Aura, and F. Saura-Calixto, "Tannins: current knowledge of food sources, intake, bioavailability and biological effects," *Molecular Nutrition and Food Research*, vol. 53, supplement 2, pp. S310–S329, 2009.

[74] C. Santos-Buelga and A. Scalbert, "Proanthocyanidins and tannin-like compounds—nature, occurrence, dietary intake and effects on nutrition and health," *Journal of the Science of Food and Agriculture*, vol. 80, no. 7, pp. 1094–1117, 2000.

[75] J. K. Prasain, N. Peng, Y. Dai et al., "Liquid chromatography tandem mass spectrometry identification of proanthocyanidins in rat plasma after oral administration of grape seed extract," *Phytomedicine*, vol. 16, no. 2-3, pp. 233–243, 2009.

[76] C. Tsang, C. Auger, W. Mullen et al., "The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats," *British Journal of Nutrition*, vol. 94, no. 2, pp. 170–181, 2005.

[77] R. R. Holt, S. A. Lazarus, M. Cameron Sullards et al., "Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa," *American Journal of Clinical Nutrition*, vol. 76, no. 4, pp. 798–804, 2002.

[78] A. Sano, J. Yamakoshi, S. Tokutake, K. Tobe, Y. Kubota, and M. Kikuchi, "Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract," *Bioscience, Biotechnology and Biochemistry*, vol. 67, no. 5, pp. 1140–1143, 2003.

[79] E. M. Brown, G. J. McDougall, D. Stewart et al., "Persistence of anticancer activity in berry extracts after simulated gastrointestinal digestion and colonic fermentation," *PLoS ONE*, vol. 7, no. 11, Article ID e49740, 2012.

[80] G. G. Mackenzie, M. P. Zago, A. G. Erlejman, L. Aimo, C. L. Keen, and P. I. Oteiza, "α-Lipoic acid and *N*-acetyl cysteine prevent zinc deficiency-induced activation of NF-κB and AP-1 transcription factors in human neuroblastoma IMR-32 cells," *Free Radical Research*, vol. 40, no. 1, pp. 75–84, 2006.

[81] J. I. Ottaviani, C. L. Keen, and C. G. Fraga, "Chocolate and health: on the vascular effects of flavanols in cocoa," *Agro Food Industry Hi-Tech*, vol. 20, no. 6, pp. 6–9, 2009.

[82] S. V. Verstraeten, L. Lanoue, C. L. Keen, and P. I. Oteiza, "Relevance of lipid polar headgroups on boron-mediated changes in membrane physical properties," *Archives of Biochemistry and Biophysics*, vol. 438, no. 1, pp. 103–110, 2005.

[83] S. Deprez, C. Brezillon, S. Rabot et al., "Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids," *Journal of Nutrition*, vol. 130, no. 11, pp. 2733–2738, 2000.

[84] M. Monagas, M. Urpi-Sarda, F. Sánchez-Patán et al., "Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites," *Food and Function*, vol. 1, no. 3, pp. 233–253, 2010.

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Review Article

Molecular Mechanisms and Therapeutic Effects of (−)-Epicatechin and Other Polyphenols in Cancer, Inflammation, Diabetes, and Neurodegeneration

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With recent insight into the mechanisms involved in diseases, such as cardiovascular disease, cancer, stroke, neurodegenerative diseases, and diabetes, more efficient modes of treatment are now being assessed. Traditional medicine including the use of natural products is widely practiced around the world, assuming that certain natural products contain the healing properties that may in fact have a preventative role in many of the diseases plaguing the human population. This paper reviews the biological effects of a group of natural compounds called polyphenols, including apigenin, epigallocatechin gallate, genistein, and (−)-epicatechin, with a focus on the latter. (−)-Epicatechin has several unique features responsible for a variety of its effects. One of these is its ability to interact with and neutralize reactive oxygen species (ROS) in the cell. (−)-Epicatechin also modulates cell signaling including the MAP kinase pathway, which is involved in cell proliferation. Mutations in this pathway are often associated with malignancies, and the use of (−)-epicatechin holds promise as a preventative agent and as an adjunct for chemotherapy and radiation therapy to improve outcome. This paper discusses the potential of some phenolic compounds to maintain, protect, and possibly reinstate health.

Dedicated to Dr. Manfred Holz

1. Introduction: Structural Characteristics of Polyphenols

Polyphenols belong to a ubiquitous family of naturally occurring compounds that encompass several other classes of compounds such as flavonoids. Flavonoids consist of several groups of compounds called anthocyanins, flavanols, flavonones, flavones, and isoflavones. These compounds are polyphenols due to the presence of multiple phenolic units in their chemical structure. Thus, phenolic compounds share structural features including an aromatic or a phenolic ring. Polyphenol compounds are most abundant in fruits, vegetables, cereals, and beverages. Fruits such as apples, grapes, pears, cherries, and berries contain 200–300 mg of polyphenols per 100 grams [1]. (−)-Epicatechin, the focus of this review article, belongs to the group of flavanols. It is most commonly found as a natural product in cacao and cacao products, such as dark chocolate, and in green tea.

2. Biological Functions

Polyphenols have various important biological properties in both plants and animals that can be divided into two main categories, with antioxidant and nonantioxidant function. These functions are discussed throughout this paper. Regarding antioxidant action, it is noteworthy that polyphenols are the most abundant antioxidants in the diet with a total daily intake as high as 1 gram, exceeding the intake of vitamin C by about 10-fold and that of vitamin E and carotenoids by about 100-fold [2]. Given the large number of studies showing beneficial effects with vitamin antioxidants, similar or better effects might be expected for polyphenols. Antioxidants, in general, have been intensely studied due to the high prevalence of oxidative stress found in numerous disease states, including Alzheimer's disease, muscular dystrophy, rheumatoid arthritis, diabetes, cancer, heart disease, and aging. For example, in a randomized clinical trial for Alzheimer's disease (AD), patients were treated for 16 weeks with vitamin E (α-tocopherol/E) 800 IU daily, 500 mg of vitamin C daily, 900 mg of α-lipoic acid (ALA) daily, and 400 mg of coenzyme Q (CoQ) three times daily or placebo [3]. The study showed, following E/C/ALA treatment only, a 19% decrease in F2-isoprostanes, which are cerebral spinal fluid (CSF) biomarkers of AD [3], suggesting the potential application of antioxidant treatment in patients with AD. Oxidative stress has also been found to play a pivotal role in the development of complications due to diabetes, such as cardiovascular and microvascular disease. Following treatment of diabetic mice with vitamins C, E, and β-carotene for 8 weeks Mekinová et al. [4] observed reductions of thiobarbituric acid reactive substances (TBARS, used to determine oxidative stress status), glutathione, and glutathione peroxidase and an increase in copper and zinc superoxide dismutase (CuZn-SOD). These examples all argue for the potential use of ROS scavengers including natural compounds with such activities in certain diseases where the redox balance and ROS load are not any longer under control, a research direction that should be pursued with polyphenol compounds in the future.

There are numerous nonantioxidant functions of polyphenols with select examples discussed later in this paper. These include effects on estrogen receptor activity, cell signaling cascades, and cell cycle control in mammalian cells. Since polyphenols are plant-derived compounds, it is not surprising that they play important roles in plant physiology. As an example related to plant signaling, flavonoids were found to greatly affect the growth pattern of *Malus x domestica*, the apple tree [5]. The authors found, following RNAi silencing of the enzyme chalcone synthase (CHS), which is responsible for flavonoid synthesis in apples, a loss in skin and leaf pigmentation and a reduction in size, with smaller leaves and shortened internode lengths [5]. This suggests that flavonoid production is important for the integrity and morphology of apples. Polyphenols also have the ability to scavenge reactive oxygen species (ROS). This is thought to be a primary function of polyphenols in mammals and therefore they are typically referred to as antioxidants.

3. Beneficial Health Effects of Selected Flavonoid Compounds

In this section we will briefly summarize the cellular and organismal effects of the selected flavonoids epigallocatechin gallate, genistein, apigenin, and (−)-epicatechin, the latter of which will be discussed in more detail.

3.1. Epigallocatechin Gallate. Epigallocatechin gallate (EGCG) (Figure 1) is the most abundant catechin found in green tea (*Camellia sinensis*) [6]. EGCG is a potent antioxidant that has various clinical applications. It is a widely studied catechin in cancer research and the potential underlying mechanisms have started to emerge. As an example, Lin et al. [7] demonstrated that treatment with EGCG inactivated the STAT3 pathway, which plays a critical role in promoting tumor formation in tumor initiating cells of nasopharyngeal carcinoma. The study showed a reduction in the stemness of tumor initiating cells by sphere formation, colony formation, cell viability, and an increased sensitivity to cisplatin, indicating that the compound directly affects growth signaling in cancer cells. Mukherjee et al. [8] reported that EGCG is able to sequester the p65 subunit of the transcription factor NF-κB and to inhibit cytokine and chemokine transcription following CpG synthetic oligodeoxynucleotide treatment in DU145, PC3, and LNCaP prostate cancer cell lines. This suggests that EGCG is able to ameliorate chronic inflammation resulting from microbial pathogens that increases the risk for prostate cancer. Poutahidis et al. [9] observed that $Apc^{Min/+}$ mutant mice, upon gastrointestinal tract infection with *Helicobacter hepaticus*, were significantly predisposed to prostate cancer, suggesting that infection-mediated inflammation can drive cancer progression. EGCG was further found to decrease protein expression of both HIF-1α and its downstream target vascular endothelial growth factor (VEGF) in MCF-7 cells in a dose-dependent manner [10]. In addition, EGCG was found to protect the cells from ionizing radiation. A recent study showed that, with a simple pretreatment of 50 μM EGCG, human epidermal keratinocytes (HaCaT cell line) were protected from radiation-induced (20 Gy) cytotoxicity [11]. This was demonstrated by a reduction in (1) apoptotic cells as analyzed by flow cytometry of annexin V/propidium iodide double staining, (2) damage to the mitochondria analyzed by MitoTracker Red staining and upregulation of superoxide dismutase 2, (3) total cellular ROS as determined by DCFDA staining, and (4) γH2AX foci, a marker of DNA damage [11]. They also observed an EGCG-dependent transcriptional induction of heme oxygenase-1, which the authors concluded to be the primary source of the protective effect. After treatment with EGCG following either siRNA knockdown or use of a specific inhibitor of heme oxygenase-1, the protective effects diminished [11].

Epidemiological and animal studies further indicate that EGCG shows protective activity in neurological disorders [12]. In vitro, EGCG was shown to inhibit the aggregation of amyloidogenic proteins including amyloid-β (Aβ) monomers, α-syn, calcitonin, hIAPP, and insulin, which are important in many neurological diseases [13–16]. Interestingly, in

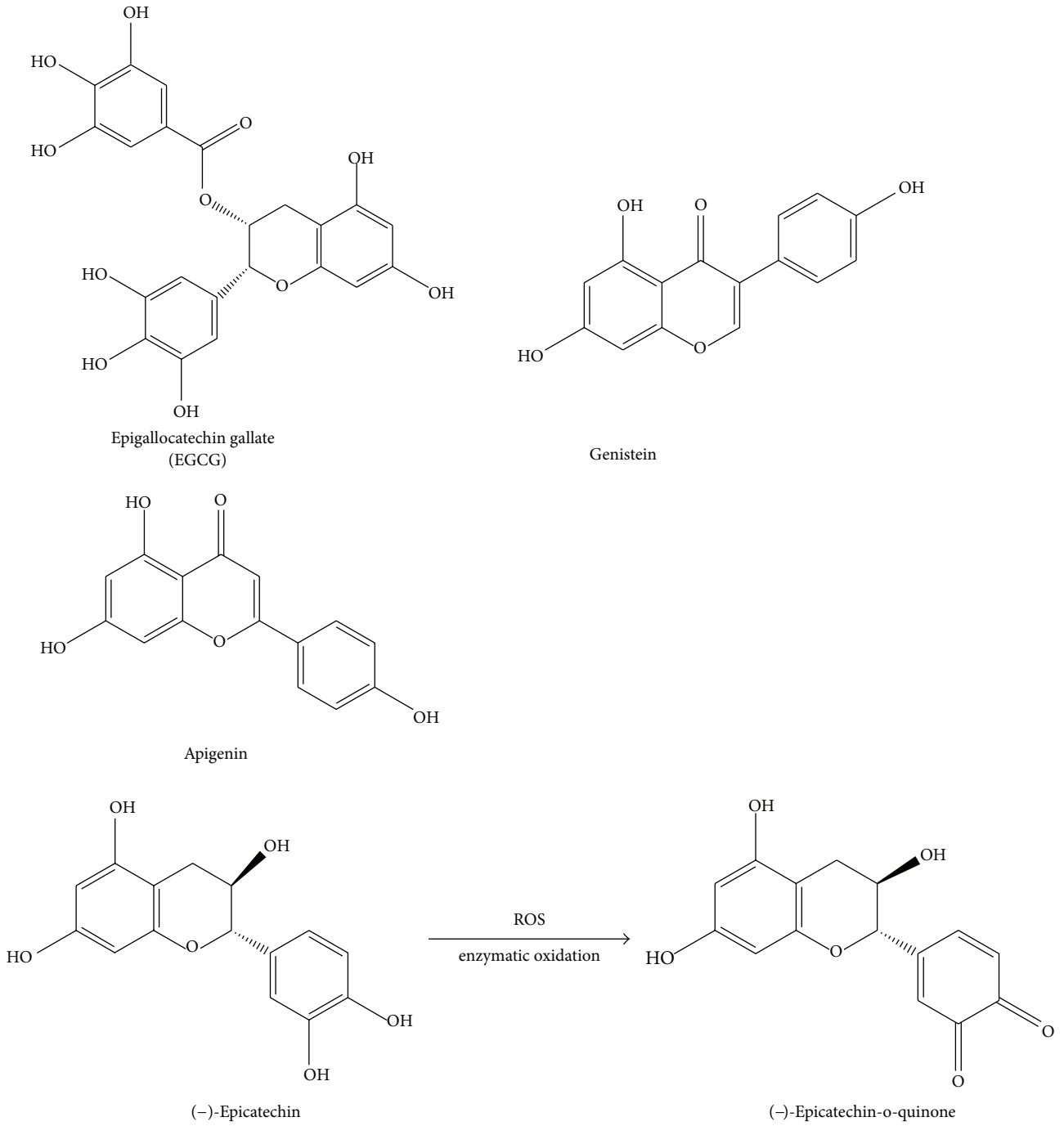

Figure 1: Chemical structures of epigallocatechin gallate, genistein, apigenin, and (−)-epicatechin and its oxidation product (−)-epicatechin-o-quinone.

the presence of EGCG, Aβ monomers adopt a new conformation with an increased inter-center-of-mass distance with reduced β-sheet content [17]. This thereby affects its inclination to form fibril-prone states that otherwise increase the severity of Alzheimer's disease [17]. Taken together, these studies indicate that EGCG has multiple cellular effects, is able to decrease the risk of tumor initiation and progression, and may be useful in preventing amyloid formation seen in neurodegenerative diseases.

3.2. Genistein. Genistein (Figure 1) belongs to the group of isoflavones and is found primarily in soybean seeds [18]. It is also a common precursor in legumes derived from the biosynthesis of antimicrobial phytoalexins and phytoanticipins [18]. Genistein may be useful in the treatment of breast cancer due to its estrogen receptor antagonist activity. It shares a structural similarity with 17β-estradiol (estrogen), allowing it to specifically interact with the estrogen receptor. Chen and Chien [19] treated malignant human breast cancer

MCF-7 cells with the phytoestrogens genistein, resveratrol, and quercetin and found that they were all individually effective at inhibiting cancer cell growth at concentrations of 10^{-4} M. Furthermore, there was a significant increase in apoptotic MCF-7 cells observed following the treatment with the three compounds, which was primarily due to a reduction in PI3K and Akt phosphorylation and an increase in Fas ligand, Fas-associated protein with death domain (FADD), truncated Bid, cytochrome *c* release, and caspases 3 and 9 activation [19]. Interestingly, when the authors treated the normal MCF-10A cell line with genistein, resveratrol, and quercetin, they observed a slight increase in cell proliferation, which was a result of an increase in PI3K and Akt phosphorylation [19], suggesting that it exerts protective effects on normal tissue. The Jordan group [20] went further and looked at the efficacy of phytoestrogens as a natural alternative to hormone replacement therapy using MCF-7 breast adenocarcinoma cells. They looked at both phytoestrogens and steroidal estrogens (17β estradiol and equilin) in fully estrogenized MCF-7 cells and long term deprived MCF-7:5C cells. Both steroidal estrogens and phytoestrogens were found to induce proliferation of MCF-7 cells while they inhibited growth and induced apoptosis in MCF-7:5C cells [20]. This effect was abated when siRNA targeted to estrogen receptor α was used, indicating that this is a direct effect of the estrogen receptor. In addition, phytoestrogens were found to induce endoplasmic reticulum stress (via *DDIT3*, *IRE1α*, and α-eIF2α) and inflammatory response stress (via caspase-4, *CEBP* β, *IL6*, and lymphotoxin-β) in MCF-7:5C cells [20]. Using the corticosteroid dexamethasone in order to inhibit inflammation, the induction of apoptosis and growth inhibition was also blocked [20], suggesting that phytoestrogens may be useful as chemopreventive compounds in patients and postmenopausal women. However, anti-inflammatory agents have antiapoptotic effects, which should be factored into the decision-making process for the treatment plan in the context of cancer therapy.

Genistein was shown to have a 20-fold higher binding affinity to estrogen receptor β than estrogen receptor α [21]. It also is 130-fold more potent than its counterpart 17β-estradiol (estrogen) to bind to estrogen receptor α [21]. Estrogen is commonly prescribed to postmenopausal women and is approved for the treatment of osteoporosis. Estrogen deficiency causes both early and late stages of osteoporosis in postmenopausal women by increasing osteoclast formation and activity [22]. In a randomized trial, hormone treatment with 0.625 mg/day of conjugated equine estrogen resulted in a 16.1% decrease in fasting insulin levels and 2.2 mg/dL lower fasting glucose level [23]. For these reasons, genistein has been explored and found to play a protective role against diabetes. For example, genistein was administered at 2, 4, and 6 mg/kg to female nonobese diabetic (NOD) insulin-dependent susceptibility 3 (Idd3) mice, which are predisposed to type 1 diabetes and maintained on a soy and alfalfa-free diet [24]. The mice treated with 2 mg/kg of genistein had a 55–79% decreased incidence of type 1 diabetes starting at 14 weeks after exposure. This effect, however, was not sustainable after 23 weeks. The two higher dose treatments had a significant decrease in incidence starting at 16 weeks [24], suggesting that the most effective dose of genistein based on this study was 2 mg/kg. The compound has also been studied in the context of preventative care of diabetes. Methylglyoxal is considered a major precursor to the so-called advanced glycation end products, which are believed to be one of the major causes of diabetes and its complications, and genistein was found to directly scavenge and thus neutralize methylglyoxal [25]. Finally, in a randomized clinical trial, at 1 year genistein treatment was found to reduce the fasting glucose and fasting insulin levels and insulin resistance compared to the placebo recipients, whose levels remained unchanged [26]. Genistein was also found to increase HDL and to lower LDL, triglycerides, the adipocyte hormone visfatin, and homocysteine blood levels [26].

3.3. Apigenin. Apigenin (Figure 1) is a flavone and is commonly found in Chinese cabbage (187 mg/kg), bell pepper (272 mg/kg), garlic (217 mg/kg), bilimbi fruit (458 mg/kg), French peas (176 kg/mg), guava (579 mg/kg), wolfberry leaves (547 mg/kg), and celery (339 mg/kg) [27]. It has been suggested in recent studies to be useful in the treatment of skin and colon cancer. According to the Birt group [28], treatment with up to 80 μM of apigenin caused a time- and dose-dependent cell cycle arrest in G2/M phase. These studies were performed on SW480, HT-29, and Caco-2 colon carcinoma cell lines. After 48 hours following treatment with 80 μM of apigenin, 64%, 42%, and 26% of SW480, HT-29, and Caco-2 cells were arrested, respectively, in contrast to only 15% of the control cells. By immune complex kinase assay, p34 (cdc2), which is a critical enzyme in the G2/M transition, was found to be inhibited in all three cell lines. Western analyses confirmed these findings showing a decrease in expression of both p34 and cyclin B1 proteins. This effect was shown to be reversible when apigenin treatment was discontinued [28]. Apigenin was also explored in breast cancer progression. An epidemiological study illustrated that apigenin at low doses (10–50 μM) was able to cause a dramatic reduction in DNA synthesis after 24 hours in all breast cancer cell lines tested (MDA-MB-468, MDA-MB-231, MCF-7, and SK-BR-3) [29]. However, the viability of these cell lines remained unchanged. Flow cytometry with Oregon Green/PI staining showed that apigenin at a concentration of 30 μM had a cytostatic effect by arresting the cells in G2/M phase [29]. Apigenin was further studied for a potential benefit to the immune system. Warat and colleagues found it to inhibit the expression of the TRAIL-R1 death receptor in RAW264.7 macrophages [30]. These studies suggest at least two significant roles that apigenin can play in antitumorigenesis, by inhibiting cell proliferation and by improving immune cell survival.

3.4. (−)-Epicatechin. The Kuna Indians, indigenous people living on islands near the coast of Panama, consume large amounts of cocoa on a daily basis [31]. They have low blood pressure and a significantly lower incidence of cardiovascular disease [32]. There is strong evidence that continuous cocoa consumption and not genetic differences causes the effect, since it is lost when cocoa consumption is discontinued.

Unfermented cocoa beans contain 120–180 g/kg of polyphenols with (−)-epicatechin being the main polyphenolic compound approximating 35% [33]. Given its high abundance it is likely that (−)-epicatechin is a key mediator of the beneficial effects of cocoa. A short-term study with healthy humans who received high-flavonoid dark chocolate containing 46 mg (−)-epicatechin daily for 2 weeks showed superior vascular function as seen by improved endothelium-dependent flow-mediated dilation of the brachial artery [34]. However, there were no measurable beneficial short-term effects on other parameters including blood pressure and lipid parameters, suggesting that continued uptake is required to achieve a higher impact on the cardiovascular system as seen in the Kuna Indians. (−)-Epicatechin has also been identified as an important bioactive compound in *Pterocarpus marsupium*, a tree that is widely distributed in central, western, and southern regions of India and used as an important traditional medication in India for the treatment of diabetes and other pathologies including those of the heart and liver (reviewed in [35]). The well-established benefits of *Pterocarpus marsupium* extract as a therapy for diabetes are likely due to the insulin-mimetic effects of (−)-epicatechin [36, 37].

Structurally, (−)-epicatechin is comprised of two aromatic rings linked by an oxygenated heterocycle with a 4-hydroxyl group (Figure 1). It is a compound with high bioactivity when analyzed in isolation. When taken orally, flavanols including (−)-epicatechin are stable during gastric transit but become glucuronidated and partially methylated in the small intestines, processes that continue in the liver, leaving only smaller levels of native (−)-epicatechin in the mesenteric circulation (reviewed in [38]). A small quantitative clinical study with human subjects consuming 80 grams of procyanidin-rich chocolate containing 137 mg (470 μmol) (−)-epicatechin showed that blood (−)-epicatechin increased 12-fold over baseline levels to 257 ± 66 nmol/L after 2 hours and then declined to baseline levels in 8 out of the ten subjects after 6 hours, while it further increased in the remaining two individuals [39]. This suggests that there is a large heterogeneity regarding the half-life and metabolism of (−)-epicatechin in humans. Bioavailability of native (−)-epicatechin is therefore smaller than for vitamins C and E, with about ~1/200 and ~1/150 bioavailability, respectively [39]. Given that most of the ingested (−)-epicatechin undergoes chemical modifications, the glucuronidated and methylated products likely play a key role for the biological effect in addition to the native compound.

3.4.1. Reactive Oxygen Species and Redox Balance. There is a large body of literature proposing that one of the main beneficial effects of (−)-epicatechin is via its ability to directly or indirectly scavenge ROS by chemically reacting with ROS or by modulating pathways that regulate ROS scavenging compounds and enzymes, respectively. Jung et al. [40] identified hydroxyl groups as the crucial structural feature of flavonoids responsible for ROS scavenging. They analyzed seven flavonoids including kaempferol, kaempferol-7-O-β-D-glucoside, (+)-catechin, dihydrokaempferol, hesperetin-5-O-β-D-glucoside, naringenin, and 7-O-β-D-glucoside and concluded that the inhibitory strength of these compounds on total ROS was increased by the number of hydroxyl groups present. Furthermore, the presence of ortho-hydroxyl groups was essential [40], as seen in the ortho- (o-) catechol moiety in (−)-epicatechin (Figure 1). In support of this concept, a recent study confirmed that the o-catechol moiety of (−)-epicatechin is essential for the direct detoxifying effects in the reaction with superoxide and hydrogen peroxide [41]. The simplest reaction product would be (−)-epicatechin-o-quinone (Figure 1) which can undergo further reactions. Interestingly, this o-quinone product is also generated via enzymatic conversion by peroxidases including myeloperoxidase, and a recent study revealed that this enzymatic reaction causes a strong inhibitory effect of (−)-epicatechin-o-quinone on macrophage migration inhibitory factor (MMIF), a key molecule for promotion and maintenance of the inflammatory response [42]. Using liquid chromatography mass spectrometry the authors found that (−)-epicatechin-o-quinone specifically reacts with the N-terminal proline residue of MMIF leading to inactivation of the protein. The authors proposed that this mechanism could explain the beneficial anti-inflammatory effects of (−)-epicatechin when taken during inflammation. It should be kept in mind that the beneficial effects of (−)-epicatechin in the pathophysiological conditions discussed below may at least in part be explained by the ROS modulating capabilities of the compound.

3.4.2. Inflammation. In addition to acute inflammation as seen in sepsis, many other more chronic diseases including diabetes and cancer have a very important inflammatory component. A central aspect of the pathogenesis of diabetes is mediated via interleukin-1β (IL-1β), which is released by infiltrating inflammatory cells in the pancreas in type I diabetes. IL-1β and other cytokines induce upregulation of the inducible form of nitric oxide synthase (iNOS), leading to the production of nitric oxide and downstream β-cell damage and death in the pancreatic islets and thus type I diabetes [43]. (−)-Epicatechin at concentrations of 0.1–1 mM was found to inhibit nitrite formation, a downstream product of nitric oxide, in a dose-dependent manner in the rat β-cell line RINm5F and in isolated islets induced with 100 pg/mL of IL-1β for 24 hours [43]. The authors showed that (−)-epicatechin inhibited the IL-1β-induced expression of iNOS by blocking the nuclear localization of the p65 subunit of NF-κB. In addition, in RINm5F cells, (−)-epicatechin was shown to block the inhibition of insulin release after addition of IL-1β [43]. It can be speculated that the antidiabetic effects of genistein (see Section 3.2) and (−)-epicatechin may primarily be a result of their nonantioxidant actions and secondarily of their antioxidant actions. Studies using vitamin E as an antioxidant did not improve outcome except for the group of patients with the haptoglobin 2-2 genotype [44]. However, increased vitamin C plasma levels are a predictive factor for a decreased risk of type II diabetes [45], and vitamin C supplementation in patients receiving metformin improved plasma vitamin C levels as well as fasting and postmeal blood glucose levels [46].

(−)-Epicatechin was also found to be effective in a mouse model of atherosclerosis at daily doses of about 110 mg/kg

body weight with an average (−)-epicatechin plasma concentration of 4.2 μM. Such treatment blocked lesion progression in ApoE3-Leiden (E3L) mice being fed an atherogenic Western-type diet (15% cocoa butter, 1% corn oil, 40.5% sucrose, 20% acid casein, 10% corn starch, and 6.2% cellulose, supplemented with 1% cholesterol) for four weeks [47]. Treatment with (−)-epicatechin was found to reduce atherosclerosis by 27% compared to the high calorie diet alone control group. Another study found that (−)-epicatechin treatment improved acute intestinal inflammatory disease. At a concentration of 10 mg/kg in rats induced with trinitrobenzenesulfonic acid, there was a reduction in colitis including less ulceration and disorganization of the tissue after histological analysis [48]. In addition, the authors observed significantly higher levels of glutathione in the colon tissue of animals treated with (−)-epicatechin.

Inflammation is also a central component of allergies [49], which are not typically thought of to be a serious condition. However, there are instances where they can be life threatening, for example during anaphylactic shock. The epidemic rise in allergies over the past few decades has been a major concern, and it is therefore important to explore better therapeutic options. A few recent reports illustrate the attenuating role polyphenols including (−)-epicatechin have in the allergic immune response. Singh et al. [50] studied mice that were sensitized to ovalbumin for 3 days and then challenged weekly with 20 mg of ovalbumin for 7 weeks. The animals were fed with pellets containing 1%, 0.3%, or 0.01% purified (−)-epicatechin for 8 days. After the treatment period the mice were again challenged with 100 mg of ovalbumin after which the authors observed a reduction in many of the clinical symptoms including scratching around the nose or head and diarrhea. They also found a reduction in ovalbumin-specific IgE present in the mice fed with the high dose (26 ± 13 ng/mL) and medium dose (89 ± 35 ng/mL) of (−)-epicatechin compared to control population (144 ± 70 ng/mL) [50]. Another polyphenol family member that is discussed above, EGCG, has also been implicated in ameliorating allergic responses. One study revealed that EGCG directly interacts with ovalbumin leading to a change in the secondary β-sheet structure of the allergen, which occurred at a 1 : 1 molar ratio, attenuating the allergic response [51]. These studies indicate that polyphenols including (−)-epicatechin have the capability to reduce inflammatory responses, for example by direct interference with proteins involved in triggering the response.

3.4.3. Cancer, Mitochondrial Metabolism, and Cell Signaling. Inflammation has been linked to cancer development, progression, invasion, and metastasis [52] and the use of anti-inflammatory agents has been proposed as an attractive adjunct therapy for the clinic in the future [53], and we here propose that application of (−)-epicatechin may be such a viable approach. In addition to its anti-inflammatory effect several studies concluded a potential for (−)-epicatechin as a novel anticancer drug mediated through additional mechanisms (Figure 2). (−)-Epicatechin was shown to cause DNA damage and apoptosis in acute myeloid leukemia cells in rats when administered orally at a dose of 40 mg/kg body weight

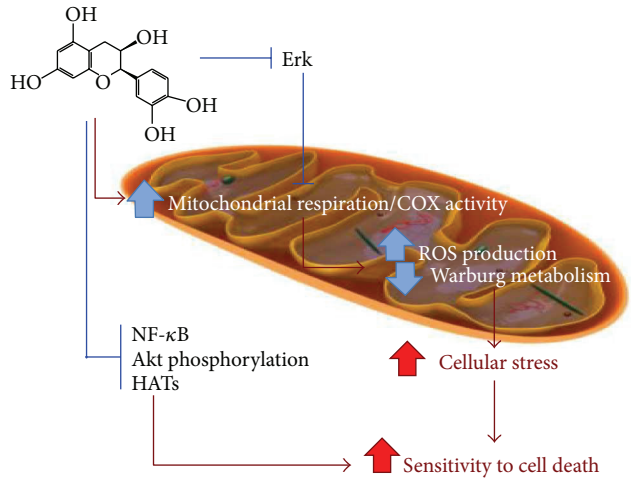

Figure 2: Proposed model of the interference of (−)-epicatechin with cancer signaling, metabolism, and proliferation. (−)-Epicatechin stimulates mitochondrial respiration and biogenesis, thus interfering with Warburg metabolism. At the cell signaling level, the compound inhibits Erk signaling, which interferes with other signaling pathways including EGFR that are known to be hyperactive in cancer. (−)-Epicatechin through Erk and/or other signaling pathways leads to an activation of mitochondrial oxidative phosphorylation, which interferes with Warburg metabolism. Other targets that are inhibited by (−)-epicatechin in cancer cells are NF-κB, Akt, and histone acetyltransferases (HATs). As a result, (−)-epicatechin interferes with cancer signaling, thus rendering the cells more susceptible to apoptosis, an effect that could be utilized to sensitize cancer cells to radiation treatment or chemotherapy. It should be noted that (−)-epicatechin exerts a distinct protective response in noncancerous normal tissue (not shown). This highlights the importance not to generalize the effects but to include detailed information including cell type and treatment regimen.

for 22 consecutive days [54]. Additionally, (−)-epicatechin was shown to inhibit the proliferation of Hodgkin's lymphoma cells and Jurkat T cells, which was attributed to the ability of (−)-epicatechin to inhibit the binding of NF-κB to DNA in these cells [55] (Figure 2). Interestingly, these effects were not associated with (−)-epicatechin's antioxidant activity, nuclear translocation of NF-κB, or p65 phosphorylation. The mechanism by which (−)-epicatechin inhibits NF-κB–DNA binding is still open to investigation. One molecular target of (−)-epicatechin that may in part explain the anticancer activity has been identified. It is the Na^+/H^+ exchanger, which is strongly inhibited by (−)-epicatechin, and it was proposed that cancer cell plasma membrane fluidity and cytosolic pH are disturbed, thus interfering with cell proliferation [56, 57].

(−)-Epicatechin was also shown to inhibit prostate cancer cell proliferation, potentially by suppressing agonist-dependent androgen receptor activation and androgen receptor-regulated gene transcription [58]. Inhibition of histone acetyltransferase activity was identified as a potential mechanism for reduced prostate cancer cell viability. However (−)-epicatechin shows a weaker potential for inhibiting histone acetyltransferase activity when compared with its analogs

EGCG and epigallocatechin. The study suggested that prostate cancer patients with androgen receptor positive tumors might specifically benefit from (−)-epicatechin and its analogs as therapy enhancing drugs. The study further suggested that (−)-epicatechin and related compounds can exploit the genetic variations that are intrinsic to cancer cells since KRAS mutant pancreatic cancer cells appear to be particularly sensitive to (−)-epicatechin's anticancer activity. Another study found that (−)-epicatechin specifically inhibited KRAS mutant pancreatic cancer cells in vitro and in vivo [59]. Here, (−)-epicatechin treatment reduced KRAS mutant pancreatic cancer cell viability but not that of normal cells, and it also reduced GTP-bound Ras protein levels, Akt phosphorylation, and NF-κB transcriptional activity.

The anticancer activity of (−)-epicatechin surpasses the mere inhibition of cancer cell proliferation into prevention of tumor promotion and development. In a mouse model for papilloma formation papilloma was induced with the carcinogen 7,12-dimethylbenz[a]anthracene and promoted using croton oil [60]. The authors showed that topical application of 100 mg (−)-epicatechin/kg body weight inhibited papilloma formation in papilloma-induced mice [60]. Furthermore, oral administration of the same dose of (−)-epicatechin to mice significantly reduced soft tissue fibrosarcoma that was induced by 20-methylcholanthrene. Interestingly, there is some indication regarding the additive or synergistic effect of certain polyphenols. A recent report suggests that panaxadiol, an active compound derived from steamed ginseng, has a synergistic effect when administered together with (−)-epicatechin [61]. Using human colorectal cancer HCT-116 cells they found that combining 20 μM panaxadiol with 150, 200, or 250 μM (−)-epicatechin resulted in growth inhibition of 51%, 97%, and 95%, respectively. The combination also increased the apoptosis level by 11.9%, 16.6%, and 25.8%, as examined by annexin V/PI staining. Thus, there is strong support for the anticancer activity of (−)-epicatechin and its analogs. However, several questions pertaining to the mechanism by which these compounds mediate their anticancer effect should be addressed in future studies including how exactly (−)-epicatechin inhibits the binding of NF-κB to DNA and Akt phosphorylation.

Patients on cancer therapy may benefit from coadministration of (−)-epicatechin together with their conventional therapy for another reason. Several studies showed that (−)-epicatechin protects normal and nontumorigenic tissues from insults by radiation or chemotherapy [62–69]. For example, pretreatment of adult rats with (−)-epicatechin reduced doxorubicin's neurotoxicity by reducing TNFα, iNOS, and NF-κB expression, as well as reducing the total nitrite levels in the brains [66]. (−)-Epicatechin's protective effect extends to other chemotherapeutic drugs such as cisplatin where it was found to inhibit mitochondrial and renal damage in vitro using cultured mouse proximal tubular cells and in vivo in mice pretreated with (−)-epicatechin prior to cisplatin administration [67]. A similar (−)-epicatechin-mediated protective effect was observed in cell lines in vitro and in a zebrafish model in vivo after cisplatin treatment, via blocking of ROS generation [68]. In another recent study, (−)-epicatechin significantly increased clonogenic survival and restored the migration ability of irradiated normal human dermal fibroblasts, in which it also inhibited radiation-induced ROS generation, mitochondrial dysfunction, and cell death [63]. Mechanistically, the authors showed that (−)-epicatechin significantly inhibited p-JNK, p38, and cleaved caspase-3 levels when combined with radiation treatment. The same group earlier reported inhibition of radiation-induced apoptosis in a human keratinocyte line (HaCaT), which is another model for normal human cells [64]. Moreover, rats given (−)-epicatechin orally showed reduction in radiation-induced oral mucositis as indicated by increased oral food intake, weight gain, and increased overall survival rates. This is an important finding suggesting that (−)-epicatechin can alleviate a common side effect of cancer therapy that is painful for the patient and significantly reduces quality of life.

Several studies have suggested the potential antiproliferative role many catechins have by acting upon DNA methyltransferases (DNMTs). These are a family of enzymes that catalyze the covalent transfer of a methyl group to the 5-position of the pyrimidine ring of cytosines predominately located within CpG islands. DNA methylation has many significant effects in the mammalian genome including transcriptional regulation, chromatin structure modulation, X chromosome inactivation, genomic imprinting, and the suppression of repetitive and parasitic DNA sequences for genome stability [70]. Flavonoids such as (−)-epicatechin are metabolized in vivo and the products include methylated, sulphated, and glucuronidated derivatives. In addition to the original flavonoids, these downstream products may also exert biological activities. A recent study showed that 4-O-methyl-epicatechin and 3-O-methyl-epicatechin had antiproliferative functions in MCF-7 breast cancer cells and BxPC-3 pancreatic cancer cells, whereas other derivatives showed limited or no activity [71], suggesting that certain metabolites could be developed as cancer therapeutics. Another recent study showed that (−)-epicatechin has the ability to directly inhibit DNA methyltransferase activity [72]. The authors used a novel electrochemical immunosensing model in order to detect the activity of the prokaryotic DNA methyltransferase M. SssI MTase. The DNA helix structure was formed on the surface of a gold nanoparticle glassy carbon electrode where it was then methylated by M. SssI MTase. Following DNA digestion with the restriction enzyme Hpall, which does not recognize methylated CpG regions, and fragment analysis the authors concluded that epicatechin was inhibitory with an IC_{50} value of 129 μM [72], whereas an earlier study reported a value of 8.4 μM [73].

In cancer cells mitochondrial metabolism and respiration are often suppressed with most solid tumors showing a 25–60% decrease in mitochondrial mass compared to healthy cells [74]. Cancer cells shift from mitochondrial respiration to glycolysis, which is known as the Warburg effect [75, 76]. Mechanistically, there is strong evidence that cancer signaling affects the oxidative phosphorylation machinery, and recent studies showed that all components of the oxidative phosphorylation system can be phosphorylated (reviewed in [77]). Among these are cytochrome *c* (Cyt c) and cytochrome *c* oxidase (COX), which catalyze the terminal and proposed

rate-limiting step in the mitochondrial electron transport chain [78, 79]. Multiple phosphorylation sites have been mapped on both enzymes by us and others [80–86]. Based on those phosphorylations that have been studied functionally, we proposed that oxidative phosphorylation is decisively regulated by cell signaling (for recent reviews see [87, 88]). As an example in the context of cancer metabolism, the activated EGF receptor was shown to translocate to the mitochondria where it directly interacts with COX catalytic subunit II leading to COX inhibition [89, 90]. A similar translocation to the mitochondria was also reported for receptor tyrosine kinase ErbB2 in cancer cell lines and cancer specimens [91]. Since the oxidative phosphorylation machinery is suppressed in most cancers it can be speculated that reactivation of mitochondrial function might be a strategy to interfere with cancer proliferation. We recently showed that (−)-epicatechin stimulates mitochondrial respiration in vitro in pancreatic cancer cells [69] and in vivo in normal mouse muscle tissues, in which it also significantly stimulated the expression of oxidative phosphorylation protein complexes [92]. Interestingly, (−)-epicatechin treatment sensitized Panc-1, U87, and MIA PaCa-2 pancreatic cancer cells to radiation treatment, significantly reducing clonogenic survival, but it had a small protective effect in normal control cells [69], ideal characteristics for a compound that could be used as an adjunct therapy with radiation or chemo treatment. Another example of the protective effect of (−)-epicatechin on healthy tissue was reported in the context of cisplatin treatment, which can cause nephropathy as a side effect. In mice, (−)-epicatechin, administered 8 hours after renal injury, which was induced by cisplatin treatment, inhibited the decrease in mitochondrial succinate dehydrogenase activity, cytochrome *c* release, mitochondrial fragmentation, and cytochrome *c* oxidase protein levels in the proximal tubular cells [67]. (−)-Epicatechin also exhibited an otoprotective effect in injury induced by cisplatin in a cochlear organ of the Corti-derived cell line HEI-OC1 in vitro and in rats in vivo by inhibiting the activation of ERK, caspase-3, JNK, and the release of cytochrome *c* [62].

The effect of (−)-epicatechin on Erk signaling has been reported by several groups. (−)-Epicatechin was shown to inhibit Erk2 phosphorylation in micromolar concentrations [62, 93]. Erk2 is part of the Ras/MAPK pathway, which is central for several cellular processes including proliferation and survival. It also crosstalks with the EGF pathway [94] and could therefore directly (via phosphorylation of oxidative phosphorylation complexes) or indirectly (via interfering with EGF signaling) regulate mitochondrial activity. Thus in cancer, MAPK pathway hyperactivation by Erk2 phosphorylation could target COX and Cyt c for phosphorylation causing Warburg metabolism. Consequently, inhibiting MAPK pathway activation—with (−)-epicatechin as we propose here—can potentially restore COX and Cyt c activity and mitochondrial respiration. However, the exact mechanism by which the MAPK pathway controls mitochondrial respiration remains to be investigated including the assignment of MAPK pathway-dependent phosphorylation sites on oxidative phosphorylation proteins and their functional effects.

There is clear evidence that (−)-epicatechin affects multiple other signaling pathways and that there are tissue-specific differences in how some of them respond to the compound. (−)-Epicatechin was found to modulate NF-κB, activator protein-1 (AP-1), and nuclear factor erythroid 2p45-related factor-2 (Nrf2) signaling, all being important in cellular detoxification, proliferation, survival, and differentiation [95]. (−)-Epicatechin also reduced p-JNK and p-38 expression in human cultured fibroblasts [63, 64], and it induced phosphorylation of Akt, HSP90, and eNOS in human coronary artery endothelial cells (HCAEC) [96]. In neurons, (−)-epicatechin at 100–300 nanomolar concentration stimulated cAMP-response element binding protein (CREB) phosphorylation [43]. Interestingly, this effect could be plotted on a bell-shaped curve, and at very low concentrations stimulation was not observed and at 30 micromolar concentrations (−)-epicatechin had an inhibitory effect. Such a behavior was also observed for ERK and Akt phosphorylation [43]. Furthermore, (−)-epicatechin promotes vascularization in healthy mouse skeletal muscle via regulation of the expression of angiogenic and antiangiogenic factors, such as VEGF and thrombospondin-1 (TSP-1), respectively [92, 97, 98]. In the same tissue it also stimulates mitochondrial biogenesis through expression of peroxisome-proliferator-activated receptor γ coactivator-1α (PGC-1α), PGC-1β, and mitochondrial transcription factor A (TFAM) [98, 99]. Increased mitochondrial biogenesis and function would also counteract the Warburg effect.

Some studies suggested that some of the effects of (−)-epicatechin are mediated through receptors. A recent study explored the use of an (−)-epicatechin-dextran conjugate, which cannot permeate through the cell wall [100]. Their findings show that treatment with both 100 nM (−)-epicatechin and 100 nM (−)-epicatechin-dextran for 10 minutes induced and activated PI3K, PDK-1, Akt, and eNOS in human coronary artery endothelial cells (HCAEC). However, the interesting finding was that (−)-epicatechin-dextran activation was significantly higher than nonconjugated (−)-epicatechin, suggesting the existence of an (−)-epicatechin cell membrane receptor. Panneerselvam et al. concluded that (−)-epicatechin is interacting with cell membrane δ-opioid receptors, promoting cardiac protection [101, 102]. In one study, the authors used mice that were treated with either control saline, 1 mg/kg (−)-epicatechin, or 5 mg/kg naltrindole, a δ-opioid receptor antagonist, by daily intraperitoneal injection for 10 days [102]. They observed that mitochondria isolated from the hearts of mice treated only with (−)-epicatechin had higher state 3 respiration rates. In contrast, heart mitochondria isolated from mice treated with naltrindole and (−)-epicatechin showed an attenuated state 3 respiration, suggesting that δ-opioid receptor activation through (−)-epicatechin augments mitochondrial function [102].

3.4.4. Ischemia/Reperfusion Injury. A pathological condition in which ROS play an essential role is ischemia/reperfusion injury as seen in ischemic stroke and myocardial infarction. These are life-threatening conditions, which are among the top causes of death and long-term disability. They are caused

by a restriction of blood flow to either the brain or heart. This process thwarts the transfer of oxygen and nutrients to these sites causing ischemia, which blocks energy production. In order to salvage the affected tissue, blood flow has to be restored, which is referred to as reperfusion. Reperfusion can increase mitochondrial and cellular damage due to excessive mitochondrial ROS production (reviewed in [103]). A key mechanism to ischemia/reperfusion injury that also involves ROS is mitochondrial permeability transition (MPT) priming. When executed, MPT causes mitochondrial membrane leakage, fatty acid accumulation, Cyt c release, antioxidant loss, and changes in intra- and extramitochondrial Ca^{2+} and pH [104]. Given its antioxidant capacity, it is not surprising that (−)-epicatechin has been studied as a cardioprotective therapy. Dose concentrations of 10 mg/kg (injected 15 minutes prior to reperfusion and/or 12 hours later) were found to be cardioprotective in rats, reducing infarct size by 27% after 48 hours and 28% after 3 weeks of reperfusion with a single administration of (−)-epicatechin [105]. Even more substantial effects were seen with the dual application of (−)-epicatechin, with a reduction in infarct size by about 80% at 48 hours and 52% at 3 weeks and improvement of other mitochondrial properties, including oxygen consumption rate and mitochondrial morphology. Protection was also observed when rats were pretreated with 20 mg/kg (−)-epicatechin daily for 21 days following induction of myocardial infarction via isoproterenol injection [106]. (−)-Epicatechin treated rats had reduced serum troponin-I (cTn-I), creatine kinase, and lactate dehydrogenase levels compared to the isoproterenol controls. In isolated heart mitochondria the activities of superoxide dismutase, glutathione peroxidase, and glutathione reductase were all significantly decreased after treatment with isoproterenol [106]. In the (−)-epicatechin pretreated group, these activities were significantly higher, as were activities of mitochondrial marker enzymes, such as succinate dehydrogenase, α-ketoglutarate dehydrogenase, and NADH dehydrogenase [106]. Another recent study looked at (−)-epicatechin-specific effects in brain ischemia/reperfusion injury following permanent distal middle cerebral artery occlusion in conjunction with (−)-epicatechin treatment 90 minutes prior to reperfusion [107]. They found a 55%, 40%, and 50% decrease in infarct size in mice treated with 5 mg/kg, 10 mg/kg, and 15 mg/kg (−)-epicatechin, respectively. They next used cultured neurons from wild-type and $Nrf2^{-/-}$ mice. Pretreatment with 50 or 100 μM (−)-epicatechin starting 6 hours prior to oxygen/glucose deprivation protected WT neuronal cultures from oxygen/glucose deprivation, but this was not observed in the neurons isolated from $Nrf2^{-/-}$ mice. This suggests that the protective effects of (−)-epicatechin are mediated through the Nrf2 antioxidant stress response pathway, which was confirmed by the observation that there was a dose-dependent increase in expression of HO-1, FTL, and BVR, which are Nrf2/ARE-regulated proteins [107].

4. Conclusion

There is a large body of literature demonstrating that several polyphenols have beneficial health effects and, based on animal models, can be used to treat acute and chronic conditions such as ischemia/reperfusion injury, neurodegeneration, diabetes, and cancer. Several signaling pathways have been implicated including Erk and Nrf2, which regulate proliferation and redox balance, respectively. It is important to note that effects mediated by compounds such as (−)-epicatechin can be distinct or even entirely opposing in different cell types such as cancer cells and normal tissues. Therefore, future work should further dissect the precise mechanisms through which the compounds act including extra- and intracellular sites in healthy and pathological conditions. Such mechanistic knowledge would raise the acceptance and help implement the utilization of these compounds in clinical practice.

Abbreviations

ALA: α-Lipoic acid
CoQ: Coenzyme Q
COX: Cytochrome c oxidase
Cyt c: Cytochrome c
EGC: Epigallocatechin
EGCG: Epigallocatechin-3-gallate
IL-1β: Interleukin-1β
Nrf22: Nuclear factor erythroid 2p45-related factor-2
ROS: Reactive oxygen species
SOD: Superoxide dismutase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] K. B. Pandey and S. I. Rizvi, "Plant polyphenols as dietary antioxidants in human health and disease," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 5, pp. 270–278, 2009.

[2] A. Scalbert, I. T. Johnson, and M. Saltmarsh, "Polyphenols: antioxidants and beyond," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, pp. 215S–217S, 2005.

[3] D. R. Galasko, E. Peskind, C. M. Clark et al., "Antioxidants for Alzheimer disease: a randomized clinical trial with cerebrospinal fluid biomarker measures," *Archives of Neurology*, vol. 69, no. 7, pp. 836–841, 2012.

[4] D. Mekinová, V. Chorváthová, K. Volkovová et al., "Effect of intake of exogenous vitamins C, E and beta-carotene on the antioxidative status in kidneys of rats with streptozotocin-induced diabetes," *Die Nahrung*, vol. 39, no. 4, pp. 257–261, 1995.

[5] A. P. Dare and R. P. Hellens, "RNA interference silencing of CHS greatly alters the growth pattern of apple (Malus x domestica)," *Plant Signaling and Behavior*, vol. 8, no. 8, 2013.

[6] B. N. Singh, S. Shankar, and R. K. Srivastava, "Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications," *Biochemical Pharmacology*, vol. 82, no. 12, pp. 1807–1821, 2011.

[7] C. H. Lin, L. K. Chao, P. H. Hung, and Y. J. Chen, "EGCG inhibits the growth and tumorigenicity of nasopharyngeal tumor-initiating cells through attenuation of STAT3 activation," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 5, pp. 2372–2381, 2014.

[8] S. Mukherjee, M. A. Siddiqui, S. Dayal, Y. Z. Ayoub, and K. Malathi, "Epigallocatechin-3-gallate suppresses proinflammatory cytokines and chemokines induced by Toll-like receptor 9 agonists in prostate cancer cells," *Journal of Inflammation Research*, vol. 7, pp. 89–101, 2014.

[9] T. Poutahidis, K. Cappelle, T. Levkovich et al., "Pathogenic intestinal bacteria enhance prostate cancer development via systemic activation of immune cells in mice," *PLoS ONE*, vol. 8, no. 8, Article ID e73933, 2013.

[10] H. Q. Luo, M. Xu, W. T. Zhong et al., "EGCG decreases the expression of HIF-1α and VEGF and cell growth in MCF-7 breast cancer cells," *Journal of B.U.ON.*, vol. 19, no. 2, pp. 435–439, 2014.

[11] W. Zhu, J. Xu, Y. Ge et al., "Epigallocatechin-3-gallate (EGCG) protects skin cells from ionizing radiation via heme oxygenase-1 (HO-1) overexpression," *Journal of Radiation Research*, vol. 55, no. 6, pp. 1056–1065, 2014.

[12] S. A. Mandel, T. Amit, L. Kalfon, L. Reznichenko, O. Weinreb, and M. B. H. Youdim, "Cell signaling pathways and iron chelation in the neurorestorative activity of green tea polyphenols: special reference to epigallocatechin gallate (EGCG)," *Journal of Alzheimer's Disease*, vol. 15, no. 2, pp. 211–222, 2008.

[13] J. Bieschke, J. Russ, R. P. Friedrich et al., "EGCG remodels mature α-synuclein and amyloid-β fibrils and reduces cellular toxicity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 17, pp. 7710–7715, 2010.

[14] F. Meng, A. Abedini, A. Plesner, C. B. Verchere, and D. P. Raleigh, "The Flavanol (-)-epigallocatechin 3-gallate inhibits amyloid formation by islet amyloid polypeptide, disaggregates amyloid fibrils, and protects cultured cells against IAPP-induced toxicity," *Biochemistry*, vol. 49, no. 37, pp. 8127–8133, 2010.

[15] Y. Porat, A. Abramowitz, and E. Gazit, "Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism," *Chemical Biology and Drug Design*, vol. 67, no. 1, pp. 27–37, 2006.

[16] N. Ferreira, M. J. Saraiva, and M. R. Almeida, "Epigallocatechin-3-gallate as a potential therapeutic drug for TTR-related amyloidosis: 'in vivo' evidence from FAP mice models," *PLoS ONE*, vol. 7, no. 1, Article ID e29933, 2012.

[17] T. Zhang, J. Zhang, P. Derreumaux, and Y. Mu, "Molecular mechanism of the inhibition of EGCG on the Alzheimer Abeta(1-42) dimer," *Journal of Physical Chemistry B*, vol. 117, no. 15, pp. 3993–4002, 2013.

[18] R. A. Dixon and D. Ferreira, "Genistein," *Phytochemistry*, vol. 60, no. 3, pp. 205–211, 2002.

[19] F.-P. Chen and M.-H. Chien, "Phytoestrogens induce differential effects on both normal and malignant human breast cells *in vitro*," *Climacteric*, vol. 17, no. 6, pp. 682–691, 2014.

[20] I. E. Obiorah, P. Fan, and V. C. O. Jordan, "Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen-deprived state," *Cancer Prevention Research*, vol. 7, no. 9, pp. 939–949, 2014.

[21] M. Gętek,, N. Czech, M. Muc-Wierzgoń, E. Grochowska-Niedworok, T. Kokot, and E. Nowakowska-Zajdel, "The active role of leguminous plant components in type 2 diabetes," *Evidence-based Complementary and Alternative Medicine*, vol. 2014, Article ID 293961, 12 pages, 2014.

[22] S. C. Manolagas, "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis," *Endocrine Reviews*, vol. 21, no. 2, pp. 115–137, 2000.

[23] M. A. Espeland, P. E. Hogan, S. E. Flneberg et al., "Effect of postmenopausal hormone therapy on glucose and insulin concentrations. PEPI Investigators. Postmenopausal Estrogen/Progestin Interventions," *Diabetes Care*, vol. 21, no. 10, pp. 1589–1595, 1998.

[24] T. L. Guo, D. R. Germolec, J. F. Zheng et al., "Genistein protects female nonobese diabetic mice from developing type 1 diabetes when fed a soy- and alfalfa-free diet," *Toxicologic Pathology*, 2014.

[25] X. Shao, H. Chen, Y. Zhu, R. Sedighi, C.-T. Ho, and S. Sang, "Essential structural requirements and additive effects for flavonoids to scavenge methylglyoxal," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 14, pp. 3202–3210, 2014.

[26] F. Squadrito, H. Marini, A. Bitto et al., "Genistein in the metabolic syndrome: results of a randomized clinical trial," *The Journal of Clinical Endocrinology & Metabolism*, vol. 98, no. 8, pp. 3366–3374, 2013.

[27] K. H. Miean and S. Mohamed, "Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 6, pp. 3106–3112, 2001.

[28] W. Wang, L. Heideman, C. S. Chung, J. C. Pelling, K. J. Koehler, and D. F. Birt, "Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines," *Molecular Carcinogenesis*, vol. 28, no. 2, pp. 102–110, 2000.

[29] M. E. Harrison, M. R. Power-Coombs, L. M. Delaney, and D. W. Hoskin, "Exposure of breast cancer cells to a subcytotoxic dose of apigenin causes growth inhibition, oxidative stress, and hypophosphorylation of Akt," *Experimental and Molecular Pathology*, vol. 97, no. 2, pp. 211–217, 2014.

[30] M. Warat, E. Szliszka, I. Korzonek-Szlacheta, W. Król, and Z. P. Czuba, "Chrysin, apigenin and acacetin inhibit tumor necrosis factor-related apoptosis-inducing ligand receptor-1 (TRAIL-R1) on activated RAW264.7 macrophages," *International Journal of Molecular Sciences*, vol. 15, no. 7, pp. 11510–11522, 2014.

[31] M. L. McCullough, K. Chevaux, L. Jackson et al., "Hypertension, the Kuna, and the epidemiology of flavanols," *Journal of Cardiovascular Pharmacology*, vol. 47, no. 2, pp. S103–S109, 2006.

[32] R. Corti, A. J. Flammer, N. K. Hollenberg, and T. F. Luscher, "Cocoa and cardiovascular health," *Circulation*, vol. 119, no. 10, pp. 1433–1441, 2009.

[33] A. Belščak, D. Komes, D. Horžić, K. K. Ganić, and D. Karlović, "Comparative study of commercially available cocoa products in terms of their bioactive composition," *Food Research International*, vol. 42, no. 5-6, pp. 707–716, 2009.

[34] M. B. Engler, M. M. Engler, C. Y. Chen et al., "Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults," *Journal of*

the American College of Nutrition, vol. 23, no. 3, pp. 197–204, 2004.

[35] M. Devgun, A. Nanda, and S. H. Ansari, "Pterocarpus marsupium Roxb.—a comprehensive review," *Pharmacognosy Reviews*, vol. 3, no. 6, pp. 359–363, 2009.

[36] E. W. Sheehan, M. A. Zemaitis, D. J. Slatkin, and P. L. Schiff Jr., "A constituent of *Pterocarpus marsupium*, (-)-epicatechin, as a potential antidiabetic agent," *Journal of Natural Products*, vol. 46, no. 2, pp. 232–234, 1983.

[37] F. Ahmad, P. Khalid, M. M. Khan, A. K. Rastogi, and J. R. Kidwai, "Insulin like activity in (-) epicatechin," *Acta Diabetologica Latina*, vol. 26, no. 4, pp. 291–300, 1989.

[38] C. G. Fraga and P. I. Oteiza, "Dietary flavonoids: role of (-)-epicatechin and related procyanidins in cell signaling," *Free Radical Biology and Medicine*, vol. 51, no. 4, pp. 813–823, 2011.

[39] D. Rein, S. Lotito, R. R. Holt, C. L. Keen, H. H. Schmitz, and C. G. Fraga, "Epicatechin in human plasma: *in vivo* determination and effect of chocolate consumption on plasma oxidation status," *The Journal of Nutrition*, vol. 130, no. 8, pp. 2109S–2114S, 2000.

[40] A. J. Jung, J. J. Mee, Y. K. Ji, Y. C. Hae, and S. C. Jae, "Inhibitory activity of flavonoids from *Prunus davidiana* and other flavonoids on total ROS and hydroxyl radical generation," *Archives of Pharmacal Research*, vol. 26, no. 10, pp. 809–815, 2003.

[41] E. J. B. Ruijters, A. R. Weseler, C. Kicken, G. R. M. M. Haenen, and A. Bast, "The flavanol (-)-epicatechin and its metabolites protect against oxidative stress in primary endothelial cells via a direct antioxidant effect," *European Journal of Pharmacology*, vol. 715, no. 1-3, pp. 147–153, 2013.

[42] N. Dickerhof, N. J. Magon, J. D. Tyndall, A. J. Kettle, and M. B. Hampton, "Potent inhibition of macrophage migration inhibitory factor (MIF) by myeloperoxidase-dependent oxidation of epicatechins," *Biochemical Journal*, vol. 462, no. 2, pp. 303–314, 2014.

[43] M.-J. Kim, G. R. Ryu, J.-H. Kang et al., "Inhibitory effects of epicatechin on interleukin-1β-induced inducible nitric oxide synthase expression in RINm5F cells and rat pancreatic islets by down-regulation of NF-κB activation," *Biochemical Pharmacology*, vol. 68, no. 9, pp. 1775–1785, 2004.

[44] H. Goldenstein, N. S. Levy, Y. T. Lipener, and A. P. Levy, "Patient selection and vitamin E treatment in diabetes mellitus," *Expert Review of Cardiovascular Therapy*, vol. 11, no. 3, pp. 319–326, 2013.

[45] A.-H. Harding, N. J. Wareham, S. A. Bingham et al., "Plasma vitamin C level, fruit and vegetable consumption, and the risk of new-onset type 2 diabetes mellitus The European prospective investigation of cancer—Norfolk prospective study," *Archives of Internal Medicine*, vol. 168, no. 14, pp. 1493–1499, 2008.

[46] G. N. Dakhale, H. V. Chaudhari, and M. Shrivastava, "Supplementation of vitamin C reduces blood glucose and improves glycosylated hemoglobin in type 2 diabetes mellitus: a randomized, double-blind study," *Advances in Pharmacological Sciences*, vol. 2011, Article ID 195271, 5 pages, 2011.

[47] M. Morrison, R. van der Heijden, P. Heeringa et al., "Epicatechin attenuates atherosclerosis and exerts anti-inflammatory effects on diet-induced human-CRP and NFκB in vivo," *Atherosclerosis*, vol. 233, no. 1, pp. 149–156, 2014.

[48] P. C. D. P. Vasconcelos, L. N. Seito, L. C. di Stasi, C. Akiko Hiruma-Lima, and C. H. Pellizzon, "Epicatechin used in the treatment of intestinal inflammatory disease: an analysis by experimental models," *Evidence-based Complementary and Alternative Medicine*, vol. 2012, Article ID 508902, 12 pages, 2012.

[49] S. Shukla, H. Shukla, S. Kumar, R. P. Aharwal, V. K. Gupta, and S. S. Sandhu, "Allergy and inflammation: an immunological and therapeutic approach," *Recent Patents on Inflammation and Allergy Drug Discovery*, vol. 7, no. 2, pp. 135–150, 2013.

[50] A. Singh, A. Demont, L. Actis-Goretta et al., "Identification of epicatechin as one of the key bioactive constituents of polyphenol-enriched extracts that demonstrate an anti-allergic effect in a murine model of food allergy," *British Journal of Nutrition*, vol. 112, no. 3, pp. 358–368, 2014.

[51] J. Ognjenović, M. Stojadinović, M. Milčić et al., "Interactions of epigallo-catechin 3-gallate and ovalbumin, the major allergen of egg white," *Food Chemistry*, vol. 164, pp. 36–43, 2014.

[52] N. B. Janakiram and C. V. Rao, "The role of inflammation in colon cancer," in *Inflammation and Cancer*, vol. 816 of *Advances in Experimental Medicine and Biology*, pp. 25–52, Springer, Basel, Switzerland, 2014.

[53] C. S. D. Roxburgh and D. C. McMillan, "Cancer and systemic inflammation: treat the tumour and treat the host," *British Journal of Cancer*, vol. 110, no. 6, pp. 1409–1412, 2014.

[54] M. A. Papiez, J. Baran, K. Bukowska-Straková, and W. Wiczkowski, "Antileukemic action of (-)-epicatechin in the spleen of rats with acute myeloid leukemia," *Food and Chemical Toxicology*, vol. 48, no. 12, pp. 3391–3397, 2010.

[55] G. G. Mackenzie and P. I. Oteiza, "Modulation of transcription factor NF-κB in Hodgkin's lymphoma cell lines: effect of (-)-epicatechin," *Free Radical Research*, vol. 40, no. 10, pp. 1086–1094, 2006.

[56] E. Matteucci, S. I. Rizvi, and O. Giampietro, "Erythrocyte sodium/hydrogen exchange inhibition by (-) epicatechin," *Cell Biology International*, vol. 25, no. 8, pp. 771–776, 2001.

[57] S. I. Rizvi and M. A. Zaid, "Impairment of sodium pump and Na/H exchanger in erythrocytes from non-insulin dependent diabetes mellitus patients: effect of tea catechins," *Clinica Chimica Acta*, vol. 354, no. 1-2, pp. 59–67, 2005.

[58] Y.-H. Lee, J. Kwak, H.-K. Choi et al., "EGCG suppresses prostate cancer cell growth modulating acetylation of androgen receptor by anti-histone acetyltransferase activity," *International Journal of Molecular Medicine*, vol. 30, no. 1, pp. 69–74, 2012.

[59] H. R. Siddique, D. J. Liao, S. K. Mishra et al., "Epicatechin-rich cocoa polyphenol inhibits Kras-activated pancreatic ductal carcinoma cell growth in vitro and in a mouse model," *International Journal of Cancer*, vol. 131, no. 7, pp. 1720–1731, 2012.

[60] C. D. Varghese, S. C. Nair, B. Panikkar, and K. R. Panikkar, "Effect of asoka on the intracellular glutathione levels and skin tumour promotion in mice," *Cancer Letters*, vol. 69, no. 1, pp. 45–50, 1993.

[61] M. Rodriguez, G.-J. Du, C.-Z. Wang, and C.-S. Yuan, "Letter to the editor: panaxadiols anticancer activity is enhanced by epicatechin," *The American Journal of Chinese Medicine*, vol. 38, no. 6, pp. 1233–1235, 2010.

[62] J. S. Lee, S. U. Kang, H. S. Hwang, J. H. Pyun, Y. H. Choung, and C. H. Kim, "Epicatechin protects the auditory organ by attenuating cisplatin-induced ototoxicity through inhibition of ERK," *Toxicology Letters*, vol. 199, no. 3, pp. 308–316, 2010.

[63] H. A. Shin, Y. S. Shin, S. U. Kang et al., "Radioprotective effect of epicatechin in cultured human fibroblasts and zebrafish," *Journal of Radiation Research*, vol. 55, no. 1, pp. 32–40, 2014.

[64] Y. S. Shin, H. A. Shin, S. U. Kang et al., "Effect of epicatechin against radiation-induced oral mucositis: in vitro and in vivo study," *PLoS ONE*, vol. 8, no. 7, Article ID e69151, 2013.

[65] S. Basu-Modak, M. J. Gordon, L. H. Dobson, J. P. E. Spencer, C. Rice-Evans, and R. M. Tyrrell, "Epicatechin and its methylated metabolite attenuate UVA-induced oxidative damage to human skin fibroblasts," *Free Radical Biology and Medicine*, vol. 35, no. 8, pp. 910–921, 2003.

[66] R. H. Mohamed, R. A. Karam, and M. G. Amer, "Epicatechin attenuates doxorubicin-induced brain toxicity: critical role of TNF-α, iNOS and NF-κB," *Brain Research Bulletin*, vol. 86, no. 1-2, pp. 22–28, 2011.

[67] K. Tanabe, Y. Tamura, M. A. Lanaspa et al., "Epicatechin limits renal injury by mitochondrial protection in cisplatin nephropathy," *American Journal of Physiology: Renal Physiology*, vol. 303, no. 9, pp. F1264–F1274, 2012.

[68] C.-H. Kim, S. U. Kang, J. Pyun, M. H. Lee, H. S. Hwang, and H. Lee, "Epicatechin protects auditory cells against cisplatin-induced death," *Apoptosis*, vol. 13, no. 9, pp. 1184–1194, 2008.

[69] H. A. Elbaz, I. Lee, D. A. Antwih, J. Liu, M. Hüttemann, and S. P. Zielske, "Epicatechin stimulates mitochondrial activity and selectively sensitizes cancer cells to radiation," *PLoS ONE*, vol. 9, no. 2, Article ID e88322, 2014.

[70] K. D. Robertson, "DNA methylation, methyltransferases, and cancer," *Oncogene*, vol. 20, no. 24, pp. 3139–3155, 2001.

[71] L. Delgado, I. Fernandes, S. González-Manzano, V. De Freitas, N. Mateus, and C. Santos-Buelga, "Anti-proliferative effects of quercetin and catechin metabolites," *Food and Function*, vol. 5, no. 4, pp. 797–803, 2014.

[72] M. Wang, Z. Xu, L. Chen, H. Yin, and S. Ai, "Electrochemical immunosensing platform for DNA methyltransferase activity analysis and inhibitor screening," *Analytical Chemistry*, vol. 84, no. 21, pp. 9072–9078, 2012.

[73] W. J. Lee, J.-Y. Shim, and B. T. Zhu, "Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids," *Molecular Pharmacology*, vol. 68, no. 4, pp. 1018–1030, 2005.

[74] P. L. Pedersen, "Tumor mitochondria and the bioenergetics of cancer cells.," *Progress in Experimental Tumor Research*, vol. 22, pp. 190–274, 1978.

[75] O. Warburg, "Über den Stoffwechsel der Carcinomzelle," *Naturwissenschaften*, vol. 12, no. 50, pp. 1131–1137, 1924.

[76] O. Warburg, "On the origin of cancer cells," *Science*, vol. 123, no. 3191, pp. 309–314, 1956.

[77] R. Covian and R. S. Balaban, "Cardiac mitochondrial matrix and respiratory complex protein phosphorylation," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 303, no. 8, pp. H940–H966, 2012.

[78] G. Villani, M. Greco, S. Papa, and G. Attardi, "Low reserve of cytochrome c oxidase capacity in vivo in the respiratory chain of a variety of human cell types," *Journal of Biological Chemistry*, vol. 273, no. 48, pp. 31829–31836, 1999.

[79] W. S. Kunz, A. Kudin, S. Vielhaber, C. E. Elger, G. Attardii, and G. Villani, "Flux control of cytochrome c oxidase in human skeletal muscle," *Journal of Biological Chemistry*, vol. 275, no. 36, pp. 27741–27745, 2000.

[80] M. Hüttemann, I. Lee, L. Samavati, H. Yu, and J. W. Doan, "Regulation of mitochondrial oxidative phosphorylation through cell signaling," *Biochimica et Biophysica Acta: Molecular Cell Research*, vol. 1773, no. 12, pp. 1701–1720, 2007.

[81] M. Hüttemann, I. Lee, A. Pecinova, P. Pecina, K. Przyklenk, and J. W. Doan, "Regulation of oxidative phosphorylation, the mitochondrial membrane potential, and their role in human disease," *Journal of Bioenergetics and Biomembranes*, vol. 40, no. 5, pp. 445–456, 2008.

[82] S. K. Prabu, H. K. Anandatheerthavarada, H. Raza, S. Srinivasan, J. F. Spear, and N. G. Avadhani, "Protein kinase A-mediated phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia-related injury," *Journal of Biological Chemistry*, vol. 281, no. 4, pp. 2061–2070, 2006.

[83] D. Guo, T. Nguyen, M. Ogbi et al., "Protein kinase C-ε coimmunoprecipitates with cytochrome oxidase subunit IV and is associated with improved cytochrome-*c* oxidase activity and cardioprotection," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 293, no. 4, pp. H2219–H2230, 2007.

[84] Z. X. Chen and S. Pervaiz, "Involvement of cytochrome c oxidase subunits Va and Vb in the regulation of cancer cell metabolism by Bcl-2," *Cell Death and Differentiation*, vol. 17, no. 3, pp. 408–420, 2010.

[85] T. H. Sanderson, G. Mahapatra, P. Pecina et al., "Cytochrome c is tyrosine 97 phosphorylated by neuroprotective insulin treatment," *PLoS ONE*, vol. 8, no. 11, Article ID e78627, 2013.

[86] K. C. Hess, J. Liu, G. Manfredi et al., "A mitochondrial CO_2-adenylyl cyclase-cAMP signalosome controls yeast normoxic cytochrome c oxidase activity," *The FASEB Journal*, vol. 28, no. 10, pp. 4369–4380, 2014.

[87] M. Hüttemann, I. Lee, L. I. Grossman, J. W. Doan, and T. H. Sanderson, "Phosphorylation of mammalian cytochrome c and cytochrome c oxidase in the regulation of cell destiny: respiration, apoptosis, and human disease," *Advances in Experimental Medicine and Biology*, vol. 748, pp. 237–264, 2012.

[88] M. Hüttemann, S. Helling, T. H. Sanderson et al., "Regulation of mitochondrial respiration and apoptosis through cell signaling: cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation," *Biochimica et Biophysica Acta*, vol. 1817, no. 4, pp. 598–609, 2012.

[89] J. L. Boerner, M. L. Demory, C. Silva, and S. J. Parsons, "Phosphorylation of Y845 on the epidermal growth factor receptor mediates binding to the mitochondrial protein cytochrome c oxidase subunit II," *Molecular and Cellular Biology*, vol. 24, no. 16, pp. 7059–7071, 2004.

[90] M. L. Demory, J. L. Boerner, R. Davidson et al., "Epidermal growth factor receptor translocation to the mitochondria: regulation and effect," *Journal of Biological Chemistry*, vol. 284, no. 52, pp. 36592–36604, 2009.

[91] Y. Ding, Z. Liu, S. Desai et al., "Receptor tyrosine kinase ErbB2 translocates into mitochondria and regulates cellular metabolism," *Nature Communications*, vol. 3, article 1271, 2012.

[92] M. Hüttemann, I. Lee, and M. H. Malek, "(-)-epicatechin maintains endurance training adaptation in mice after 14 days of detraining," *The FASEB Journal*, vol. 26, no. 4, pp. 1413–1422, 2012.

[93] W.-Y. Zhang, H.-Q. Liu, K.-Q. Xie et al., "Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells," *Biochemical and Biophysical Research Communications*, vol. 345, no. 1, pp. 508–515, 2006.

[94] T. Marchbank, A. Mahmood, and R. J. Playford, "Pancreatic secretory trypsin inhibitor causes autocrine-mediated migration and invasion in bladder cancer and phosphorylates the EGF receptor, Akt2 and Akt3, and ERK1 and ERK2," *American*

Journal of Physiology—Renal Physiology, vol. 305, no. 3, pp. F382–F389, 2013.

[95] A. B. Granado-Serrano, M. A. Martín, G. Haegeman, L. Goya, L. Bravo, and S. Ramos, "Epicatechin induces NF-kappaB, activator protein-1 (AP-1) and nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) via phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signalling in HepG2 cells," *British Journal of Nutrition*, vol. 103, no. 2, pp. 168–179, 2010.

[96] I. Ramirez-Sanchez, H. Aguilar, G. Ceballos, and F. Villarreal, "(-)-Epicatechin-induced calcium independent eNOS activation: roles of HSP90 and AKT," *Molecular and Cellular Biochemistry*, vol. 370, no. 1-2, pp. 141–150, 2012.

[97] I. Ramirez-Sanchez, L. Nogueira, A. Moreno et al., "Stimulatory effects of the flavanol (-)-epicatechin on cardiac angiogenesis: additive effects with exercise," *Journal of Cardiovascular Pharmacology*, vol. 60, no. 5, pp. 429–438, 2012.

[98] M. Hüttemann, I. Lee, G. A. Perkins, S. L. Britton, L. G. Koch, and M. H. Malek, "(-)-epicatechin is associated with increased angiogenic and mitochondrial signalling in the hindlimb of rats selectively bred for innate low running capacity," *Clinical Science*, vol. 124, no. 11, pp. 663–674, 2013.

[99] L. Nogueira, I. Ramirez-Sanchez, G. A. Perkins et al., "(-)-Epicatechin enhances fatigue resistance and oxidative capacity in mouse muscle," *Journal of Physiology*, vol. 589, no. 18, pp. 4615–4631, 2011.

[100] A. Moreno-Ulloa, D. Romero-Perez, F. Villarreal, G. Ceballos, and I. Ramirez-Sanchez, "Cell membrane mediated (−)-epicatechin effects on upstream endothelial cell signaling: evidence for a surface receptor," *Bioorganic & Medicinal Chemistry Letters*, vol. 24, no. 12, pp. 2749–2752, 2014.

[101] M. Panneerselvam, Y. M. Tsutsumi, J. A. Bonds et al., "Dark chocolate receptors: Epicatechin-induced cardiac protection is dependent on δ-opioid receptor stimulation," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 299, no. 5, pp. H1604–H1609, 2010.

[102] M. Panneerselvam, S. S. Ali, J. C. Finley et al., "Epicatechin regulation of mitochondrial structure and function is opioid receptor dependent," *Molecular Nutrition and Food Research*, vol. 57, no. 6, pp. 1007–1014, 2013.

[103] T. H. Sanderson, C. A. Reynolds, R. Kumar, K. Przyklenk, and M. Hüttemann, "Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation," *Molecular Neurobiology*, vol. 47, no. 1, pp. 9–23, 2013.

[104] H. M. Honda, P. Korge, and J. N. Weiss, "Mitochondria and ischemia/reperfusion injury," *Annals of the New York Academy of Sciences*, vol. 1047, pp. 248–258, 2005.

[105] K. G. Yamazaki, A. Y. Andreyev, P. Ortiz-Vilchis et al., "Intravenous (-)-epicatechin reduces myocardial ischemic injury by protecting mitochondrial function," *International Journal of Cardiology*, vol. 175, no. 2, pp. 297–306, 2014.

[106] P. S. M. Prince, "(-) epicatechin attenuates mitochondrial damage by enhancing mitochondrial multi-marker enzymes, Adenosine triphosphate and lowering calcium in isoproterenol induced myocardial infarcted rats," *Food and Chemical Toxicology*, vol. 53, pp. 409–416, 2013.

[107] C. C. Leonardo, M. Agrawal, N. Singh, J. R. Moore, S. Biswal, and S. Doré, "Oral administration of the flavanol (-)-epicatechin bolsters endogenous protection against focal ischemia through the Nrf2 cytoprotective pathway," *European Journal of Neuroscience*, vol. 38, no. 11, pp. 3659–3668, 2013.

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Review Article

Development of Antiatherosclerotic Drugs on the basis of Natural Products Using Cell Model Approach

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Atherosclerosis including its subclinical form is one of the key medical and social problems. At present, there is no therapy available for widespread use against subclinical atherosclerosis. The use of synthetic drugs for the prevention of arteriosclerosis in its early stages is not sufficient because of the limited indications for severe side effects and high cost of treatment. Obviously, effective antiatherosclerotic drugs based on natural products would be a preferred alternative. Simple cell-based models for testing different natural products have been developed and the ability of natural products to prevent intracellular lipid accumulation in primary cell culture was evaluated. This approach utilizing cell models allowed to test effects of such direct antiatherosclerotic therapy, analyzing the effects mimicking those which can occur "at the level" of arterial wall via the inhibition of intracellular lipid deposition. The data from the carried out clinical trials support a point of view that the identification of antiatherosclerotic activity of natural products might offer a great opportunity for the prevention and treatment of atherosclerotic disease, reducing cardiovascular morbidity and mortality.

1. Introduction

Atherosclerosis is one of the key medical and social problems, since its clinical manifestations take a leading place in "the structure" of overall morbidity and mortality [1]. Atherosclerosis is a multifactorial disease, which is characterized, in general, by the development of degenerative changes in the wall of large arteries, followed by occlusion of the lumen and that limits blood supply to organs and tissues. Subclinical (asymptomatic) atherosclerosis is the most widespread pathology. It is well known that atherosclerotic lesions exist already in young people, steadily progressing over decades until clinical manifestations occur [1–4]. In the middle age, individuals are usually free from clinical manifestations of atherosclerosis while, as a matter of fact, the incidence of atherosclerotic lesions accounts for nearly 100% [2–4].

At present, there are no methods of "direct" antiatherosclerotic prevention and therapy available for widespread use in subclinical atherosclerosis. The main reason for this is the still existing uncertainty about the exact mechanisms of human atherogenesis and, as a result, about the most appropriate therapeutic targets. In epidemiological studies, a number of factors, associated with the increased risk of vascular occlusion, have been identified [5]; those conventional risk factors for atherosclerosis development include several clinical and biochemical syndromes, which contribute to the development of pathology. It is not surprising, therefore, that the elimination of risk factors is the most extensively studied

area and is a widely used approach for the primary prevention of atherosclerosis [6]. However, this approach provides just an indirect impact, since it is directed to the changing of several conditions which are not "immediately" and directly related to the molecular and cellular mechanisms of atherogenesis but are hindering the emergence and progression of atherosclerotic lesions.

On the opposite side, an idea of a so called "direct" antiatherosclerotic impact of therapy does exist and is thought to be a pathogenetic approach to prevent the onset and progression of atherosclerotic lesions by inhibiting the molecular and cellular mechanisms of atherogenesis thus causing prevention or regression of atherosclerosis [7]. Currently, such approach to pathogenetic atherosclerosis prevention and treatment is under development.

It should be noted here that a wide use of synthetic drugs for the prevention of arteriosclerosis in the early stages of the disease development may be impractical, because of the limited indications for use, severe side effects, and high cost of treatment. Currently, discussion about drugs possessing antiatherosclerotic action focuses primarily on statins [8]. However, it is known that regular long-term statin therapy leads to a cessation of development and regression of existing atherosclerotic lesions. It has become clear that the widespread use of statins for the prevention of atherosclerosis in its early stages seems to be unlikely because of the narrow indications for prescription and severity of side effects. Effective antiatherosclerotic drugs based on natural products can be a preferred alternative [9]. For early prevention of atherosclerosis, nonpharmaceutical medicines of natural origin may be suggested, as they have virtually no side effects, may have physiological regulatory effect, and, as a consequence, may allow for a lifetime appointment. The remedies of natural origin may have a wider range of effects than drugs, affecting a number of risk factors for atherosclerosis, thus possessing not only a direct antiatherosclerotic action at the cellular level, but also indirect effects (e.g., cholesterol lowering and blood pressure regulation). The basic principle for the use of drugs and nonpharmaceutical remedies for the prevention of atherosclerosis should be the pathogenetic mechanism of action and the effectiveness confirmed in clinical studies.

Using natural products for the prevention of atherosclerosis should be based on their ability to prevent the accumulation of cholesterol in the cells of the arterial wall that could be prevented at the initial stage of atherogenesis at cellular level. Currently, parapharmaceuticals based on botanicals and other natural products are increasingly attracting researcher interest. Nevertheless, understanding their possible cardioprotective effects and limited appreciation of their possible impact on risk factors for cardiovascular disease such as lowering of blood cholesterol and blood pressure regulation [7, 10–12] are still limited. It is clear that the development of a new methodology for assessing the therapeutic potential of natural products would be obviously beneficial. Until recently there were no methodological approaches to the assessment of antiatherosclerotic potential of natural substances. One of the main obstacles for the development of pathogenetic therapy of atherosclerosis, especially against the early subclinical stages of the disease development, is the lack of adequate pathophysiological models and the absence of a proper algorithm for the development of drug and parapharmaceuticals that would possess direct antiatherosclerotic action.

In this review, we highlight achievements in the development and use of models based on primary cultures of human aortic cells which were already utilized for search of antiatherosclerotic agents of natural origin. In order to explain the peculiarities and usefulness of such models for testing properties of natural substances against atherosclerotic process, we firstly here briefly describe some key atherosclerotic mechanisms which can and should be targeted.

2. Mechanisms of Human Atherogenesis

Current understanding of cellular and molecular mechanisms of atherogenesis is based on the classical lipid theory of atherosclerosis, postulating that the most important event in the development of atherosclerosis is the accumulation of extracellular and intracellular lipids in the arterial intima [13, 14]. The major source of lipids accumulating in intimal cells is low-density lipoprotein (LDL). It should be stressed here that chemical modification of lipoprotein particles is evidently necessary for the manifestation of atherogenic effect, since native (intact) LDL does not cause the accumulation of lipids in cells populating the arterial wall. The range of exactly known atherogenic LDL modifications is limited to three options: desialylation, the change of the total surface charge, and the change of hydrated density of lipoprotein particles; all of them may be accompanied by oxidation [15–18]. In fact, in all cases we actually deal with the same type of multiple atherogenic modifications, but differently evaluated by different methods of laboratory diagnostics [15]. If there is a sufficient amount of modified LDL in the circulation, the additional mechanisms enhancing LDL atherogenic potential come into action. The main one is the formation of large lipid-containing complexes. It is known that modified LDL due to the changes in surface charge acquires the ability to spontaneous self-association. Additionally, modified LDL possesses antigenic properties, thus inducing the production of anti-apoB autoantibodies, which ultimately leads to the formation of LDL-containing circulating immune complexes. Modified LDL also has high avidity for connective tissue matrix components. All the above mentioned processes lead to the appearance of large LDL-containing aggregates, the metabolism of which at the cellular level is operated by alternative pathways other than the classical receptor pathway. The main mechanism of internalization of such particles by vascular cells is uncontrolled phagocytosis. The pathways of intracellular degradation of LDL-containing phagocytized particles differ significantly from the classic native LDL metabolism; therefore, it occurs in "a short time" and leads to massive intracellular accumulation of the residues of lipoprotein particles, mainly in the form of lipid droplets. Under histological examination, these lipid-laden cells are defined as foam cells, the presence of which is an essential feature and key attribute of atherosclerotic lesions.

A scheme, presented in Figure 1, is based on findings produced during studies of human atherosclerosis; this scheme incorporates the key events of atherogenesis (Figure 1). Such a scheme not only explains the accumulated facts about the development of atherosclerosis but also might suggest how to develop novel approaches for the prevention and treatment of atherosclerosis. For the development of initial focal lesion in arterial wall, at least two events must cooccur, namely, atherogenic modification of LDL circulating in the blood and the local change in endothelial permeability. It is important to mention that modification of LDL can occur also directly in the intima, after penetration of LDL via the luminal endothelium. The causes of LDL modification are the processes occurring in both the blood and the vascular wall leading to multiple changes in the physical and chemical characteristics of lipoprotein and consequently to a disturbance of the functions. One of the earliest events of multiple modifications of LDL is a desialylation of lipoprotein glycoconjugates caused by the transsialidase circulating in the blood [15]. Local changes in endothelial permeability might be associated with the heterogeneity of the endothelial lining, that is, the presence of clusters of multinucleated giant endothelial cells and small endothelial cells which are located mosaically [19]. Undoubtedly, different structural types of the endothelium are present along the luminal surface and differ functionally, even though the peculiarities of heterogeneity of the endothelium in human arteries are still poorly understood. One cannot exclude the possibility that mosaicism in the distribution of endothelial clusters possessing different functional properties might determine mosaicism and "focality" of the development of the atherosclerotic lesions in the arterial system. Penetration of modified LDL into the subendothelial space in loci with increased endothelial permeability leads to the formation of focal intimal lipid infiltrations. Both in the blood and in the intima modified LDLs form associates with each other or with autoantibodies against modified LDL (circulating immune complexes). Additionally, in the intima LDLs might associate with extracellular matrix components, complicating the characteristics of modified LDLs [16–18]. Modified LDLs and LDL associates interact with cells populating the subendothelial intima. Intimal cells comprise resident mesenchymal origin cells and cells that intrude the intima from the circulation (hematogenous cells). Conformation of modified LDL as a part of associate is fundamentally different from that of native lipoprotein particle, that is why LDL associates do not interact with LDL-receptors on the cell surface but rather enter the intimal cell by nonreceptor pathway. According to Goldstein and Brown [20], nonreceptor LDL uptake leads to the accumulation of intracellular lipids and foam cell formation.

Phagocytosis of LDL associates might be understood as a result of innate immunity response. An intimal cell cannot percept a LDL associate as a LDL particle, for which such cell has well-preserved mechanism of receptor interaction, internalization, and degradation. Associate, containing LDL, is likely perceived by the intimal cell as a pathogen, a subject for the destruction by phagocytosis. Phagocyte (monocyte-derived macrophage or resident pluripotent mesenchymal cell) takes up the associate, containing LDL, perceived as pathogen switching on the mechanism of innate immunity by secreting the signaling molecules which attract neighboring arterial cells and blood-origin inflammatory cells in the focus (locus) of inflammation [21]. In response to inflammatory signals, resident and inflammatory cells that accumulated in the intima also begin to take up the LDL associates and thus accumulating excess lipids and transforming into foam cells. Accumulation of lipids in arterial cells not only generates foam cells but also triggers the processes typical of reparative phase of inflammation, namely, proliferation and fibrosis (extracellular matrix synthesis); besides interaction with the LDL associates leads to a decrease in cell-to-cell contacts between resident cells [22].

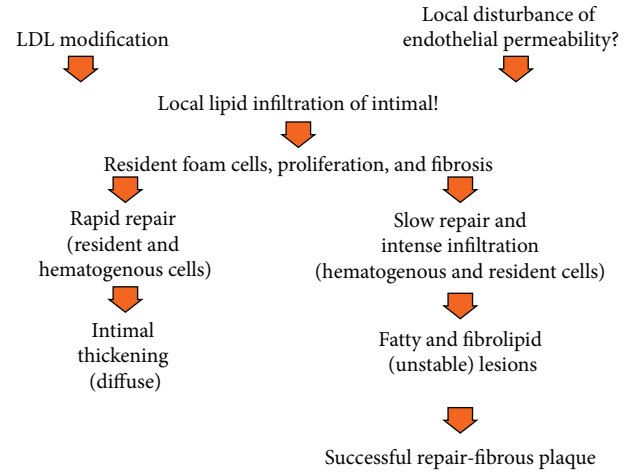

Figure 1: Scheme showing an association of consecutive events relating to the initiation and development of atherosclerotic lesions.

With favorable developments the reparation process completes rapidly. In the areas of the intima, where such events happened, focal increase in cell number occurs, along with the increase in excessive production of extracellular matrix components. Their events occur in human arterial intima throughout life. Over time, focal fibrotic thickening, which is also characterized by an increase in cellularity, develops throughout the arterial bed and becomes diffused. Apparently, the formation of diffuse intimal thickening is typical for adult arteries.

However, not always the innate immune response (an inflammatory reaction) completes promptly and successfully. If, for some reasons, the local lipid infiltration of the intima does not cease immune cells will be "forced" to participate in the development of an inflammatory response, with increased intensity. Local accumulation of cells will increase and cell proliferation and fibrosis will intensify. In this scenario, reparation will proceed slowly and inefficiently, and the process would become chronic. Local chronic inflammation will be accompanied by increased lipidosis because the cells, populating this locus of intima, would not be able to effectively cope with the continuously increased lipid infiltration of the intima. Development of cellular lipidosis will thus lead to a loss of intercellular contacts, increased proliferation,

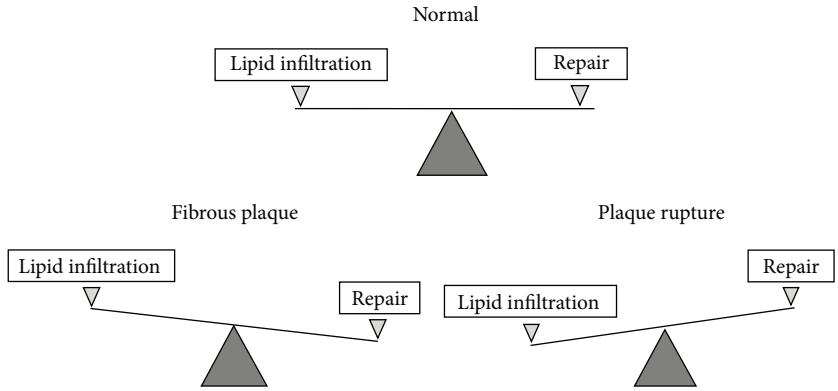

Figure 2: Scheme showing delicate balance between infiltrative and reparative phases in fatty atherosclerotic lesion.

and a sharp intensification of fibrosis [22]. As a result, in this area of the intima, an atherosclerotic lesion would form, initially fatty streak and eventually fibrolipid plaque. The inability of cells to effectively cope with prolonged local inflammation in the subendothelial intima will cause tissue reaction, accompanied by the formation fibrous cap in order to isolate the center of inflammation and create a barrier to prevent the further penetration of lipoproteins and immune cells from the bloodstream, which in chronic inflammatory response would contribute to and determine the pathological process. A favorable outcome in this situation would be a complete separation of the focus of inflammation with the suppression of the inflammatory response and the gradual and partial restoration of tissue functions with the formation of fibrous plaques (scar). Alternatively, unfavorable outcome with severe or even fatal consequences for the organism would be the development of fibrolipid plaque which would be prone to plaque rupture and thrombus formation [23]. Fibrolipid plaque as a stage of atherosclerotic lesion development is characterized by the occurrence of two opposing processes, namely, by infiltration and reparation (Figure 2). This equilibrium is unstable (Figure 2). If the balance is shifted toward reparation, fibrous plaque is formed which, from clinical point of view, is a favorable outcome. If reparation occurs inefficiently and lipid infiltration prevails, plaque rupture could rather occur. Such an outcome kills every second human. Thus, at both the cellular and tissue levels the most crucial event for the initiation and development of atherosclerotic lesions is lipidosis, that is, lipid infiltration of arterial intima accompanied by lipid accumulation in intimal cells. Lipidosis is a trigger for the development of atherosclerotic plaques. Appreciation of the importance of lipid accumulation in intimal cells encourages the search for approaches to prevent such event.

3. Cellular Models for Development of Antiatherosclerotic Drugs: Why Do We Need Such Models?

Pathogenetic approach to the prevention and treatment of atherosclerosis should be directed to search approaches that would prevent intracellular accumulation of lipids. There are at least four theoretical possibilities of how to process the intracellular accumulation of lipids; these include the following possibilities: (1) the removal of modified LDL from circulation, (2) inhibition of proatherogenic modification of native LDL, (3) the suppression of trapping of modified LDL in tissues, and (4) the removal of accumulated lipids from cells. Integrated measure of different antiatherosclerotic effects is the reduction of the rate of accumulation of intracellular lipids and the decrease of intracellular pool of cholesterol esters [24–27].

Currently, there are no drugs that would be claimed to have a direct antiatherosclerotic action. It is known that some drugs may help to reduce atherogenic potential of serum of patients with atherosclerosis [24–27]. The concept of "atherogenic potential" ("atherogenicity") refers to the ability of the serum or its components to cause cholesterol accumulation in cells cultured from unaffected human aortic atherosclerotic intima or other types of cultured cells. The phenomenon of serum atherogenicity was first detected in patients with coronary atherosclerosis [26]. Targeted reduction of serum atherogenic potential may help prevent accumulation of lipids in cells of the arterial wall and thereby suppress atherogenesis in its initial stage [26]. Therefore, cell culture test is thought to be the most optimal and appropriate way to model the early processes of atherogenesis at the cellular level [24–27]. There is an urgent need to develop cellular models adequate for the evaluation of antiatherogenic potential of different drugs and substances. Such an approach would make it possible to perform streaming screening of drugs with potential antiatherosclerotic effect and the estimation of clinical efficacy of antiatherosclerotic therapy.

4. *In Vitro* Model

For the screening of antiatherosclerotic substances, a cellular model based on a primary culture of human aortic cells was developed. In this model, cells were isolated from the subendothelial part of the normal (unaffected by atherosclerosis) human aortic intima, that is, a part of the aorta which is localized between the endothelial lining and the tunica media [24–27]. For living cell isolation collagenase and elastase

Table 1: Proportion of cell types (identified by cell-defining marker) in primary culture of cells isolated from human aortic subendothelial intima.

Smooth muscle α-actin$^+$	3G5$^+$	2A7$^+$	CD45$^+$	CD68$^+$
89.6 ± 6.7%	45.8 ± 10.9%	24.1 ± 9.9%	3.6 ± 0.4%	5.2 ± 1.3%

were used [24–26]. Using monoclonal antibodies, it has been established that cells, which were obtained from the intima and then were cultured *in vitro,* represented a mixture of different cell types [24–26], including smooth muscle cells (20–50%), pericyte-like cells (30–70%), and blood-origin cells and tissue macrophages (10%). Table 1 shows the proportion of cell types in such primary culture. It is necessary to note here that antibodies to smooth muscle α-actin identify smooth muscle cells and pericytes. Antibodies 3G5 and 2A7 identify resting and active pericytes, respectively. Thus, smooth muscle cells and stellate-shaped pericyte-like cells represented the major portion of the in cell culture. Cells of hematogenous origin detectable by antibodies against leukocytes (CD45+) and tissue macrophages (CD68+) represented only a minor portion of the cultured cells.

To stimulate cellular lipidosis atherogenic serum of patients (with assessed atherosclerosis) was added to primary culture of aortic cells. Such serum has been shown to increase intracellular cholesterol approximately twofold after 24 hours of incubation in cell culture [24–26]. Together with atherogenic serum aqueous solution of substance under examination is added to the culture. If the substance reduces intracellular cholesterol accumulation caused by atherogenic serum this substance is regarded to be antiatherosclerotic agent. Antiatherosclerotic effect is expressed as percentage of suppression of intracellular cholesterol accumulation, caused by atherogenic serum.

Using this model, the effects of various drugs and chemicals were examined. Some of the substances have been found to cause antiatherosclerotic effects on cultured cells while others were ineffective in this respect; some substances have been found to possess proatherogenic effect, which is manifested in intensified accumulation of intracellular cholesterol induced by atherogenic serum (Table 2).

Table 2: Substances tested with *in vitro* cellular model*.

Substance
Antiatherosclerotic
Cyclic AMP
Prostacyclin
Prostaglandin E_2
Artificial high-density lipoprotein (HDL)
Antioxidants
Calcium antagonists
Trapidil and its derivatives
Lipoxygenase inhibitors
Lipostabil
Mushroom extracts
Proatherogenic
Beta-blockers
Thromboxane A_2
Phenothiazine
Indifferent
Nitrates
Cholestyramine
Sulfonylureas

*Adapted from [31], with permission from Bentham Science Publisher.

5. *Ex Vivo* Model

On the basis of primary culture of cell cultivated from the normal (unaffected by atherosclerosis) human aortic intima *ex vivo* model was also developed [24–27]. The main difference of *ex vivo* model from *in vitro* model is that not a substance but blood serum of patients after drug administration was investigated. Thereby, changes of atherogenic properties of the blood serum under the action of various preparations are evaluated. Blood is taken before and in a certain time after single dose drug administration. Sera obtained from blood samples are added to the primary culture of aortic cells.

Ex vivo model allows for testing not only drugs but also natural products [27]. An essential feature of the *ex vivo* model is a possibility to assess antiatherosclerotic potential of various substances and their active metabolites after digestion, distribution, and biotransformation in human organism. It is thus possible to obtain specific pharmacodynamic properties. It is known that many of the molecules present in plants are metabolized once consumed and that they are present in circulation in different chemical structures than in food. These metabolites often present different biological effects. In the case of *ex vivo* model we do not face such problems because after taking the natural product any of its metabolites are in the blood serum which we actually investigate.

In *ex vivo* model, a number of studies have been performed to test various natural products, mainly botanicals [27]. In screening studies volunteers (groups of 4–8 people; men and women aged 45–60 years) who had atherogenic serum were involved. The effect of a single dose of investigated natural product on atherogenicity of blood serum was evaluated. The results of the evaluation obtained in the *ex vivo* model when antiatherosclerotic effects of onion after a single dose of capsulated bulb powder (300 mg) were analyzed are presented in Figure 3. Onion powder has been found to possess antiatherogenic effect in *ex vivo* model; this effect was manifested in a moderate reduction of serum atherogenicity by 12%, 28%, and 24% from baseline after 2, 4, and 6 hours after a single dose of the preparation, respectively.

Wheat seedlings have been found to possess a prolonged and pronounced antiatherosclerotic effect in *ex vivo* model; the effect was manifested in lowering of serum atherogenicity after a single dose of 300 mg preparation (Figure 4). It should be noted that 4 hours after administration of the preparation atherogenicity was completely eliminated. Results of the

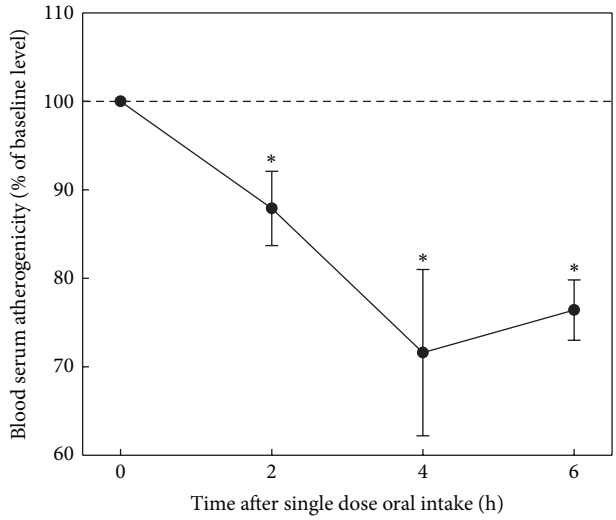

Figure 3: Antiatherosclerotic effect of onion in *ex vivo* model. The study involved 4 volunteers (3 males, 1 female, mean age 57 ± 5 years) whose blood serum induced 1.3–1.5-fold increase in cholesterol content of cells cultured from unaffected human aortic intima (the average level of serum atherogenicity was 141 ± 4%). Intracellular cholesterol in control cultures was 38.4 ± 1.1 mg/mg cell protein. Baseline serum atherogenicity was taken as 100%. The average values of changes of serum atherogenicity with indication of standard errors are presented.

Table 3: Integral estimation of antiatherogenic actions of natural products*.

Botanical and its source	The mean efficiency of atherogenic reduction, %	Maximum effect, %
Spirulina platensis powder	50.7%	61
Onion (*Allium cepa*) bulb powder	21.4%	28
Beet (*Beta vulgaris*) juice powder	30.7%	40
Wheat (*Triticum vulgaris*) seedlings powder	70.0%	100
Licorice (*Glycyrrhiza glabra*) root powder	54.6%	32
Salsola collina leaf powder	10.9%	28
Garlic (*Allium sativum*) bulbs powder	76.6%	100
Pine (*Pinus sylvestris*) needles extract	52.1%	62

*The integrated effect was calculated as a mean reduction in serum atherogenicity for 6 hours after a single oral dose.

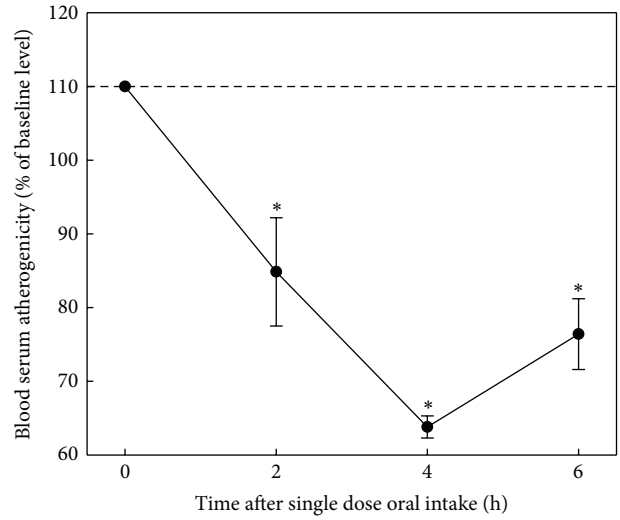

Figure 4: Antiatherosclerotic effect of wheat seedlings in *ex vivo* model. The study involved 8 volunteers (5 males, 3 females, mean age 51 ± 2 years) whose blood serum induced 1.7–2.3-fold increase in cholesterol content of cells cultured from unaffected human aortic intima (the average level of serum atherogenicity was 199 ± 6%). Intracellular cholesterol in control cultures was 28.0 ± 1.2 mg/mg cell protein. Baseline serum atherogenicity was taken as 100%. The average values of changes of serum atherogenicity with indication of standard errors are presented. *Significant decrease of serum atherogenicity, $p < 0.05$.

evaluation of antiatherosclerotic effect of dry beet juice in *ex vivo* model after single dose capsuled preparation (300 mg) are presented in Figure 5. Dry beet juice possessed moderate but prolonged antiatherosclerotic effect in the *ex vivo* model. Garlic powder possessed a pronounced and prolonged antiatherosclerotic effect in the *ex vivo* model [27]; the effect was manifested in lowering of serum atherogenicity after a single dose of 300 mg preparation; 4 hours after administration atherogenicity was completely eliminated (Figure 6). In addition to the above results antiatherosclerotic effects of other natural products have also been revealed. Integral estimation data of antiatherogenic effects of the tested natural products is presented in Table 3. According to the data presented in Table 3, wheat seedlings and garlic powder possess the most pronounced antiatherosclerotic effects in the *ex vivo* model. After a single 300 mg dose they reduced atherogenicity serum 3-fold and the biological effect was observed for 6 hours. However, dynamics of atherogenicity reducing was more pronounced in garlic powder.

To develop antiatherosclerotic therapy which would be based on a natural product, it is essential to determine the effective dose, adequate regime, and course of treatment. For this purpose, patients whose serum possessed atherogenic potential took natural product and their blood was collected after 2 and 4 hours. Patient's serum was incubated with cultured subendothelial cells isolated from unaffected human aortic intima and then intracellular cholesterol was determined. Dose-dependent effect was revealed by comparing the efficacy of two doses. Efficacy of each dose was assessed by the analysis of at least six different sera obtained from patients. Thus, it was found that the garlic powder has antiatherosclerotic effect in a dose range of 50–300 mg and half-maximal effect was achieved at a dose of 100 mg and the maximal effect was achieved at a dose of 150 mg [27]. Thus, the data obtained in experiments utilizing the *ex vivo* model allowed to conclude that natural products including

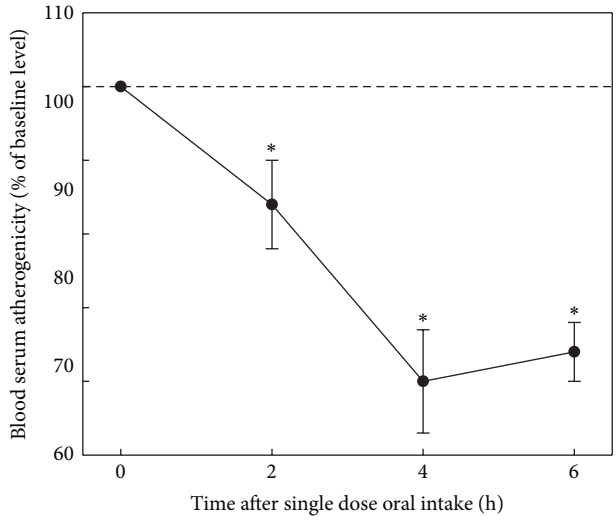

Figure 5: Antiatherosclerotic effect of beet juice in *ex vivo* model. The study involved 8 volunteers (6 males, 2 females, mean age 53 ± 5 years) whose blood serum induced 1.3–2.2-fold increase in cholesterol content of cells cultured from unaffected human aortic intima (the average level of serum atherogenicity was 161 ± 8%). Intracellular cholesterol in control cultures was 37.0 ± 3.6 mg/mg cell protein. Baseline serum atherogenicity was taken as 100%. The average values of changes of serum atherogenicity with indication of standard errors are presented. *Significant decrease of serum atherogenicity, $p < 0.05$.

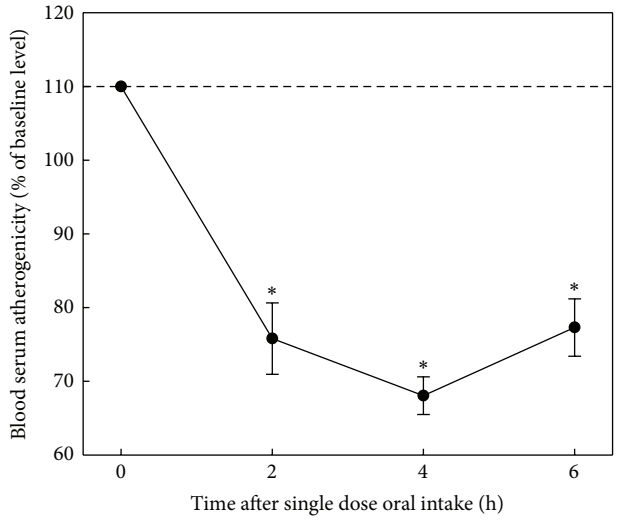

Figure 6: Antiatherosclerotic effect of garlic powder in the *ex vivo* model. The study involved 8 volunteers (6 males, 2 females, mean age 53 ± 5 years) whose blood serum induced 1.3–2.7-fold increase in cholesterol content of cells cultured from unaffected human aortic intima (the average level of serum atherogenicity was 164 ± 9%). Intracellular cholesterol in control cultures was 39.0 ± 4.2 mg/mg cell protein. Baseline serum atherogenicity was taken as 100%. The average values of changes of serum atherogenicity with indication of standard errors are presented. *Significant decrease of serum atherogenicity, $p < 0.05$.

botanicals may be regarded as potential drug substances for the development of direct antiatherosclerotic therapy. Eventually, several preparations that have been registered as dietary supplements have been developed. Clinical studies were conducted to evaluate the antiatherosclerotic efficacy of the three preparations.

6. Clinical Studies

In an open-label prospective pilot study performed in 28 apparently healthy men aged 46–58 (mean age 52.0, SD = 9.0) the effect of garlic-based dietary supplement (Allicor, INAT-Farma, Russia) on carotid intima-media thickness (cIMT) was estimated. The study participants were normolipidemic or mildly hyperlipidemic and had no clinical signs of coronary heart disease (CHD). Diffused intimal thickening without elevated atherosclerotic lesions was diagnosed by ultrasound B-mode examination of common carotid arteries by the method described elsewhere [28]; the cut-off cIMT value of 0.7 mm in the distal segment of at least one common carotid artery was taken for diagnostics of diffused intimal thickening. Study participants had no chronic diseases requiring continuous use of vasoactive drugs, diuretics, and lipid-lowering or antidiabetic drugs. The mean cIMT value at the baseline was 0.832 ± 0.024 mm. Study participants were divided into 2 groups: 16 of them received 600 mg Allicor daily, and 12 were in the control group. Interview and ultrasound examination of the carotid arteries were held every 3 months, and the total duration of follow-up was

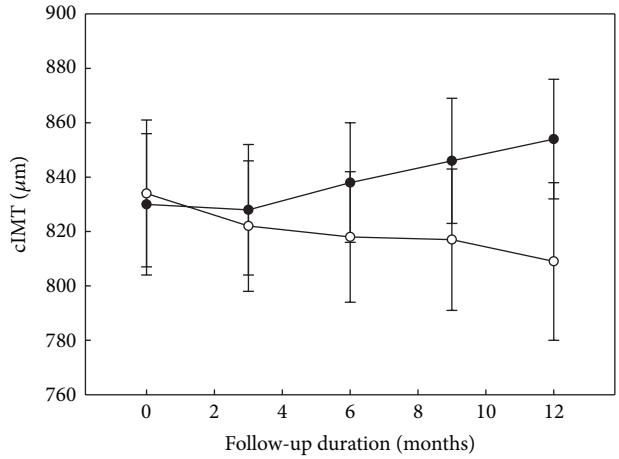

Figure 7: The dynamics of cIMT in open-label pilot study on antiatherosclerotic effects of garlic-based drug Allicor. Open circles, Allicor recipients; solid circles, control subjects. The data are presented in the terms of means and S.E.M.

12 months. During the follow-up, no adverse effects were observed, and tolerability was good. The dynamics of cIMT changes are shown in Figure 7. There were no statistically significant changes in cIMT, and by the end of 12-month follow-up the two groups did not differ in cIMT. However, regression analysis revealed a significant difference between the trends in cIMT dynamics ($p < 0.05$). The control group showed a tendency to cIMT increase, which was significantly

different from that of null hypothesis of no change (F-test, 31.72, $p = 0.011$). In the Allicor-treated group, the tendency to cIMT decrease was revealed, which was also significantly different from that of null hypothesis (F-test, 28.81, $p = 0.013$).

The obtained results indicated that the therapy with Allicor might potentially stop the development and induce the regression of subclinical atherosclerosis; the statistical power of this pilot study was insufficient to avoid type 2 error. Therefore, the results of this pilot study allowed us to design the further prospective clinical study with the assessment of a number of clinical and biochemical parameters associated with atherogenesis and the risk of atherosclerosis, including the assessment of the dynamics of serum atherogenicity. It was necessary to increase the number of study participants to achieve statistical power >80%, standardize inclusion and exclusion criteria, use placebo control, and increase the duration of follow-up.

This double-masked placebo-controlled clinical study was designed to estimate the effect of time-released garlic powder tablets Allicor on the progression of cIMT in 211 asymptomatic men aged 40–74. The primary outcome was the rate of progression of subclinical atherosclerosis, estimated by B-mode ultrasonography as the increase in cIMT (Clinicaltrials.gov identifier NCT01734707). By the end of the first 12 months of follow-up, in Allicor-treated group the decrease of cIMT by 0.028 ± 0.008 mm was observed, whereas in placebo group there was a moderate progression at the rate of 0.014 ± 0.009 mm per 12 months ($p = 0.002$ for the difference). Serum atherogenicity (the ability of serum to induce cholesterol accumulation in cultured cells) was lowered in Allicor-treated patients by 45% from the baseline level, on an average. In the placebo group, serum atherogenic potential did not change significantly. These results demonstrated that long-term treatment with Allicor provides a direct antiatherosclerotic effect on subclinical carotid atherosclerosis, and this effect may be due to serum atherogenicity inhibition [7].

By the end of 24-month follow-up, 196 evaluable study participants remained, since 15 discontinued their participation. The mean rate of cIMT decreases in Allicor-treated group accounted for 0.022 ± 0.007 mm per year, which was significantly different ($p = 0.002$) from the placebo group, in which there was a moderate but statistically significant progression of 0.015 ± 0.008 mm at the overall mean baseline cIMT of 0.931 ± 0.009 mm [7]. Within Allicor-treated group, cIMT significant reduction was observed in 47.3% study participants versus 30.1% in placebo group ($p < 0.05$). The further significant cIMT increase was registered in 32.2% study participants in Allicor-treated group versus 47.3% in the placebo group ($p < 0.05$). At the baseline, serum taken from study participants induced 1.56-fold increase in intracellular cholesterol content in cell culture test, on an average. Serum atherogenicity (the ability of serum to induce cholesterol accumulation in cultured cells) was lowered in Allicor-treated study participants by 30% on an average. In the placebo group, serum atherogenic potential did not change significantly during the study. A significant correlation has been revealed between the changes in blood serum atherogenicity during the study and the changes in intima-media thickness of common carotid arteries ($r = 0.144$; $p = 0.045$). The data on the changes in cIMT and serum atherogenicity are shown in Figures 8 and 9. Thus, the results of the above two-year placebo-controlled double-masked study demonstrated that long-term treatment with garlic-based drug Allicor has a direct antiatherosclerotic effect on subclinical carotid atherosclerosis, and this effect may be attributed to serum atherogenicity inhibition [7].

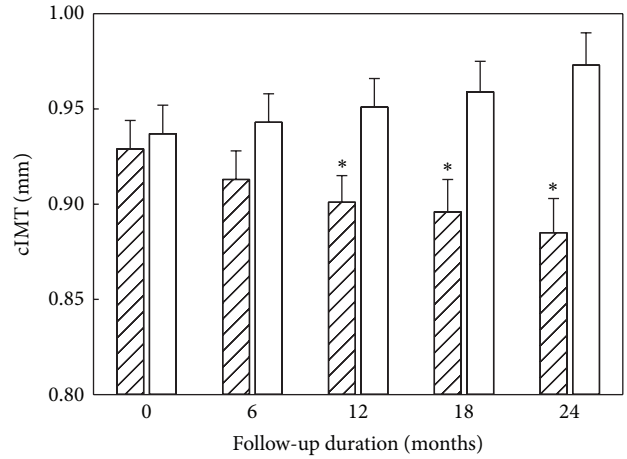

Figure 8: The dynamics of cIMT in double-masked placebo-controlled study on antiatherosclerotic effects of garlic-based drug Allicor. Hatched bars, Allicor recipients; open bars, placebo recipients. The data are presented in terms of means and S.E.M. *Significant difference between groups, $p < 0.05$.

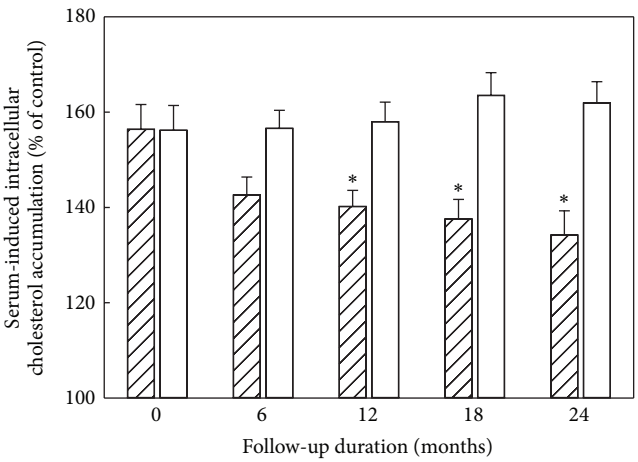

Figure 9: The dynamics of serum atherogenicity in double-masked placebo-controlled study on antiatherosclerotic effects of garlic-based drug Allicor. Hatched bars, Allicor recipients; open bars, placebo recipients. The data are presented in terms of means and S.E.M. *Significant difference between groups, $p < 0.05$.

Atherosclerosis is regarded as a pathological process with the elements of local aseptic inflammation, while inflammatory cytokines play a role at every stage of atherosclerosis development [29, 30]. Therefore, the drugs possessing systemic anti-inflammatory action may be effective for the

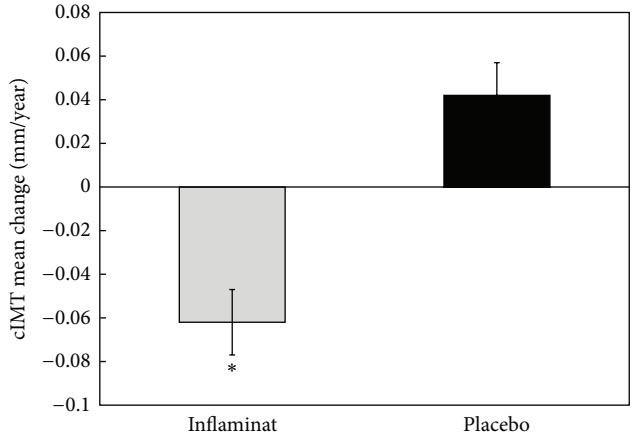

Figure 10: The changes of cIMT in double-masked placebo-controlled study on antiatherosclerotic effects of Inflaminat. The data are presented in terms of means and S.D. *Significant difference between groups, $p < 0.05$. Adapted from [37], with permission from Bentham Science Publisher.

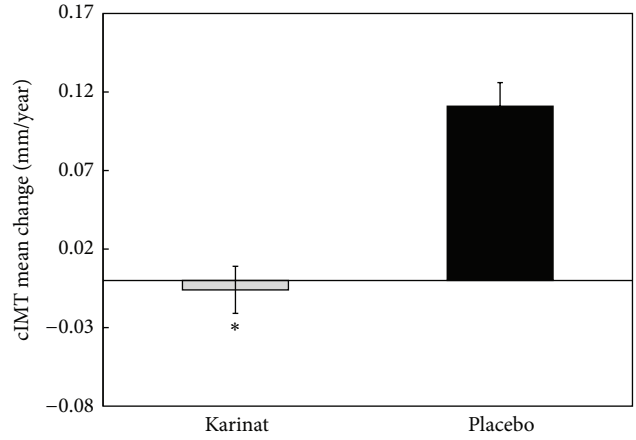

Figure 11: The changes of cIMT in double-masked placebo-controlled study on antiatherosclerotic effects of Karinat. The data are presented in terms of means and S.D. *Significant difference between groups, $p < 0.05$. Adapted from [37], with permission from Bentham Science Publisher.

prevention of atherosclerosis. It has been demonstrated that several natural compounds possess not only anti-inflammatory effect, but also antiatherogenic one: among them are calendula, elder, and violet [31]. On the basis of combination of these three herbs, the dietary supplement has been developed (Inflaminat, INAT-Farma, Russia) [31]. The pilot placebo-controlled double-blinded study has been performed to investigate the effect of Inflaminat on cIMT dynamics in 67 asymptomatic men (Clinicaltrials.gov identifier NCT01743404) [31]. The protocol of the study was similar to that reported above; the duration of follow-up accounted for 12 months. It has been demonstrated that Inflaminat induced cIMT regression in subclinical atherosclerosis, and cIMT changes were statistically significant compared to the baseline as placebo group (Figure 10). Thus, Inflaminat has anti-inflammatory and antiatherosclerotic effects on cellular level revealed in cell culture and induces regression of subclinical atherosclerosis in asymptomatic men.

We screened several natural phytoestrogen-rich components for their antiatherogenic activity using *in vitro* and *ex vivo* test systems [31, 32]. The most promising of these compounds were garlic powder, extract of grape seeds, green tea leafs, and hop cones; all of them produced a significant antiatherogenic effect. On the basis of their combination, isoflavonoid-rich dietary supplement was developed (Karinat, INAT-Farma, Russia). This combination produces the most efficient antiatherogenic effect in cell culture models and is characterized by improved phytoestrogen profile, providing additional amounts of biologically active polyphenols including resveratrol, genistein, and daidzein that are claimed to produce beneficial effects on atherosclerosis development. A randomized double-blinded placebo-controlled pilot clinical study on atherosclerotic effect of Karinat was performed in 157 asymptomatic postmenopausal women to understand the risks and benefits of phytoestrogen therapy in relation to atherosclerosis progression (Clinicaltrials.gov identifier NCT01742000) [31]. The primary endpoint was the annual rate of changes in cIMT. The protocol of the study was similar to that reported above; the duration of follow-up accounted for 12 months.

In the placebo group, an increase in the mean cIMT of more than 100 μm per year was observed. Thus, the rate of cIMT progression in postmenopausal women was high, as it accounted for 13% per year and growth of atherosclerotic plaques of 40% per year. On the opposite side, in Karinat recipients the mean cIMT did not change; there was statistically insignificant increase of 6 μm per year, that is, less than 1% (Figure 11). In this clinical study, the results of quantitative measurements of the degree of subclinical atherosclerosis in the dynamics have shown that the use of phytoestrogen complex in postmenopausal women almost completely suppresses the formation of new atherosclerotic lesions [31].

7. Conclusions

A discovery of the phenomenon of serum atherogenicity significantly promoted the development of simple cell-based models for testing of different types of natural products and drugs with respect to their antiatherosclerotic effects. This approach allowed the development of so called "direct" antiatherosclerotic therapy, the effect of which is realized at the level of the arterial wall, predominantly, via the inhibition of intracellular lipid deposition. To be of potential benefit in patients with established atherosclerosis, a drug should produce regression or slow the progression of atherosclerosis. Further examination of antiatherogenic and antiatherosclerotic effects of various natural products would help the development of novel cardiovascular drugs, possessing mechanistic mode of action on the processes of atherogenesis.

No doubt, there are commonly accepted understanding and appreciation of the fact that any experimental model,

including any cell culture model, has some limitations [33–39]. The problem of limitations of experimental models, in particular in atherosclerosis research, has been well highlighted and discussed in a number of comprehensive reviews [36–42]. Despite the existence of unavoidable limitations of any model approach, including the cell culture model approach, the development and principles of which are highlighted in the present review, the data obtained during the clinical trials strongly support a point of view that arterial wall cell models offer a suitable instrument for analysis of effects of drugs and that the discovery of antiatherosclerotic activity of natural products offers great opportunities for the prevention and treatment of atherosclerotic disease, reducing cardiovascular morbidity and mortality.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgment

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References

[1] A. Simmons, K. Steffen, and S. Sanders, "Medical therapy for peripheral arterial disease," *Current Opinion in Cardiology*, vol. 27, no. 6, pp. 592–597, 2012.

[2] G. S. Berenson, S. R. Srinivasan, W. Bao, W. P. Newman III, R. E. Tracy, and W. A. Wattigney, "Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults," *The New England Journal of Medicine*, vol. 338, no. 23, pp. 1650–1656, 1998.

[3] H. C. McGill Jr., E. E. Herderick, C. A. McMahan et al., "Atherosclerosis in youth," *Minerva Pediatrica*, vol. 54, no. 5, pp. 437–447, 2002.

[4] E. M. Tuzcu, S. R. Kapadia, E. Tutar et al., "High prevalence of coronary atherosclerosis in asymptomatic teenagers and young adults: evidence from intravascular ultrasound," *Circulation*, vol. 103, no. 22, pp. 2705–2710, 2001.

[5] K. M. Anderson, P. W. F. Wilson, P. M. Odell, and W. B. Kannel, "An updated coronary risk profile. A statement for health professionals," *Circulation*, vol. 83, no. 1, pp. 356–362, 1991.

[6] F. G. R. Fowkes, D. Rudan, I. Rudan et al., "Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis," *The Lancet*, vol. 382, no. 9901, pp. 1329–1340, 2013.

[7] A. N. Orekhov, I. A. Sobenin, N. V. Korneev et al., "Antiatherosclerotic therapy based on botanicals," *Recent Patents on Cardiovascular Drug Discovery*, vol. 8, no. 1, pp. 56–66, 2013.

[8] E. A. Stein and F. J. Raal, "New therapies for reducing low-density lipoprotein cholesterol," *Endocrinology Metabolism Clinics of North America*, vol. 43, pp. 1007–1033, 2014.

[9] A. K. Rai, P. Debetto, and F. D. Sala, "Molecular regulation of cholesterol metabolism: HDL-based intervention through drugs and diet," *Indian Journal of Experimental Biology*, vol. 51, no. 11, pp. 885–894, 2013.

[10] N. Al-Waili, K. Salom, A. Al-Ghamdi, M. J. Ansari, A. Al-Waili, and T. Al-Waili, "Honey and cardiovascular risk factors, in normal individuals and in patients with diabetes mellitus or dyslipidemia," *Journal of Medicinal Food*, vol. 16, no. 12, pp. 1063–1078, 2013.

[11] K. Ried, C. Toben, and P. Fakler, "Effect of garlic on serum lipids: an updated meta-analysis," *Nutrition Reviews*, vol. 71, no. 5, pp. 282–299, 2013.

[12] A. L. Hopkins, M. G. Lamm, J. L. Funk, and C. Ritenbaugh, "*Hibiscus sabdariffa* L. in the treatment of hypertension and hyperlipidemia: a comprehensive review of animal and human studies," *Fitoterapia*, vol. 85, no. 1, pp. 84–94, 2013.

[13] M. Schönfelder, "Ortologie und patologie der Langhans-zellen der aortenintima des menschen," *Pathologia et Microbiologia (Basel)*, vol. 33, pp. 129–145, 1969.

[14] I. E. Konstantinov, N. Mejevoi, and N. M. Anichkov, "Nikolai N. Anichkov and his theory of atherosclerosis," *Texas Heart Institute Journal*, vol. 33, no. 4, pp. 417–423, 2006.

[15] O. Jaakkola, T. Solakivi, V. V. Tertov, A. N. Orekhov, T. A. Miettinen, and T. Nikkari, "Characteristics of low-density lipoprotein subfractions from patients with coronary artery disease," *Coronary Artery Disease*, vol. 4, no. 4, pp. 379–385, 1993.

[16] A. G. Kacharava, V. V. Tertov, and A. N. Orekhov, "Autoantibodies against low-density lipoprotein and atherogenic potential of blood," *Annals of Medicine*, vol. 25, no. 6, pp. 551–555, 1993.

[17] V. V. Tertov and A. N. Orekhov, "Metabolism of native and naturally occurring multiple modified low density lipoprotein in smooth muscle cells of human aortic intima," *Experimental and Molecular Pathology*, vol. 64, no. 3, pp. 127–145, 1997.

[18] A. N. Orekov, O. S. Kalenich, V. V. Tertov, and I. D. Novikov, "Lipoprotein immune complexes as markers of atherosclerosis," *International Journal of Tissue Reactions*, vol. 13, no. 5, pp. 233–236, 1991.

[19] A. S. Antonov, M. A. Nikolaeva, T. S. Klueva et al., "Primary culture of endothelial cells from atherosclerotic human aorta. Part 1. Identification, morphological and ultrastructural characteristics of two endothelial cell subpopulations," *Atherosclerosis*, vol. 59, no. 1, pp. 1–19, 1986.

[20] J. L. Goldstein and M. S. Brown, "Regulation of low-density lipoprotein receptors: implications for pathogenesis and therapy of hypercholesterolemia and atherosclerosis," *Circulation*, vol. 76, no. 3, pp. 504–507, 1987.

[21] A. Gratchev, I. Sobenin, A. Orekhov, and J. Kzhyshkowska, "Monocytes as a diagnostic marker of cardiovascular diseases," *Immunobiology*, vol. 217, no. 5, pp. 476–482, 2012.

[22] A. N. Orekhov, V. A. Koshkh, V. S. Repin, and V. N. Smirnov, "Cell proliferation in normal and atherosclerotic human aorta. I. Flow cytofluorometric determination of cellular deoxyribonucleic acid content," *Laboratory Investigation*, vol. 48, no. 4, pp. 395–398, 1983.

[23] L. Badimon and G. Vilahur, "Thrombosis formation on atherosclerotic lesions and plaque rupture," *Journal of Internal Medicine*, vol. 276, no. 6, pp. 618–632, 2014.

[24] S. E. Akopov, A. N. Orekhov, V. V. Tertov, K. A. Khashimov, E. S. Gabrielyan, and V. N. Smirnov, "Stable analogues of prostacyclin and thromboxane A_2 display contradictory influences on atherosclerotic properties of cells cultured from human aorta: the effect of calcium antagonists," *Atherosclerosis*, vol. 72, no. 2-3, pp. 245–248, 1988.

[25] A. N. Orekhov, V. V. Tertov, K. A. Khashimov, S. S. Kudryashov, and V. N. Smirnov, "Evidence of antiatherosclerotic action of

verapamil from direct effects on arterial cells," *The American Journal of Cardiology*, vol. 59, no. 5, pp. 495–496, 1987.

[26] A. N. Orekhov, V. V. Tertov, K. A. Khashimov, S. A. Kudryashov, and V. N. Smirnov, "Antiatherosclerotic effects of verapamil in primary culture of human aortic intimal cells," *Journal of Hypertension*, vol. 4, supplement 6, pp. S153–S155, 1986.

[27] I. A. Sobenin, V. V. Pryanishnikov, L. M. Kunnova, Y. A. Rabinovich, D. M. Martirosyan, and A. N. Orekhov, "The effects of time-released garlic powder tablets on multifunctional cardiovascular risk in patients with coronary artery disease," *Lipids in Health and Disease*, vol. 9, article 119, 2010.

[28] R. Salonen, K. Nyyssönen, E. Porkkala et al., "Kuopio Atherosclerosis Prevention Study (KAPS): a population-based primary preventive trial of the effect of LDL lowering on atherosclerotic progression in carotid and femoral arteries," *Circulation*, vol. 92, no. 7, pp. 1758–1764, 1995.

[29] P. Libby, "Inflammation in atherosclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, pp. 2045–2051, 2012.

[30] D. Wolf, P. Stachon, C. Bode, and A. Zirlik, "Inflammatory mechanisms in atherosclerosis," *Hamostaseologie*, vol. 34, no. 1, pp. 63–71, 2014.

[31] A. N. Orekhov, "Direct anti-atherosclerotic therapy; development of natural anti-atherosclerotic drugs preventing cellular cholesterol retention," *Current Pharmaceutical Design*, vol. 19, no. 33, pp. 5909–5928, 2013.

[32] V. A. Myasoedova and I. A. Sobenin, "Background, rationale and design of clinical study of the effect of isoflavonoid-rich botanicals on natural history of atherosclerosis in women," *Atherosclerosis Supplements*, vol. 9, no. 1, 171 pages, 2008.

[33] T. Hartung and G. Daston, "Are in vitro tests suitable for regulatory use?" *Toxicological Sciences*, vol. 111, no. 2, pp. 233–237, 2009.

[34] B. T. Hill, "In vitro human tumour model systems for investigating drug resistance," *Cancer Surveys*, vol. 5, no. 1, pp. 129–149, 1986.

[35] G. Camenisch, G. Folkers, and H. van de Waterbeemd, "Review of theoretical passive drug absorption models: historical background, recent developments and limitations," *Pharmaceutica Acta Helvetiae*, vol. 71, no. 5, pp. 309–327, 1996.

[36] T. M. A. Bocan, "Animal models of atherosclerosis and interpretation of drug intervention studies," *Current Pharmaceutical Design*, vol. 4, no. 1, pp. 37–52, 1998.

[37] P. Carmeliet, L. Moons, and D. Collen, "Mouse models of angiogenesis, arterial stenosis, atherosclerosis and hemostasis," *Cardiovascular Research*, vol. 39, no. 1, pp. 8–33, 1998.

[38] G. J. Johnson, T. R. Griggs, and L. Badimon, "The utility of animal models in the preclinical study of interventions to prevent human coronary artery restenosis: analysis and recommendations. On behalf of the Subcommittee on Animal, Cellular and Molecular Models of Thrombosis and Haemostasis of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis," *Thrombosis and Haemostasis*, vol. 81, no. 5, pp. 835–843, 1999.

[39] M. H. Moghadasian, J. J. Frohlich, and B. M. McManus, "Advances in experimental dyslipidemia and atherosclerosis," *Laboratory Investigation*, vol. 81, no. 9, pp. 1173–1183, 2001.

[40] R. Kones, "Primary prevention of coronary heart disease: integration of new data, evolving views, revised goals, and role of rosuvastatin in management. A comprehensive survey," *Drug Design, Development and Therapy*, vol. 5, pp. 325–380, 2011.

[41] X. Peng, "Transgenic rabbit models for studying human cardiovascular diseases," *Comparative Medicine*, vol. 62, no. 6, pp. 472–479, 2012.

[42] G. S. Getz and C. A. Reardon, "Animal models of atherosclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, pp. 1104–1115, 2012.

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Research Article

Protective Pleiotropic Effect of Flavonoids on NAD^+ Levels in Endothelial Cells Exposed to High Glucose

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NAD^+ is important for oxidative metabolism by serving as an electron transporter. Hyperglycemia decreases NAD^+ levels by activation of the polyol pathway and by overactivation of poly(ADP-ribose)-polymerase (PARP). We examined the protective role of three structurally related flavonoids (rutin, quercetin, and flavone) during high glucose conditions in an *in vitro* model using human umbilical vein endothelial cells (HUVECs). Additionally we assessed the ability of these flavonoids to inhibit aldose reductase enzyme activity. We have previously shown that flavonoids can inhibit PARP activation. Extending these studies, we here provide evidence that flavonoids are also able to protect endothelial cells against a high glucose induced decrease in NAD^+. In addition, we established that flavonoids are able to inhibit aldose reductase, the key enzyme in the polyol pathway. We conclude that this protective effect of flavonoids on NAD^+ levels is a combination of the flavonoids ability to inhibit both PARP activation and aldose reductase enzyme activity. This study shows that flavonoids, by a combination of effects, maintain the redox state of the cell during hyperglycemia. This mode of action enables flavonoids to ameliorate diabetic complications.

1. Introduction

Worldwide more than 400 million people suffer from diabetes. This number will only grow due to the rapid increase in the incidence of the disease caused by population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity [1]. A hallmark of diabetes is hyperglycemia [2]. A number of epidemiological studies have shown a relationship between hyperglycemia and an increased risk of cardiovascular diseases, including microvascular pathologies in the eye, kidney, and peripheral nerves. As a consequence, diabetes is a leading cause of blindness, renal disease, and a variety of debilitating neuropathies (e.g., diabetic foot) [3, 4].

Nicotinamide adenine dinucleotide (NAD) is found in all living cells in an oxidized form (NAD^+) and a reduced form (NADH). The main function of NAD in cells is modulating cellular redox status by carrying electrons from one reaction to another. Additionally, it is also involved in other cellular processes (e.g., acting as a substrate for enzymes involved in posttranslational modification) [5]. Hyperglycemia decreases NAD^+ levels by an increased flux of glucose through the polyol pathway. This pathway becomes active when intracellular glucose levels are elevated [6]. During normoglycemia only ~3% of all glucose will enter the polyol pathway. Most of the glucose will be phosphorylated to glucose-6-phosphate by hexokinase. However, under hyperglycemic conditions ten times more glucose enters the polyol pathway [7], mainly due to a saturation of hexokinase [8]. Aldose reductase, the first and rate-limiting enzyme in the pathway, reduces glucose to sorbitol using NADPH as a cofactor. Then, sorbitol is reduced to fructose by sorbitol dehydrogenase which uses NAD^+ as a cofactor. The osmotic stress that accompanies sorbitol accumulation and the redox imbalance following the depletion of NADPH and NAD^+ contributes to cell damage and organ injury, ultimately leading to cataract genesis, neuropathy, and other diabetic complications [9–11].

Poly(ADP-ribose)-polymerase (PARP) activation can also lead to NAD^+ depletion. The nuclear enzyme PARP has

Table 1: Primer sequences for genes used for gene expression analysis.

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
Beta-actin (β-actin)	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT
Aldose reductase	TACACATGGGCACAGTCGAT	GGGGTTGGGTACCTGGAA
PARP-1	GCCAGTTCAGGACCTCATCAA	CGGCCTGGATCTGCCTTT

been implicated in the regulation of many important cellular functions like DNA repair, gene transcription, cell cycle progression, cell death, chromatin function, and genomic stability [12]. PARP detects and signals single-strand DNA breaks (SSB), which can be induced by hyperglycemia. Upon detection of a SSB, PARP binds to the DNA and synthesizes a poly(ADP-ribose) (PAR) chain as a signal for DNA repair enzymes. NAD^+ is required as a substrate for the synthesis of these PAR monomers. Overactivation of PARP therefore depletes cellular NAD^+ stores [13]. Several studies have suggested an important role of PARP activation in the pathogenesis of diabetic complications like nephropathy, neuropathy, and retinopathy [14–16].

Previously we have established that dietary flavonoids inhibit PARP both *in vitro* and *in vivo* [17–19]. Flavonoids are polyphenolic compounds which are found in fruits, vegetables, and plant-derived products like red wine and tea [18]. Flavonoids have been shown to display positive health effects, for example, reduced risks for cardiovascular and chronic inflammatory diseases [20–23], which have been ascribed to their antioxidant and anti-inflammatory properties [22, 24]. We now studied the effect on NAD^+ levels in endothelial cells after exposing the cells to high glucose in the presence or absence of flavonoids. In addition we determined whether three structurally related flavonoids are also able to inhibit aldose reductase, the most important enzyme of the polyol pathway.

2. Material and Methods

2.1. Chemicals. All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless stated otherwise. F12K medium, Hank's balanced salt solution (HBSS), trypsin-EDTA, non-heat-inactivated fetal calf serum (FCS), and penicillin/streptomycin were obtained from Gibco (Breda, The Netherlands). Endothelial cell growth supplement (ECGS) was obtained from BD Bioscience (Breda, The Netherlands). Heparin was purchased from Leo Pharmaceuticals (Amsterdam, The Netherlands).

2.2. Cell Culture. Human umbilical vein endothelial cells (HUVECs) (CRL-1730) were obtained from ATCC. HUVECs were cultured in F12K medium with 10% FCS, 1% penicillin/streptomycin, 0.05 mg/mL endothelial cell growth supplement (ECGS), and 0.1 mg/mL heparin. Cells were maintained in collagen coated flasks at 37°C in a 5% CO_2 atmosphere. For experiments, cells were seeded in 6- or 96-well plates and allowed to attach overnight. Next, medium was removed and cells were washed with HBSS. Additionally, fresh medium was added containing glucose (30 mM final concentration) or vehicle (medium) and flavonoids (5 μM final concentration), sorbinil (0.5 μM final concentration), or its vehicle (DMSO).

2.3. Gene Expression Analysis. RNA was isolated from QIAzol suspended cells according to the manufacturer's protocol and quantified spectrophotometrically with a NanoDrop. RNA (500 ng) was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands). Next, real time PCR was performed with a Bio-Rad MyIQ iCycler Single Color RT-PCR detection system using Sensimix Plus SYBR and Fluorescein (Quantace-Bioline, Alphen a/d Rijn, The Netherlands), 5 μL diluted (10x) cDNA, and 0.3 μM primers in a total volume of 25 μL. PCR was conducted as follows: denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. After PCR, a melt curve (60–95°C) was produced for product identification and purity. β-actin was included as internal control. Primer sequences are shown in Table 1. Data were analysed using the MyIQ software system (Bio-Rad) and were expressed as relative gene expression (fold change) using the $2^{\Delta\Delta Ct}$ method.

2.4. Determination of NAD^+ Levels. Cells were lysed with 1% dodecyltrimethylammonium bromide (DTAB) in 0.2 N NaOH. To ensure that only NAD^+ levels were measured 0.4 M HCl was added and samples were incubated at 60°C for 15 minutes. Afterwards, cells were incubated at room temperature for 10 minutes and 0.5 M Trizma base was added to the cells after which NAD^+ levels were determined with the NAD^+/NADH cell based assay kit from Cayman Chemical (Ann Arbor, MI, USA).

2.5. Preparation of Lens Aldose Reductase. Porcine lenses were used as a source of aldose reductase enzyme. Porcine eyes were obtained from a local slaughterhouse. Lenses were removed and stored at −20°C until use. Lens homogenate was freshly prepared for every experiment. Lenses were homogenized in 1.25 mL homogenization buffer (20 mM potassium phosphate buffer, pH 7.5 containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol). The homogenate was centrifuged at 10.000 ×g for 10 minutes at 4°C.

2.6. Aldose Reductase Assay. Aldose reductase activity was determined spectrophotometrically. The reaction mixture (0.7 mL) contained 30 mM potassium phosphate buffer (pH 6.2), 0.2 mM NADPH, 0.2 M lithium sulphate, and the substrate DL-glyceraldehyde (0–2 mM). Flavonoids (flavone,

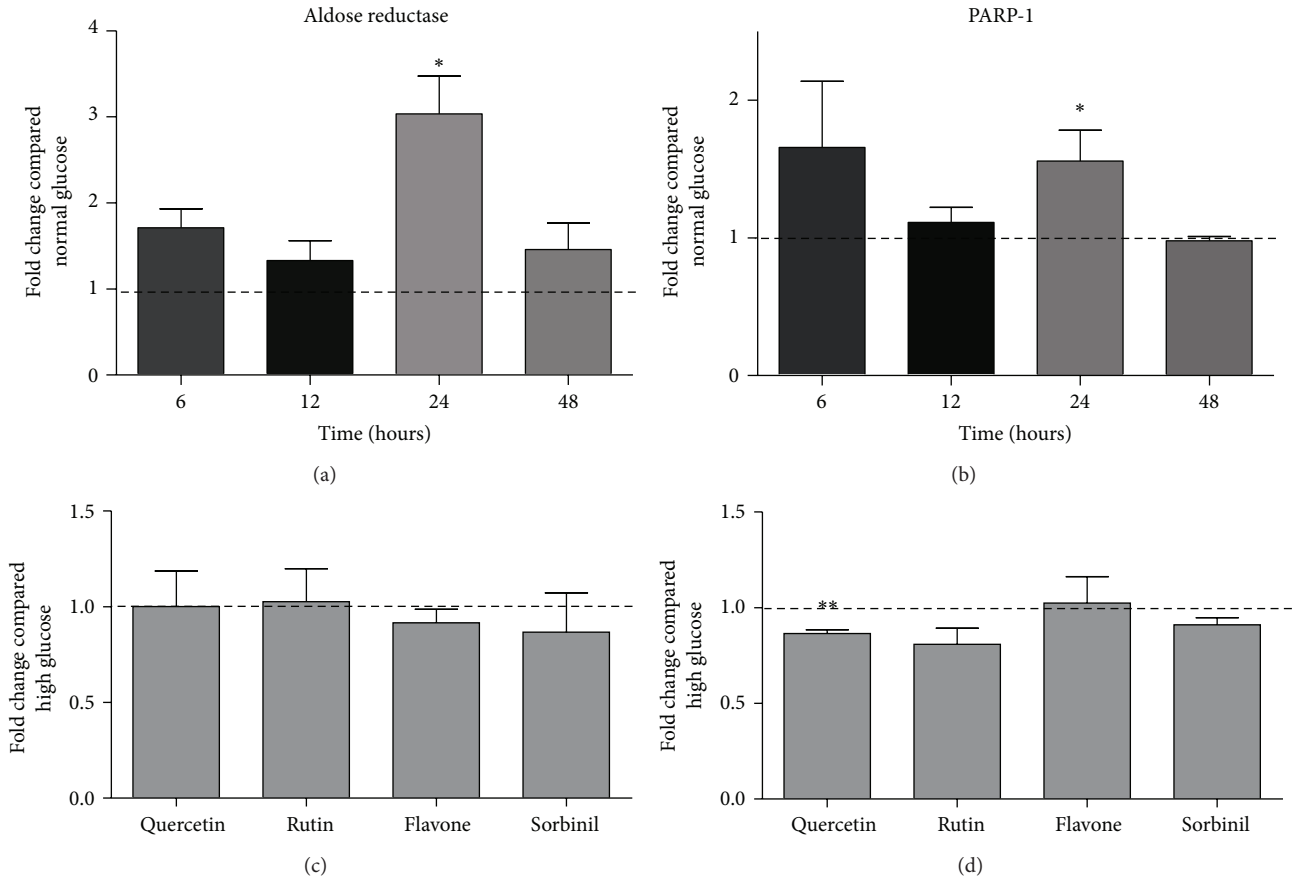


Figure 1: Effect of incubation with 30 mM glucose on the expression of aldose reductase (a) and PARP-1 (b) after several incubation times. Effect of addition of flavonoids (compared to high glucose incubation) after 24 hours incubation is shown in (c) (aldose reductase) and (d) (PARP-1). Data are expressed as mean ± standard error from three independent experiments. $^{*}P < 0.05$ compared to normal glucose incubation; $^{**}P < 0.05$ compared to incubation with high glucose alone.

quercetin, and rutin) were added to the reaction mixture (final concentration 0.5 or 5 μM). As a positive control, the known aldose reductase inhibitor sorbinil was used in a concentration of 0.5 μM. Reaction was initiated by addition of NADPH. The consumption of NADPH was followed by the decrease in absorbance at 340 nm for 5 minutes at 37°C.

2.7. Statistical Analysis. The effect of HG incubation and effects of flavonoids were tested using Student's t-test for independent samples or the Mann-Whitney U test when not normally distributed. P values < 0.05 were considered statistically significant and P values < 0.1 were considered statistical trends. Statistical analyses were analyzed with SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL, USA).

3. Results

In Figure 1, the effect of incubating HUVECs with 30 mM of glucose on gene expression of aldose reductase and PARP-1 is presented. It is visible that both aldose reductase and PARP-1 have a significant higher expression after 24-hour incubation compared to normal glucose. When flavonoids are coincubated during these 24 hours, there is no effect on aldose reductase expression compared to only high glucose incubation. Only quercetin seems to lower PARP-1 expression compared to high glucose incubation.

The effect of incubation with 30 mM glucose on the NAD^+ status of HUVECs is depicted in Figure 2. High glucose incubation leads to a significant decrease in NAD^+ levels after 24 hours. This decrease is attenuated when the cells are coincubated with flavone or quercetin (trend) but not with rutin. Incubation with the known aldose reductase inhibitor sorbinil led to an even larger decrease in NAD^+ levels.

Quercetin, rutin, and flavone at a concentration of 5 μM decreased the V_{max} of the aldose reductase catalysed conversion of DL-glyceraldehyde to glycerol. Sorbinil was used as a control and decreased both the V_{max} and K_m at a concentration of 0.5 μM. Rutin also showed a small but significant decrease of K_m compared to the control (Figure 3).

4. Discussion

In epidemiological studies, the intake of flavonoids has been related to a reduced risk for various diseases, including

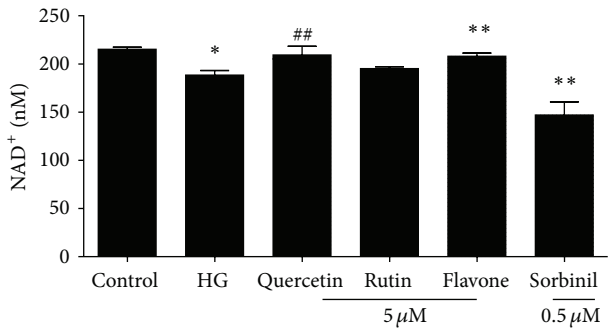


Figure 2: Effect of 24-hour incubation with 7 (control) or 30 mM (HG) glucose on the NAD^+ level of HUVECs with or without coincubation with flavonoids. Data are expressed as mean ± standard error from four independent experiments. $^*P < 0.05$ compared to control; $^{**}P < 0.05$ compared to incubation with high glucose alone; $^{\#\#}P < 0.1$ compared to incubation with high glucose alone.

diabetes [23, 25, 26]. Many complications that arise from diabetes are attributed to a redox imbalance. In previous studies we established that flavonoids were able to attenuate NAD^+ depletion by inhibiting PARP overactivation both *in vitro* and *in vivo* [17–19]. Extending these studies, we here provide evidence that flavonoids are also able to protect endothelial cells against a decrease in NAD^+ due to high glucose. In addition we show that flavonoids are able to inhibit the key enzyme of the polyol pathway, aldose reductase. From previous (unpublished) experiments we know that flavonoids at the concentration used in this study (5 μM) do not influence glucose uptake. The flavonoids' concentration needs to be at least 10-fold higher to influence the uptake of glucose.

In this study three structurally related flavonoids were studied: flavone, the core structure of the flavonoid subgroup flavones, a compound that is present in many cereal grains as well as in dill weed [27]; quercetin, one of the most prominent dietary flavonoids present in many foods including citrus fruit and berries [28]; and rutin, a glycoside of quercetin which is found in buckwheat [29]. These compounds are usually conjugated to sugar moieties but are certainly of interest as protectors during inflammatory conditions when the pH and glucuronidase appear favorable for deconjugation as has been shown by [30].

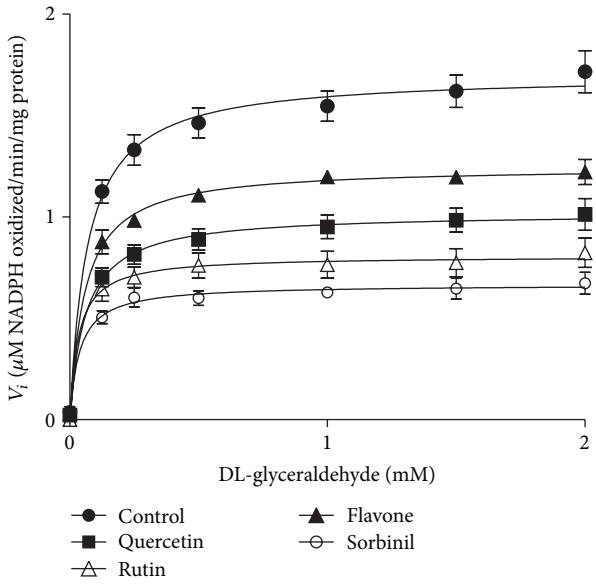
Gene expression of aldose reductase and PARP was investigated in endothelial cells exposed to 30 mM glucose. A higher expression of aldose reductase in peripheral blood mononuclear cells has been linked to an increased risk for kidney disease in diabetic patients [31]. Furthermore, in transgenic mice, it was found that human aldose reductase expression increased atherosclerosis lesion size which could be attenuated by aldose reductase inhibitors [32, 33]. An increase in PARP mRNA expression was found in patients with type 2 diabetes and microangiopathy [34]. We found an increase in the expression of both genes when endothelial cells were exposed to 30 mM glucose for 24 hours. Coincubation with flavone, rutin, or sorbinil did not affect this increase.

NAD^+ is a cofactor in numerous critical oxidation reactions. Because of the involvement in redox signalling, NAD^+ : NADH is regarded as one of the most important redox couples of the cells and therefore an important determinant of redox status of cells. We found a slight decrease in NAD^+ levels after incubating HUVECs with 30 mM glucose for 24 hours. This change is most likely a combination of the two previously described pathways: a decrease in NAD^+ due to activation of the polyol pathway and overactivation of PARP-1. Therefore we also investigated the potential of flavonoids to inhibit aldose reductase. The flavonoids' ability to inhibit aldose reductase has been described previously [35]. In our study, it was found that all tested flavonoids were able to inhibit aldose reductase enzyme activity at a concentration of 5 μM. Quercetin and flavone appear to be noncompetitive inhibitors because only the $V_{\max}$ of the reaction is decreased. Conversely, not only did rutin decrease the $V_{\max}$, but it also decreased the K_M slightly. This would indicate a slightly higher reaction rate at very low substrate concentrations but a much lower rate at higher substrate concentrations. Rutin contains rutinose, which is a disaccharide composed of rhamnose and glucose. The latter is a substrate of aldose reductase; however the affinity of aldose reductase for DL-glyceraldehyde is higher [7]. Rutin as a competitive inhibitor is further supported by the results of sorbinil, which is a known competitive inhibitor of aldose reductase [36]. Sorbinil was tested at a lower concentration (0.5 μM) but shows the same results as rutin, a decrease in both $V_{\max}$ and K_M. Of the tested flavonoids, rutin showed the strongest inhibition, while flavone had the least effect. This is contrary to their capacity to inhibit PARP overactivation, where flavone is the most potent inhibitor and rutin is not able to inhibit PARP (Table 2). In both reactions quercetin is an intermediate inhibitor compared to rutin and flavone.

These findings indicate that the overactivation of PARP-1 plays a larger role than the polyol pathway in the decrease of NAD^+ levels in HUVECs. When cells were coincubated with flavonoids, we observed that flavone was able to attenuate the decrease in NAD^+ concentration. Flavone is the most potent PARP-1 inhibitor but did not have a large effect on aldose reductase activity. This finding is also supported by the observation that rutin, the most potent aldose reductase activity inhibitor, did not show an effect on NAD^+ levels. Quercetin, an average inhibitor of both pathways, showed a trend towards increasing NAD^+ levels to normal. The influence of the polyol pathway on the lower NAD^+ level seems to be small. Most likely the activation of this pathway has a more pronounced effect on the levels of NADPH. By lowering the levels of this essential cofactor for glutathione, the cells get more susceptible to oxidative stress [37]. This in turn can lead to more reactive oxygen species that can damage DNA, inducing activation of PARP-1, which subsequently can lead to a decrease in NAD^+ levels as we observed in HUVECs. This might also be the reason why coincubation with sorbinil leads to an extra decrease in NAD^+ levels in HUVECs. By inhibiting the aldose reductase almost completely, unlike the flavonoids which show a mild inhibition, other pathways involved in the pathogenesis of diabetic complications may

	K_m (mM)	V_{max} (μM NADPH oxidized/min/mg protein)
Control	0.065 ± 0.02	1.68 ± 0.31
0.5 μM sorbinil	0.038 ± 0.003*	0.67 ± 0.06*
0.5 μM flavone	0.071 ± 0.04	1.61 ± 0.18
5 μM flavone	0.058 ± 0.02	1.25 ± 0.04*
0.5 μM quercetin	0.084 ± 0.01	1.78 ± 0.33
5 μM quercetin	0.062 ± 0.02	1.02 ± 0.18*
0.5 μM rutin	0.056 ± 0.02	1.53 ± 0.29
5 μM rutin	0.034 ± 0.01*	0.81 ± 0.10*

(a)



(b)

Figure 3: (a) Kinetics of porcine aldose reductase in the absence and presence of flavonoids. Data are expressed as mean ± standard deviation of at least three separate experiments. * $P < 0.05$ compared to control. (b) An example of a Michaelis Menten plot of aldose reductase in absence (filled circles) and presence of 5 μM quercetin (filled squares), 5 μM rutin (open triangles), 5 μM flavone (filled triangles), or 0.5 μM sorbinil (open circles). Data are expressed as mean ± standard error of at least three experiments.

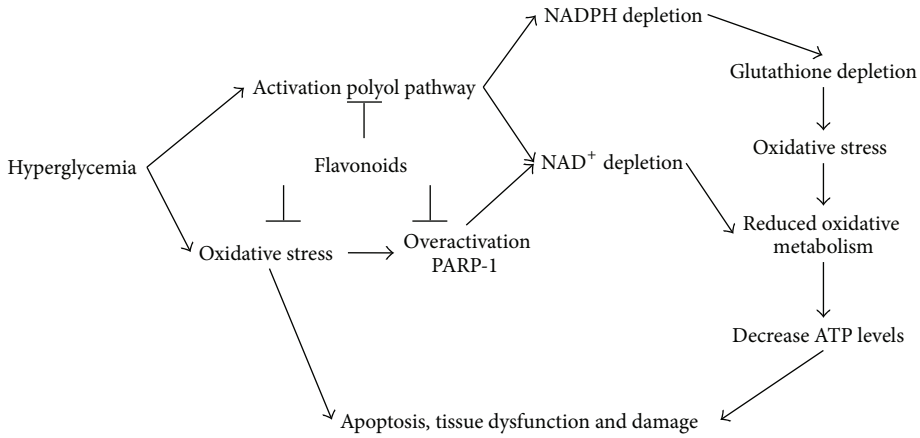


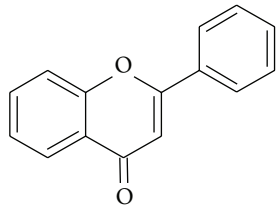
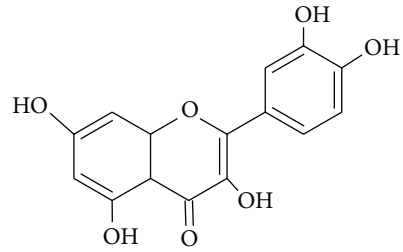
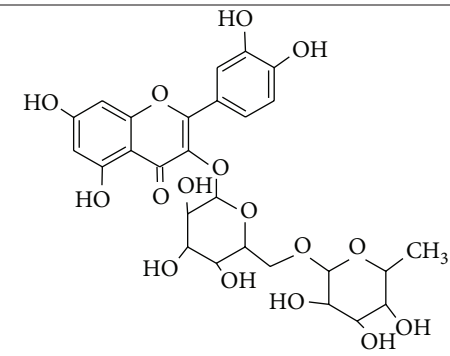
Figure 4: Flavonoids can protect cells under hyperglycemic stress in several ways. First, flavonoids are able to inhibit overactivation of PARP-1, preventing a decrease in NAD^+ levels. Furthermore, flavonoids are able to inhibit aldose reductase activity, preventing an additional decrease in NAD^+ and NADH levels. Also, because of their antioxidant properties, flavonoids are able to prevent damaging effects of oxidative stress. By a combination of all these effects flavonoids are able to protect cells against high glucose induced damage.

become more activated (e.g., activation PKC); this then can lead to more oxidative stress and activation of PARP-1 [37].

5. Conclusions

We conclude that flavonoids are able to exert pleiotropic protective effects under high glucose conditions (Figure 4). We observed that flavonoids were able to inhibit overactivation of PARP-1, thereby preventing a fall in NAD^+ levels. Furthermore we observed that flavonoids are able to inhibit aldose reductase activity, preventing an additional decrease in NAD^+ levels. Moreover, because of the known antioxidant properties of flavonoids they are also able to prevent the deleterious effects of reactive oxygen species which can be formed when a redox imbalance is present. In conclusion,

Table 2: Overview of structure and PARP inhibiting capacity of flavonoids used in this study [17, 18].

Name	Flavone	Quercetin	Rutin
Structure			
PARP inhibiting capacity	Strong, also at low concentrations	Strong, less at low concentrations	No inhibiting capacity

the combination of all these effects is most likely the reason why flavonoids were able to protect endothelial cells against a high glucose induced drop in NAD^+ levels in an *in vitro* system.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] IDF, *IDF Diabetes Atlas*, International Diabetes Federation, 6th edition, 2013.

[2] E. Crimi, L. J. Ignarro, and C. Napoli, "Microcirculation and oxidative stress," *Free Radical Research*, vol. 41, no. 12, pp. 1364–1375, 2007.

[3] D. Aronson, "Hyperglycemia and the pathobiology of diabetic complications," *Advances in Cardiology*, vol. 45, pp. 1–16, 2008.

[4] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[5] Y. Ido, "Pyridine nucleotide redox abnormalities in diabetes," *Antioxidants and Redox Signaling*, vol. 9, no. 7, pp. 931–942, 2007.

[6] K. H. Gabbay, "The sorbitol pathway and the complications of diabetes," *The New England Journal of Medicine*, vol. 288, no. 16, pp. 831–836, 1973.

[7] A. Bhatnagar and S. K. Srivastava, "Aldose reductase: congenial and injurious profiles of an enigmatic enzyme," *Biochemical Medicine and Metabolic Biology*, vol. 48, no. 2, pp. 91–121, 1992.

[8] C. Yabe-Nishimura, "Aldose reductase in glucose toxicity: a potential target for the prevention of diabetic complications," *Pharmacological Reviews*, vol. 50, no. 1, pp. 21–33, 1998.

[9] T. Oyama, Y. Miyasita, H. Watanabe, and K. Shirai, "The role of polyol pathway in high glucose-induced endothelial cell damages," *Diabetes Research and Clinical Practice*, vol. 73, no. 3, pp. 227–234, 2006.

[10] G. B. Reddy, A. Satyanarayana, N. Balakrishna et al., "Erythrocyte aldose reductase activity and sorbitol levels in diabetic retinopathy," *Molecular Vision*, vol. 14, pp. 593–601, 2008.

[11] S. K. Srivastava, K. V. Ramana, and A. Bhatnagar, "Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options," *Endocrine Reviews*, vol. 26, no. 3, pp. 380–392, 2005.

[12] P. Pacher and C. Szabo, "Role of the peroxynitrite-poly(ADP-ribose) polymerase pathway in human disease," *The American Journal of Pathology*, vol. 173, no. 1, pp. 2–13, 2008.

[13] H. J. J. Moonen, L. Geraets, A. Vaarhorst, A. Bast, E. F. M. Wouters, and G. J. Hageman, "Theophylline prevents NAD^+ depletion via PARP-1 inhibition in human pulmonary epithelial cells," *Biochemical and Biophysical Research Communications*, vol. 338, no. 4, pp. 1805–1810, 2005.

[14] F. Li, C. Szabó, P. Pacher et al., "Evaluation of orally active poly(ADP-ribose) polymerase inhibitor in streptozotocin-diabetic rat model of early peripheral neuropathy," *Diabetologia*, vol. 47, no. 4, pp. 710–717, 2004.

[15] A. G. Minchenko, M. J. Stevens, L. White et al., "Diabetes-induced overexpression of endothelin-1 and endothelin receptors in the rat renal cortex is mediated via poly(ADP-ribose) polymerase activation," *The FASEB Journal*, vol. 17, no. 11, pp. 1514–1516, 2003.

[16] C. Szabó, A. Biser, R. Benko, E. Böttinger, and K. Suszták, "Poly(ADP-ribose) polymerase inhibitors ameliorate nephropathy of type 2 diabetic Leprdb/db mice," *Diabetes*, vol. 55, no. 11, pp. 3004–3012, 2006.

[17] L. Geraets, H. J. J. Moonen, K. Brauers et al., "Flavone as PARP-1 inhibitor: its effect on lipopolysaccharide induced gene-expression," *European Journal of Pharmacology*, vol. 573, no. 1–3, pp. 241–248, 2007.

[18] L. Geraets, H. J. J. Moonen, K. Brauers, E. F. M. Wouters, A. Bast, and G. J. Hageman, "Dietary flavones and flavonoles are inhibitors of poly(ADP-ribose) polymerase-1 in pulmonary epithelial cells," *Journal of Nutrition*, vol. 137, no. 10, pp. 2190–2195, 2007.

[19] A. R. Weseler, L. Geraets, H. J. J. Moonen et al., "Poly (ADP-ribose) polymerase-1-inhibiting flavonoids attenuate cytokine release in blood from male patients with chronic obstructive pulmonary disease or type 2 diabetes," *Journal of Nutrition*, vol. 139, no. 5, pp. 952–957, 2009.

[20] M. G. L. Hertog, E. J. M. Feskens, P. C. H. Hollman, M. B. Katan, and D. Kromhout, "Dietary antioxidant flavonoids and risk of

coronary heart disease: the Zutphen Elderly Study," *The Lancet*, vol. 342, no. 8878, pp. 1007–1011, 1993.

[21] R. J. Nijveldt, E. van Nood, D. E. C. van Hoorn, P. G. Boelens, K. van Norren, and P. A. M. van Leeuwen, "Flavonoids: a review of probable mechanisms of action and potential applications," *The American Journal of Clinical Nutrition*, vol. 74, no. 4, pp. 418–425, 2001.

[22] A. Scalbert, I. T. Johnson, and M. Saltmarsh, "Polyphenols: antioxidants and beyond," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, pp. 215S–217S, 2005.

[23] Z. Bahadoran, P. Mirmiran, and F. Azizi, "Dietary polyphenols as potential nutraceuticals in management of diabetes: a review," *Journal of Diabetes & Metabolic Disorders*, vol. 12, p. 43, 2013.

[24] C. A. Rice-Evans, N. J. Miller, and G. Paganga, "Structure-antioxidant activity relationships of flavonoids and phenolic acids," *Free Radical Biology & Medicine*, vol. 20, no. 7, pp. 933–956, 1996.

[25] M. G. L. Hertog, P. C. H. Hollman, M. B. Katan, and D. Kromhout, "Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands," *Nutrition and Cancer*, vol. 20, no. 1, pp. 21–29, 1993.

[26] N. M. Wedick, A. Pan, A. Cassidy et al., "Dietary flavonoid intakes and risk of type 2 diabetes in US men and women," *The American Journal of Clinical Nutrition*, vol. 95, no. 4, pp. 925–933, 2012.

[27] U. Wenzel, S. Kuntz, M. D. Brendel, and H. Daniel, "Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells," *Cancer Research*, vol. 60, no. 14, pp. 3823–3831, 2000.

[28] S. H. Hakkinen, S. O. Kärenlampi, I. M. Heinonen, H. M. Mykkanen, and A. R. Torronen, "Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 6, pp. 2274–2279, 1999.

[29] S. Kreft, M. Knapp, and I. Kreft, "Extraction of rutin from buckwheat (*Fagopyrum esculentum* moench) seeds and determination by capillary electrophoresis," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 11, pp. 4649–4652, 1999.

[30] R. Bartholomé, G. Haenen, C. H. Hollman et al., "Deconjugation kinetics of glucuronidated phase II flavonoid metabolites by β-glucuronidase from neutrophils," *Drug Metabolism and Pharmacokinetics*, vol. 25, no. 4, pp. 379–387, 2010.

[31] V. O. Shah, R. I. Dorin, Y. Sun, M. Braun, and P. G. Zager, "Aldose reductase gene expression is increased in diabetic nephropathy," *Journal of Clinical Endocrinology and Metabolism*, vol. 82, no. 7, pp. 2294–2298, 1997.

[32] R. K. Vikramadithyan, Y. Hu, H.-L. Noh et al., "Human aldose reductase expression accelerates diabetic atherosclerosis in transgenic mice," *The Journal of Clinical Investigation*, vol. 115, no. 9, pp. 2434–2443, 2005.

[33] S. Vedantham, H. Noh, R. Ananthakrishnan et al., "Human aldose reductase expression accelerates atherosclerosis in diabetic apolipoprotein E-/-Mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 8, pp. 1805–1813, 2011.

[34] A. Adaikalakoteswari, M. Rema, V. Mohan, and M. Balasubramanyam, "Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 9, pp. 1673–1684, 2007.

[35] S. D. Varma, I. Mikuni, and J. H. Kinoshita, "Flavonoids as inhibitors of lens aldose reductase," *Science*, vol. 188, no. 4194, pp. 1215–1216, 1975.

[36] S.-Q. Liu, A. Bhatnagar, and S. K. Srivastava, "Does sorbinil bind to the substrate binding site of aldose reductase?" *Biochemical Pharmacology*, vol. 44, no. 12, pp. 2427–2429, 1992.

[37] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.

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Review Article

Resveratrol: A Focus on Several Neurodegenerative Diseases

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Molecules of the plant world are proving their effectiveness in countering, slowing down, and regressing many diseases. The resveratrol for its intrinsic properties related to its stilbene structure has been proven to be a universal panacea, especially for a wide range of neurodegenerative diseases. This paper evaluates (in vivo and in vitro) the various molecular targets of this peculiar polyphenol and its ability to effectively counter several neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's diseases and amyotrophic lateral sclerosis. What emerges is that, in the deep heterogeneity of the pathologies evaluated, resveratrol through a convergence on the protein targets is able to give therapeutic responses in neuronal cells deeply diversified not only in morphological structure but especially in their function performed in the anatomical district to which they belong.

1. Introduction

Resveratrol (RV), or 3,5,4′-trihydroxy-trans-stilbene, is an antifungal molecule of the stilbene family produced in a variety of plant species in response to pathogen attack or under stress conditions such as UV radiation and exposure to heavy metal ions [1]. It is a natural phenol found in red grapes, mulberries, peanuts, wines, and tea and it can be extracted from red wine during fermentation of grape skin. RV exists in two isoforms: trans-RV, the more stable form, and cis-isomer, both produced as a branch from the phenylpropanoid pathway [2]. In plants, RV biosynthesis starts by the coupling of *p*-coumaric acid, an intermediate in lignin production, to coenzyme A (CoA) by the action of 4-coumarate CoA ligase (4CL); see Figure 1. Subsequently, coumaroyl-CoA is converted into RV with release of carbon dioxide, by sequential addition of three units of malonyl-CoA by the action of stilbene synthase (STS) [3].

In in vitro and in vivo experiments, RV displays a wide range of beneficial effects on human diseases but the mechanisms by which RV exerts its action have not yet been clarified. After oral administration, RV is transported to the circulatory system and is distributed to all organs where it remains detectable for some hours after administration; it can also rapidly cross the blood-brain enriching the brain tissue [4]. However, one of the main limitations of this drug is its low oral bioavailability, due to rapid excretion and extensive metabolism into variants of glucuronide and sulfonated conjugates of unknown potential biological activities [5].

RV shows several mechanisms of action and interacts with a significant number of molecular targets, but its positive effect on the human health seems to be related mainly to its antioxidant activity. Since oxidative stress appears to be closely related to major neuronal pathologies, RV treatment has been tested with positive results in neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), prion, cerebral ischemia, Parkinson's disease (PD), epilepsy, and amyotrophic lateral sclerosis (ALS) [6], but there are quite a few studies to describe the dose dependency of the drug towards these health benefits.

This review aims to give an overview of the beneficial effects of RV on several human neurodegenerations as AD,

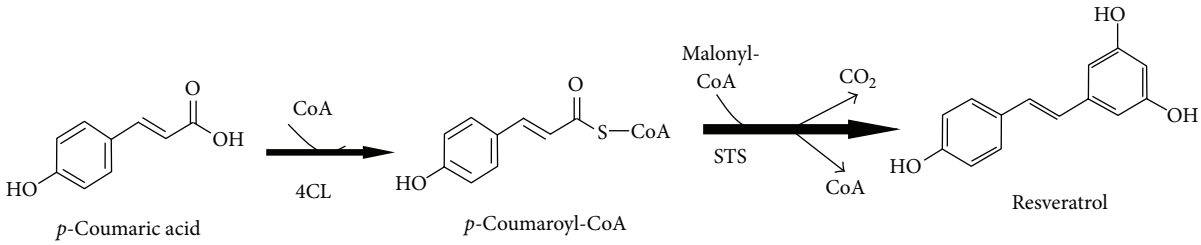

Figure 1: Biosynthetic pathway of RV.

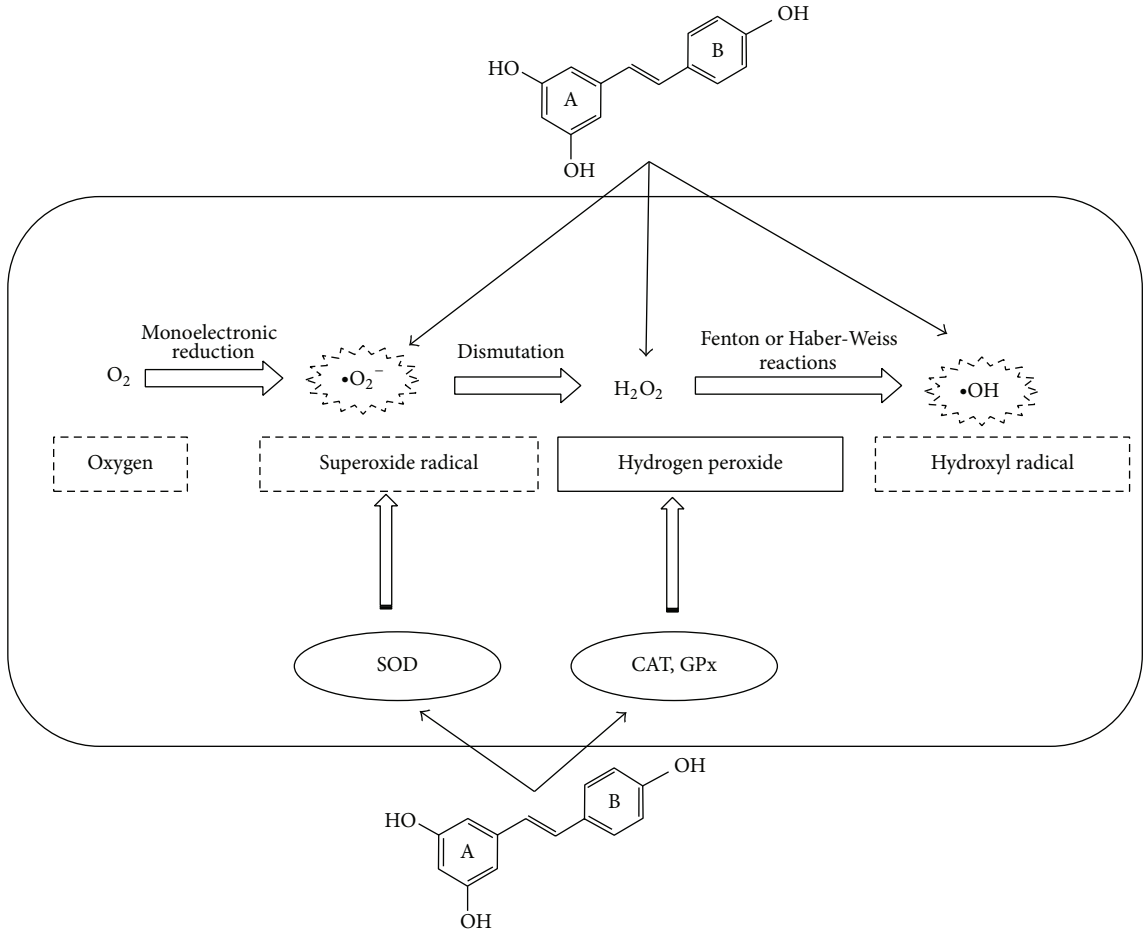

Figure 2: Antioxidant activity of RV against ROS.

PD, HD and ALS trying to highlight the mechanisms by which the polyphenol exerts its specific activity.

1.1. Oxidative Stress and Neurodegenerative Diseases. A suitable amount of free radicals is essential for life because they are involved in cell signaling and are used by phagocytes for their bactericidal action [7]. However, nonessential production of reactive oxygen species (ROS) is suggested to be strongly associated with the aging process and certain degenerative disorders. So to human health, the balance is very important between free radicals produced by metabolism or derived from environmental sources and the antioxidant defense systems such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) able to promptly scavenge and neutralize free radicals (Figure 2).

Among the pathologies linked to oxidative stress, the neurodegenerative disorders occupy a relevant side because neurons are particularly vulnerable to attack by free radical attacks and oxidative stress is one of the major pathogenic mechanisms in the etiology of a variety of late onset diseases. The high vulnerability of the nervous system, including the brain, spinal cord, and peripheral nerves, to oxidative stress is due to its elevated bioenergetics and oxygen requirements.

In fact neurons with axons and multiple synapses have high ATP demand and they are largely responsible for the brain's massive consumption of oxygen in the respiratory chain; this coupled with the high content of lipid and easily mobilizable iron from several areas of the brain can stimulate the generation of ROS (Fenton and/or Haber-Weiss reactions) [8]. Since age is related to the reduced capacity to counteract oxidative stress, also this can lead to irreversible damages that can contribute to the pathogenesis of neurodegenerative disorders. At the pathological level, almost all neurodegenerative diseases share common features such as the generation of misfolded protein deposits, metal ion deregulation, and exposure to oxidative stress [9–13]. Generally, the protein aggregates are primarily composed of typical proteins in different diseases. For example, in AD the extracellular senile plaques are predominantly consisted of amyloid-β (Aβ) peptides derived from the mutations in genes encoding the amyloid precursor protein (APP), while the intracellular tangles are from hyperphosphorylated Tau protein; HD is caused by the gene mutation that affects the conformation and aggregation propensity of the huntingtin protein (htt) [14]; in PD the accumulation of intracytoplasmic Lewy bodies is mainly composed of α-synuclein and ubiquitin [15]; similarly the protein products of the associated diverse set of genes including SOD1, TDP43, FUS, UBQLN2, and C9OKF72 have also been found in neuronal aggregates from ALS patients [16–18].

Besides, iron changes have been detected in multiple sclerosis, spastic paraplegia, and ALS, reinforcing the belief that iron accumulation is a secondary alteration associated with neurodegeneration, probably due to the changes in the integrity of the blood brain caused by abnormal vascularization of tissue or by inflammatory events [19].

It is demonstrated yet that markers of oxidative stress precede pathologic lesions in AD, including senile plaques and neurofibrillary tangles [20]. Furthermore, ATP depletion or lipid and protein peroxidation induced by ROS is also implicated in PD and kills neurons by necrotic processes [21]; protein oxidative damage in the form of protein carbonyls and increased levels of 8-hydroxydeoxyguanosine are also present in PD brain and some evidences suggest a role for nitration and nitrosylation of certain proteins due to reactive nitrogen species [22, 23]. In this context, the linkage between neurodegenerative diseases and oxidative stress is largely investigated by researchers. Support for this curiosity comes from increasing attention to the efficacy of therapies with antioxidants and to the scavenger substances as protectors of nervous tissue from damage by oxidative stress. Clearly, strategies aimed at limiting free radical production, oxidative stress, and damage may slow the progression of neurodegenerative diseases. Actually there is a great interest to study the neuroprotective effects of natural products obtained from plants. There are several natural compounds with antioxidant properties which may contribute to counteracting oxidative stress by working to neutralize the excess free radicals and stopping them from starting the chain reactions that contribute to disease. In this context, RV for its chemical properties may be a very promising lead compound to counteract neuronal pathologies (Figure 3).

1.2. Antioxidant and Prooxidant Effects of RV. The antioxidant and prooxidant activities of RV appear to be dose and cell type dependent. In particular, antioxidant properties of RV seem to be enhanced with increasing concentration of the drug and Cavallaro et al. [24] demonstrated that RV inhibited superoxide anion generation both in low and high concentrations.

RV's activity as antioxidant and free radical scavenger is related to its ability to transfer hydrogen atoms or electrons to the free radicals [25, 26]. In this context, the characteristic position of hydroxyl groups plays a major role, among which the 4′-hydroxyl group is the most reactive one [27, 28]. RV antioxidant properties result from its chemical structure; in fact the molecule contains two phenol groups in which the presence of conjugated double bond makes the electrons more delocalized. Support of this hypothetic mechanism of action comes from studies on oxyresveratrol (oxy-RV), demonstrating in the modified drug a more effective antioxidant activity than RV probably due to the extra hydroxyl group on oxy-RV which makes oxy-RV a better hydrogen donor enhancing its antioxidant activity [29]. So RV may donate hydrogen to free radicals inhibiting the peroxidation and protecting cellular DNA, lipids, and proteins from oxidative damage.

Recently, using planar lipid bilayer and liposome models, it has been shown that RV at low doses interacts with the surface polar groups and at higher doses localizes in the outer leaflet of the lipid bilayer. Interestingly, RV localization is strictly related to the antioxidant properties of the drug, because the polyphenol localization in the membrane bilayer prevents lipid peroxidation [30] and intraerythrocyte RV, by interacting with hemoglobin, may protect the protein against oxidative damage [31]. The drug breaks the chain-reaction process of lipid peroxidation by scavenging free radicals and forming phenoxy radicals that are stabilized by resonance. For the global reactivity of RV toward $^{\bullet}OH$ radical, the most electrophilic radical is the sequential electron proton transfer (SEPT): $RV + {}^{\bullet}OH \rightarrow RV^{+\bullet} + OH^{-} \leftrightarrow RV(-H)^{\bullet} + H_2O$ [26].

RV shows a moderate antioxidant activity towards the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, induces a significant reduction of superoxide anion, and decreases oxidation of hemoglobin, contributing to decreasing the superoxide concentration [31, 32]. In particular, RV action against oxidation of hemoglobin may be due to the action of its phenol groups which are able to reduce Fe^{3+} to Fe^{2+}. It is also known that RV prevents low density lipoprotein (LDL) oxidation, responsible of atheromatous plaques in atherosclerosis disease [33]; in fact RV was shown to be more potent than flavonoids in preventing copper-catalyzed oxidation [34] and contributed to maintaining the levels of antioxidant enzymes like GPx, glutathione-S-transferase (GST) and reductase (GR), SOD, and CAT [35, 36]. Most likely part of beneficial properties of RV is probably related to concomitant downregulation of the expression of inducible NO synthase (iNOS) and upregulation of vasorelaxant endothelial NO synthase (eNOS) as observed by several studies [37–39]. Interestingly, RV acting as an antioxidant prevents the formation of toxic Aβ oligomers and protofibrillar intermediates, delaying

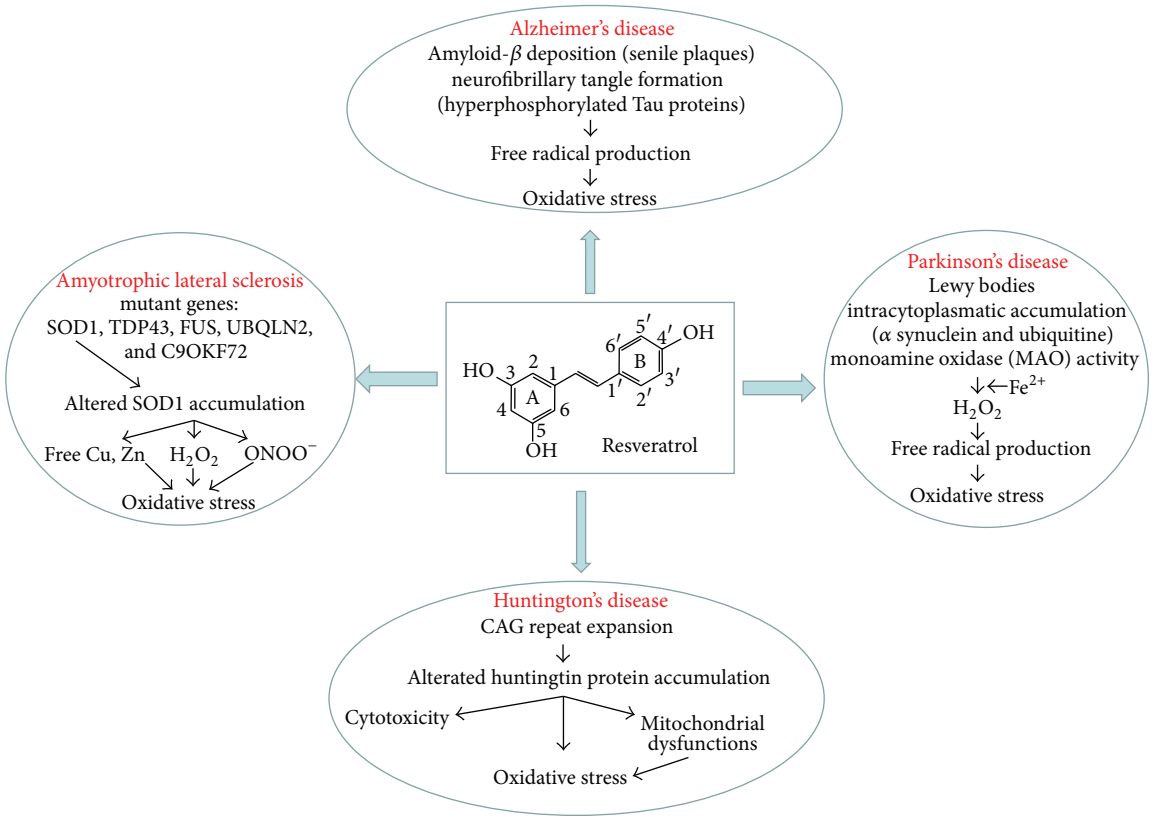

Figure 3: RV and neurodegenerative disease.

the induced Aβ toxicity in different neuronal culture models [40]. These studies contributed to shedding light on the molecular mechanisms potentially involved in the beneficial effect of RV intake against AD (Figure 4) [41].

On the other hand, Ahmad results suggest prooxidant properties of RV at low concentration due to an increase in intracellular superoxide production and in the presence of copper ions [42, 43]. In detail, RV promotes the reduction of copper (II) to copper (I) [36] but its binding with copper promotes prooxidant activity of the drug [44].

1.3. RV Molecular Targets. Although the interest on RV was initially focused on its antioxidant properties, it has been reported that the drug affects a wide range of signaling transduction pathways.

Several studies using both in vitro and in vivo model systems have illustrated RV capacity to modulate a multitude of biological activity associated with cellular growth and differentiation, apoptosis, angiogenesis, and metastasis [45–47]. Thus, RV modulates multiple signaling pathways that interrupt the carcinogenic process and is also able to extend one or more stages of this process. Also, RV has been shown to inhibit a plethora of enzymes belonging to different classes, including (but not limited to) kinases, lipo- and cyclooxygenases, sirtuins, and other proteins. Furthermore, RV is reported to induce cell cycle arrest in many cancer cell lines, probably through the modulation of cyclin dependent kinase (CDK) associated proteins and through the activity of the tumor suppressor protein p53 dependent and independent pathways [48–50]. p53 is a key mediator in the prevention of carcinogenesis because it is involved in the regulation of cell proliferation and apoptosis [51].

In addition, RV has been shown to mediate the activation of sirtuin-1 (SIRT1). Sirtuin enzymes are a family of highly conserved deacetylase proteins with potential therapeutic targets in a variety of human diseases including diabetes, inflammatory disorders, and neurodegenerative diseases [18].

RV antagonizes calcium cytoplasmic elevation and neurotoxicity generated by ASL [52, 53] and shows many antioxidant properties. RV has been proven to exert neuroprotection against glutamate toxicity in neuronal cultures [54] and through P13K/Akt pathway by downregulating the expression of glycogen synthase kinase 3 (GSK-3β) [55]. GSK-3β is involved in multiple signaling pathways and has several phosphorylation targets; it is mainly localized in the cytosol, but lower amounts are expressed in the nucleus and mitochondria, where it has a regulatory role in the cell death pathway elicited by stress conditions [56, 57]. A number of studies on cerebral blood flow (CBF) and cognitive performance in humans provide evidences that RV administration can modulate brain functions improving glucose metabolism [58] and vasorelaxation by promoting eNOS and/or NO synthesis [59, 60].

RV positively influences telomeres length promoting the expression of Werner syndrome ATP-dependent-helicase, a telomere maintenance factor [61, 62]; this protection

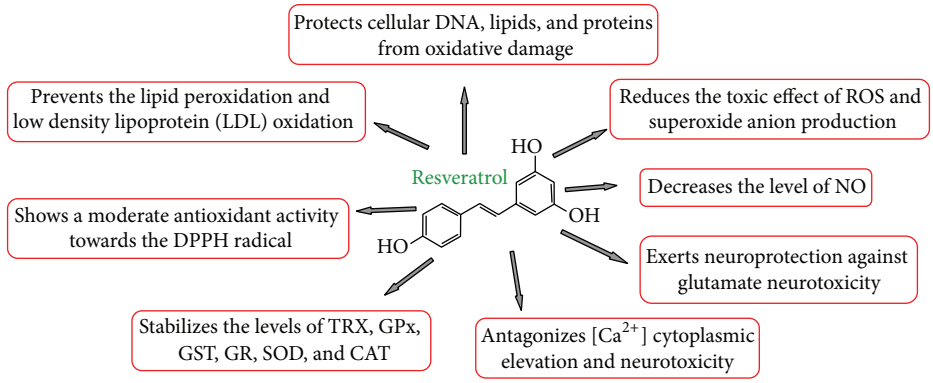

Figure 4: Main effects of RV.

is important for mitochondrial efficiency and oxidative stress defenses because telomere shortening, activating p53 represses the transcription of the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) and impairs mitochondrial function [63]. But RV stimulates PGC-1α also through its interaction with SIRT1 where deacetylating activates PGC-1α. PGC-1α is a potent stimulator of mitochondrial biogenesis and respiration because it induces the transcription of nuclear respiratory factor (NRF)1 and NRF2, leading to the increased expression of mitochondrial transcription factor A (mtTFA) [64] as well as other nuclear-encoded mitochondria subunits of the electron transport chain complex [65].

RV increased cAMP and modulated Akt pathway in cell model studies [66]; besides, RV activates AMP protein kinase-SIRT1 autophagy pathway in PD cell model studies [67], upregulates antiapoptotic Bcl-2 protein, and downregulates Bax protein expression [68] and also acts as mitochondrial antioxidant by elevating the levels of antioxidants thioredoxin-2 (TRX2) and X-chromosome-linked inhibitor of apoptosis protein [69]. Another study has shown that RV increased expression of Bcl-2, thus preventing neuronal apoptosis [70]. RV appears to be effective in reducing the inflammatory status; the drug attenuates the activation of immune cells and subsequent synthesis and release of inflammatory mediators through the inhibition of transcription factors such as nuclear factor-kappaB (NF-κB) [71].

2. RV and Alzheimer's Disease

AD is a progressive, age dependent neurodegenerative disorder leading to the most common form of dementia in elderly people. Histopathological studies of the AD brain revealed in the cortex and hippocampus the presence of ultrastructural changes triggered by two classical lesions, the extracellular senile plaques mainly composed of Aβ peptides and intracellular neurofibrillary tangles composed of hyperphosphorylated Tau proteins [72, 73]. Tau is a multifunctional microtubule-associated protein that plays major role in assembly of microtubules and in bridging these polymers with other cytoskeletal filaments [74]. The earliest modification found in AD brains consists of hyperphosphorylation on Tau by the action of different protein kinase and phosphatase systems that lead to structural and conformational changes in this protein, affecting its binding with tubulin and the capacity to promote microtubule assembly [75]. The most relevant protein kinases involved in Tau modification in neurofibrillary degeneration are GSK3β [76]. GSK3β would increase Tau hyperphosphorylation at sites that transform Tau into a protein lacking the ability to associate with cytoskeleton.

Although most of the AD cases are sporadic with an obscure etiology, some forms are inherited and several genes encoding APP, presenilin 1 (PS1), and presenilin 2 (PS2) were found to be implicated in familial forms of the disease. In both cases (familial and sporadic) Aβ peptides were regarded as a causative event in the pathogenesis of the APP by β and γ secretases. The formation of diffusible Aβ oligomers that can aggregate and form fibril and amyloid deposition plaques is a process that initiates the synaptic malfunction and the AD toxic effects [77]. Neuropathologic studies show an increased rate of apoptotic neurons in postmortem sample from AD patients [78]. Apoptosis, is due to a number of cascades of cellular events involving caspase activation that actively kills the cell. In the nervous system, apoptosis appears to be triggered by trophic factor deprivation. The lack of activation of intracellular pathway transducing trophic factor leads to caspase activation. Trophic factor deprivation in neurons may result in dephosphorylation of BAD that interacts with Bcl-2 facilitating the release from mitochondria of cytochrome C and apoptosis-activating factor (apaf) which finally leads to caspase 3 activation [79]. These findings in motor neurons induce oxidative stress involving the production of nitric oxide, superoxide, and peroxynitrite which also activate caspase 3, suggesting a more general role of the oxidants as mediator of apoptosis. At this regard, we should not forget that also presenilins seem to play a role as modulators of neuronal apoptosis too. RV has been shown to inhibit Aβ fibrils formation [80, 81] by degreasing Aβ production through sirtuin dependent activation; the drug potentiates SIRT1 activity via an allosteric mechanism [82, 83]. In detail, neuronal SIRT1 expression decreased levels of ROCK1, a serine/threonine Rho-kinase previously shown to regulate Aβ metabolism and this effect enhanced α-secretase activity, an enzyme which process APP along a nonamyloidogenic

pathway [84, 85]. Additionally in vitro observations indicated that SIRT1 can directly deacetylate Tau protein at multiple residues. The removal of these acetyl groups may expose Lys residues to ubiquitin ligases so that Tau protein could be marked for proteasomal degradation [86, 87].

Hooper et al. [88] reported that p53 is upregulated approximately 2-fold in the superior temporal gyrus of AD and that p53 induces Tau indirect phosphorylation. Thus, p53 seems to play a pivotal role in AD implying that modulation of cell death pathways might be of therapeutic benefit (and indeed in other age related neurological disorders). The identification of p53 as a SIRT1 substrate highlights a further protective role of RV in AD-related cognitive decline. In fact, allosteric modulation of RV on SIRT1 activating deacetylation of p53 attenuates its activity [89]. Additionally, inhibition of p53 by RV might alter and in some way partially inhibit the GSK3β and p53 interaction. Since p53 and GSK3β are both involved in the apoptotic pathway (GSK3β overactivity leads to increased levels of plaques and tangles and p53 activity induces Tau phosphorylation), a strong RV effect may be speculated on AD against several molecular targets. Besides, Vingtdeux et al. [90] demonstrated the antiamyloidogenic effect of RV through activation of AMP-protein kinase (AMPK). AMPK is a heterotrimeric Ser/Thr protein kinase activated by different upstream kinases among which calcium/calmodulin-dependent protein kinase kinase-β (CamKKβ) is predominantly expressed in neuronal tissue [91]. AMPK signaling controls Aβ metabolism and RV increasing intracellular calcium levels promote AMPK activation by the CamKKβ pathway [90, 92–94]. Alterations of mitochondrial functioning followed by ROS generation are two alarming conditions known in aging and early stages of AD [95]. RV efficiently counteracts both pathological conditions, on the one hand through activation of SIRT1 and the PGC1α pathway that lead to improved mitochondrial function and efficiency and on the other hand through its antioxidant activity reducing ROS generation [31, 32, 96–98] (Figure 5).

3. RV and Huntington's Disease

HD is an autosomal-dominant neurological disorder; the most striking pathological manifestation of the disease is a gradual loss of neurons predominantly in the striatum causing motor abnormalities and cognitive decline [99]. HD genesis is caused by an unstable trinucleotide CAG repeat expansion at the N-terminus of the gene encoding htt [100]. The mutation leads to the production of the htt with an abnormal protein-protein interaction named mutant polyglutamine htt (m-htt) which forms cytotoxic aggregates in neurons [101, 102]. Overexpression of htt fragment in neurons results in a gain of function mechanosensory defect that is the cause of the HD pathology.

RV beneficial effects against 3-nitropropionic acid suggest a role of the drug in protecting by neurotoxins in HD because 3-nitropropionic acid is an inhibitor of complex II of the electron transport chain, which causes HD's like symptoms. RV inhibits cyclooxygenase I (COX) activity significantly improving motor and cognitive impairments in the 3-nitropropionic acid-induced model of HD [103]. In addition, RV protects neurons against cytotoxicity of the mutant polyglutamine htt acting through SIRT1 activation [104]. Several mechanisms have been proposed by which m-htt may trigger striatal neurodegeneration, including mitochondrial dysfunction, oxidative stress, and apoptosis. In this context, p53 activation plays a crucial role in mediating m-htt toxic effects in human neuronal cells. The tumor suppressor p53 mediates dysfunctions and cytotoxicity in HD cells and in transgenic mouse whereas its inhibition prevents these phenotypes [105]. RV protects cells by toxic effects of m-htt potentiating SIRT1 activity and inducing an indirect inhibition of p53 because SIRT1 interacts with and deacetylates p53 [106, 107]. The deacetylation of p53 attenuates its activity and inhibits p53 dependent apoptosis. In general, p53 activation which happens in HD has been linked to enhanced mitochondrial oxidation [108, 109], while activation of SIRT1 as happens in presence of RV allows the cell to adapt to situations of energy stress [89].

RV can effectively interject in the mitochondrial oxidation through its antioxidant properties and counteract impaired mitochondrial function through the activation of the SIRT1-PGC1α pathway [110–112]. In fact, PGC1α regulates the expression and activities of ROS scavenging antioxidant enzymes and therefore counteracts oxidative stress [113].

4. RV and Parkinson's Disease

PD is the second most common neurodegenerative disorder after AD, affecting nearly 2% of individuals over the age of 65 in industrialized countries [114]. Although the etiology of sporadic PD is poorly understood, there is evidence that both environmental factors and genetic predisposition contribute to its development. Rare missense mutations and more frequent multiplications of a large genomic region including the α-synuclein gene cause autosomal dominant Parkinsonian syndromes [115]. Clinically, PD is characterized by a progressive neurodegenerative disorders showing invalidating neurological symptoms: increasing muscle rigidity, tremor, bradykinesia, and in extreme cases a nearly complete loss of movements. Motor symptoms originate from the degeneration of dopaminergic neurons of the substantia nigra with a consequent loss of dopamine and accumulation of intracytoplasmic Lewy bodies, inclusions that contain α-synuclein and ubiquitin [15]. Dopamine is inactivated by the monoamine oxidase enzyme (MAO), a reaction that yields significant amounts of hydrogen peroxide that must be continuously detoxified by intracellular antioxidants.

Dopaminergic cells are believed to die by apoptosis rather than necrosis, but even this basic concept is disputed [116]; there is no doubt that oxidative and nitrative stress occurring in substantia nigra is prominent features of this disease [117].

The source of nitrogen species (nitric oxide and peroxynitrite) is clearly related to alterations in iNOS activity. The origin of oxygen radicals is much less clear and is based mainly on indirect biochemical changes, such as increased iron levels, alterations in antioxidant mechanisms, and mitochondrial dysfunction.

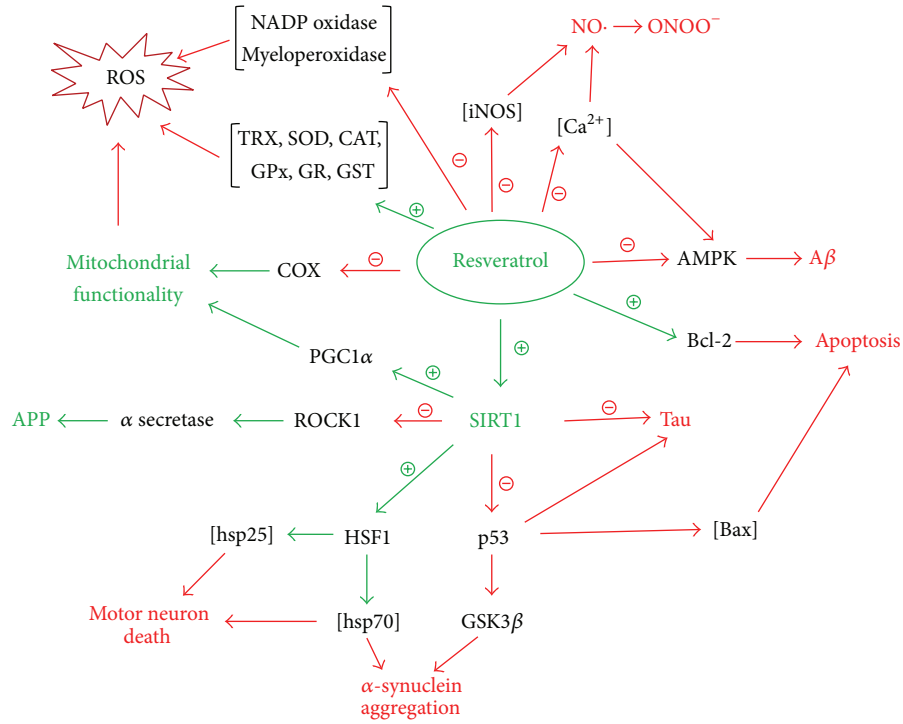

Figure 5: Global representation of RV targets in neurodegenerative protection.

The involvement of mitochondrial impairment in PD pathogenesis has been established for over two decades. Complex I inhibition is known to be the major source of free radicals, and it is thought that the alteration in its functionally could, above and beyond the declining production of ATP, give rise to increased oxidative stress, thus explaining the emergence of the disease [118].

RV treatment ameliorates the mitochondrial respiratory capacities via a pathway in which SIRT1-AMPK and PGC-1α play a pivotal role. In detail, the activation of AMPK-SIRT1 signaling by RV results in the induction of the PGC-1α activity [119]. The impact of PGC-1α activation on mitochondrial respiratory capacities leads to an increase of mitochondrial biogenesis and improves mitochondrial function.

An interesting hypothesis for the vulnerability of certain neuronal groups in PD is the relation between the decline in ATP and the calcium intracellular oscillations. MAO induced metabolism of dopamine produces calcium signaling in astrocytes through ROS (hydrogen peroxide, principally) [120]. This creates a metabolic stress because the repeated and persistent entry of calcium into cells needs to be counterbalanced by ATP demanding pumps to restore the calcium homeostasis [23]. It has been demonstrated that the opening of L-type calcium channels in the mitochondria of such neurons makes them highly vulnerable to disease process [121]. RV can effectively interject the progression of PD preventing calcium elevation [52, 122, 123].

In experimental models of PD, treatment with RV exerts neuroprotective effects on dopaminergic neurons probably related to antioxidant properties of the drug [118, 124]. In this context, the RV scavenger activity against hydrogen peroxide (H_2O_2) may be particularly efficient; the drug at 100 μg/mL exhibited 60% of its effect [25]. Besides, RV inducing activation and expression of SIRT1 protects against pathological α-synuclein aggregation [125]; in detail, SIRT1 can deacetylate and activate heat shock factor 1 (HSF1), which affects transcription of molecular chaperons including heat shock proteins 70 (hsp70). Hsp70 regulate homeostasis of cellular proteins decreasing the formation of abnormal protein aggregates [18, 126, 127].

Studies have shown that GSK-3β inhibition protects the dopaminergic neurons from various stress-induced injuries, indicating the involvement of GSK-3β in PD pathogenesis because α-synuclein is a substrate for GSK-3β phosphorylation [128]. RV may decrease α-synuclein protein expression in cellular model of PD through its downregulation and partially inhibition of GSK-3β [129].

5. RV and Amyotrophic Lateral Sclerosis

ALS is a progressive and fatal neurodegenerative disease, characterized by the selective loss of motor neurons in brain, brainstem, and spinal cord [130]. In human patients ALS symptoms onset is varied but usually begins with muscle weakness, muscle atrophy, and spasticity leading to paralysis, respiratory insufficiency, and death with a median survival time of less than 5 years. Although the selective mechanism of motor neuron death is still unknown, two ALS forms have been identified: sporadic (SALS) with no known genetic component and familial (FALS) with a positive familial history and a genetic component [131]. Currently, several genes have been identified as possible causes of onset for

FALS but curiously, although these genes control different cellular mechanisms, the progression of the disease leads inexorably to motor neuron degeneration. About 20–40% of FALS forms have one of over 150 mutations in the gene for Cu, Zn superoxide dismutase 1 (SOD1) [17], while unexpectedly mutations in the TDP-43 gene, which codes for RNA binding protein, are responsible for about 5% of both FALS and SALS [132]. Really, SALS and FALS are clinically indistinguishable suggesting a common pathogenesis of the disease; in fact, the protein products of genes associated with ALS as mutant SOD1, TDP43, or FUS were found in neuronal aggregates from ALS patients and observed to coincide with the manifestation of disease symptoms in all mouse models [16, 133] suggesting that, in addition to playing a role in FALS, these proteins may be altered also in SALS forms of the disease [134, 135]. The current consensus is that most causes may converge to the motor neuron damage typified by ALS [136], from which the most studied are the following.

SOD1 Mutations. SOD1 is a gene that codes for SOD, an enzyme which helps to convert superoxide radicals into less harmful molecules. If SOD is damaged, free radicals accumulation could contribute to ALS. In addition, accumulation of abnormal SOD molecules may be (the seed for large) the trigger for misfolded protein that are toxic to neurons [137].

Glutamate Toxicity. Under normal conditions, glutamate is an important neurotransmitter but in patients with ALS glutamate is accumulated in the synapse. These elevated levels of glutamate-mediated excitation can kill motor neurons [138].

Oxidative Stress. Studies have found elevated levels of oxidative stress within the central neurons system in ALS [139]. This condition causing injury of adjacent neurons promotes the propagating of the disease and may be linked to the inability of mutant SOD1 to complex Cu and Zn [10]. Diminished metal binding (by SOD1) could also enhance the release of copper and zinc and trigger metal-mediated neurotoxicity.

Mitochondrial Dysfunction. Studies of both human and animal neurons have found extensive mitochondrial dysfunction associated with ALS [140–142]. In such cases biochemical analyses have delineated defects in the respiratory chain complexes I and IV in muscle [143], but the main morphological damage is the presence of vacuolated mitochondria derived from a detachment between the inner and the outer membrane [144]. Several observations linked mutant SOD1 with mitochondrial damage because SOD1 has been found in the mitochondria intermembrane space, in the matrix, and in the cytosolic face of the outer membrane [145]. The presence of SOD1 protects mitochondrial functionality defending proteins from oxidation but abnormal protein aggregation of mutant SOD1 could directly damage mitochondria triggering cell death [146, 147].

Calcium Dysregulation. Ca^{2+} dyshomeostasis has been implicated in the pathogenesis of motoneurone death in ALS [148, 149]. Calcium accumulation in intracellular compartments can lead to an increase in the production of nitric oxide and peroxynitrite both of which could be lethal to the cell [150, 151]. Yet there is considerable evidence that calcium overload and mitochondrial abnormalities are early events in toxicity at least some SOD1 mutants [152, 153].

In this context, RV has shown several benefic effects which virtually can efficiently counteract ALS molecular targets. Mitochondrial impairment and ROS generation may be reduced by antioxidant properties of RV; in this regard, Song et al. [154] demonstrated the repression of ROS level after RV treatment in the ALS mice. Besides, as mentioned before, RV protects against mitochondrial fragmentation by the activation of PGC1α mediated by RV-SIRT1 interaction [18, 96, 155]. Zhao et al. reported that RV through the overexpression of PGC1α improved motor performance and survival in a mouse model of ALS [156].

Activation of SIRT1 by RV treatment has been shown also to decrease proteotoxic stress derived from misfolded SOD1 aggregates. The proposed mechanism is that SIRT1 activated by RV can deacetylate HSF1, inducing the transcription of molecular chaperones such as hsp70 and hsp25 and decreasing motor neuron death [18, 157, 158]. Song et al. correlated the strong inhibition effects of RV on apoptosis with the potential effects of the drug to prevent the motor neurons from degeneration in ALS [154]. In detail, RV-SIRT1 interaction mediated deacetylation and inhibition of p53 ability to induce the expression of the proapoptotic factor Bax [159].

RV has already been proven to exert neuroprotection against glutamate neurotoxicity in neuronal cultures [54] and to prevent the $[Ca^{2+}]$ elevation [52].

6. Conclusions

In the past few years, it has become clear that the dysfunction of mitochondrial metabolism and ROS dyshomeostasis are the main contributing factors in the progression of many neurodegenerative diseases. However, whether such events are a primary cause or consequences of the disease progression is still an unanswered question. Evaluating the safety and efficacy of small molecules which exhibit remarkable multipotent ability to control and modulate ROS, metal toxicity, and abnormal protein aggregations may be important elements in the development of new therapeutic strategies to treat neurodegenerative diseases.

RV as a multitarget compound with several neuroprotective roles represents an intriguing candidate for potential application in the treatment of neurological impairments. Particularly attractive are recent studies showing the role of RV in improved mitochondrial functions and biogenesis through SIRT1/AMPK/PGC1α pathway which highlight RV benefits not only limited to the antioxidant and anti-inflammatory properties.

It is right to remember the potential problems related to a possible therapeutic use of the RV, because it is not much soluble in water [160].

Despite this, RV may be considered as a very promising "model compound" [161] starting from which useful and more effective derivatives could be obtained by appropriate

chemical modifications and decorations of the stilbene scaffold. Recently, it is also worth noting that piceid, a precursor of RV, exhibited higher scavenging activity against hydroxyl radicals than RV in vitro [162]. Consequently, the synthesis of analogues of the RV with improved bioavailability and solubility could help raise the number of targets affected by biological molecule and better delineate the pathways of action, opening new perspectives in the search and synthesis of novel agents to treat neurodegenerative diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] L. Frémont, "Biological effects of resveratrol," *Life Sciences*, vol. 66, no. 8, pp. 663–673, 2000.

[2] F. Sparvoli, C. Martin, A. Scienza, G. Gavazzi, and C. Tonelli, "Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.)," *Plant Molecular Biology*, vol. 24, no. 5, pp. 743–755, 1994.

[3] O. Choi, J. K. Lee, S.-Y. Kang et al., "Construction of artificial biosynthetic pathways for resveratrol glucoside derivativesS," *Journal of Microbiology and Biotechnology*, vol. 24, no. 5, pp. 614–618, 2014.

[4] Q. Wang, J. Xu, G. E. Rottinghaus et al., "Resveratrol protects against global cerebral ischemic injury in gerbils," *Brain Research*, vol. 958, no. 2, pp. 439–447, 2002.

[5] T. Walle, F. Hsieh, M. H. DeLegge, J. E. Oatis Jr., and U. K. Walle, "High absorption but very low bioavailability of oral resveratrol in humans," *Drug Metabolism and Disposition*, vol. 32, no. 12, pp. 1377–1382, 2004.

[6] H. I. Rocha-González, M. Ambriz-Tututi, and V. Granados-Soto, "Resveratrol: a natural compound with pharmacological potential in neurodegenerative diseases," *CNS Neuroscience and Therapeutics*, vol. 14, no. 3, pp. 234–247, 2008.

[7] H. J. Forman, "Reactive oxygen species and α,β-unsaturated aldehydes as second messengers in signal transduction," *Annals of the New York Academy of Sciences*, vol. 1203, pp. 35–44, 2010.

[8] A. C. Andorn, R. S. Britton, and B. R. Bacon, "Evidence that lipid peroxidation and total iron are increased in Alzheimer's brain," *Neurobiology of Aging*, vol. 11, pp. 316–320, 1990.

[9] A. Granzotto and P. Zatta, "Resveratrol and Alzheimer's disease: message in a bottle on red wine and cognition," *Frontiers in Aging Neuroscience*, vol. 6, article 95, 2014.

[10] S. Boillée, C. Vande Velde, and D. Cleveland, "ALS: a disease of motor neurons and their nonneuronal neighbors," *Neuron*, vol. 52, no. 1, pp. 39–59, 2006.

[11] J. A. Duce and A. I. Bush, "Biological metals and Alzheimer's disease: implications for therapeutics and diagnostics," *Progress in Neurobiology*, vol. 92, no. 1, pp. 1–18, 2010.

[12] B. R. Roberts, T. M. Ryan, A. I. Bush, C. L. Masters, and J. A. Duce, "The role of metallobiology and amyloid-beta peptides in Alzheimer's disease," *Journal of Neurochemistry*, vol. 120, no. 1, pp. 149–166, 2012.

[13] R. González-Domínguez, T. García-Barrera, and J. L. Gómez-Ariza, "Characterization of metal profiles in serum during the progression of Alzheimer's disease," *Metallomics*, vol. 6, no. 2, pp. 292–300, 2014.

[14] A. R. La Spada, "Finding a sirtuin truth in Huntington's disease," *Nature Medicine*, vol. 18, no. 1, pp. 24–26, 2012.

[15] J. Blesa, S. Phani, V. Jackson-Lewis, and S. Przedborski, "Classic and new animal models of Parkinson's disease," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 845618, 10 pages, 2012.

[16] L. I. Bruijn, T. M. Miller, and D. W. Cleveland, "Unraveling the mechanisms involved in motor neuron degeneration in ALS," *Annual Review of Neuroscience*, vol. 27, pp. 723–749, 2004.

[17] D. R. Rosen, T. Siddique, D. Patterson et al., "Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis," *Nature*, vol. 362, no. 6415, pp. 59–62, 1993.

[18] A. Z. Herskovits and L. Guarente, "Sirtuin deacetylases in neurodegenerative diseases of aging," *Cell Research*, vol. 23, no. 6, pp. 746–758, 2013.

[19] J. M. C. Gutteridge, "Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides," *FEBS Letters*, vol. 201, no. 2, pp. 291–295, 1986.

[20] R. J. Castellani, R. K. Rolston, and M. A. Smith, "Alzheimer disease," *Disease-a-Month*, vol. 56, no. 9, pp. 484–546, 2010.

[21] W. G. Tatton, R. Chalmers-Redman, D. Brown, and N. Tatton, "Apoptosis in Parkinson's disease: signals for neuronal degradation," *Annals of Neurology*, vol. 53, supplement 3, pp. S61–S72, 2003.

[22] R. C. S. Seet, C.-Y. J. Lee, E. C. H. Lim et al., "Oxidative damage in Parkinson disease: measurement using accurate biomarkers," *Free Radical Biology and Medicine*, vol. 48, no. 4, pp. 560–566, 2010.

[23] S. Gandhi and A. Y. Abramov, "Mechanism of oxidative stress in neurodegeneration," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 428010, 11 pages, 2012.

[24] A. Cavallaro, T. Ainis, C. Bottari, and V. Fimiani, "Effect of resveratrol on some activities of isolated and in whole blood human neutrophils," *Physiological Research*, vol. 52, no. 5, pp. 555–562, 2003.

[25] M. A. Hussein, "A convenient mechanism for the free radical scavenging activity of resveratrol," *International Journal of Phytomedicine*, vol. 3, no. 4, pp. 459–469, 2011.

[26] C. Iuga, J. R. Alvarez-Idaboy, and N. Russo, "Antioxidant activity of trans-resveratrol toward hydroxyl and hydroperoxyl radicals: a quantum chemical and computational kinetics study," *Journal of Organic Chemistry*, vol. 77, no. 8, pp. 3868–3877, 2012.

[27] L. A. Stivala, M. Savio, F. Carafoli et al., "Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol," *The Journal of Biological Chemistry*, vol. 276, no. 25, pp. 22586–22594, 2001.

[28] S. Petralia, C. Spatafora, C. Tringali, M. C. Foti, and S. Sortino, "Hydrogen atom abstraction from resveratrol and two lipophilic derivatives by tert-butoxyl radicals. A laser flash photolysis study," *New Journal of Chemistry*, vol. 28, no. 12, pp. 1484–1487, 2004.

[29] J. Chao, M. S. Yu, Y. S. Ho, M. Wang, and R. C. C. Chang, "Dietary oxyresveratrol prevents parkinsonian mimetic 6-hydroxydopamine neurotoxicity," *Free Radical Biology and Medicine*, vol. 45, no. 7, pp. 1019–1026, 2008.

[30] S. Selvaraj, A. Mohan, S. Narayanan, S. Sethuraman, and U. M. Krishnan, "Dose-dependent interaction of trans-resveratrol with biomembranes: effects on antioxidant property," *Journal of Medicinal Chemistry*, vol. 56, no. 3, pp. 970–981, 2013.

[31] E. Tellone, M. C. de Rosa, D. Pirolli et al., "Molecular interactions of hemoglobin with resveratrol: potential protective antioxidant role and metabolic adaptations of the erythrocyte," *Biological Chemistry*, vol. 395, no. 3, pp. 347–354, 2014.

[32] A. Galtieri, E. Tellone, S. Ficarra et al., "Resveratrol treatment induces redox stress in red blood cells: A possible role of caspase 3 in metabolism and anion transport," *Biological Chemistry*, vol. 391, no. 9, pp. 1057–1065, 2010.

[33] S. Pervaiz, "Resveratrol: from grapevines to mammalian biology," *The FASEB Journal*, vol. 17, no. 14, pp. 1975–1985, 2003.

[34] E. N. Frankel, A. L. Waterhouse, and J. E. Kinsella, "Inhibition of human LDL oxidation by resveratrol," *The Lancet*, vol. 341, no. 8852, pp. 1103–1104, 1993.

[35] G.-C. Yen, P.-D. Duh, and C.-W. Lin, "Effects of resveratrol and 4-hexylresorcinol on hydrogen peroxide-induced oxidative DNA damage in human lymphocytes," *Free Radical Research*, vol. 37, no. 5, pp. 509–514, 2003.

[36] C. A. de La Lastra and I. Villegas, "Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications," *Biochemical Society Transactions*, vol. 35, no. 5, pp. 1156–1160, 2007.

[37] S.-K. Tsai, L.-M. Hung, Y.-T. Fu et al., "Resveratrol neuroprotective effects during focal cerebral ischemia injury via nitric oxide mechanism in rats," *Journal of Vascular Surgery*, vol. 46, no. 2, pp. 346–353, 2007.

[38] C. Centeno-Baez, P. Dallaire, and A. Marette, "Resveratrol inhibition of inducible nitric oxide synthase in skeletal muscle involves AMPK but not SIRT1," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 301, no. 5, pp. E922–E930, 2011.

[39] T. C. Huang, K. T. Lu, Y. Y. P. Wo, Y. J. Wu, and Y. L. Yang, "Resveratrol protects rats from Aβ-induced neurotoxicity by the reduction of iNOS expression and lipid peroxidation," *PLoS ONE*, vol. 6, no. 12, Article ID e29102, 2011.

[40] E. Savaskan, G. Olivieri, F. Meier, E. Seifritz, A. Wirz-Justice, and F. Müller-Spahn, "Red wine ingredient resveratrol protects from β-amyloid neurotoxicity," *Gerontology*, vol. 49, no. 6, pp. 380–383, 2003.

[41] V. Vingtdeux, U. Dreses-Werringloer, H. Zhao, P. Davies, and P. Marambaud, "Therapeutic potential of resveratrol in Alzheimer's disease," *BMC Neuroscience*, vol. 9, supplement 2, article S6, 2008.

[42] K. A. Ahmad, M.-V. Clement, and S. Pervaiz, "Pro-oxidant activity of low doses of resveratrol inhibits hydrogen peroxide—induced apoptosis," *Annals of the New York Academy of Sciences*, vol. 1010, pp. 365–373, 2003.

[43] A. Ahmad, F. A. Syed, S. Singh, and S. M. Hadi, "Prooxidant activity of resveratrol in the presence of copper ions: mutagenicity in plasmid DNA," *Toxicology Letters*, vol. 159, no. 1, pp. 1–12, 2005.

[44] I. Muqbil, F. W. J. Beck, B. Bao et al., "Old wine in a new bottle: the Warburg effect and anticancer mechanisms of resveratrol," *Current Pharmaceutical Design*, vol. 18, no. 12, pp. 1645–1654, 2012.

[45] J. K. Kundu and Y.-J. Surh, "Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives," *Cancer Letters*, vol. 269, no. 2, pp. 243–261, 2008.

[46] Y. Bai, Q.-Q. Mao, J. Qin et al., "Resveratrol induces apoptosis and cell cycle arrest of human T24 bladder cancer cells in vitro and inhibits tumor growth in vivo," *Cancer Science*, vol. 101, no. 2, pp. 488–493, 2010.

[47] P. Parekh, L. Motiwale, N. Naik, and K. V. K. Rao, "Downregulation of cyclin D1 is associated with decreased levels of p38 MAP kinases, Akt/PKB and Pak1 during chemopreventive effects of resveratrol in liver cancer cells," *Experimental and Toxicologic Pathology*, vol. 63, no. 1-2, pp. 167–173, 2011.

[48] E. Fan, S. Jiang, L. Zhang, and Y. Bai, "Molecular mechanism of apoptosis induction by resveratrol, a natural cancer chemopreventive agent," *International Journal for Vitamin and Nutrition Research*, vol. 78, no. 1, pp. 3–8, 2008.

[49] E. Madan, S. Prasad, P. Roy, J. George, and Y. Shukla, "Regulation of apoptosis by resveratrol through JAK/STAT and mitochondria mediated pathway in human epidermoid carcinoma A431 cells," *Biochemical and Biophysical Research Communications*, vol. 377, no. 4, pp. 1232–1237, 2008.

[50] P. Roy, N. Kalra, S. Prasad, J. George, and Y. Shukla, "Chemopreventive potential of resveratrol in mouse skin tumors through regulation of mitochondrial and PI3K/AKT signaling pathways," *Pharmaceutical Research*, vol. 26, no. 1, pp. 211–217, 2009.

[51] M. Farnebo, V. J. N. Bykov, and K. G. Wiman, "The p53 tumor suppressor: a master regulator of diverse cellular processes and therapeutic target in cancer," *Biochemical and Biophysical Research Communications*, vol. 396, no. 1, pp. 85–89, 2010.

[52] M. Yáñez, L. Galán, J. Matías-Guiu, A. Vela, A. Guerrero, and A. G. García, "CSF from amyotrophic lateral sclerosis patients produces glutamate independent death of rat motor brain cortical neurons: protection by resveratrol but not riluzole," *Brain Research*, vol. 1423, pp. 77–86, 2011.

[53] F. Orallo, "Trans-resveratrol: a magical elixir of eternal youth?" *Current Medicinal Chemistry*, vol. 15, no. 19, pp. 1887–1898, 2008.

[54] L. M. Vieira de Almeida, C. C. Piñeiro, M. C. Leite et al., "Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures," *Cellular and Molecular Neurobiology*, vol. 27, no. 5, pp. 661–668, 2007.

[55] F. Simão, A. Matté, A. S. Pagnussat, C. A. Netto, and C. G. Salbego, "Resveratrol prevents CA1 neurons against ischemic injury by parallel modulation of both GSK-3β and CREB through PI3-K/Akt pathways," *European Journal of Neuroscience*, vol. 36, no. 7, pp. 2899–2905, 2012.

[56] T. D. King, B. Clodfelder-Miller, K. A. Barksdale, and G. N. Bijur, "Unregulated mitochondrial GSK3β activity results in NADH: ubiquinone oxidoreductase deficiency," *Neurotoxicity Research*, vol. 14, no. 4, pp. 367–382, 2008.

[57] D.-W. Li, Z.-Q. Liu, W. Chen, M. Yao, and G.-R. Li, "Association of glycogen synthase kinase-3β with Parkinson's disease (Review)," *Molecular Medicine Reports*, vol. 9, no. 6, pp. 2043–2050, 2014.

[58] A. V. Witte, L. Kerti, D. S. Margulies, and A. Flöel, "Effects of resveratrol on memory performance, hippocampal functional connectivity, and glucose metabolism in healthy older adults," *Journal of Neuroscience*, vol. 34, no. 23, pp. 7862–7870, 2014.

[59] D. O. Kennedy, E. L. Wightman, J. L. Reay et al., "Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation," *American Journal of Clinical Nutrition*, vol. 91, no. 6, pp. 1590–1597, 2010.

[60] E. L. Wightman, J. L. Reay, C. F. Haskell, G. Williamson, T. P. Dew, and D. O. Kennedy, "Effects of resveratrol alone or in combination with piperine on cerebral blood flow parameters and cognitive performance in human subjects: a randomised, double-blind, placebo-controlled, cross-over investigation," *British Journal of Nutrition*, vol. 112, no. 2, pp. 203–213, 2014.

[61] F. Uchiumi, T. Watanabe, S. Hasegawa, T. Hoshi, Y. Higami, and S.-I. Tanuma, "The effect of resveratrol on the Werner syndrome RecQ helicase gene and telomerase activity," *Current Aging Science*, vol. 4, no. 1, pp. 1–7, 2011.

[62] T. Jayasena, A. Poljak, G. Smythe, N. Braidy, G. Münch, and P. Sachdev, "The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease," *Ageing Research Reviews*, vol. 12, no. 4, pp. 867–883, 2013.

[63] E. Sahin, S. Colla, M. Liesa et al., "Telomere dysfunction induces metabolic and mitochondrial compromise," *Nature*, vol. 470, no. 7334, pp. 359–365, 2011.

[64] Z. Wu, P. Puigserver, U. Andersson et al., "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1," *Cell*, vol. 98, no. 1, pp. 115–124, 1999.

[65] R. C. Scarpulla, "Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1813, no. 7, pp. 1269–1278, 2011.

[66] X. Mu, G. He, Y. Cheng, X. Li, B. Xu, and G. Du, "Baicalein exerts neuroprotective effects in 6-hydroxydopamine-induced experimental parkinsonism *in vivo* and *in vitro*," *Pharmacology Biochemistry and Behavior*, vol. 92, no. 4, pp. 642–648, 2009.

[67] Y. Wu, X. Li, J. X. Zhu et al., "Resveratrol-activated AMPK/SIRT1/autophagy in cellular models of Parkinson's disease," *NeuroSignals*, vol. 19, no. 3, pp. 163–174, 2011.

[68] Z. Li, L. Pang, F. Fang et al., "Resveratrol attenuates brain damage in a rat model of focal cerebral ischemia via up-regulation of hippocampal Bcl-2," *Brain Research*, vol. 1450, pp. 116–124, 2012.

[69] M. Kairisalo, A. Bonomo, A. Hyrskyluoto et al., "Resveratrol reduces oxidative stress and cell death and increases mitochondrial antioxidants and XIAP in PC6.3-cells," *Neuroscience Letters*, vol. 488, no. 3, pp. 263–266, 2011.

[70] F. Jin, Q. Wu, Y.-F. Lu, Q.-H. Gong, and J.-S. Shi, "Neuroprotective effect of resveratrol on 6-OHDA-induced Parkinson's disease in rats," *European Journal of Pharmacology*, vol. 600, no. 1–3, pp. 78–82, 2008.

[71] F. Zhang, J.-S. Shi, H. Zhou, B. Wilson, J.-S. Hong, and H.-M. Gao, "Resveratrol protects dopamine neurons against lipopolysaccharide-induced neurotoxicity through its anti-inflammatory actions," *Molecular Pharmacology*, vol. 78, no. 3, pp. 466–477, 2010.

[72] D. J. Selkoe, "Alzheimer's disease is a synaptic failure," *Science*, vol. 298, no. 5594, pp. 789–791, 2002.

[73] R. E. Tanzi, "The synaptic Aβ hypothesis of Alzheimer disease," *Nature Neuroscience*, vol. 8, no. 8, pp. 977–979, 2005.

[74] L. Saragoni, P. Hernández, and R. B. Maccioni, "Differential association of tau with subsets of microtubules containing posttranslationally-modified tubulin variants in neuroblastoma cells," *Neurochemical Research*, vol. 25, no. 1, pp. 59–70, 2000.

[75] M. J. Smith, R. A. Crowther, and M. Goedert, "The natural osmolyte trimethylamine N-oxide (TMAO) restores the ability of mutant tau to promote microtubule assembly," *FEBS Letters*, vol. 484, no. 3, pp. 265–270, 2000.

[76] A. Takashima, T. Honda, K. Yasutake et al., "Activation of tau protein kinase I/glycogen synthase kinase-3β by amyloid β peptide (25–35) enhances phosphorylation of tau in hippocampal neurons," *Neuroscience Research*, vol. 31, no. 4, pp. 317–323, 1998.

[77] A. C. Cuello, "Intracellular and extracellular Aβ, a tale of two neuropathologies," *Brain Pathology*, vol. 15, no. 1, pp. 66–71, 2005.

[78] D. W. Ethell and L. A. Buhler, "Fas ligand-mediated apoptosis in degenerative disorders of the brain," *Journal of Clinical Immunology*, vol. 23, no. 6, pp. 439–446, 2003.

[79] H. Dudek, S. R. Datta, T. F. Franke et al., "Regulation of neuronal survival by the serine-threonine protein kinase Akt," *Science*, vol. 275, no. 5300, pp. 661–665, 1997.

[80] Y. Feng, X.-P. Wang, S.-G. Yang et al., "Resveratrol inhibits beta-amyloid oligomeric cytotoxicity but does not prevent oligomer formation," *NeuroToxicology*, vol. 30, no. 6, pp. 986–995, 2009.

[81] Y. Porat, A. Abramowitz, and E. Gazit, "Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism," *Chemical Biology and Drug Design*, vol. 67, no. 1, pp. 27–37, 2006.

[82] M. Gertz, G. T. T. Nguyen, F. Fischer et al., "A molecular mechanism for direct sirtuin activation by resveratrol," *PLoS ONE*, vol. 7, no. 11, Article ID e49761, 2012.

[83] B. P. Hubbard, A. P. Gomes, H. Dai et al., "Evidence for a common mechanism of SIRT1 regulation by allosteric activators," *Science*, vol. 339, no. 6124, pp. 1216–1219, 2013.

[84] W. Qin, T. Yang, L. Ho et al., "Neuronal SIRT1 activation as a novel mechanism underlying the prevention of alzheimer disease amyloid neuropathology by calorie restriction," *The Journal of Biological Chemistry*, vol. 281, no. 31, pp. 21745–21754, 2006.

[85] Y. Zhou, Y. Su, B. Li et al., "Non-steroidal anti-inflammatory drugs can lower amyloidogenic Ab42 by inhibiting Rho," *Science*, vol. 302, pp. 1215–1217, 2003.

[86] T. J. Cohen, J. L. Guo, D. E. Hurtado et al., "The acetylation of tau inhibits its function and promotes pathological tau aggregation," *Nature Communications*, vol. 2, no. 1, article 252, 2011.

[87] S.-W. Min, S.-H. Cho, Y. Zhou et al., "Acetylation of tau inhibits its degradation and contributes to tauopathy," *Neuron*, vol. 67, no. 6, pp. 953–966, 2010.

[88] C. Hooper, E. Meimaridou, M. Tavassoli, G. Melino, S. Lovestone, and R. Killick, "p53 is upregulated in Alzheimer's disease and induces tau phosphorylation in HEK293a cells," *Neuroscience Letters*, vol. 418, no. 1, pp. 34–37, 2007.

[89] C. Cantó and J. Auwerx, "Targeting sirtuin 1 to improve metabolism: all you need is NAD^+?" *Pharmacological Reviews*, vol. 64, no. 1, pp. 166–187, 2012.

[90] V. Vingtdeux, L. Giliberto, H. Zhao et al., "AMP-activated protein kinase signaling activation by resveratrol modulates amyloid-beta peptide metabolism," *The Journal of Biological Chemistry*, vol. 285, no. 12, pp. 9100–9113, 2010.

[91] S. Fogarty, S. A. Hawley, K. A. Green, N. Saner, K. J. Mustard, and D. G. Hardie, "Calmodulin-dependent protein kinase kinase-β activates AMPK without forming a stable complex: Synergistic effects of Ca^{2+} and AMP," *Biochemical Journal*, vol. 426, no. 1, pp. 109–118, 2010.

[92] S.-J. Park, F. Ahmad, A. Philp et al., "Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases," *Cell*, vol. 148, supplement 3, pp. 421–433, 2012.

[93] S. A. Hawley, F. A. Ross, C. Chevtzoff et al., "Use of cells expressing γ subunit variants to identify diverse mechanisms of AMPK activation," *Cell Metabolism*, vol. 11, no. 6, pp. 554–565, 2010.

[94] C. Cantó and J. Auwerx, "AMP-activated protein kinase and its downstream transcriptional pathways," *Cellular and Molecular Life Sciences*, vol. 67, no. 20, pp. 3407–3423, 2010.

[95] X. Wang, W. Wang, L. Li, G. Perry, H. G. Lee, and X. Zhu, "Oxidative stress and mithocondrial dysfunction in Alzheimer's disease," *Biochimica et Biophysica Acta*, vol. 1842, no. 8, pp. 1240–1247, 2014.

[96] K. Higashida, S. H. Kim, S. R. Jung, M. Asaka, J. O. Holloszy, and D.-H. Han, "Effects of resveratrol and SIRT1 on PGC-1α activity and mitochondrial biogenesis: a reevaluation," *PLoS Biology*, vol. 11, no. 7, Article ID e1001603, 2013.

[97] A. P. Gomes, N. L. Price, A. J. Y. Ling et al., "Declining NAD^+ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging," *Cell*, vol. 155, no. 7, pp. 1624–1638, 2013.

[98] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The hallmarks of aging," *Cell*, vol. 153, no. 6, pp. 1194–1217, 2013.

[99] J. P. G. Vonsattel and M. DiFiglia, "Huntington disease," *Journal of Neuropathology and Experimental Neurology*, vol. 57, no. 5, pp. 369–384, 1998.

[100] M. E. MacDonald, C. M. Ambrose, M. P. Duyao et al., "A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group," *Cell*, vol. 72, no. 6, pp. 971–983, 1993.

[101] P. Lajoie and E. L. Snapp, "Formation and toxicity of soluble polyglutamine oligomers in living cells," *PLoS ONE*, vol. 5, no. 12, Article ID e15245, 2010.

[102] G. Cisbani and F. Cicchetti, "An in vitro perspective on the molecular mechanisms underlying mutant huntingtin protein toxicity," *Cell Death and Disease*, vol. 3, no. 8, article e380, 2012.

[103] P. Kumar, S. S. V. Padi, P. S. Naidu, and A. Kumar, "Cyclooxygenase inhibition attenuates 3-nitropropionic acid-induced neurotoxicity in rats: possible antioxidant mechanisms," *Fundamental and Clinical Pharmacology*, vol. 21, no. 3, pp. 297–306, 2007.

[104] X. Feng, N. Liang, D. Zhu et al., "Resveratrol inhibits β-amyloid-induced neuronal apoptosis through regulation of SIRT1-ROCK1 signaling pathway," *PLoS ONE*, vol. 8, no. 3, Article ID e59888, 2013.

[105] B.-I. Bae, H. Xu, S. Igarashi et al., "p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease," *Neuron*, vol. 47, no. 1, pp. 29–41, 2005.

[106] J. Luo, A. Y. Nikolaev, S.-I. Imai et al., "Negative control of p53 by Sir2α promotes cell survival under stress," *Cell*, vol. 107, no. 2, pp. 137–148, 2001.

[107] H. Vaziri, S. K. Dessain, E. N. Eaton et al., "hSIR2SIRT1 functions as an NAD-dependent p53 deacetylase," *Cell*, vol. 107, no. 2, pp. 149–159, 2001.

[108] S. Zhou, S. Kachhap, and K. K. Singh, "Mitochondrial impairment in p53-deficient human cancer cells," *Mutagenesis*, vol. 18, no. 3, pp. 287–292, 2003.

[109] S. Matoba, J.-G. Kang, W. D. Patino et al., "p53 regulates mitochondrial respiration," *Science*, vol. 312, no. 5780, pp. 1650–1653, 2006.

[110] A. Granzotto, S. Bolognin, J. Scancar, R. Milacic, and P. Zatta, "β-amyloid toxicity increases with hydrophobicity in the presence of metal ions," *Monatshefte fur Chemie*, vol. 142, no. 4, pp. 421–430, 2011.

[111] K. M. Choi, H. L. Lee, Y. Y. Kwon, M. S. Kang, S. K. Lee, and C. K. Lee, "Enhancement of mitochondrial function correlates with the extension of lifespan by caloric restriction and caloric restriction mimetics in yeast," *Biochemical and Biophysical Research Communications*, vol. 441, no. 1, pp. 236–242, 2013.

[112] V. Desquiret-Dumas, N. Gueguen, G. Leman et al., "Resveratrol induces a mitochondrial complex i-dependent increase in nadh oxidation responsible for sirtuin activation in liver cells," *The Journal of Biological Chemistry*, vol. 288, no. 51, pp. 36662–36675, 2013.

[113] D. Lettieri Barbato, S. Baldelli, B. Pagliei, K. Aquilano, and M. R. Ciriolo, "Caloric restriction and the nutrient-sensing PGC-1α in mitochondrial homeostasis: new perspectives in neurodegeneration," *International Journal of Cell Biology*, vol. 2012, Article ID 759583, 11 pages, 2012.

[114] M. C. De Rijk, C. Tzourio, M. M. B. Breteler et al., "Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON collaborative study. European Community Concerted Action on the Epidemiology of Parkinson's disease," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 62, no. 1, pp. 10–15, 1997.

[115] T. Gasser, "Genetics of Parkinson's disease," *Current Opinion in Neurology*, vol. 18, pp. 363–369, 2005.

[116] Y. He, T. Lee, and S. K. Leong, "6-Hydroxydopamine induced apoptosis of dopaminergic cells in the rat substantia nigra," *Brain Research*, vol. 858, no. 1, pp. 163–166, 2000.

[117] B. I. Giasson, H. Ischiropoulos, V. M.-Y. Lee, and J. Q. Trojanowski, "The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases," *Free Radical Biology and Medicine*, vol. 32, no. 12, pp. 1264–1275, 2002.

[118] D. Alvira, M. Yeste-Velasco, J. Folch et al., "Comparative analysis of the effects of resveratrol in two apoptotic models: inhibition of complex I and potassium deprivation in cerebellar neurons," *Neuroscience*, vol. 147, no. 3, pp. 746–756, 2007.

[119] A. Ferretta, A. Gaballo, P. Tanzarella et al., "Effect of resveratrol on mitochondrial function: implications in parkin-associated familiar Parkinson's disease," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1842, no. 7, pp. 902–915, 2014.

[120] A. Vaarmann, S. Gandhi, and A. Y. Abramov, "Dopamine induces Ca^{2+} signaling in astrocytes through reactive oxygen species generated by monoamine oxidase," *Journal of Biological Chemistry*, vol. 285, no. 32, pp. 25018–25023, 2010.

[121] D. J. Surmeier, J. N. Guzman, J. Sanchez-Padilla, and J. A. Goldberg, "The origins of oxidant stress in Parkinson's disease and therapeutic strategies," *Antioxidants and Redox Signaling*, vol. 14, no. 7, pp. 1289–1301, 2011.

[122] R. S. Duncan, D. L. Goad, M. A. Grillo, S. Kaja, A. J. Payne, and P. Koulen, "Control of intracellular calcium signaling as a neuroprotective strategy," *Molecules*, vol. 15, no. 3, pp. 1168–1195, 2010.

[123] A. E. McCalley, S. Kaja, A. J. Payne, and P. Koulen, "Resveratrol and calcium signaling: molecular mechanisms and clinical relevance," *Molecules*, vol. 19, no. 6, pp. 7327–7340, 2014.

[124] J. Blanchet, F. Longpré, G. Bureau et al., "Resveratrol, a red wine polyphenol, protects dopaminergic neurons in MPTP-treated mice," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 32, no. 5, pp. 1243–1250, 2008.

[125] G. Donmez, A. Arun, C. Y. Chung, P. J. Mclean, S. Lindquist, and L. Guarente, "SIRT1 protects against alpha-synuclein aggregation by activating molecular chaperones," *The Journal of Neuroscience*, vol. 32, no. 1, pp. 124–132, 2012.

[126] R. Raynes, B. D. Leckey Jr., K. Nguyen, and S. D. Westerheide, "Heat shock and caloric restriction have a synergistic effect on the heat shock response in a sir2.1-dependent manner in *Caenorhabditis elegans*," *The Journal of Biological Chemistry*, vol. 287, no. 34, pp. 29045–29053, 2012.

[127] S. D. Westerheide, J. Anckar, S. M. Stevens Jr., L. Sistonen, and R. I. Morimoto, "Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT," *Science*, vol. 323, no. 5917, pp. 1063–1066, 2009.

[128] D.-W. Li, Z.-Q. Liu, W. Chen, M. Yao, and G.-R. Li, "Association of glycogen synthase kinase-3β with Parkinson's disease (Review)," *Molecular Medicine Reports*, vol. 9, no. 6, pp. 2043–2050, 2014.

[129] F. Simão, A. Matté, A. S. Pagnussat, C. A. Netto, and C. G. Salbego, "Resveratrol prevents CA1 neurons against ischemic injury by parallel modulation of both GSK-3β and CREB through PI3-K/Akt pathways," *European Journal of Neuroscience*, vol. 36, no. 7, pp. 2899–2905, 2012.

[130] L. P. Rowland and N. A. Shneider, "Amyotrophic lateral sclerosis," *The New England Journal of Medicine*, vol. 344, no. 22, pp. 1688–1700, 2001.

[131] D. W. Mulder, L. T. Kurland, K. P. Offord, and M. Beard, "Familial adult motor neuron disease: amyotrophic lateral sclerosis," *Neurology*, vol. 36, no. 4, pp. 511–517, 1986.

[132] S. Da Cruz and D. W. Cleveland, "Understanding the role of TDP-43 and FUS/TLS in ALS and beyond," *Current Opinion in Neurobiology*, vol. 21, no. 6, pp. 904–919, 2011.

[133] M. Prudencio, P. J. Hart, D. R. Borchelt, and P. M. Andersen, "Variation in aggregation propensities among ALS-associated variants of SOD1: correlation to human disease," *Human Molecular Genetics*, vol. 18, no. 17, pp. 3217–3226, 2009.

[134] M. DeJesus-Hernandez, I. R. Mackenzie, B. F. Boeve et al., "Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS," *Neuron*, vol. 72, no. 2, pp. 245–256, 2011.

[135] A. E. Renton, E. Majounie, A. Waite et al., "A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD," *Neuron*, vol. 72, no. 2, pp. 257–268, 2011.

[136] J. D. Rothstein, "Current hypotheses for the underlying biology of amyotrophic lateral sclerosis," *Annals of Neurology*, vol. 65, no. 1, pp. S3–S9, 2009.

[137] C. M. Karch, M. Prudencio, D. D. Winkler, P. J. Hart, and D. R. Borchelt, "Role of mutant SOD1 disulfide oxidation and aggregation in the pathogenesis of familial ALS," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 19, pp. 7774–7779, 2009.

[138] J. Dunlop, H. Beal McIlvain, Y. She, and D. S. Howland, "Impaired spinal cord glutamate transport capacity and reduced sensitivity to riluzole in a transgenic superoxide dismutase mutant rat model of amyotrophic lateral sclerosis," *Journal of Neuroscience*, vol. 23, no. 5, pp. 1688–1696, 2003.

[139] F. J. Miana-Mena, E. Piedrafita, C. González-Mingot et al., "Levels of membrane fluidity in the spinal cord and the brain in an animal model of amyotrophic lateral sclerosis," *Journal of Bioenergetics and Biomembranes*, vol. 43, no. 2, pp. 181–186, 2011.

[140] P. Shi, J. Gal, D. M. Kwinter, X. Liu, and H. Zhu, "Mitochondrial dysfunction in amyotrophic lateral sclerosis," *Biochimica et Biophysica Acta*, vol. 1802, no. 1, pp. 45–51, 2010.

[141] M. Cozzolino and M. T. Carrì, "Mitochondrial dysfunction in ALS," *Progress in Neurobiology*, vol. 97, no. 2, pp. 54–66, 2012.

[142] L. Faes and G. Callewaert, "Mitochondrial dysfunction in familial amyotrophic lateral sclerosis," *Journal of Bioenergetics and Biomembranes*, vol. 43, no. 6, pp. 587–592, 2011.

[143] A. Kumar, D. Ghosh, and R. L. Singh, "Amyotrophic lateral sclerosis and metabolomics: clinical implication and therapeutic approach," *Journal of Biomarkers*, vol. 2013, Article ID 538765, 15 pages, 2013.

[144] P. Pasinelli and R. H. Brown, "Molecular biology of amyotrophic lateral sclerosis: insights from genetics," *Nature Reviews Neuroscience*, vol. 7, no. 9, pp. 710–723, 2006.

[145] M. T. Carrì and M. Cozzolino, "SOD1 and mitochondria in ALS: a dangerous liaison," *Journal of Bioenergetics and Biomembranes*, vol. 43, no. 6, pp. 593–599, 2011.

[146] A. Igoudjil, J. Magrané, L. R. Fischer et al., "In vivo pathogenic role of mutant SOD1 localized in the mitochondrial intermembrane space," *Journal of Neuroscience*, vol. 31, no. 44, pp. 15826–15837, 2011.

[147] R. A. Saccon, R. K. A. Bunton-Stasyshyn, E. M. C. Fisher, and P. Fratta, "Is SOD1 loss of function involved in amyotrophic lateral sclerosis?" *Brain*, vol. 136, no. 8, pp. 2342–2358, 2013.

[148] M. K. Jaiswal, "Selective vulnerability of motoneuron and perturbed mitochondrial calcium homeostasis in amyotrophic lateral sclerosis: implications for motoneurons specific calcium dysregulation," *Molecular and Cellular Therapies*, vol. 2, no. 1, article 26, 2014.

[149] J. Lautenschläger, T. Prell, J. Ruhmer, L. Weidemann, O. W. Witte, and J. Grosskreutz, "Overexpression of human mutated G93A SOD1 changes dynamics of the ER mitochondria calcium cycle specifically in mouse embryonic motor neurons," *Experimental Neurology*, vol. 247, pp. 91–100, 2013.

[150] S. D. Rao and J. H. Weiss, "Excitotoxic and oxidative cross-talk between motor neurons and glia in ALS pathogenesis," *Trends in Neurosciences*, vol. 27, no. 1, pp. 17–23, 2004.

[151] C. Raoul, A. G. Estévez, H. Nishimune et al., "Motoneuron death triggered by a specific pathway downstream of fas: potentiation by ALS-linked SOD1 mutations," *Neuron*, vol. 35, no. 6, pp. 1067–1083, 2002.

[152] H. Kawamata, S. K. Ng, N. Diaz et al., "Abnormal intracellular calcium signaling and SNARE dependent exocytosis contributes to SOD1G93A astrocyte- mediated toxicity in amyotrophic lateral sclerosis," *The Journal of Neuroscience*, vol. 34, no. 6, pp. 2331–2348, 2014.

[153] M. L. Tradewell, Z. Yu, M. Tibshirani, M.-C. Boulanger, H. D. Durham, and S. Richard, "Arginine methylation by prmt1 regulates nuclear-cytoplasmic localization and toxicity of FUS/TLS harbouring ALS-linked mutations," *Human Molecular Genetics*, vol. 21, no. 1, Article ID ddr448, pp. 136–149, 2012.

[154] L. Song, L. Chen, X. Zhang, J. Li, and W. Le, "Resveratrol ameliorates motor neuron degeneration and improves survival in SOD1G93A mouse model of amyotrophic lateral sclerosis," *BioMed Research International*, vol. 2014, Article ID 483501, 10 pages, 2014.

[155] S. Davinelli, N. Sapere, M. Visentin, D. Zella, and G. Scapagnini, "Enhancement of mitochondrial biogenesis with polyphenols: combined effects of resveratrol and equol in human endothelial cells," *Immunity & Ageing*, vol. 10, no. 1, article 28, 2013.

[156] W. Zhao, M. Varghese, S. Yemul et al., "Peroxisome proliferator activator receptor gamma coactivator-1alpha (PGC-1α) improves motor performance and survival in a mouse model of amyotrophic lateral sclerosis," *Molecular Neurodegeneration*, vol. 6, no. 1, article 51, 2011.

[157] S. M. Han, H. Tsuda, Y. Yang et al., "Secreted VAPB/ALS8 major sperm protein domains modulate mitochondrial localization and morphology via growth cone guidance receptors," *Developmental Cell*, vol. 22, no. 2, pp. 348–362, 2012.

[158] G. Donmez, A. Arun, C.-Y. Chung, P. J. Mclean, S. Lindquist, and L. Guarente, "SIRT1 protects against α-synuclein aggregation by activating molecular chaperones," *Journal of Neuroscience*, vol. 32, no. 1, pp. 124–132, 2012.

[159] D. Kim, M. D. Nguyen, M. M. Dobbin et al., "SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis," *The EMBO Journal*, vol. 26, no. 13, pp. 3169–3179, 2007.

[160] T. Walle, "Bioavailability of resveratrol," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 9–15, 2011.

[161] A. Scala, S. Ficarra, A. Russo et al., "A new erythrocyte-based biochemical approach to predict the antiproliferative effects of heterocyclic scaffolds: the case of indolone," *Biochimica et Biophysica Acta—General Subjects*, vol. 1850, no. 1, pp. 73–79, 2015.

[162] D. Su, Y. Cheng, M. Liu et al., "Comparision of piceid and resveratrol in antioxidation and antiproliferation activities in vitro," *PLoS ONE*, vol. 8, no. 1, Article ID e54505, 2013.

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Review Article

An Organ System Approach to Explore the Antioxidative, Anti-Inflammatory, and Cytoprotective Actions of Resveratrol

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Resveratrol is a phenolic phytochemical, with a stilbene backbone, derived from edible plants such as grape and peanut. It is a bioactive molecule with physiological effects on multiple organ systems. Its effects range from the neuroprotective to the nephroprotective, including cardiovascular, neuronal, and antineoplastic responses as a part of its broad spectrum of action. In this review, we examine the effects of resveratrol on the following organ systems: the central nervous system, including neurological pathology such as Parkinson's and Alzheimer's disease; the cardiovascular system, including disorders such as atherosclerosis, ischemia-reperfusion injury, and cardiomyocyte hypertrophy; the kidneys, including primary and secondary nephropathies and nephrolithiasis; multiple forms of cancer; and metabolic syndromes including diabetes. We emphasize commonalities in extracellular matrix protein alterations and intracellular signal transduction system induction following resveratrol treatment. We summarize the known anti-inflammatory, antioxidative, and cytoprotective effects of resveratrol across disparate organ systems. Additionally, we analyze the available literature regarding the pharmacokinetics of resveratrol formulations used in these studies. Finally, we critically examine select clinical trials documenting a lack of effect following resveratrol treatment.

1. Introduction

Resveratrol is a polyphenolic phytochemical that is biosynthesized by certain edible plants such as grape, peanut, and berry in response to phytogenic insults or pathogens. As shown in Figure 1, chemically it is 3,5,4′-trihydroxystilbene, a stilbene derivative, which is a phenylpropanoid with a C6-C2-C6 general structural formula. It is found as two isomeric structures in nature, the *cis*- and *trans*-isoforms, the *trans*-isoform being convertible to *cis*-isoform by heating. Its biosynthetic pathway begins with coumaroyl-CoA and 3-malonyl CoA and involves the enzyme stilbene synthase, which leads to a general biological yield of 50 to 400 μg resveratrol per g wet weight of leaves [1, 2]. Since it is biosynthesized to a greater extent in the lignified parts of these plants, the process of crushing and mashing grapes for the purpose of wine making results in relative enrichment of resveratrol in wine, making the latter a significant dietary source of resveratrol. The high resveratrol level in red wine was postulated as a factor in the "French Paradox", where epidemiological data revealed an apparent disconnect between French patterns of low rates of cardiovascular disease despite their high saturated fat consumption. Interestingly, based on its function in plants, resveratrol may be classified in the general category of low molecular weight compounds known as phytoalexins, which are phytochemicals possessing an antimicrobial and antioxidant function.

However, in the context of therapeutic engagement, resveratrol exhibits a problem shared by many other potential drugs, that of its hydrophobicity, which ameliorates its bioavailability. Indeed its pharmacokinetic properties, discussed in detail later, engender a short residence time in the body, making this an unlikely therapeutic agent due to pharmaceutical and bioavailability issues. However, taking into consideration its overall multifaceted biological activity that ranges from anti-inflammatory to cardio-, neuro-, and nephroprotective effects and to antineoplastic attributes, a lot of innovative work has gone into the design and formulation

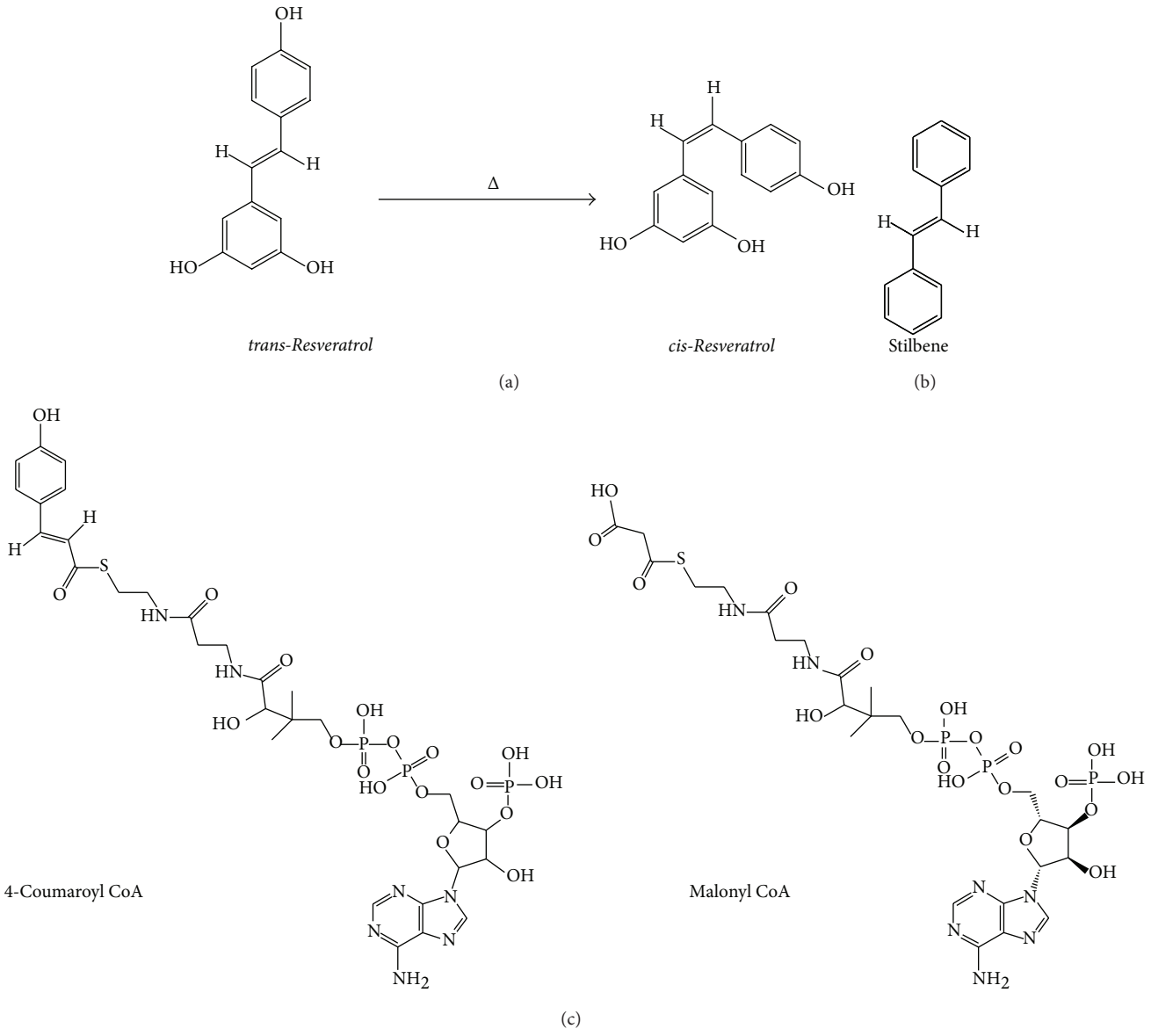

Figure 1: The chemistry of resveratrol. (a) shows the *trans*- and *cis*-isomers of resveratrol. (b) shows the stilbene C6-C2-C6 backbone, which is chemically modified in resveratrol. (c) describes the overall biosynthetic process in plants, beginning with a reaction of coumaroyl-CoA and malonyl-CoA catalyzed by stilbene synthase.

of nano- and microemulsion-based formulation systems for resveratrol. These pharmaceutical formulation techniques enhance the bioavailability of this drug and prolong its duration of action. Neves et al. have provided a detailed review and analysis of some of these resveratrol formulations and the interested reader is referred to this work [1].

This review focuses on providing a broad overview of the experimental studies and clinical trials conducted to analyze the systemic anti-inflammatory, antioxidative multiorgan protective effects of resveratrol. We substantiate this information by summarizing molecular pharmacology mechanisms and specific second messenger pathways, along with pharmacokinetic data with reference to resveratrol.

2. SiRT1 and Resveratrol: Heading towards a Centralized Mechanism

Sirtuins are a family of proteins involved in a number of processes in cells, but some are particularly associated with cellular and organismal longevity. Multiple mammalian isoforms, SIRT 1–7, exist, with SIRT1 being the molecular target of small molecule activators such as resveratrol, which can induce its expression by 10-fold. Generally induced by restricted calorie diet, these molecules have been proposed to be the molecular connecting link between the physiological effects of extraneous chemicals and subsequent genetic responses in a variety of tissue and cell types across

various organs. Although Howitz et al. suggested that SIRT1 was the intracellular target of resveratrol [11], there was considerable controversy about this claim, as later studies conducted by two groups, Kaeberlein et al., and Borra et al. seemed to suggest that activation depended on the fluorescent moiety (aminomethylcoumarin, or AMC) attached to the peptide substrate in the assay [12, 13]. However, later mice experiments using gene knockout technology confirmed not only the role of SIRT-1, but also the role of its downstream second messengers such as AMPK [14]. Thus, it appears that in many of the tissues tested, such as the kidney or cardiovascular tissue, SIRT1 is the molecular switch through which resveratrol mediates its effect.

3. Resveratrol in Kidney Disease

In the field of nephrology, resveratrol has shown great promise in ameliorating the effects of renal cytotoxicity in animal-based experiments. In general, renal disease may be classified as acute or chronic, each with multifactorial etiology, and may be of primary or secondary causation depending on whether the kidney is the major site of origin. For instance, nephropathy resulting from complications of uncontrolled diabetes over a long period of time may be considered a secondary manifestation, whereas glomerulonephritis due to a toxicant, which may lead to acute nephropathy, may be classified as a primary disease. In addition to extraneous agents, the nephropathy may arise from genetic or metabolic causes, such as the development of kidney stones and some forms of polycystic disease. Interestingly, a body of work developed over the past decade documents the advantage of using resveratrol in alleviating symptomatology or the pathological progression of many of the renal disease states introduced above.

Acute kidney injury (AKI) may become the precursor to chronic disease, if the toxicant remains in prolonged contact with the kidney or the pattern of pathological lesions becomes recurrent. In these situations, AKI may lead to renal fibrosis, which in turn may eventually lead to renal failure. Although kidney function may linearly deteriorate with progressive renal disease, clinically significant gains may be made by the therapeutic reversal of fibrosis and prevention of further damage to the tissue. Alterations in the renal microenvironment, such as those that occur as a consequence of advancing renal disease, generally manifest as altered expression of extracellular matrix (ECM) proteins in the affected tissue [15]. Resveratrol has been shown in rat models of renal fibrosis to effect changes in ECM protein expression. Liang et al. examined the effects of resveratrol at 20 mg/kg/day in murine experiments, where experimental renal fibrosis was induced by unilateral ureteral obstruction. The study showed resveratrol to be effective in decreasing the renal cortical mRNA levels of the ECM proteins, specifically the cell adhesion molecules, fibronectin, and ICAM-1. Additionally, it also reduced TNF-α and TGF-β, which may be involved in renal inflammation and the production of reactive oxygen species (ROS) such as malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG) [16]. Detailed sequence of the underlying molecular mechanism resulting in reduced TGF-β expression following resveratrol treatment was elucidated in a study by Huang et al. Using cultured mesangial cells and kidney pieces from nephrectomized rats, these authors demonstrated that the nephroprotective effect of resveratrol was mediated by the activation of SIRT1, a member of the sirtuin family of proteins capable of downregulating p53, which resulted in an inhibition of the TFG-β/Smad3 pathway [15].

As introduced above, renal damage may also occur as a consequence of other primary diseases such as diabetes. Resveratrol ameliorated nephropathy in a rat model of streptozotocin-induced diabetes, normalizing creatinine and adiponectin levels and reducing polyuria and proteinuria. This was mediated through counteracting ROS activity via augmenting enzymatic activities of superoxide dismutase and glutathione reductase and enhancing the expression of the transcription factor nuclear factor erythroid factor 2 (Nrf2) [17, 18]. A number of independent studies further explored the molecular mechanisms of resveratrol-mediated nephroprotection. Ding et al. showed that resveratrol delayed the progression of diabetic nephropathy by activating AMPK signaling, while suppressing phosphorylation of 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 in diabetic rat kidneys [19]. Wu et al. demonstrated increased SIRT1/FOXO1 activity in the diabetic rat kidney, effectively attenuating renal oxidative stress [20].

The nephroprotective effects of resveratrol were not limited to an amelioration of pathological renal fibrosis but extended to other renal morbidities such as nephrolithiasis, also known as the deposition of kidney stones. Hong et al. investigated the effect of resveratrol in rats with ethylene glycol-induced kidney stones. Resveratrol exhibited anti-nephrolithic properties by both inhibiting free radical formation and attenuating the expression of a number of inflammatory mediators and ECM proteins such as TGβ-1, osteopontin, hyaluronan, and MCP-1 [21].

In summary, resveratrol has shown protective effects against several types of kidney injuries mostly due to its antioxidant effect and activation of SIRT1. Further studies should be undertaken to examine the potential effect of resveratrol in human and animals models of kidney injuries.

4. Resveratrol in CNS Disease

The molecular pharmacology of resveratrol in the central nervous system follows the general themes for other organ systems, in that it produces a wide spectrum of physiological responses by affecting diverse functions. Equally interesting is the orchestration of second messenger systems in the brain, which parallels the activation of similar, if not the same, second messengers in other organs, following resveratrol treatment. It is interesting to note that a polyphenol phytochemical such as resveratrol elicits an influence over disparate neurological phenomena ranging from developmental neuronal processes to those involved in aging and dementia, probably by acting as a small molecule regulator of multiple intraneuronal processes.

The past two decades have seen tremendous effort in the elucidation of the molecular mechanisms of progressive

dementia such as Alzheimer's disease (AD), with multiple breakthroughs in molecular research leading to novel possible therapeutic interventions. Approaching AD from the perspective of protein misfolding and dysfunction has shed new light on pathological mechanisms. For instance, the "tau" protein is important for mitochondrial transport and therefore energy production in axons; its phosphorylation is associated with the progression of AD. Tau acetylation is increased in early to moderate Braak stages of tauopathy, which describe the degree of neurofibrillary tangles (NFTs) involved in AD. Therefore, modulating tau acetylation can serve as an effective new target in ameliorating neurodegeneration in relation to AD. Min et al. demonstrated that *in vitro* treatment with resveratrol leads to modulation of tau acetylation and thus attenuated tau-mediated neurodegeneration [22]. Additionally, in the context of AD, resveratrol was demonstrated to enhance the intracellular degradation of β amyloid peptides through a mechanism that implicated the proteasome [23]. Extracellular accumulation of β amyloid plaques was identified as an early pathological lesion in AD development. The beneficial effects of resveratrol for AD were not limited to reduction of β amyloid plaque but in a different study resveratrol improved mitochondrial function, reduced ROS activity, and promoted neuronal cell survival via activating the SIRT1 pathway [24]. As noted, involvement of SIRT1 downstream of resveratrol is a feature shared by the resveratrol-mediated intracellular protection pathway in multiple organs, for instance as discussed above for the kidney.

Telomeres are located at the end of chromosomes and become shorter in length with cell division. Telomere shortening is correlated with aging and is believed to play a key role in the pathogenesis of AD. Shorter telomeres are associated with increased neuronal cell death, as they are more susceptible to stressors such as ROS and UV radiation. Resveratrol mediated neuroprotective effects *in vitro*, by promoting the expression of telomere-maintenance factors such as WRN helicase gene [25]. Additionally, the neuroprotective effects of resveratrol included anti-inflammatory regulation of β amyloid-triggered microglial activation. Microglial cells serve a macrophage-like scavenger function in the CNS and remove infected and dying cells by phagocytosis. The anti-inflammatory effect of resveratrol is associated with the inhibition of the nuclear factor κ-light chain enhancer of activated B cells (NFκB), STAT, and TLR4 signaling pathways [26].

Neuronal inflammation may also be associated with other CNS disease states. For instance, inflammatory mediators and activation of glial cells seem to contribute to dopaminergic cell death in Parkinson's disease (PD). Such selective neurotoxicity, followed by apoptosis, is considered to be hallmark of the pathogenesis of PD, which is believed to originate from an imbalance in acetylcholine and dopamine in the CNS. The protein, suppressor of cytokine signaling (SOCS-1), regulates several CNS processes including glial cell activation and inflammatory reactions. The neuroprotective action of resveratrol was observed in murine models of Parkinson-like disease, where treatment with this polyphenol resulted in the expression of SOCS-1, effectively impairing microglial activation and inflammatory reactions [27]. Further, resveratrol administration to PD rats at 20 mg/kg/day for 14 days resulted in a reduction in all the following: (1) abnormal rotational behavior [28], (2) nigral cell death, and (3) ROS species [27]; the first two are directly linked to the phenotypic manifestation of PD in humans and may offer relief from debilitating tardive kinesis and dyskinesia. In this experimental model, liposomal delivery of resveratrol augmented its neuroprotective effects [28]. Finally, oxidative stress is one of the mechanisms thought to induce apoptotic death of dopaminergic neurons in PD. Resveratrol treatment ameliorated the effect on MPP+ (Parkinsonian toxin) induced oxidative stress on dopaminergic neurons. Pretreatment with resveratrol diminished neuronal cell death by modulating pro- and antiapoptotic proteins such as Bax and Bcl-2 [29].

In addition to anti-inflammatory effects, resveratrol induced the production of neurotrophic factors, such as the glial cell line-derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF), which play an important role in the survival of neurons and oligodendrocytes. Neurotrophic factors originate from the astroglia and may be a promising therapy for neurodegenerative diseases like PD. Resveratrol was effective in increasing production of neurotrophic factors in astroglial-enriched culture mediums, four- to sixfold, compared with control cultures. It was postulated that resveratrol mediated the release of neurotrophic factors via the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and cAMP responsive element-binding protein (CREB) in the astroglia [30].

In relation to other types of neurological stressors, the recent literature has found a strong correlation between hyperglycemia and neurodegeneration. Resveratrol ameliorated the effects of hyperglycemia-induced oxidative stress and subsequent apoptosis of dopaminergic neurons, by regulating the genes modulating the apoptotic cascade and reducing p53 and superoxide anion expression [31].

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by selective loss of motor neurons in the brain and spinal cord. Using a mice model of ALS, resveratrol was found to delay the disease onset, extend life span of animals, ameliorate loss of motor neurons, and inhibit mitochondrial dysfunction and muscle atrophy [32, 33]. Such beneficial effects of resveratrol were associated with enhanced expression and activation of SIRT1 and AMPK in vertebral spinal cord, improved lipid peroxidation, and inhibition of P53 and its downstream apoptotic pathway.

In summary, the above *in vitro* and animal studies provide growing evidence that resveratrol has beneficial effects in several neurodegenerative diseases. These effects are mostly attributed to its antioxidant effect, activation of SIRT1, and anti-inflammatory effect. Although many clinical trials have been conducted recently to investigate the potential effect of resveratrol in neurodegenerative disorders, there is no complete large scale clinical trial coming to a conclusive evidence [34, 35].

5. Resveratrol and Cancer

Resveratrol is similar to many bioactive phenolic phytochemicals in eliciting an antineoplastic response. Its antineoplastic properties may be classified into three broad areas: (1) antiproliferative, (2) antitumorigenic, and (3) antimetastatic. Though the following studies detail the specific molecular mechanisms of action, which may differ slightly, the sheer breadth of experimental cancer models and cancer cells that resveratrol is active against provides compelling evidence for conducting clinical trials in humans and for advocating its use in combination chemotherapy as a possible adjuvant. However, a careful analysis of its mechanism of action must be completed before any such endeavor is instituted, as exemplified by the estrogenic studies done on resveratrol that are outlined below. Interestingly, overall resveratrol treatment greatly benefits carcinoma therapy, suggesting the involvement of similar intracellular molecular signaling pathways downstream of resveratrol treatment that result in tumor regression in carcinomas.

5.1. Resveratrol and Cancer Pathogenesis

5.1.1. Colon Cancer. Resveratrol has been found to possess anticancer activity against colon cancer both in human clinical trials and in experimental animal models in the laboratory.

Nguyen et al. conducted a phase I pilot clinical trial examining the effect of plant-derived resveratrol and grape powder on Wnt gene expression in colonic mucosa and colon cancer. The Wnt signaling pathway is implicated in the pathogenesis of colon cancer, with activating mutations seen in over 85% of cases. This clinical trial involving colon cancer patients showed that resveratrol administration reduced Wnt gene expression in normal mucosa cells but had no effect on cancerous cells [36].

In an experimental rat model, Tessitore et al. supplemented the drinking water supply to a final resveratrol dose of 200 μg/kg/day and demonstrated significant reduction in large aberrant crypt foci (ACF) in rat colons. ACF are clusters of abnormal glands that line the colon and rectum and form before the development of colorectal polyps. The regulation of Bax and p21 is pertinent to the pathogenesis of colon cancer since Bax is a proapoptotic member of the Bcl-2 family and p21 is a cyclin-dependent kinase inhibitor and thereby involved in cell cycle progression. The protective role of resveratrol in colonic carcinogenesis was achieved by modulating Bax and p21 expression in the ACF but not in the rest of the colon mucosa [37].

5.1.2. Skin Tumors. In a similar vein, treatment of mice with resveratrol led to a decline in 7,12-dimethylbenz[$\propto$]anthracene- (DMBA-) initiated skin tumors. Resveratrol was moderately effective in reducing DMBA induced tumor formation through induction of apoptosis, which was mediated via upregulation of p53, Bax, and apoptotic protease activating factor-1 (APAF-1), cytochrome c release from the mitochondria, and inhibition of Bcl-2 [38, 39].

Ultraviolet radiation is a major cause of nonmelanoma skin cancer and resveratrol treatment; both pre- and post-UVB exposure in mice resulted in a decrease in skin tumor formation. Resveratrol modulated its UVB protective effects via suppressing mRNA levels of protein survivin and increasing proapoptotic proteins like Smac [40].

5.1.3. Breast Cancer. Breast cancer is another area of oncology research where resveratrol treatment has shown beneficial responses in experimental models and where direct evidence of the molecular mechanisms of resveratrol has accumulated over the years.

Banerjee et al. showed that treatment of mice with resveratrol at dietary levels led to a reduction in the formation of DMBA-induced mammary tumors and effectively prolonged the latency period in mice. Its antitumorigenic effects were elicited through an inhibition of COX-2, matrix metaloproteinase-9 (MMP-9), and NFκB activation in breast tumor, since the enzymes MMP-9 and COX-2 are involved in the tumor metastasis, and NF-κB mediates tumor cell proliferation [41]. However, in addition to downregulating these enzymes, Bhat et al. [42] demonstrated heterogeneity in resveratrol-mediated estrogenic engagement using disparate experimental models of breast cancer. Bhat and colleagues demonstrated that resveratrol possessed both weak estrogenic potential as well as strong antagonism when coadministered with estrogen to breast cancer cells. The cell type appeared to influence the ratio of agonism to antagonism of resveratrol, indicating a tissue specific response, suggesting that resveratrol behaved as a selective estrogen receptor modulator or SERM drug. Further, by inducing apoptosis, suppressing angiogenesis through reducing VEGF extracellular levels and modulating progesterone receptor expression, resveratrol supplementation in mice positively impacted breast cancer development [43]. Provinciali et al. demonstrated that supplementation of the drinking water of experimental mice resulted in a reduction of mammary tumor formation and decreased the average number and size of mammary tumors. In the context of cellular mechanisms, these findings were associated with a downregulation of HER-2/neuexpression and increased apoptosis in mammary gland tumors and in other tumor cell lines [44].

5.1.4. Prostate Cancer. Mechanistic analysis of the action of resveratrol in prostate cancer was carried out in different studies. Harper and colleagues used a dose of 625 mg resveratrol per kg body weight and showed that resveratrol suppressed prostate cancer progression in a male transgenic adenocarcinoma mouse prostate cancer model. Resveratrol mediated chemopreventive effects against prostate cancer by reducing cell proliferation, insulin-like growth factor-1, and simultaneously downregulating the phosphorylated and activated MAP kinases called extracellular regulating kinase, ERK-1 and ERK-2 [45], which are involved in pathogenesis of prostate cancer [45]. In transgenic rats, Seeni et al. found that resveratrol inhibited prostate tumor growth via modulating apoptosis through androgen receptor downregulation and inhibiting androgen responsive glandular kallikrein (an ortholog of human prostate specific antigen) [46].

5.1.5. Hepatocellular Carcinoma. Resveratrol induced apoptosis of tumor cells in rats modeling hepatocellular cancer via facilitating an increase in cells in the G2-M phase and upregulating Nrf2 expression [47, 48]. According to Carbó et al., resveratrol administration resulted in a 25% reduction in tumor cell content [47]. Furthermore, Bishayee et al. noticed that resveratrol supplementation at 100 and 300 mg/kg decreased the appearance and number of hepatocyte nodules in Sprague-Dawley rats. In this study, the effect of resveratrol was evaluated in hepatitis B infected mice, which later spontaneously developed hepatocellular carcinoma (HCC) [48]. Resveratrol administration at 30 mg/kg/day for 4 months in transgenic mice significantly decreased the incidence of hepatitis B virus-mediated HCC by 5.3-fold. Resveratrol suppressed ROS production and enhanced hepatocyte proliferation, thereby replacing damaged cells in the transgenic liver and promoting regeneration. Furthermore, hepatocyte proliferation returned to basal levels after 30 days of resveratrol supplementation when mice recovered from the liver pathology [49].

5.1.6. Pancreatic Cancer. Cancer of the pancreas may originate from a variety of cellular sources within the pancreas, but the more common is pancreatic adenocarcinoma which, like the carcinomas of the breast and the prostate gland discussed above, originates from the ductal epithelium. Resveratrol caused a decline in pancreatic tumor growth in rats in a dose-dependent manner [50, 51]. The antitumor effects of resveratrol were linked to decreased activity of the inflammatory enzyme leukotriene A4 hydrolase and a promotion of cell cycle arrest through the activation of the FOXO transcription factor [50, 51]. Resveratrol was successful in inducing apoptosis in pancreatic cancer cells *in vitro*, through suppressing mIR-21 expression which is linked with the downregulation of Bcl-2 expression [52].

5.1.7. Gastric Cancer. The physiological effects of resveratrol were also evaluated in congenic mice. Congenic mice provide an experimental animal model that contains mutated adenomatous polyposis coli (APC) genes, as a result becoming predisposed to developing intestinal tumors. Resveratrol supplementation in congenic mice led to a 70% decline in small intestinal tumor formation through the downregulation of genes involved in the cell cycle progression such as cyclins D1 and D2 and the DP-1 transcription factor. Furthermore, resveratrol promoted suppression of the carcinogenesis process by activating cytotoxic T cells, leukemia inhibitory factor receptor, and monocyte chemotactic protein-3 [53]. Resveratrol inhibited the growth of human primary gastric carcinoma cells in nude mice, which lack the ability to mount an immune response, by promoting apoptosis via attenuating the expression of *BCL-2*, while enhancing *BAX* gene expression [54].

5.1.8. Lung Cancer. Resveratrol treatment reduced tumor volume (42%) and weight (44%) in a lung cancer model at doses of 2.5 and 10 mg/kg. Resveratrol also reduced metastasis (56%) by significantly decreasing the number of tumor cell colonies that metastasized to the lung in Lewis lung carcinoma bearing mice [55]. The authors suggested that the antitumor activities of resveratrol might be linked to increased apoptosis via reduction in the S phase population and inhibition of neovascularization [55]. Furthermore, Lee et al. also noticed antitumor effects of resveratrol via caspase-mediated apoptosis, increased expression of caspase-9 and caspase-3, and suppression of tumor proliferation through inhibition of basic fibroblast growth factor (BFGF) [56].

Combination therapy may be an effective approach in the treatment of cancer. Radiotherapy is one of the most widely used interventions in cancer treatment; however, its success is limited by the development of resistant cancer cells. Thus, using resveratrol as an adjuvant drug, along with the use of increased ionizing radiotherapy, induced apoptosis in nonsmall cell lung cancer (NSCLC) cells via suppressing the formation of ROS [57]. Similarly, resveratrol enhanced the *in vivo* antitumor effects of 5-fluorouracil in mice inoculated with H22. Resveratrol instigated S phase arrest of H22 cells and also ameliorated the toxic effects of 5-FU [58].

5.1.9. Resveratrol and Metastases. A phase I randomized double-blind clinical trial involving the administration of micronized resveratrol (SRT501) for 14 days to patients with hepatic metastases found resveratrol to be well tolerated, detectable in hepatic tissue, and also found significantly increased levels of caspase-3 in malignant hepatic tissue, suggesting resveratrol-mediated apoptosis of metastasized cancer cells [3]. Further evidence for resveratrol-mediated amelioration of metastases was reported by Brown et al. They documented that resveratrol treatment in 40 healthy volunteers for 29 days resulted in a reduction of circulating insulin-like growth factor-1 (IGF-1) and IGFBP-3 in all volunteers. Increases in IGF-1 and IGFBP-3 had been associated with metastasis, leading the authors to suggest that resveratrol may serve as a potential chemotherapeutic adjuvant [59].

In summary, several studies using animal models and different cancer cells *in vitro* have indicated that resveratrol appears to have several antitumor mechanisms. Considering similarities in the origin, pathogenesis, and progression of carcinomas, the tumor suppressing activity of resveratrol suggests the targeting of similar intracellular oncogenic molecular patterns by this phenolic phytochemical. Compared to these positive effects, the data from the limited human clinical trials has sown inconsistent outcomes of resveratrol supplementation. It should be noted that most of these clinical trials have small patient sample size and used different doses and different routes of resveratrol administration. It is worthy to note that some reports have shown adverse effects of resveratrol in certain cancer patients. For example, in a phase II clinical trial using a dose of 5 g/day in myeloma patients, resveratrol was found to cause nausea, diarrhea, and nephrotoxicity which may have led to one death [60]. Together, both *in vitro* and *in vivo* data suggest a need for more extensive and large clinical trials to emphasize the efficacy and safety of resveratrol as an anticancer agent. Currently, the most promising use of resveratrol is mostly as a cancer chemopreventive agent.

6. Resveratrol and Metabolic Disorders

Obesity, insulin resistance, and disorders of lipid metabolism are multifactorial diseases that may develop due to both intrinsic and extrinsic factors. Due to the complicated etiology and pathogenesis of metabolic disease, in many instances, therapy requires both behavioral modification and combination therapy, in addition to the use of dietary adjuvants which allow pharmacological synergism and an enhanced physiological response. Resveratrol, being a dietary constituent is an excellent candidate for adjuvant therapy for disorders with metabolic origin.

Since several studies using animal models of diet-induced obesity have shown beneficial effect of resveratrol on improving insulin sensitivity and obesity, many clinical trials were performed to speculate its effect as an antidiabetic agent in humans. For example, Brasnyó et al. evaluated the effects of 10 mg resveratrol supplementation for 4 weeks in a randomized, double-blind, placebo-controlled study involving patients with type 2 diabetes. The results of this this study reveal that resveratrol was effective in attenuating insulin resistance that was shown by a reduction in blood glucose levels and a delay in the time for peak postprandial glucose in patients receiving resveratrol compared to placebo [61]. Similarly, Labbé et al. demonstrated a positive effect on insulin sensitivity and blood glucose levels in mice following resveratrol treatment. Furthermore, resveratrol treated mice had a 33% reduction in inguinal fat in comparison to the control, even though the daily food and water intake was similar between the two groups [62].

Resveratrol supplementation significantly improved glycemic control, in clinical trials involving patients with impaired glucose tolerance. Resveratrol was effective in reducing hemoglobin-A1c levels, postprandial glucose levels, and postprandial insulin AUC by approximately 18% [63, 64].

Several studies have been conducted to investigate the mechanisms responsible for eliciting the positive blood glucose regulating effects of resveratrol. For example, Um et al. administered resveratrol to AMPK deficient mice and control mice for 13 weeks. Although resveratrol improved insulin sensitivity, metabolic rate, and glucose tolerance in the control mice, it failed to evoke any effects in AMPK deficient mice. The results of this study suggested that resveratrol mediated its metabolic effects primarily through activating AMPK [65]. Other postulated mechanisms include anti-inflammatory, antioxidant, and SIRT1-dependant suppression of protein-tyrosine phosphate 1B that acts on insulin receptors.

Resveratrol was also shown to exhibit favorable effects in obese mice on an atherogenic diet by ameliorating steatohepatitis via modulating the genes involved in lipid metabolism [66]. In a study by Gómez-Zorita et al., resveratrol decreased both whole body and liver weight in obese rats through reducing oxidative stress [67]. Although resveratrol administration to rodent models elicited positive effects on metabolic syndrome, resveratrol supplementation in obese men and nonobese postmenopausal women did not yield any significant metabolic benefits [68, 69].

Poulsen et al. reported a randomized, placebo-controlled clinical trial involving high-dose supplementation of resveratrol for obese men [68]. Specifically, following resveratrol administration, the substrate metabolism, insulin sensitivity, and body composition of 24 obese but otherwise healthy men enrolled in the 4-week long clinical trial were evaluated. Interestingly, the group did not notice an improvement in any of the study parameters, which may be attributed to the specific formulation of resveratrol used for administration. We contend that, given the pharmacokinetics of resveratrol, there may have been dramatic fluctuations in its bioavailability, a parameter that did not appear to be controlled across the enrolled group. Further, ethnicity data, which may affect pharmacogenomic variance, are not reported in their study and may contribute a cause for deviation from expected results [68].

Yoshino et al. conducted a similar study in nonobese Caucasian postmenopausal women with normal glucose tolerance, where they tested the effect of resveratrol administration for 12 weeks on various metabolic parameters in this study population. Additionally, they also investigated the effect of resveratrol on selected signal transduction molecules, such as Adenosine monophosphate kinase (AMPK), which is known to be activated in response to resveratrol treatment, but found no changes in any of the selected second messengers. However, in addition to the variability in the bioavailability of resveratrol as explained for the clinical study above, resveratrol was administered with food, which may lead to lower absorption [69]. The study conducted by this author group also suffered from another design drawback which may explain the lack of differences in the induction of signal transduction molecules. Of note is the fact that the biological samples were collected from skeletal muscle biopsies and subcutaneous adipose tissue harvests from all individuals enrolled in the study. Since the authors only examined signal transduction molecules from these limited tissue type, it is conceivable that the physiological effect of resveratrol was overlooked.

Thus, although both clinical trials indicate a paucity of resveratrol-mediated profound effects on metabolic markers such as insulin sensitivity, blood pressure, basal metabolic rate, plasma lipids, or body weight, the study design in both cases did not take into consideration some of the flaws discussed above.

In contrast, Dash et al. evaluated the effects of resveratrol administration for 2 weeks in obese men and reported a decrease in intestinal and hepatic lipoprotein particle production [70]. It is well established that elevated levels of hepatic apolipoprotein B (apoB-100) and intestinal apoB-48 particles are linked to the pathogenesis of hypertriglyceridemia [70]. Resveratrol's ability to modulate the production of intestinal and hepatic lipoproteins can alleviate hypertriglyceridemia.

Dal-Pan et al. postulated resveratrol's facilitation of weight loss in non-human primate models of obesity by increasing metabolic rate and suppressing torpor expression. In fact, resveratrol administration in primate models led to a 13% reduction in energy intake while increasing resting metabolic rate by 29% [71]. This study is further supported by Timmers et al. where, in a randomized, double-blind

crossover study, resveratrol supplementation at 150 mg/day in 11 obese men mimicked the effects of caloric restriction by significantly reducing intramyocellular lipid content, hepatic lipid synthesis, blood glucose levels, and triglycerides in the subjects. The effects were linked to mechanisms involving increased AMPK, SIRT1, PGC-1∝ protein levels, and citrate synthase activity [72].

It is noteworthy that hypoglycemia, one of the major adverse effects associated with most of the antidiabetic agents, has not been reported with resveratrol in any animal or human studies, making resveratrol a viable option once its efficacy is thoroughly demonstrated.

7. Resveratrol and Cardiovascular Disease

One of the principal protective effects of resveratrol is on the cardiovascular system, where its use improves a variety of cardiac and hemodynamic functions. In fact, discovery of its cardioprotective effects was the primary reason that augmentation of therapy with resveratrol became a well-accepted notion.

Cao et al. reported resveratrol to mediate antihypertensive effects in angiotensin II induced hypertensive mice via activating AMPK and reducing expression of RhoA/ROCK genes. Furthermore, in spontaneously hypertensive rats, resveratrol treatment decreased blood pressure in comparison to control rats and ameliorated the progression of hypertension. These antihypertensive effects are linked with mechanisms involving the prevention of eNOS uncoupling and NO scavenging [73, 74].

Interestingly, resveratrol appeared to orchestrate multiple cardiac responses by a direct effect on cardiac function and restructuring. Kanamori et al. reported that high dose resveratrol improved cardiac performance via the AMPK pathway. In their experiments, resveratrol reduced left ventricular end-diastolic pressure and dilation, while preserving systolic pressure. In another study involving hypertensive rat models, resveratrol supplementation at 18 mg/kg/day for 8 weeks resulted in improved survival rate, reduction in HDL and LDL cholesterol levels, and reduction in markers of cardiac remodeling such as atrial natriuretic factor (ANF) [75]. These effects were linked to the upregulation of PGC-1a and PPAR∝ cardiac expression [76]. Resveratrol was also found to attenuate cardiac hypertrophy in another study of hypertensive rats through modulating the LKB1/AMPK signaling pathway [77].

Resveratrol mitigated platelet aggregation *in vitro* by reducing thromboxane A2 receptor levels, phospholipase C activity, and GPIIb/IIIa expression [78, 79]. The inhibitory effects of resveratrol on platelets were also evident *in vivo* through increased cGMP and Nitric Oxide (NO) levels. Activation of NO inhibited phospholipase C [80, 81]. The cardiovascular protective effect of resveratrol was further demonstrated in high-risk cardiac patients with aspirin resistance, where resveratrol significantly attenuated aspirin-resistant platelet aggregation, while exhibiting minimal effects on aspirin sensitive platelets [82].

The ischemia-reperfusion model has emerged as an excellent experimental system to validate the effects of various therapies on the heart and to elicit the molecular biology of the process. Pretreatment of rats with resveratrol followed by experimentally induced ischemia reperfusion, led to significant cardioprotection as evidenced by attenuated NOS (nitric oxide synthase) induction, decreased myocardial infarct size, and reduced numbers of cardiomyocytes. Resveratrol elicited cardioprotective effects through mediating NO-dependent pathways and inducing HO-1 expression, which involved p38 MAP Kinase and the PI-3 Kinase signaling systems [83, 84]. In separate experiments, resveratrol administration in rats improved ischemia-reperfusion injury through reduced calcium overload as a direct consequence of the inhibition of the Wnt5a/Frizzled-2 pathway. It also improved postischemic ventricular function via upregulated superoxide dismutase and peroxidase activities and downregulated catalase enzyme activity [85, 86]. Its cardioprotective effects were further validated in postmyocardial infarction patients, where resveratrol improved left ventricular ejection fraction and reduced LDL levels [87].

Resveratrol supplementation at 3 mg/kg/day in a hypercholesterolemic rabbit model led to a reduction in size, density, and mean area of atherosclerotic plaques and a decrease in the intimal layer thickness [88]. Resveratrol and a resveratrol containing mixture also elicited antiatherogenic properties in apoE-deficient mice. The mechanisms for resveratrol mediated antiatherogenic effects include the reduction of chemokines such as MCP-1 and the downregulation of intracellular adhesion molecule-1 (ICAM-1) and of vascular cell adhesion molecule-1 (VCAM-1) in the atherosclerotic vessels [89, 90].

In conclusion, resveratrol treatment provides far ranging cardiovascular protection by improving both cardiac function and architecture, while decreasing vascular effects such as platelet clumping, ischemia-reperfusion injury, and atherosclerosis. Several direct and indirect molecular targets mediating these cardiovascular effects have been identified and include AMP-activated protein kinase, cyclooxygenase 1, thromboxane A2, ICAM, NF-*k*B, and SIRT-1.

In brief, plethora of reports from *in vitro* and laboratory animal studies in the past decade has suggested that resveratrol has considerable potential for therapy and/or protection for numerous acute and chronic diseases. These data were supported, in most part, by the emerging results from limited human clinical trials. Larger controlled human clinical trials are still needed to investigate dose- and formulation-dependent efficacy and safety of resveratrol and to study the effect of long-term effect of resveratrol supplementation. Figure 2 provides a summary of the aforementioned health benefits of resveratrol with potential underlying molecular mechanisms.

8. Pharmacokinetics of Resveratrol

All the studies discussed thus far suggest that inclusion of resveratrol in therapy augments health benefits through multiple mechanisms of action. However, an important question that remains to be answered before translating *in*

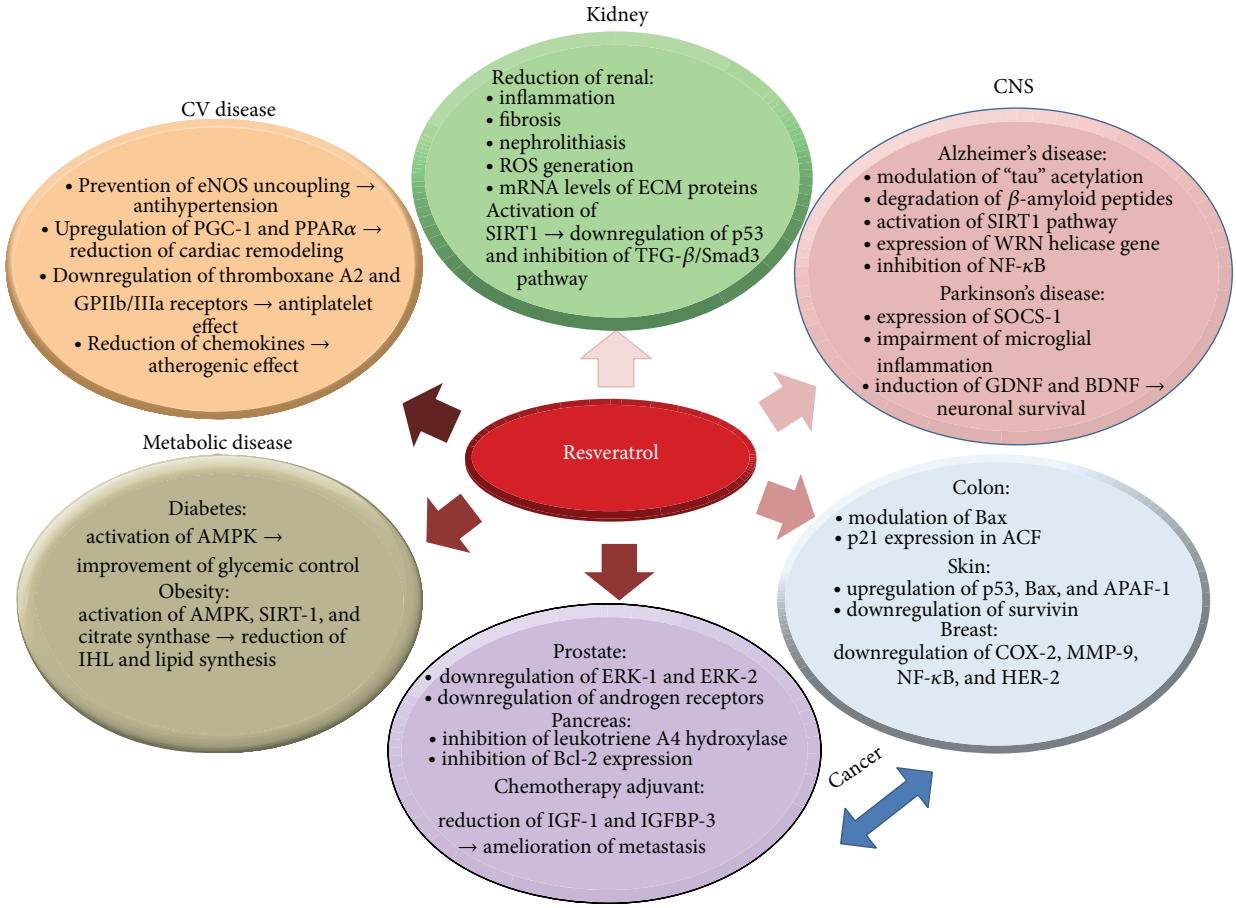

Figure 2: A summary of the published health benefits of resveratrol with the potential underlying molecular mechanisms. (See the Abbreviations section for complete definition of the abbreviations.)

vitro and preclinical research into therapeutics is a consideration of the dose of resveratrol that would yield optimal response. Resveratrol doses that have been utilized in most of the published studies in animal models range from 0.1 to 1000 mg/kg. To add to the complexity, dose-dependent effects of resveratrol have been observed. For example, although high dose (400 mg/kg/day) causes a significant weight loss in mice, low dose (5 mg/kg) was found to induce weight gain [91]. Understanding the pharmacokinetics of resveratrol may significantly help answering this question.

Pharmacokinetic studies in human and animal models indicate that resveratrol bioavailability shows a high interindividual variation, which may limit its clinical application. The main reason found to contribute significantly to such variability is the role of intestinal microbial flora in resveratrol metabolism [92]. Although studies in rats report that 15–50% of orally ingested resveratrol is bioavailable, higher bioavailability (20–70%) was reported in human studies [93–95]. Comparison of bioavailability after a single oral dose to daily dose for 14 days did not show any evidence of saturation-limited absorption [95], suggesting perhaps that the uptake of resveratrol does not depend on a receptor-mediated process. Achieving adequate and consistent bioavailability at the desired doses is generally the major issue limiting future therapeutic application of resveratrol. Table 1 summarizes strategies that have been utilized to enhance resveratrol bioavailability after oral administration.

Interestingly, dietary constituents were found to affect the rate and extent of resveratrol absorption. High fat meal significantly decreased the exposure level of resveratrol, as indicated by AUC and C_{max} [96]. Although rapid uptake of resveratrol in humans resulted in a peak plasma concentration at 0.5 hours, administration under fasting conditions delayed the peak to 1.5–2 hours [93, 94]. It should be noted that none of these studies have included control groups where subjects administered resveratrol by itself; therefore, it may be difficult to conclude whether high fat meal or fasting condition significantly affects AUC and C_{max} compared to resveratrol alone.

Resveratrol was quickly metabolized, with an average elimination half-life of 130–180 minutes, with glucuronide and sulfate conjugates, resulting in mixed sulfate-glucuronides, which were the primary metabolites [95]. It was suggested that these phase II conjugates underwent enterohepatic recycling where they got secreted back to the intestine, deconjugated, and subsequently either reabsorbed or

Table 1: Approaches to enhance resveratrol's bioavailability.

Strategy	Examples	Effect on absorption pharmacokinetics	Reference
Solubility	(1) SRT501 formulation: a mixture of micronized resveratrol with particle size <5 μm, flavors, colors, and emulsifiers.	More than 4-fold increase in C_{max}, AUC and 2-fold increase in T_{max}.	[3]
	(2) Using hydroxypropyl-β-cyclodextrin.	Significant increase in rate of absorption with a negligible impact on bioavailability.	[4]
1st-pass effect	(1) Coadministration with piperine, a polyphenol from black pepper.	1000% increase in C_{max} and delaying the major glucuronide metabolite.	[5]
	(2) Coadministration with quercetin, a plant flavonoid with potent antioxidant action.	Quercetin reduces the rate of resveratrol sulfate conjugation, but with no significant improvement on C_{max} and AUC.	[6]
Resveratrol precursors/prodrugs	Acetylation of the hydrophilic hydroxyl groups on resveratrol that are the major targets for sulfation and glucuronidation.	A significant increase in C_{max} and AUC in a single animal study.	[7]
Nanoformulation	(1) Lipid-core nanocapsules.	Nanoformulation improves resveratrol's solubility, decreases gastrointestinal damage, and enhances distribution to tissues.	[8]
	(2) Bovine serum albumin nanoparticles.		[9]
	(3) Loaded solid nanoparticles.		[10]

Table 2: Average resveratrol pharmacokinetic parameters in both rats and human.

Bioavailability F (%)		Volume of distribution V_d (L/kg)		Systemic clearance Cl (mL/min/kg)		Elimination half-life $t_{1/2}$ (min)	
Rats	Human	Rats	Human	Rats	Human	Rats	Human
29	70	35	30	184	157	130	180

excreted in feces [93]. Such extensive metabolism and rapid elimination of its conjugated metabolites may explain the low bioavailability of resveratrol.

Modulation of drug-metabolizing enzymes by resveratrol has been extensively studied *in vitro* and *in vivo*. Inhibition of benzo[a]pyrene-induced carcinogenesis by resveratrol was attributed to the ability of the latter to inhibit CYP1A1, an enzyme that is involved in the bioactivation of many carcinogens [97]. Administration of 1 g resveratrol for 4 weeks to forty-two healthy volunteers was found to inhibit the activity of CYP3A, CYP2D6, and CYP2C9 and to induce the activity of CYP1A2 [98]. As these P450 enzymes metabolize more than 75% of the drugs on the market, coadministration of resveratrol may influence the pharmacokinetics of drugs that are significantly metabolized by these enzymes. The same study revealed that resveratrol intervention induced the activity of phase II enzymes, especially glutathione-S-transferases (GST) in patients with low baseline enzyme activity. As phase II enzymes mediate detoxification of reactive metabolites and carcinogens, induction of this pathway by resveratrol could explain the chemopreventive effects described in the preceding sections. Comparison of the metabolism kinetics of resveratrol after a single dose and chronic administration provided no clear evidence of nonlinear kinetics [99]. Table 2 summarizes the average pharmacokinetic parameters for resveratrol based on limited published data from animal and human studies [94, 95, 100, 101].

9. Conclusion

Resveratrol affects multiple cellular processes and is an excellent candidate for use in human disorders. However, its physicochemical properties, coupled with its vagrant metabolism, may limit its bioavailability necessitating the design and fabrication of novel pharmaceutical delivery systems.

Abbreviations

ACF: Aberrant crypt foci
AD: Alzheimer's disease
AKI: Acute kidney injury
ALS: Amyotrophic lateral sclerosis
AMPK: Adenosine monophosphate kinase
APAF-1: Apoptotic protease activating factor-1
ANF: Atrial natriuretic factor
APAF-1: Apoptotic protease activating factor-1
APC: Adenomatous polyposis coli
ApoB: Apolipoprotein B
AUC: Area under the curve

BCL-2: B-cell lymphoma 2
BDNF: Brain derived neurotrophic factor
BFGF: Basic fibroblast growth factor
cAMP: Cyclic adenosine monophosphate
cGMP: Cyclic guanosine monophosphate
CREB: cAMP-response-element-binding protein
DMBA: Dimethylbenz[α]anthracene
ECM: Extracellular matrix
eNOS: Endothelial nitric oxide synthase
ERK-1: Extracellular regulating kinase 1
GDNF: Glial cell line-derived neurotrophic factor
GST: Glutathione-S-transferases
HCC: Hepatocellular carcinoma
HDL: High-density lipoprotein
HER-2: Human epidermal growth factor 2
HO-1: Heme-oxygenase-1
H22: Hepatocarcinoma cells 22
ICAM-1: Intracellular adhesion molecule-1
IGFBP-3: Insulin-like growth factor binding protein-3
ILC: Intramyocellular lipid content
ILGF-1: Insulin-like growth factor-1
LDL: Low-density lipoprotein
LKB1: Liver kinase B1
MAP: Mitogen-activated protein
MCP-1: Monocyte chemoattractant protein-1
MDA: Malondialdehyde
MMP-9: Matrix metaloproteinase-9
MPP^+: 1-Methyl-4-phenylpyridinium
NFR2: Nuclear factor erythroid factor
NFkB: Nuclear factor kappa B
NFT: Neurofibrillary tangles
NOS: Nitric oxide synthase
NSCLC: Nonsmall cell lung cancer
PD: Parkinson's disease
PPAR-γ: Peroxisome proliferator-activated receptor gamma
ROCK: Rho-associated, coiled coil containing protein kinase
ROS: Reactive oxygen species
SERM: Selective estrogen receptor modulator
SIRT-1: Sirtuin 1
SOCS-1: Suppressor of cytokine signaling 1
STAT: Signal transduction and activator of transcription
TGF-β: Transforming growth factor beta
TLR4: Toll-like receptor 4
TNF-α: Tumor necrosis factor alpha
VCAM-1: Vascular cell adhesion molecule-1
VEGF: Vascular endothelial growth factor
4E-BP1: 4E-binding protein 1
5-FU: Fluorouracil
8-OHdG: 8-Hydroxydeoxyguanosine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] A. R. Neves, M. Lúcio, J. L. C. Lima, and S. Reis, "Resveratrol in medicinal chemistry: a critical review of its pharmacokinetics, drug-delivery, and membrane interactions," *Current Medicinal Chemistry*, vol. 19, no. 11, pp. 1663–1681, 2012.

[2] V. Cucciolla, A. Borriello, A. Oliva, P. Galletti, V. Zappia, and F. D. Ragione, "Resveratrol: from basic science to the clinic," *Cell Cycle*, vol. 6, no. 20, pp. 2495–2510, 2007.

[3] L. M. Howells, D. P. Berry, P. J. Elliott et al., "Phase I randomized, double-blind pilot study of micronized resveratrol (SRT501) in patients with hepatic metastases—safety, pharmacokinetics, and pharmacodynamics," *Cancer Prevention Research*, vol. 4, no. 9, pp. 1419–1425, 2011.

[4] S. Das, H.-S. Lin, P. C. Ho, and K.-Y. Ng, "The impact of aqueous solubility and dose on the pharmacokinetic profiles of resveratrol," *Pharmaceutical Research*, vol. 25, no. 11, pp. 2593–2600, 2008.

[5] J. J. Johnson, M. Nihal, I. A. Siddiqui et al., "Enhancing the bioavailability of resveratrol by combining it with piperine," *Molecular Nutrition and Food Research*, vol. 55, no. 8, pp. 1169–1176, 2011.

[6] C. de Santi, A. Pietrabissa, F. Mosca, and G. M. Pacifici, "Glucuronidation of resveratrol, a natural product present in grape and wine, in the human liver," *Xenobiotica*, vol. 30, no. 11, pp. 1047–1054, 2000.

[7] L. Liang, X. Liu, Q. Wang, S. Cheng, S. Zhang, and M. Zhang, "Pharmacokinetics, tissue distribution and excretion study of resveratrol and its prodrug 3,5,4′-tri-*O*-acetylresveratrol in rats," *Phytomedicine*, vol. 20, no. 6, pp. 558–563, 2013.

[8] R. L. Frozza, A. Bernardi, K. Paese et al., "Characterization of trans-resveratrol-loaded lipid-core nanocapsules and tissue distribution studies in rats," *Journal of Biomedical Nanotechnology*, vol. 6, no. 6, pp. 694–703, 2010.

[9] L. Guo, Y. Peng, J. Yao, L. Sui, A. Gu, and J. Wang, "Anticancer activity and molecular mechanism of resveratrol-bovine serum albumin nanoparticles on subcutaneously implanted human primary ovarian carcinoma cells in nude mice," *Cancer Biotherapy and Radiopharmaceuticals*, vol. 25, no. 4, pp. 471–477, 2010.

[10] S. Jose, S. S. Anju, T. A. Cinu, N. A. Aleykutty, S. Thomas, and E. B. Souto, "In vivo pharmacokinetics and biodistribution of resveratrol-loaded solid lipid nanoparticles for brain delivery," *International Journal of Pharmaceutics*, vol. 474, no. 1-2, pp. 6–13, 2014.

[11] K. T. Howitz, K. J. Bitterman, H. Y. Cohen et al., "Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan," *Nature*, vol. 425, no. 6954, pp. 191–196, 2003.

[12] M. Kaeberlein, T. McDonagh, B. Heltweg et al., "Substrate-specific activation of sirtuins by resveratrol," *The Journal of Biological Chemistry*, vol. 280, no. 17, pp. 17038–17045, 2005.

[13] M. T. Borra, M. R. Langer, J. T. Slama, and J. M. Denu, "Substrate specificity and kinetic mechanism of the Sir2 family of NAD^+-dependent histone/protein deacetylases," *Biochemistry*, vol. 43, no. 30, pp. 9877–9887, 2004.

[14] N. L. Price, A. P. Gomes, A. J. Y. Ling et al., "SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function," *Cell Metabolism*, vol. 15, no. 5, pp. 675–690, 2012.

[15] X.-Z. Huang, D. Wen, M. Zhang et al., "Sirt1 activation ameliorates renal fibrosis by inhibiting the TGF-β/Smad3 pathway," *Journal of Cellular Biochemistry*, vol. 115, no. 5, pp. 996–1005, 2014.

[16] J. Liang, S. Tian, J. Han, and P. Xiong, "Resveratrol as a therapeutic agent for renal fibrosis induced by unilateral ureteral obstruction," *Renal Failure*, vol. 36, no. 2, pp. 285–291, 2014.

[17] S. Sharma, M. Anjaneyulu, S. K. Kulkarni, and K. Chopra, "Resveratrol, a polyphenolic phytoalexin, attenuates diabetic nephropathy in rats," *Pharmacology*, vol. 76, no. 2, pp. 69–75, 2006.

[18] P. Palsamy and S. Subramanian, "Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1812, no. 7, pp. 719–731, 2011.

[19] D.-F. Ding, N. You, X.-M. Wu et al., "Resveratrol attenuates renal hypertrophy in early-stage diabetes by activating AMPK," *American Journal of Nephrology*, vol. 31, no. 4, pp. 363–374, 2010.

[20] L. Wu, Y. Zhang, X. Ma, N. Zhang, and G. Qin, "The effect of resveratrol on FoxO1 expression in kidneys of diabetic nephropathy rats," *Molecular Biology Reports*, vol. 39, no. 9, pp. 9085–9093, 2012.

[21] S. H. Hong, H.-J. Lee, E. J. Sohn et al., "Anti-nephrolithic potential of resveratrol via inhibition of ROS, MCP-1, hyaluronan and osteopontin in vitro and in vivo," *Pharmacological Reports*, vol. 65, no. 4, pp. 970–979, 2013.

[22] S.-W. Min, S.-H. Cho, Y. Zhou et al., "Acetylation of tau inhibits its degradation and contributes to tauopathy," *Neuron*, vol. 67, no. 6, pp. 953–966, 2010.

[23] P. Marambaud, H. Zhao, and P. Davies, "Resveratrol promotes clearance of Alzheimer's disease amyloid-β peptides," *The Journal of Biological Chemistry*, vol. 280, no. 45, pp. 37377–37382, 2005.

[24] R. S. Khan, Z. Fonseca-Kelly, C. Callinan, L. Zuo, M. M. Sachdeva, and K. S. Shindler, "SIRT1 activating compounds reduce oxidative stress and prevent cell death in neuronal cells," *Frontiers in Cellular Neuroscience*, vol. 6, article 63, 2012.

[25] F. Uchiumi, T. Watanabe, S. Hasegawa, T. Hoshi, Y. Higami, and S.-I. Tanuma, "The effect of resveratrol on the werner syndrome recq helicase gene and telomerase activity," *Current Aging Science*, vol. 4, no. 1, pp. 1–7, 2011.

[26] H. Capiralla, V. Vingtdeux, H. Zhao et al., "Resveratrol mitigates lipopolysaccharide- and Aβ-mediated microglial inflammation by inhibiting the TLR4/NF-κB/STAT signaling cascade," *Journal of Neurochemistry*, vol. 120, no. 3, pp. 461–472, 2012.

[27] D. D. Lofrumento, G. Nicolardi, A. Cianciulli et al., "Neuroprotective effects of resveratrol in an MPTP mouse model of Parkinson's-like disease: possible role of SOCS-1 in reducing pro-inflammatory responses," *Innate Immunity*, vol. 20, no. 3, pp. 249–260, 2014.

[28] Y. Wang, H. Xu, Q. Fu, R. Ma, and J. Xiang, "Protective effect of resveratrol derived from Polygonum cuspidatum and its liposomal form on nigral cells in Parkinsonian rats," *Journal of the Neurological Sciences*, vol. 304, no. 1-2, pp. 29–34, 2011.

[29] J. Bournival, P. Quessy, and M.-G. Martinoli, "Protective effects of resveratrol and quercetin against MPP^+-induced oxidative stress act by modulating markers of apoptotic death in dopaminergic neurons," *Cellular and Molecular Neurobiology*, vol. 29, no. 8, pp. 1169–1180, 2009.

[30] F. Zhang, Y. F. Lu, Q. Wu, J. Liu, and J. S. Shi, "Resveratrol promotes neurotrophic factor release from astroglia," *Experimental Biology and Medicine*, vol. 237, no. 8, pp. 943–948, 2012.

[31] J. Renaud, J. Bournival, X. Zottig, and M.-G. Martinoli, "Resveratrol protects DAergic PC12 cells from high glucose-induced oxidative stress and apoptosis: effect on p53 and GRP75 localization," *Neurotoxicity Research*, vol. 25, no. 1, pp. 110–123, 2014.

[32] L. Song, L. Chen, X. Zhang, J. Li, and W. Le, "Resveratrol ameliorates motor neuron degeneration and improves survival in $SOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis," *BioMed Research International*, vol. 2014, Article ID 483501, 11 pages, 2014.

[33] R. Mancuso, J. del Valle, L. Modol et al., "Resveratrol improves motoneuron function and extends survival in SOD1(G93A) ALS mice," *Neurotherapeutics*, vol. 11, no. 2, pp. 419–432, 2014.

[34] T. Nunes, L. Almeida, J.-F. Rocha et al., "Pharmacokinetics of trans-resveratrol following repeated administration in healthy elderly and young subjects," *Journal of Clinical Pharmacology*, vol. 49, no. 12, pp. 1477–1482, 2009.

[35] C. H. Cottart, V. Nivet-Antoine, C. Laguillier-Morizot, and J. L. Beaudeux, "Resveratrol bioavailability and toxicity in humans," *Molecular Nutrition and Food Research*, vol. 54, no. 1, pp. 7–16, 2010.

[36] A. V. Nguyen, M. Martinez, M. J. Stamos et al., "Results of a phase I pilot clinical trial examining the effect of plant-derived resveratrol and grape powder on wnt pathway target gene expression in colonic mucosa and colon cancer," *Cancer Management and Research*, vol. 1, pp. 25–37, 2009.

[37] L. Tessitore, A. Davit, I. Sarotto, and G. Caderni, "Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression," *Carcinogenesis*, vol. 21, no. 8, pp. 1619–1622, 2000.

[38] N. Kalra, P. Roy, S. Prasad, and Y. Shukla, "Resveratrol induces apoptosis involving mitochondrial pathways in mouse skin tumorigenesis," *Life Sciences*, vol. 82, no. 7-8, pp. 348–358, 2008.

[39] G. J. Soleas, L. Grass, P. D. Josephy, D. M. Goldberg, and E. P. Diamandis, "A comparison of the anticarcinogenic properties of four red wine polyphenols," *Clinical Biochemistry*, vol. 35, no. 2, pp. 119–124, 2002.

[40] M. H. Aziz, F. Afaq, and N. Ahmad, "Prevention of ultraviolet-B radiation damage by resveratrol in mouse skin is mediated via modulation in survivin," *Photochemistry and Photobiology*, vol. 81, no. 1, pp. 25–31, 2005.

[41] S. Banerjee, C. Bueso-Ramos, and B. B. Aggarwal, "Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloprotease 9," *Cancer Research*, vol. 62, no. 17, pp. 4945–4954, 2002.

[42] K. P. L. Bhat, D. Lantvit, K. Christov, R. G. Mehta, R. C. Moon, and J. M. Pezzuto, "Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models," *Cancer Research*, vol. 61, no. 20, pp. 7456–7463, 2001.

[43] S. Garvin, K. Öllinger, and C. Dabrosin, "Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo," *Cancer Letters*, vol. 231, no. 1, pp. 113–122, 2006.

[44] M. Provinciali, F. Re, A. Donnini et al., "Effect of resveratrol on the development of spontaneous mammary tumors in HER-2/neu transgenic mice," *International Journal of Cancer*, vol. 115, no. 1, pp. 36–45, 2005.

[45] C. E. Harper, B. B. Patel, J. Wang, A. Arabshahi, I. A. Eltoum, and C. A. Lamartiniere, "Resveratrol suppresses prostate cancer progression in transgenic mice," *Carcinogenesis*, vol. 28, no. 9, pp. 1946–1953, 2007.

[46] A. Seeni, S. Takahashi, K. Takeshita et al., "Suppression of prostate cancer growth by resveratrol in the transgenic rat for adenocarcinoma of prostate (TRAP) model," *Asian Pacific journal of cancer prevention*, vol. 9, no. 1, pp. 7–14, 2008.

[47] N. Carbó, P. Costelli, F. M. Baccino, F. J. López-Soriano, and J. M. Argilés, "Resveratrol, a natural product present in wine, decreases tumour growth in a rat tumour model," *Biochemical and Biophysical Research Communications*, vol. 254, no. 3, pp. 739–743, 1999.

[48] A. Bishayee, K. F. Barnes, D. Bhatia, A. S. Darvesh, and R. T. Carroll, "Resveratrol suppresses oxidative stress and inflammatory response in diethylnitrosamine-initiated rat hepatocarcinogenesis," *Cancer Prevention Research*, vol. 3, no. 6, pp. 753–763, 2010.

[49] H.-C. Lin, Y.-F. Chen, W.-H. Hsu, C.-W. Yang, C.-H. Kao, and T.-F. Tsai, "Resveratrol helps recovery from fatty liver and protects against hepatocellular carcinoma induced by hepatitis B virus X protein in a mouse model," *Cancer Prevention Research*, vol. 5, no. 7, pp. 952–962, 2012.

[50] N. Oi, C.-H. Jeong, J. Nadas et al., "Resveratrol, a red wine polyphenol, suppresses pancreatic cancer by inhibiting leukotriene A(4) hydrolase," *Cancer Research*, vol. 70, no. 23, pp. 9755–9764, 2010.

[51] S. K. Roy, Q. Chen, J. Fu, S. Shankar, and R. K. Srivastava, "Resveratrol inhibits growth of orthotopic pancreatic tumors through activation of FOXO transcription factors," *PLoS ONE*, vol. 6, no. 9, Article ID e25166, 2011.

[52] P. Liu, H. Liang, Q. Xia et al., "Resveratrol induces apoptosis of pancreatic cancers cells by inhibiting miR-21 regulation of BCL-2 expression," *Clinical and Translational Oncology*, vol. 15, no. 9, pp. 741–746, 2013.

[53] Y. Schneider, B. Duranton, F. Gossé, R. Schleiffer, N. Seiler, and F. Raul, "Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis," *Nutrition and Cancer*, vol. 39, no. 1, pp. 102–107, 2001.

[54] H.-B. Zhou, J.-J. Chen, W.-X. Wang, J.-T. Cai, and Q. Du, "Anticancer activity of resveratrol on implanted human primary gastric carcinoma cells in nude mice," *World Journal of Gastroenterology*, vol. 11, no. 2, pp. 280–284, 2005.

[55] Y. Kimura and H. Okuda, "Resveratrol isolated from Polygonum cuspidatum root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in lewis lung carcinoma-bearing mice," *Journal of Nutrition*, vol. 131, no. 6, pp. 1844–1849, 2001.

[56] E.-O. Lee, H.-J. Lee, H.-S. Hwang et al., "Potent inhibition of Lewis lung cancer growth by heyneanol A from the roots of *Vitis amurensis*through apoptotic and anti-angiogenic activities," *Carcinogenesis*, vol. 27, no. 10, pp. 2059–2069, 2006.

[57] H. Luo, L. Wang, B. A. Schulte, A. Yang, S. Tang, and G. Y. Wang, "Resveratrol enhances ionizing radiation-induced premature senescence in lung cancer cells," *International Journal of Oncology*, vol. 43, no. 6, pp. 1999–2006, 2013.

[58] S.-L. Wu, Z.-J. Sun, L. Yu, K.-W. Meng, X.-L. Qin, and C.-E. Pan, "Effect of resveratrol and in combination with 5-FU on murine liver cancer," *World Journal of Gastroenterology*, vol. 10, no. 20, pp. 3048–3052, 2004.

[59] V. A. Brown, K. R. Patel, M. Viskaduraki et al., "Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics, and effect on the insulin-like growth factor axis," *Cancer Research*, vol. 70, no. 22, pp. 9003–9011, 2010.

[60] R. Popat, T. Plesner, F. Davies et al., "A phase 2 study of SRT501 (resveratrol) with bortezomib for patients with relapsed and or refractory multiple myeloma," *British Journal of Haematology*, vol. 160, no. 5, pp. 714–717, 2013.

[61] P. Brasnyó, G. A. Molnár, M. Mohás et al., "Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2 diabetic patients," *British Journal of Nutrition*, vol. 106, no. 3, pp. 383–389, 2011.

[62] A. Labbé, C. Garand, V. C. Cogger et al., "Resveratrol improves insulin resistance hyperglycemia and hepatosteatosis but not hypertriglyceridemia, inflammation, and life span in a mouse model for werner syndrome," *Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 66, no. 3, pp. 264–278, 2011.

[63] J. K. Bhatt, S. Thomas, and M. J. Nanjan, "Resveratrol supplementation improves glycemic control in type 2 diabetes mellitus," *Nutrition Research*, vol. 32, no. 7, pp. 537–541, 2012.

[64] J. P. Crandall, V. Oram, G. Trandafirescu et al., "Pilot study of resveratrol in older adults with impaired glucose tolerance," *Journals of Gerontology, Series A: Biological Sciences and Medical Sciences*, vol. 67, no. 12, pp. 1307–1312, 2012.

[65] J.-H. Um, S.-J. Park, H. Kang et al., "AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol," *Diabetes*, vol. 59, no. 3, pp. 554–563, 2010.

[66] J. Ahn, I. Cho, S. Kim, D. Kwon, and T. Ha, "Dietary resveratrol alters lipid metabolism-related gene expression of mice on an atherogenic diet," *Journal of Hepatology*, vol. 49, no. 6, pp. 1019–1028, 2008.

[67] S. Gómez-Zorita, A. Fernández-Quintela, M. T. MacArulla et al., "Resveratrol attenuates steatosis in obese Zucker rats by decreasing fatty acid availability and reducing oxidative stress," *British Journal of Nutrition*, vol. 107, no. 2, pp. 202–210, 2012.

[68] M. M. Poulsen, P. F. Vestergaard, B. F. Clasen et al., "High-dose resveratrol supplementation in obese men an investigator-initiated, randomized, placebo-controlled clinical trial of substrate metabolism, insulin sensitivity, and body composition," *Diabetes*, vol. 62, no. 4, pp. 1186–1195, 2013.

[69] J. Yoshino, C. Conte, L. Fontana et al., "Resveratrol supplementation does not improve metabolic function in nonobese women with normal glucose tolerance," *Cell Metabolism*, vol. 16, no. 5, pp. 658–664, 2012.

[70] S. Dash, C. Xiao, C. Morgantini, L. Szeto, and G. F. Lewis, "High-dose resveratrol treatment for 2 weeks inhibits intestinal and hepatic lipoprotein production in overweight/obese men," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 12, pp. 2895–2901, 2013.

[71] A. Dal-Pan, S. Blanc, and F. Aujard, "Resveratrol suppresses body mass gain in a seasonal non-human primate model of obesity," *BMC Physiology*, vol. 10, no. 1, article 11, 2010.

[72] S. Timmers, E. Konings, L. Bilet et al., "Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans," *Cell Metabolism*, vol. 14, no. 5, pp. 612–622, 2011.

[73] S. R. Bhatt, M. F. Lokhandwala, and A. A. Banday, "Resveratrol prevents endothelial nitric oxide synthase uncoupling and attenuates development of hypertension in spontaneously hypertensive rats," *European Journal of Pharmacology*, vol. 667, no. 1"3, pp. 258–264, 2011.

[74] X. Cao, T. Luo, X. Luo, and Z. Tang, "Resveratrol prevents AngII-induced hypertension via AMPK activation and RhoA/ROCK suppression in mice," *Hypertension Research*, vol. 37, no. 9, pp. 803–810, 2014.

[75] H. Kanamori, G. Takemura, K. Goto et al., "Resveratrol reverses remodeling in hearts with large, old myocardial infarctions through enhanced autophagy-activating AMP kinase pathway," *The American Journal of Pathology*, vol. 182, no. 3, pp. 701–713, 2013.

[76] S. Rimbaud, M. Ruiz, J. Piquereau et al., "Resveratrol improves survival, hemodynamics and energetics in a rat model of hypertension leading to heart failure," *PLoS ONE*, vol. 6, no. 10, Article ID e26391, 2011.

[77] V. W. Dolinsky, A. Y. M. Chan, I. R. Frayne, P. E. Light, C. D. Rosiers, and J. R. B. Dyck, "Resveratrol prevents the prohypertrophic effects of oxidative stress on LKB1," *Circulation*, vol. 119, no. 12, pp. 1643–1652, 2009.

[78] Y.-M. Yang, J.-Z. Chen, X.-X. Wang, S.-J. Wang, H. Hu, and H.-Q. Wang, "Resveratrol attenuates thromboxane A2 receptor agonist-induced platelet activation by reducing phospholipase C activity," *European Journal of Pharmacology*, vol. 583, no. 1, pp. 148–155, 2008.

[79] I. K. Toliopoulos, Y. V. Simos, S. Oikonomidis, and S. C. Karkabounas, "Resveratrol diminishes platelet aggregation and increases susceptibility of K562 tumor cells to natural killer cells," *Indian Journal of Biochemistry and Biophysics*, vol. 50, no. 1, pp. 14–18, 2013.

[80] Z. Wang, J. Zou, Y. Huang, K. Cao, Y. Xu, and J. M. Wu, "Effect of resveratrol on platelet aggregation in vivo and in vitro," *Chinese Medical Journal*, vol. 115, no. 3, pp. 378–380, 2002.

[81] M. Y. Shen, G. Hsiao, C. L. Liu et al., "Inhibitory mechanisms of resveratrol in platelet activation: Pivotal roles of p38 MAPK and NO/cyclic GMP," *British Journal of Haematology*, vol. 139, no. 3, pp. 475–485, 2007.

[82] G. Stef, A. Csiszar, K. Lerea, Z. Ungvari, and G. Veress, "Resveratrol inhibits aggregation of platelets from high-risk cardiac patients with aspirin resistance," *Journal of Cardiovascular Pharmacology*, vol. 48, no. 2, pp. 1–5, 2006.

[83] S. Das, C. G. Fraga, and D. K. Das, "Cardioprotective effect of resveratrol via HO-1 expression involves p38 map kinase and PI-3-kinase signaling, but does not involve NFκB," *Free Radical Research*, vol. 40, no. 10, pp. 1066–1075, 2006.

[84] L. M. Hung, M. J. Su, and J. K. Chen, "Resveratrol protects myocardial ischemia-reperfusion injury through both NO-dependent and NO-independent mechanisms," *Free Radical Biology and Medicine*, vol. 36, no. 6, pp. 774–781, 2004.

[85] X. Wu, S. Zhou, N. Zhu et al., "Resveratrol attenuates hypoxia/reoxygenationinduced Ca2+ overload by inhibiting the Wnt5a/Frizzled2 pathway in rat H9c2 cells," *Molecular Medicine Reports*, vol. 10, no. 5, pp. 2542–2548, 2014.

[86] M. Mokni, S. Hamlaoui, I. Karkouch et al., "Resveratrol provides cardioprotection after ischemia/reperfusion injury via modulation of antioxidant enzyme activities," *Iranian Journal of Pharmaceutical Research*, vol. 12, no. 4, pp. 867–875, 2013.

[87] K. Magyar, R. Halmosi, A. Palfi et al., "Cardioprotection by resveratrol: a human clinical trial in patients with stable coronary artery disease," *Clinical Hemorheology and Microcirculation*, vol. 50, no. 3, pp. 179–187, 2012.

[88] Z. Wang, J. Zou, K. Cao, T. C. Hsieh, Y. Huang, and J. M. Wu, "Dealcoholized red wine containing known amounts of resveratrol suppresses atherosclerosis in hypercholesterolemic rabbits without affecting plasma lipid levels," *International Journal of Molecular Medicine*, vol. 16, no. 4, pp. 533–540, 2005.

[89] G.-M. Do, E.-Y. Kwon, H.-J. Kim et al., "Long-term effects of resveratrol supplementation on suppression of atherogenic lesion formation and cholesterol synthesis in apo E-deficient mice," *Biochemical and Biophysical Research Communications*, vol. 374, no. 1, pp. 55–59, 2008.

[90] G. D. Norata, P. Marchesi, S. Passamonti, A. Pirillo, F. Violi, and A. L. Catapano, "Anti-inflammatory and anti-atherogenic effects of cathechin, caffeic acid and trans-resveratrol in apolipoprotein E deficient mice," *Atherosclerosis*, vol. 191, no. 2, pp. 265–271, 2007.

[91] J. M. Smoliga, O. Vang, and J. A. Baur, "Challenges of translating basic research into therapeutics: resveratrol as an example," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 67, no. 2, pp. 158–167, 2012.

[92] J. Tomé-Carneiro, M. Larrosa, A. González-Sarrías, F. A. Tomás-Barberán, M. T. García-Conesa, and J. C. Espín, "Resveratrol and clinical trials: the crossroad from in vitro studies to human evidence," *Current Pharmaceutical Design*, vol. 19, no. 34, pp. 6064–6093, 2013.

[93] J.-F. Marier, P. Vachon, A. Gritsas, J. Zhang, J.-P. Moreau, and M. P. Ducharme, "Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model," *Journal of Pharmacology and Experimental Therapeutics*, vol. 302, no. 1, pp. 369–373, 2002.

[94] T. Walle, F. Hsieh, M. H. DeLegge, J. E. Oatis Jr., and U. K. Walle, "High absorption but very low bioavailability of oral resveratrol in humans," *Drug Metabolism and Disposition*, vol. 32, no. 12, pp. 1377–1382, 2004.

[95] I. M. Kapetanovic, M. Muzzio, Z. Huang, T. N. Thompson, and D. L. McCormick, "Pharmacokinetics, oral bioavailability, and metabolic profile of resveratrol and its dimethylether analog, pterostilbene, in rats," *Cancer Chemotherapy and Pharmacology*, vol. 68, no. 3, pp. 593–601, 2011.

[96] C. la Porte, N. Voduc, G. Zhang et al., "Steady-state pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (ethanol) in healthy human subjects," *Clinical Pharmacokinetics*, vol. 49, no. 7, pp. 449–454, 2010.

[97] A. Revel, H. Raanani, E. Younglai et al., "Resveratrol, a natural aryl hydrocarbon receptor antagonist, protects lung from DNA damage and apoptosis caused by benzo[a]pyrene," *Journal of Applied Toxicology*, vol. 23, no. 4, pp. 255–261, 2003.

[98] H.-H. S. Chow, L. L. Garland, C.-H. Hsu et al., "Resveratrol modulates drug- and carcinogen-metabolizing enzymes in a healthy volunteer study," *Cancer Prevention Research*, vol. 3, no. 9, pp. 1168–1175, 2010.

[99] D. J. Boocock, G. E. S. Faust, K. R. Patel et al., "Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent," *Cancer Epidemiology Biomarkers and Prevention*, vol. 16, no. 6, pp. 1246–1252, 2007.

[100] O. F. Iwuchukwu, S. Sharan, D. J. Canney, and S. Nagar, "Analytical method development for synthesized conjugated metabolites of trans-resveratrol, and application to pharmacokinetic studies," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 63, pp. 1–8, 2012.

[101] S. Sharan and S. Nagar, "Pulmonary metabolism of resveratrol: in vitro and in vivo evidence," *Drug Metabolism and Disposition*, vol. 41, no. 5, pp. 1163–1169, 2013.

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Research Article

Antioxidative Effects and Inhibition of Human Low Density Lipoprotein Oxidation *In Vitro* of Polyphenolic Compounds in *Flammulina velutipes* (Golden Needle Mushroom)

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Dietary polyphenolic compounds mediate polynomial actions in guarding against multiple diseases. Atherosclerosis is an oxidative stress driven pathophysiological complication where free radical induced oxidative modification of low density lipoprotein (LDL) plays the ground breaking role. Mushrooms have been highly regarded for possessing an antioxidant arsenal. Polyphenolic compounds present in dietary mushrooms seem pertinent in withstanding LDL oxidation en route to controlling atherosclerosis. In this study, the antioxidative effect of five solvent fractions consisting of methanol : dichloromethane (M : DCM), hexane (HEX), dichloromethane (DCM), ethyl acetate (EA), and aqueous residue (AQ) of *Flammulina velutipes* was evaluated. M : DCM fraction showed the most potent 2,2-diphenyl-1-picrylhydrazyl radical scavenging effect with IC_{50} of 0.86 mg/mL and total phenolic content of 56.36 gallic acid equivalent/g fraction. In LDL oxidation inhibitory tests, M : DCM fraction at 1 μg/mL concentration mostly lengthened the lag time (125 mins) of conjugated diene formation and inhibited the formation of thiobarbituric acid reactive substances (48.71%, at 1 mg/mL concentration). LC-MS/MS analyses of M : DCM fraction identified the presence of polyphenolic substances protocatechuic acid, *p*-coumaric, and ellagic acid. These chain-breaking polyphenolics might impart the antioxidative effects of *F. velutipes*. Thus, mushroom-based dietary polyphenolic compounds might be implicated in slowing down the progression of atherosclerosis.

1. Introduction

The implication of dietary polyphenols and their effects upon cell biochemistry and pathophysiology emanates from their prophylactic role against multiple diseases. Polyphenols entail a diverse array of plant and fungal secondary metabolites having pluripotent actions at the cellular level [1]. Their mode of action is as diverse as their structures are and both ameliorating and null effects have been noticed while considering antioxidative role of dietary polyphenols [2, 3]. Substantial evidences support their involvement in cellular signaling processes that ultimately result in reduced production of inflammatory mediators and increased generation of vasodilators [4, 5]. Although no direct antioxidative function of dietary polyphenols has been noticed in human cardiovascular health studies, polyphenols have been implicated in inducing antioxidant defense by stimulating the Nrf2/Keap1 signaling pathway [6, 7].

Actually, free radicals are being constantly produced in our body as byproducts or defensive weapons against pathogens and also removed accordingly with a view to maintaining normal cellular biochemistry and physiology [8, 9]. However, the imbroglio of their excessive production and accumulation afflicts the cellular and physiological normalcy resulting in pathophysiological consequences [10]. Atherosclerosis being such a free radical-induced and

oxidative stress-governed predicament imparts the major morbidity and mortality associated with cardiovascular diseases (CVD) [11]. Etiologically, free radicals oxidatively attack the low density lipoproteins (LDL) and the oxidized LDL molecules (ox-LDL) become modified both structurally and functionally [12]. This occurs in the arterial wall and the ox-LDL manifests multiple proatherogenic potential including its stimulatory role towards excessive production of proinflammatory cytokines, formation of foam cells, impairment of the endothelial vasculature, and endothelial dysfunction [13]. The ox-LDL itself is cytotoxic towards endothelial cells as it generates many free radicals only to worsen the atherogenic situation [14]. Thus, controlling of atherosclerosis warrants proactive action of therapeutic strategies capable of slowing down the generation of ox-LDL.

Prodigious strive for mitigating oxidative modification of LDL has flared towards boosting antioxidative defense arsenal up. Natural food and nutraceutical-based approaches come forward in this regard [15]. Edible macrofungi have been highly recognized for possessing numerous bioactive components of both nutritional and medicinal importance [16, 17]. Among different varieties, *Flammulina velutipes* (Curtis) Singer (also known as the golden needle mushroom, lily mushroom, enokitake, enokidake, and/or enoki) is a white, thin, and long mushroom species. It is popular as a delicate cuisine. Its immune-stimulating effects have been well recognized and the associated fungal immune-modulatory protein (FIP), FVE, has been reported [18, 19]. Its immune-modulating polysaccharides have also been identified and structure-activity relationship has been elucidated [20, 21]. Compared to other mushroom species, its antioxidative status and corresponding implication upon atherosclerosis have been less documented. Thus, *Flammulina velutipes* had been chosen under the present study to evaluate its effect upon *in vitro* atherosclerosis attenuation through mitigation of *in vitro* oxidative stress followed by the identification of responsible bioactive components, especially the polyphenolics. To our knowledge, this is the first report indicating the presence of polyphenolic compounds in *F. velutipes* solvent partitioned fractions and relating those seminal biocomponents with antiatherosclerotic venture.

2. Materials and Methods

2.1. Solvent Partitioning and Preparation of Liquid-Liquid Fractions. Mushroom bioactive components warrant appropriate separation, recovery, and purification processes for obtaining maximum output from their usage. Solvent partitioning, also called liquid-liquid partitioning, is a steady-state procedure for fractioning biocomponents [22]. It enables the separation of biocompounds based on their relative solubility in two different immiscible solvents [23]. In the present study, the modified method of Mayakrishnan et al. (2013) was applied for solvent partitioning and fractionation of *F. velutipes* [24]. Briefly, each two-hundred-gram powder was fractioned with 4 L of methanol : dichloromethane (M : DCM) (2 : 1) using conical flasks at room temperature with occasional stirring and shaking, followed by filtration through Whatman number 1 filter paper. The total organic solution was evaporated using a rotary evaporator (Büchi Rotavapor R-114, Switzerland) that yielded the M : DCM fraction. An aliquot of M : DCM fraction was dissolved in 90% aqueous methanol and partitioned with hexane (3 × 100 mLs). Separating funnel-based assay of the upper hexane layer was followed by the rotaevaporation of hexane and the hexane fraction collected. The aqueous methanolic layer left at the bottom was rotaevaporated and the semisolid fraction redissolved in distilled water (100 mL). Then successive partitioning with dichloromethane (DCM, 3 × 100 mLs) and rotaevaporation resulted in the DCM fraction. The bottom-layered aqueous fraction was repartitioned with ethyl acetate (EA, 3 × 100 mLs). Ethyl acetate was rotaevaporated and the lowered aqueous part freeze-dried to gain the respective fraction.

2.2. Sources of the Chemicals. Analytical grade chemicals were used in the present study. All of them had been purchased from Sigma-Aldrich (USA).

2.3. Investigation into the Antioxidant Activities of the Fractions. The following standard assays were performed to delve out the antioxidant potentiality of the various fractions of *F. velutipes* and compared with the natural antioxidant, quercetin.

2.3.1. Scavenging Effect on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical. The method previously described by Abdullah et al. (2012) was followed [25]. In short, 0.1 mL of each solvent fraction (1.0 mg/mL conc.) was mixed with 3.9 mL of 0.06 mM DPPH dissolved in methanol. The solution was shaken vigorously under darkness and absorbance was taken at 515 nm. Methanol was used as the blank. The percentage of DPPH free radical scavenging was calculated using the following equation:

$$\text{radical scavenging activity}\,(\%) = \frac{(A_0 - A_s)}{A_0} \times 100, \quad (1)$$

where A_0 is the absorbance of the 0.06 mM methanolic DPPH only and A_s is the absorbance of the reaction mixture. IC_{50} value (concentration of the fraction to produce half maximal inhibition/scavenging) of the most potent solvent fraction was calculated from the graph of the radical scavenging activity against fraction concentration.

2.3.2. Folin-Ciocalteu Assay. The modified methodology of Slinkard and Singleton (1977) was used to determine the reducing potential of the fractions [26]. In brief, 250 μL of each of the solvent fractions was added to an equal volume of 10% Folin-Ciocalteu reagent and kept at darkness for 3 minutes while shaking. Then, 500 μL of 10% sodium carbonate was added to the reagent mixture and kept at dark again. After 1 h, absorbance was taken at 750 nm. The calibration curve of gallic acid (2–10 μg/mL) was used to express the performance of the Folin-Ciocalteu assay as gallic acid equivalents (GAE) per gram of fraction.

2.3.3. Inhibition of Lipid Peroxidation of Buffered Egg Yolk. A slightly modified version of the method described by Daker et al. (2008) was used to determine the inhibitory effect of each of the fractions on lipid peroxidation of the buffered egg yolk [27]. In a nutshell, 0.5 g of fowl egg yolk was emulsified with 0.1 M phosphate buffer (pH 7.4) so that the final volume becomes 25 g/L. Ferrous sulphate (1 M, 100 μL) was added to the mixture to induce lipid peroxidation. Thereafter, fractions of various concentrations were introduced to the peroxidation-prone milieu at the volume of 100 μL and shaken vigorously. After 1 h incubation at room temperature, 0.5 mL of 15% trichloroacetic acid (TCA) and 1 mL of thiobarbituric acid (TBA), both freshly prepared, were added. After incubation on the boiling water bath for 10 minutes, the tubes containing the reaction mixtures were cooled at room temperature and centrifuged at 3,500 g for 10 minutes to precipitate the proteins. Supernatant (100 μL) was taken to measure the formation of thiobarbituric acid reactant substances (TBARS) through studying absorbance at 532 nm. Buffered egg yolk with Fe^{+2} only was used as the control. The percentage inhibition of lipid peroxidation was calculated using the following equation:

$$\text{inhibition (\%)} = \frac{(A_0 - A_s)}{A_0} \times 100, \quad (2)$$

where A_0 is the absorbance of the control and A_s is the absorbance of the reaction mixture containing the fraction. IC_{50} value (concentration of the fraction to produce half maximal inhibition) of the most potent solvent fraction was calculated from the graph of the inhibition of lipid peroxidation against fraction concentration.

2.3.4. $FeSO_4$-Induced LDL Oxidation and F. velutipes Fractions-Mediated Inhibition. Two features of LDL oxidation were measured: lag time extension during conjugated diene (CD) formation and inhibition of thiobarbituric acid reactive substances (TBARS) production.

(a) Measurement of the Lag Time Extension during CD Formation. The method described by Rahman et al. (2014) was applied to measure the fractions' inhibitory effect on the human LDL oxidation through extension of lag period of CD formation [9]. Human LDL concentration was adjusted to 150 μg/mL and the reaction volume totaled to 200 μL with 0.1 M phosphate buffer, pH 7.4. Transition metal ion induced oxidative stress to LDL was exerted by freshly prepared $FeSO_4$ solution (50 μg/mL) at room temperature. LDL oxidation kinetics of Fe^{+2} and antioxidant effect of the fractions were studied at 234 nm at 20-minute intervals for a period of 3 h. $FeSO_4$ in ultrapure water only, at pH 7.4, was used as the blank. *F. velutipes* fractions-mediated protection period of the LDL oxidation was dubbed as the "lengthened lag time of CD formation" and was measured until the amount of the CD began to increase.

(b) Inhibitory Effect upon the Formation of TBARS. For determining the inhibitory effects of *F. velutipes* upon TBARS formation, the method developed by Rahman et al. (2014) was employed [9]. Ferrous sulphate (10 mM, 191 μL) was applied upon human LDL (9 μL) to generate oxidative modification of LDL. *F. velutipes* fraction at 1 μg/mL (100 μL) was added with the oxidized LDL. Freshly prepared 500 μL of 15% trichloroacetic acid (TCA) and 1 mL of 1% thiobarbituric acid (TBA) were added and incubated at 100°C for 10 minutes followed by cooling at room temperature. Finally, an aliquot (300 μL) was taken to the ELISA reader and the absorbance read at 532 nm. For blank, $FeSO_4$ in water, pH 7.4, was used. The percentage inhibition of TBARS formation was calculated using the following equation:

$$\text{inhibition (\%)} = \frac{(A_0 - A_s)}{A_0} \times 100, \quad (3)$$

where A_0 is the absorbance of the control and A_s is the absorbance of the reaction mixture containing the fraction. IC_{50} value (concentration of the fraction to produce half maximal inhibition) of the most potent solvent fraction was calculated from the graph of the inhibition of TBARS against fraction concentration.

2.3.5. Identification of Polyphenols and Other Bioactive Components by LC-MS/MS. Liquid chromatography-mass spectroscopy (LC-MS/MS) full scan analysis was performed to determine the presence of polyphenolic compounds in the M : DCM fraction of *F. velutipes*. The system setup involved ionization mode: negative; column: Zorbax C18, 150 mm × 4.6 mm × 5 μM; buffer: (a) water with 0.1% formic acid and 5 mM ammonium formate and (b) acetonitrile with 0.1% formic acid and 5 mM ammonium formate; run time: 15-minute rapid screening. The apparatus utilized was AB Sciex 3200QTrap LCMS/MS with Perkin Elmer FX 15 uHPLC System (Perkin Elmer, USA). MS setting included voltage IS: −4500 V, source temperature: 500°C, desolvation gas: 40 psi, source gas: 40 psi, scan range: 100–1200 *m/z* for full scan and 50–1200 *m/z* for MS/MS scan, declustering potential: 40 V, entrance potential: 10 V, and collision energy: spread of 35 eV ± 15 eV. Sample fractions were diluted in appropriate solvent such as methanol, dichloromethane and substitution with acetonitrile in preparation for LCMS/MS analysis, filtered with nylon 0.22 μM, and injected at volume 20 μL.

2.3.6. Statistical Analyses. We conducted all the experiments in triplicate and presented the data as mean ± SD. Using statistical package SPSS version 16, we performed one-way analysis of variance (ANOVA). The differences among means were further analyzed by least significance difference (LSD) test at 95% level ($P \leq 0.05$).

3. Results and Discussion

3.1. DPPH Free Radical Scavenging Activity. The antioxidant property of natural compounds was most commonly evaluated on the basis of DPPH free radical scavenging property. Although the test is not any single antioxidant-specific but rather measures the cumulative antioxidant potential of any sample, the procedure is simple, rapid, and cost effective. Specifically, the scavenging antioxidants

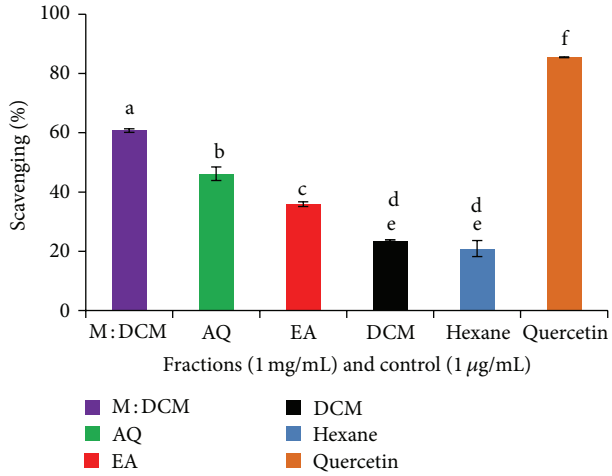


Figure 1: DPPH free radical scavenging effect of the *F. velutipes* fractions compared to the positive control, quercetin. Data presented as mean ± SD of triplicate determinations. Mean values with different lowercase superscripts (a–f) represent statistically significant difference at 95% level ($P \leq 0.05$) with post hoc least significance difference (LSD) test.

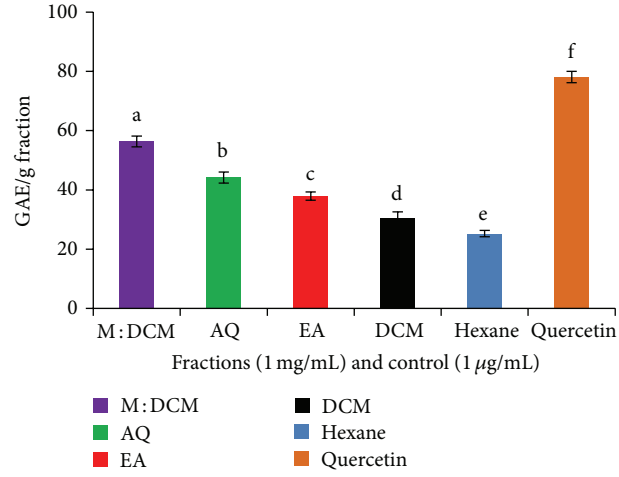


Figure 2: Folin-Ciocalteu assay of the *F. velutipes* fractions compared to positive control, quercetin. Data presented as mean ± SD of triplicate determinations. Mean values with different lowercase superscripts (a–f) represent statistically significant difference at 95% level ($P \leq 0.05$) with post hoc least significance difference (LSD) test.

reduce the stable, purple radical DPPH$^{\bullet}$ through bond pairing between its odd electron and scavenging antioxidant's hydrogen atom and convert it into yellow colored, nonradical form (DPPH-H). As shown in Figure 1, at 1 mg/mL concentration, the methyl : DCM fraction of *F. velutipes* performed the best DPPH free radical scavenging effect (60.75%, IC_{50} 0.86 mg/mL).

3.2. Folin-Ciocalteu Assay. The antioxidative prowess of mushrooms depends largely on their biochemical composition involving phenolics, flavonoids, carotenoids, ascorbic acid, amino acids, and various enzymes that impart reducing effect on the oxidant-laden molecules or ions. Through Folin-Ciocalteu assay, the reducing capacities of each of the fractions had been measured and the results had been expressed as gallic acid equivalents (GAE) per gram of the dried fraction. As is evident from Figure 2, the M : DCM fraction contained the highest reducing capacity (56.36 GAE/g fraction), followed by that of the aqueous one (44.16 GAE/g fraction).

3.3. Lipid Peroxidation Inhibition Test. The peroxidative modification of lipid structures and the resulting products are among the key factors initiating the pathogenesis of atherosclerosis. The unsaturated portions of lipids, especially the double bonds of fatty acids present in lipid molecules, are most vulnerable to oxidative attack by free radicals and ions that lead to altered lipid structures resulting in the breakdown products like malondialdehyde (MDA). Thus, estimation of whether mushroom fractions could inhibit, albeit reduce, the formation of malondialdehyde, equivalent to the reduced lipid peroxidation, would be of high importance in assessing the antioxidative potential of the mushroom fractions in concern [28]. Based on this paradigm, we induced egg yolk

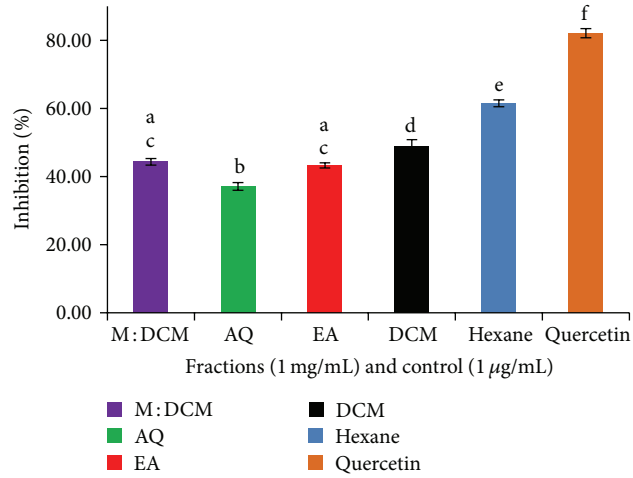


Figure 3: Lipid peroxidation inhibitory effect of *F. velutipes* fractions compared to positive control, quercetin. Data presented as mean ± SD of triplicate determinations. Mean values with different lowercase superscripts (a–f) represent statistically significant difference at 95% level ($P \leq 0.05$) with post hoc least significance difference (LSD) test.

lipid peroxidation by Fe^{+2} at low pH and elevated temperature and tested the action of each fraction. We found that the hexane fraction had the most potent inhibitory effect on lipid peroxidation (61.52%), followed by DCM (48.79%). M : DCM fraction stood third in position (44.33%) (Figure 3). The reason may be that the polar, hydrophilic polyphenolics were not in appropriate amount to baffle the nonpolar, hydrophobic milieu of the lipid peroxidation. Similarly, the high inhibitory effect of the nonpolar hexane fraction might be attributed to its lipophilic and/or biocomponents contents.

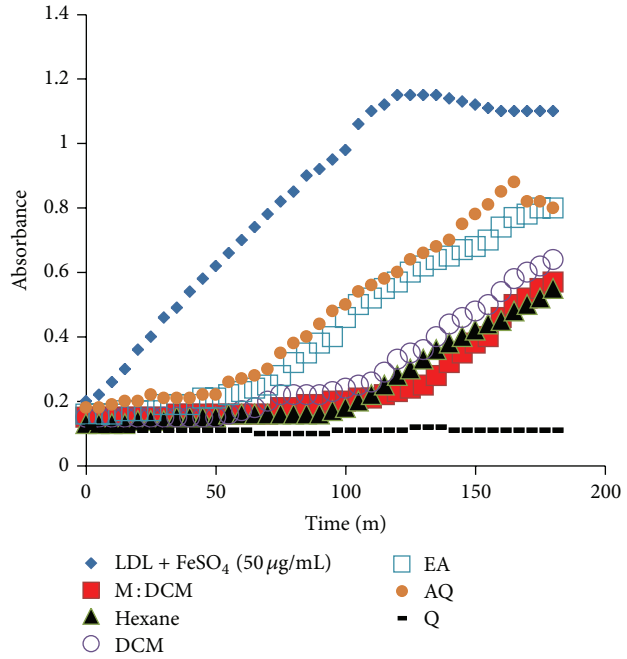


Figure 4: Effect of *F. velutipes* fractions upon lag time of conjugated diene (CD) formation. The gradual increase in the lag time of CD formation at 234 nm indicates the extent of ox-LDL inhibition by the respective fraction against the $FeSO_4$-induced LDL oxidation. The positive control, quercetin, and all the fractions were used at 1 μg/mL conc.

3.4. Inhibition of LDL Oxidation by F. velutipes Fractions

3.4.1. Effect of F. velutipes Fractions upon Lag Time of CD Formation. Fe^{+2}-ions exerted peroxidative modification of the polyunsaturated fatty acids (PUFAs) present in the LDL molecule and caused molecular rearrangement, resulting in conjugated dienes (CDs) formation. As the LDL molecule's integral antioxidant, α-tocopherol protects LDL by withstanding the oxidative stress initially, causing a slowed oxidation, known as the lag period. Oxidation withstanding prowess of an antioxidant is directly proportional to the lengthening of the lag phase [14]. When *F. velutipes* solvent partitioned fractions were applied, the lag time was lengthened even up to 120 minutes for the M : DCM and 95 minutes for the hexane fraction, respectively (Figure 4).

3.4.2. Effect of F. velutipes Fractions upon TBARS Formation. During LDL oxidation, the lag phase is followed by the propagation phase of rapid LDL oxidation giving rise to lipid peroxides. Lipid peroxides undergo decomposition phase when breaking of the double bonds gives rise to malondialdehydes (MDA). Nucleophilic substitution reaction between MDA and TBA used in the experimentation produced "MDA:TBA adduct," also called "thiobarbituric acid reactive substances (TBARS)" [29]. The amount of TBARS production is inversely proportional to the antioxidant capacity of a biocomponent. All the solvent fractions of *F. velutipes* were capable of inhibiting the formation of TBARS, but the M : DCM fraction inhibited it the most (48.71%) (Figure 5).

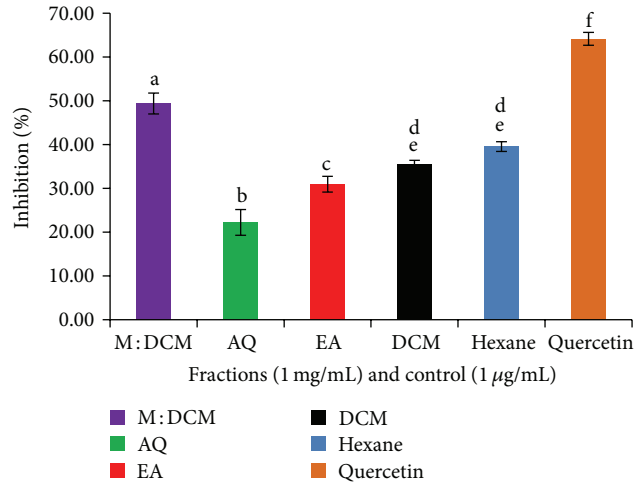


Figure 5: TBARS formation inhibitory effect of *F. velutipes* fractions as compared to positive control, quercetin. Data presented as mean ± SD of triplicate determinations. Mean values with different lowercase superscripts (a–f) represent statistically significant difference at 95% level ($P \leq 0.05$) with post hoc least significance difference (LSD) test.

3.5. Identification of Polyphenolic Compounds in the M : DCM Fraction by LC-MS/MS. The polyphenolic representative antioxidants present in the M : DCM fraction of *F. velutipes* were protocatechuic acid, *p*-hydroxycinnamic acid moiety (*p*-coumaric acid), and ellagic acid (Figures 6, 7, and 8 and Table 1).

3.5.1. Protocatechuic Acid. Protocatechuic acid is an important polyphenolic component having therapeutic potential against oxidative stress, atherosclerosis, microbial infection, analgesic, and neurological and nephrological complications [30]. It has structural and functional similarity with other well-known antioxidants like caffeic acid, gallic acid, syringic acid, and vanillic acid [30]. Barros et al. (2009) reported the presence of protocatechuic acid in some Portuguese wild mushrooms [31]. Mattila et al. also observed the presence of protocatechuic acid in *Agaricus bisporus* and *Lentinula edodes* [32].

Protocatechuic acid is a unique antioxidant in the sense that it can prevent *in vitro* oxidative stress in both aqueous and nonaqueous media and can chelate transition metal ions and also scavenge free radicals [33]. Lende et al. (2011) reported the *in vitro* antioxidative effects of protocatechuic acid where their DPPH free radical scavenging effects and other antioxidative performances were better than the positive control, trolox [34].

Protocatechuic acid may exert antioxidative action by the following two modes.

(A) Free radical scavenging:

(i) protocatechuic acid (PCA) accepts a hydrogen atom ($H^{\bullet}$) from the DPPH free radical ($DPPH^{\bullet}$) forming the stable DPPH-H and the unstable anion $PCA^{\bullet}$ (A);

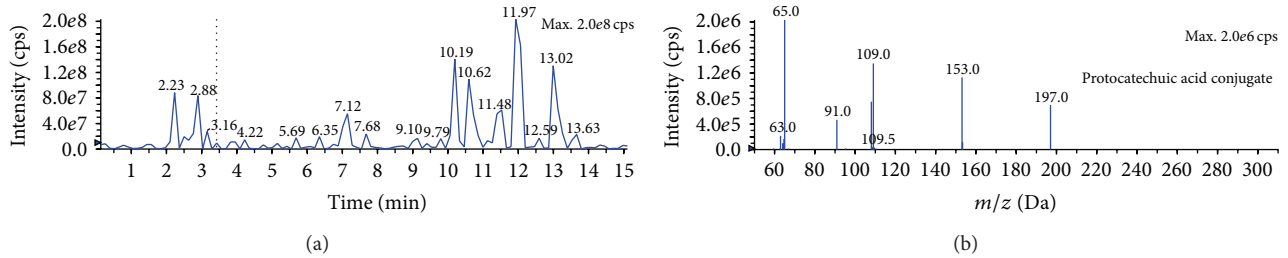


Figure 6: Full LC-MS/MS chromatogram (a) and the presence of protocatechuic acid in the M : DCM fraction (b) of *F. velutipes*.

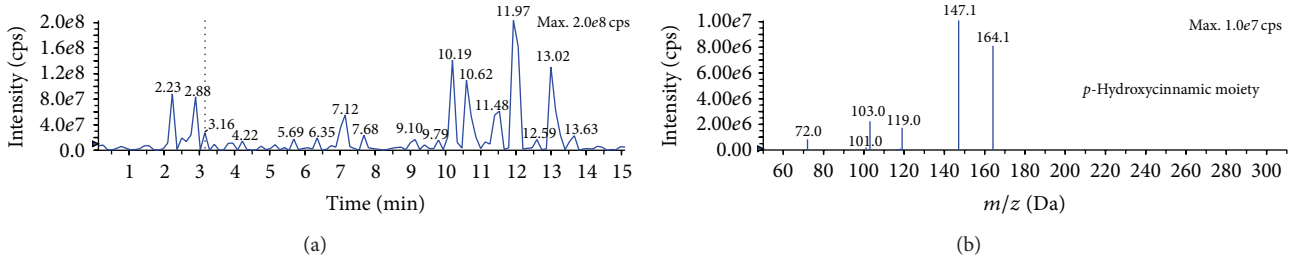


Figure 7: Full LC-MS/MS chromatogram (a) and the presence of *p*-hydroxycinnamic acid moiety (*p*-coumaric acid) in the M : DCM fraction (b) of *F. velutipes*.

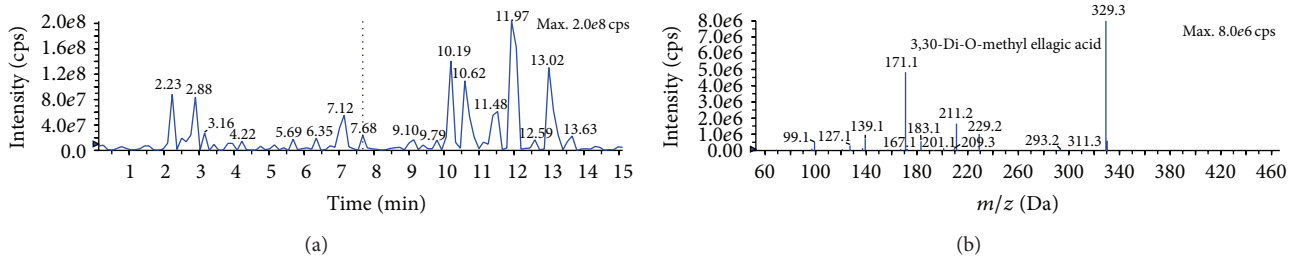


Figure 8: Full LC-MS/MS chromatogram (a) and the presence of ellagic acid in the M : DCM fraction (b) of *F. velutipes*.

(ii) the unstable anion PCA$^{\bullet}$ (A) can withdraw another hydrogen atom (H$^{\bullet}$) to form a stable quinone (see Figure 9).

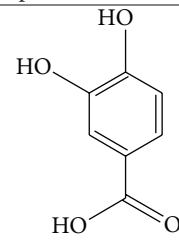
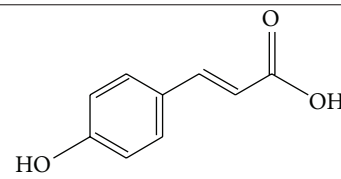
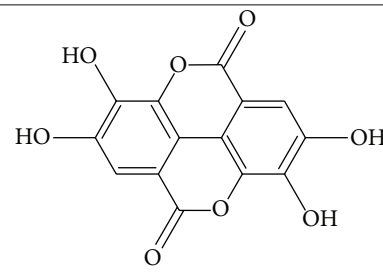
(B) Metal ion chelating: the orthodihydroxyl group present in PCA aid it in chelating metal ion, in this experiment Fe^{+2} (see Figure 10).

Antiatherosclerotic effect of protocatechuic acid is also mediated via its anti-inflammatory effects. It inhibits vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) expression and reduces NF-κB binding activity. Thus, it prevents binding of the monocytes to the endothelial wall and further atherosclerotic processes [31]. It has been reported to attenuate platelet derived growth factor-(PDGF-) induced migration and proliferation of VSMC. The molecular mechanism involved might be the downregulation of the phosphatidylinositol 3-kinase (PI3 K)/Akt and the mitogen-activated protein kinase (MAPK) pathways [34]. It also has antihyperlipidemic effects [35].

3.5.2. p-Hydroxycinnamic Acid Moiety (p-Coumaric Acid). Barros et al. (2009) reported the presence of *p*-coumaric acid in some Portuguese wild mushrooms [31]. *p*-Coumaric acid has been regarded to inhibit LDL oxidation both at *in vitro* and at *in vivo* condition [36]. The proposed mechanism involves scavenging of $^{\bullet}$OH (a representative reactive oxygen species, ROS) [36]. Its antioxidative effect upon human colon cell culture (HT-29) has also been reported [37]. Besides, *p*-coumaric acid has dually been recognized as an anti-inflammatory and platelet aggregation inhibitor in *in vivo* studies [38].

3.5.3. Ellagic Acid. Ellagic acid is a flavonoid type of phenolic compound having antioxidant, anticarcinogenic, and immune-modulatory effects. Festa et al. reported the *in vitro* radical scavenging effect of ellagic acid to be stronger than that of ascorbic acid and melatonin while studying H_2O_2- and bleomycin-induced DNA damage [39]. Ellagic acid derivatives isolated from other sources also showed potent antioxidant effects [40]. Antioxidant studies involving colon cell lines (HCT 16) also showed similar results for ellagic acid [41]. It can enhance the production of phase II enzymes of xenobiotic metabolism and thus increase the detoxification of free radicals in hepatic tissues [42]. *In vivo* studies show

Table 1: Polyphenolic compounds present in the M : DCM fraction of *F. velutipes*.

Number	Retention time (m)	Mode (+/−)	Compound name and structure	Molecular formula	Molecular weight (g)
1	3.4	−	 Protocatechuic acid	$C_7H_6O_4$	154.12
2	5.2	−	 *p*-Coumaric acid	$C_9H_8O_3$	164.16
3	7.68	−	 Ellagic acid	$C_{14}H_6O_8$	302.197

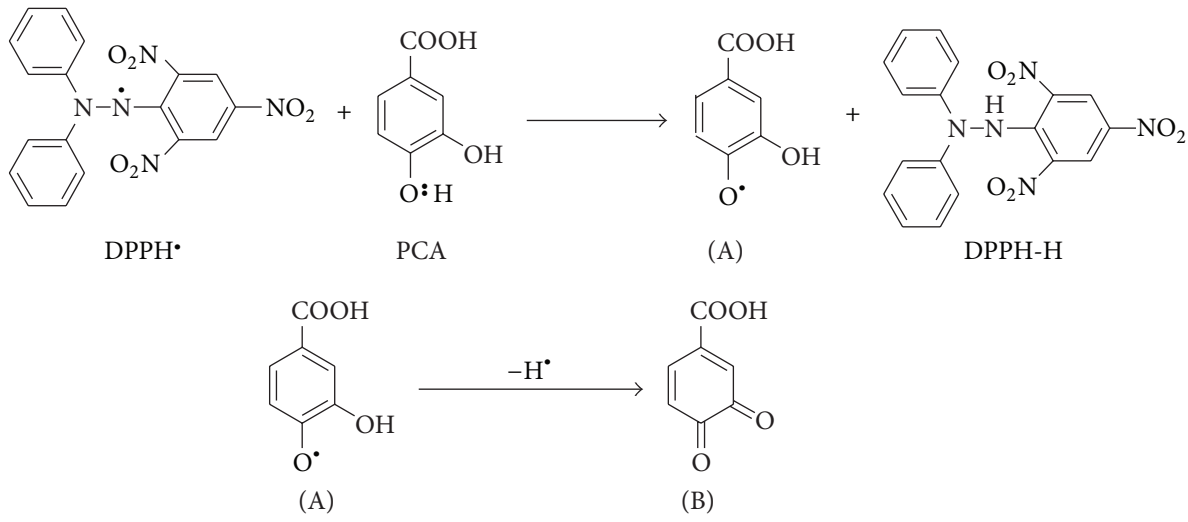


Figure 9

that ellagic acid can bind with the carcinogens and inactivate them and can prevent the damage of p53 protein [42]. Ellagic acid can aid in overcoming the host immune tolerance and has therapeutic potential for the HBV carriers [43].

During free radical-induced oxidative modification of LDL, peroxidation of polyunsaturated fatty acids generates lipid hydroperoxides. Further decomposition of hydroperoxides augments the peroxidation process until there occurs a deflection through reduction of the alkoxyl and/or peroxyl radicals to alkoxides and/or hydroperoxides, respectively. Chain-breaking phenolic antioxidants come into play in this situation and shield against the vicious cycle of oxidation

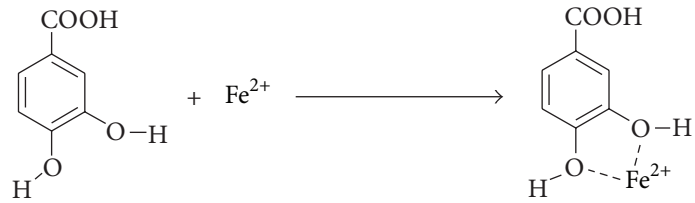


Figure 10

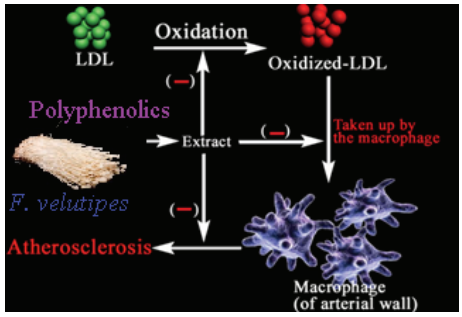


Figure 11: Antiatherosclerotic implications of the polyphenolic compounds of *F. velutipes*.

and peroxidation [44]. The antioxidant capacity of phenolic antioxidants is dependent upon their structural feature, the presence of phenolic group and the potency of it to stabilize the resulting phenoxyl radical, and their redox potential: the higher the redox potential, the lower the antioxidant capacity [45]. Besides, the antioxidative effects of dietary polyphenols might be attributed to their effects upon free radical scavenging, chelating of the transition metal ions, stimulation of antioxidative enzymes, inhibition of prooxidative enzymes, and cumulative effects with other antioxidants.

As described in the present study, the phenolic (ellagic acid) and polyphenolic compounds (protocatechuic acid and *p*-coumaric acid) present in the *F. velutipes* spur high in withstanding oxidative ramification of LDL that might be implicated as this mushroom's antioxidative led antiatherosclerotic accomplishment (Figure 11). Notwithstanding to surmise, *in vivo* studies incorporating these polyphenolic substances are quintessential to decipher the relevant mechanism of action.

4. Conclusion

As LDL oxidation is the initiating step in atherosclerotic pathogenesis, intervention at this level sounds imperative in halting the onset of atherosclerosis. Polyphenolic compounds present in the *F. velutipes* impart this mushroom's antiatherosclerotic pursuance under the aegis of antioxidative panoply. These findings, novel in case of this mushroom species, would pave a new vista for functional food-based therapeutic intervention of atherosclerosis and cardiovascular diseases and thus be of monumental importance to the humanity.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] M. A. Soobrattee, V. S. Neergheen, A. Luximon-Ramma, O. I. Aruoma, and T. Bahorun, "Phenolics as potential antioxidant therapeutic agents: Mechanism and actions," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 579, no. 1-2, pp. 200–213, 2005.

[2] T. L. Zern and M. L. Fernandez, "Cardioprotective effects of dietary polyphenols," *The Journal of Nutrition*, vol. 135, no. 10, pp. 2291–2294, 2005.

[3] Y. Kishimoto, M. Tani, and K. Kondo, "Pleiotropic preventive effects of dietary polyphenols in cardiovascular diseases," *European Journal of Clinical Nutrition*, vol. 67, no. 5, pp. 532–535, 2013.

[4] R. J. Williams, J. P. E. Spencer, and C. Rice-Evans, "Flavonoids: antioxidants or signalling molecules?" *Free Radical Biology and Medicine*, vol. 36, no. 7, pp. 838–849, 2004.

[5] I. Rahman, S. K. Biswas, and P. A. Kirkham, "Regulation of inflammation and redox signaling by dietary polyphenols," *Biochemical Pharmacology*, vol. 72, no. 11, pp. 1439–1452, 2006.

[6] P. C. H. Hollman, A. Cassidy, B. Comte et al., "The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established," *The Journal of Nutrition*, vol. 141, no. 5, pp. 989S–1009S, 2011.

[7] B. Holst and G. Williamson, "Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants," *Current Opinion in Biotechnology*, vol. 19, no. 2, pp. 73–82, 2008.

[8] B. Halliwell, *Free Radicals and Other Reactive Species in Disease*, John Wiley & Sons, 2005.

[9] M. A. Rahman, N. Abdullah, and N. Aminudin, "Inhibitory effect on *in vitro* LDL oxidation and HMG Co-a reductase activity of the liquid-liquid partitioned fractions of *Hericium erinaceus* (Bull.) persoon (Lion's Mane Mushroom)," *BioMed Research International*, vol. 2014, Article ID 828149, 9 pages, 2014.

[10] B. Halliwell, "The antioxidant paradox: less paradoxical now?" *British Journal of Clinical Pharmacology*, vol. 75, no. 3, pp. 637–644, 2013.

[11] American Heart Association (AHA), "AHA statistical update heart disease and stroke statistics—2012 update, a report from the American Heart Association," *Circulation*, vol. 125, pp. e2–e220, 2012.

[12] D. Li and J. L. Mehta, "Oxidized LDL, a critical factor in atherogenesis," *Cardiovascular Research*, vol. 68, no. 3, pp. 353–354, 2005.

[13] P. Libby, "Inflammation in atherosclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 9, pp. 2045–2051, 2012.

[14] H. Yoshida and R. Kisugi, "Mechanisms of LDL oxidation," *Clinica Chimica Acta*, vol. 411, no. 23-24, pp. 1875–1882, 2010.

[15] V. Lobo, A. Patil, A. Phatak, and N. Chandra, "Free radicals, antioxidants and functional foods: impact on human health," *Pharmacognosy Reviews*, vol. 4, no. 8, pp. 118–126, 2010.

[16] S. P. Wasser, "Current findings, future trends, and unsolved problems in studies of medicinal mushrooms," *Applied Microbiology and Biotechnology*, vol. 89, no. 5, pp. 1323–1332, 2011.

[17] S. P. Wasser, "Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides," *Applied Microbiology and Biotechnology*, vol. 60, no. 3, pp. 258–274, 2002.

[18] H.-H. Chang, K.-Y. Hsieh, C.-H. Yeh, Y.-P. Tu, and F. Sheu, "Oral administration of an Enoki mushroom protein FVE activates innate and adaptive immunity and induces anti-tumor activity against murine hepatocellular carcinoma," *International Immunopharmacology*, vol. 10, no. 2, pp. 239–246, 2010.

[19] Y. Ding, S. V. Seow, C. H. Huang et al., "Coadministration of the fungal immunomodulatory protein FIP-Fve and a tumour-associated antigen enhanced antitumour immunity," *Immunology*, vol. 128, no. 1, part 2, pp. e881–e894, 2009.

[20] H. L. Chang, L. S. Lei, C. L. Yu, Z. G. Zhu, N. N. Chen, and S. G. Wu, "Effect of Flammulina velutipes polysaccharides on production of cytokines by murine immunocytes and serum levels of cytokines in tumor-bearing mice," *Journal of Chinese Medicinal Materials*, vol. 32, no. 4, pp. 561–563, 2009.

[21] H. Yin, Y. Wang, Y. Wang, T. Chen, H. Tang, and M. Wang, "Purification, characterization and immuno-modulating properties of polysaccharides isolated from *Flammulina velutipes* mycelium," *The American Journal of Chinese Medicine*, vol. 38, no. 1, pp. 191–204, 2010.

[22] T. Gu, "Liquid-liquid partitioning methods for bioseparations," in *The Handbook of Bioseparations-2*, A. Ahuja, Ed., pp. 329–364, Academic Press, New York, NY, USA, 2000.

[23] S. K. Sikdar, K. D. Cole, R. M. Stewart, D. C. Szlag, P. Todd, and H. Cabezas Jr., "Aqueous two-phase extraction in bioseparations: an assessment," *Nature Biotechnology*, vol. 9, no. 3, pp. 252–256, 1991.

[24] V. Mayakrishnan, N. Abdullah, M. H. Z. Abidin et al., "Investigation of the antioxidative potential of various solvent fractions from fruiting bodies of *Schizophyllum commune* (Fr.) mushrooms and characterization of phytoconstituents," *Journal of Agricultural Science*, vol. 5, no. 6, p. 58, 2013.

[25] N. Abdullah, S. M. Ismail, N. Aminudin, A. S. Shuib, and B. F. Lau, "Evaluation of selected culinary-medicinal mushrooms for antioxidant and ACE inhibitory activities," *Evidence-based Complementary and Alternative Medicine*, vol. 2012, Article ID 464238, 12 pages, 2012.

[26] K. Slinkard and V. L. Singleton, "Total phenol analysis: automation and comparison with manual methods," *American Journal of Enology and Viticulture*, vol. 28, no. 1, pp. 49–55, 1977.

[27] M. Daker, N. Abdullah, S. Vikineswary, P. C. Goh, and U. R. Kuppusamy, "Antioxidant from maize and maize fermented by *Marasmiellus* sp. as stabiliser of lipid-rich foods," *Food Chemistry*, vol. 107, no. 3, pp. 1092–1098, 2008.

[28] E. M. Z. Michielin, L. P. De Lemos Wiese, E. A. Ferreira, R. C. Pedrosa, and S. R. S. Ferreira, "Radical-scavenging activity of extracts from *Cordia verbenacea* DC obtained by different methods," *The Journal of Supercritical Fluids*, vol. 56, no. 1, pp. 89–96, 2011.

[29] G. Lefèvre, M. Beljean-Leymarie, F. Beyerle et al., "Evaluation of lipid peroxidation by assaying the thiobarbituric acid-reactive substances," *Annales de Biologie Clinique*, vol. 56, no. 3, pp. 305–319, 1998.

[30] S. Kakkar and S. Bais, "A Review on protocatechuic acid and its pharmacological potential," *ISRN Pharmacology*, vol. 2014, Article ID 952943, 9 pages, 2014.

[31] L. Barros, M. Dueñas, I. C. F. R. Ferreira, P. Baptista, and C. Santos-Buelga, "Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species," *Food and Chemical Toxicology*, vol. 47, no. 6, pp. 1076–1079, 2009.

[32] P. Mattila, P. Salo-Väänänen, K. Könkö, H. Aro, and T. Jalava, "Basic composition and amino acid contents of mushrooms cultivated in Finland," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 22, pp. 6419–6422, 2002.

[33] X. Li, X. Wang, S. Chen, and D. Chen, "Antioxidant activity and mechanism of protocatechuic acid *in vitro*," *Functional Foods in Health and Disease*, vol. 7, pp. 232–244, 2011.

[34] A. B. Lende, A. D. Kshirsagar, A. D. Deshpande et al., "Anti-inflammatory and analgesic activity of protocatechuic acid in rats and mice," *Inflammopharmacology*, vol. 19, no. 5, pp. 255–263, 2011.

[35] A. R. Borate, A. A. Suralkar, S. S. Birje, P. V. Malusare, and P. A. Bangale, "Antihyperlipidemic effect of protocatechuic acid in fructose induced hyperlipidemia in rats," *International Journal of Pharma and Bio Sciences*, vol. 2, no. 4, pp. 456–460, 2011.

[36] L.-Y. Zang, G. Cosma, H. Gardner, X. Shi, V. Castranova, and V. Vallyathan, "Effect of antioxidant protection by p-coumaric acid on low-density lipoprotein cholesterol oxidation," *The American Journal of Physiology—Cell Physiology*, vol. 279, no. 4, pp. C954–C960, 2000.

[37] L. R. Ferguson, S.-T. Zhu, and P. J. Harris, "Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29 cells," *Molecular Nutrition and Food Research*, vol. 49, no. 6, pp. 585–593, 2005.

[38] C. Luceri, L. Giannini, M. Lodovici et al., "p-Coumaric acid, a common dietary phenol, inhibits platelet activity *in vitro* and *in vivo*," *British Journal of Nutrition*, vol. 97, no. 3, pp. 458–463, 2007.

[39] F. Festa, T. Aglitti, G. Duranti, R. Ricordy, P. Perticone, and R. Cozzi, "Strong antioxidant activity of ellagic acid in mammalian cells *in vitro* revealed by the Comet assay," *Anticancer Research*, vol. 21, no. 6, pp. 3903–3908, 2001.

[40] Atta-Ur-Rahman, F. N. Ngounou, M. I. Choudhary et al., "New antioxidant and antimicrobial ellagic acid derivatives from *Pteleopsis hylodendron*," *Planta Medica*, vol. 67, no. 4, pp. 335–339, 2001.

[41] N. P. Seeram, L. S. Adams, S. M. Henning et al., "*In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice," *The Journal of Nutritional Biochemistry*, vol. 16, no. 6, pp. 360–367, 2005.

[42] D. Ahn, D. Putt, L. Kresty, G. D. Stoner, D. Fromm, and P. F. Hollenberg, "The effects of dietary ellagic acid on rat hepatic and esophageal mucosal cytochromes P450 and phase II enzymes," *Carcinogenesis*, vol. 17, no. 4, pp. 821–828, 1996.

[43] E. H. Kang, T. Y. Kown, G. T. Oh et al., "The flavonoid ellagic acid from a medicinal herb inhibits host immune tolerance induced by the hepatitis B virus-e antigen," *Antiviral Research*, vol. 72, no. 2, pp. 100–106, 2006.

[44] C. A. Rice-Evans, N. J. Miller, and G. Paganga, "Antioxidant properties of phenolic compounds," *Trends in Plant Science*, vol. 2, no. 4, pp. 152–159, 1997.

[45] J. Teixeira, A. Gaspar, E. M. Garrido, J. Garrido, and F. Borges, "Hydroxycinnamic acid antioxidants: an electrochemical overview," *BioMed Research International*, vol. 2013, Article ID 251754, 11 pages, 2013.

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Research Article

Tiliacora triandra, an Anti-Intoxication Plant, Improves Memory Impairment, Neurodegeneration, Cholinergic Function, and Oxidative Stress in Hippocampus of Ethanol Dependence Rats

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Oxidative stress plays an important role in brain dysfunctions induced by alcohol. Since less therapeutic agent against cognitive deficit and brain damage induced by chronic alcohol consumption is less available, we aimed to assess the effect of *Tiliacora triandra* extract, a plant possessing antioxidant activity, on memory impairment, neuron density, cholinergic function, and oxidative stress in hippocampus of alcoholic rats. Male Wistar rats were induced ethanol dependence condition by semivoluntary intake of alcohol for 15 weeks. Alcoholic rats were orally given *T. triandra* at doses of 100, 200, and 400 mg·kg^{-1} BW for 14 days. Memory assessment was performed every 7 days while neuron density, activities of AChE, SOD, CAT, and GSH-Px and, MDA level in hippocampus were assessed at the end of study. Interestingly, the extract mitigated the increased escape latency, AChE and MDA level. The extract also mitigated the decreased retention time, SOD, CAT, and GSH-Px activities, and neurons density in hippocampus induced by alcohol. These data suggested that the extract improved memory deficit in alcoholic rats partly via the decreased oxidative stress and the suppression of AChE. Therefore, *T. triandra* is the potential reagent for treating brain dysfunction induced by alcohol. However, further researches are necessary to understand the detail mechanism and possible active ingredient.

1. Introduction

Alcohol (ethanol) consumption in Thailand is dramatically increased. Data obtained from alcohol consumption collected by World Health Organization have demonstrated that Thailand is the top ethanol-consuming country in Asia and 40% of drinkers are in North-East region [1]. Chronic ethanol exposure can produce multiple and durable changes in the central nervous system. It has been reported that chronic ethanol consumption produces a significant loss of brain tissues especially in forebrain and hippocampus [2] together with the neurodegeneration of cholinergic neurons in basal forebrain [3]. Several lines of evidence from animal study have also revealed that a specific neuronal loss in the dentate gyrus, increased arborizations of the dendritic spines of the granule cells, a reduction of the number of spines of the CA3-pyramidal cells, and a reorganization of synaptic formations [4–7] are also presented. In addition, chronic ethanol consumption also induces memory impairment. This impairment has been demonstrated to relate with hippocampus degeneration and cholinergic function [8].

Accumulative lines of evidence have demonstrated that neurodegeneration induced by chronic ethanol consumption is associated with the elevation of oxidative stress [9, 10].

The elevation of oxidative stress induced by chronic ethanol consumption is reported to occur both via the increased free radical formation and via the decreased antioxidant enzyme activities [11]. In addition, these changes can be mitigated by the substances possessing antioxidant activity [10]. Currently, drugs which target at protecting against brain damage and memory impairment in alcoholism are less available. Therefore, the therapeutic benefit of herbal medicine has gained much attention especially in Asian countries [12].

Tiliacora triandra (Colebr.) Diels or Ya-nang in Thai belongs to the family of Menispermaceae. It is the native plant of Southeast Asia and widely used in the cuisines of northeast Thailand and Laos. Ya-nang is used not only as food but also as medicine in traditional folklore. According to the traditional medicine of many countries in Southeast Asia, it has been used as anti-pyretic, detoxication agent, anti-inflammation, anticancer, antibacterial, and immune modulator. In addition, it also possesses antioxidant activity [13]. Recent toxicity study has revealed that water extract of *T. triandra* leaves shows no toxicity up to 5000 mg·kg^{-1} in single administration. Moreover, no adverse effects were observed following the subchronic administration of the water extract of this plant at doses of 300, 600, and 1200 mg·kg^{-1} [14]. Based on the antioxidant effect together with the detoxification reputation of this herb and the benefit of substance possessing antioxidant activity against ethanol neurotoxicity mentioned earlier, the health benefit against neurotoxicity of *T. triandra* extract has been considered. Thus, this study was carried out to determine the effect of water extract of *T. triandra* on memory impairment, neurodegeneration, cholinergic function, and oxidative stress in hippocampus of ethanol dependence rats.

2. Materials and Methods

2.1. Experimental Animals. Adult male Wistar rats, 8 weeks old, were used as experimental animals. They were purchased from National Laboratory Animal Center, Salaya, Nakorn Pathom. All animals were acclimatized for two weeks on normal diet of rat chow, with water given ad libitum at room temperature with a 12-hour light and dark cycle before the commencement of the experiment. The weights of the animals on the first day of experiment were 180–220 g. The experiments were performed to minimize animals' suffering and the experiment protocols were approved by the Institutional Animal Care and Unit Committee Khon Kaen University, Thailand.

2.2. Plant Material and Extract Preparation. The aerial parts of *T. triandra* were collected from Khon Kaen province, Thailand. The plant was authenticated by Associate Professor Panee Siri-sa-ard, Faculty of Pharmacy, Chiangmai University, Chiangmai, Thailand (authentication number 023160). They were cleaned dried and ground to fine powder. Then, the ground powder was boiled with distilled water at a ratio of 1 : 6 (w/v) for 5 minutes with a continuous stirring. After being left at room temperature for 24 hours, the extract was filtered using Whatman No. 1. The filtrate was evaporated under reduced pressure using a rotary evaporator [15]. The percent yield of the extract was 5.5.

Table 1: Gradient program of HPLC analysis.

Times (minutes)	Solvents (%)	
	A (methanol)	B (2.5% acetic acid)
0	10	90
17	70	30
18	100	—
20	100	—
20.5	10	90
25	10	90

2.3. Sample Analysis. The total phenolic compounds content was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE) [16].

The finger print of *T. triandra* leaves extract was carried out by using gradient high performance liquid chromatography (HPLC) system. The system consists of 515 HPLC pump and 2998 Photodiode array detector of Waters company, USA. Chromatographic separation was performed using Purospher STAR, C-18 encapped (5 μm), LiChroCART 250-4.6, and HPLC-Cartridge, Sorbet Lot number HX255346 (Merk, Germany). Two mobile phases consisting of methanol and 2.5% acetic acid in deionized (DI) water were used to induce gradient elution. The injection volume was 20 μL and the flow rate was 1.0 mL/min. During HPLC analysis the solvent gradient was programmed as shown in Table 1 and data analysis was performed using Empower 3.

2.4. Experimental Protocol. The animals were induced ethanol dependence by using a semivoluntary intermittent intake method [17]. In brief, rats were exposed to ethanol in drinking water. The ethanol concentration in drinking water was gradually increased in a stepwise fashion from 5% at a rate of 5% per week until reaching a 20% ethanol wihin 4 weeks in order to allow the animals to get used to the taste of alcohol and mimic the condition of alcohol addiction in human. Then, the ethanol concentration was raised to 30% ethanol and maintained at this concentration between the fifth and the fifteenth weeks. The hyperexcitability symptoms which reflected ethanol dependence condition including the increased response to environmental stimuli such as startle, irritability, and hypervigilance were evaluated [17]. In addition, all ethanol dependence rats used in this study showed the average ethanol concentration at 9.89 ± 0.86 mg/dL prior to the intervention. The ethanol dependence rats were selected for further study and randomly assigned to 7 groups of 6 animals each as follows.

Group I: control group: rats in this group received no treatment.

Group II: ethanol + vehicle: alcoholic rats were orally administered vehicle (distilled water).

Group III: ethanol + Aricept (donepezil): ethanol dependence rats were administered donepezil via oral route at dose of 1 mg/kg^{-1}BW (this group served as positive control because this drug has been used as

standard drug for treating memory deficit patients) [18].

Group IV: ethanol + vitamin C: ethanol dependence rats were administered vitamin C at dose of 250 mg/kg^{-1}BW. (This group also served as positive control based on the previous findings that the substances possessing antioxidant effect exert neuroprotective effect against alcohol neurotoxicity of vitamin C [19].)

Group V: ethanol + *T. triandra* 100: ethanol dependence rats were orally administered *T. triandra* extract at dose of 100 mg·kg^{-1}BW.

Group VI: ethanol + *T. triandra* 200: ethanol dependence rats were orally administered *T. triandra* extract at dose of 200 mg·kg^{-1}BW,

Group VII: ethanol + *T. triandra* 400: ethanol dependence rats were orally administered *T. triandra* extract at dose of 400 mg·kg^{-1}BW.

All animals received the assigned treatments at a period of 14 days after the induction of ethanol dependence by a 15-week alcohol treatment. They were assessed spatial memory using Morris water maze test after the single intervention, 7 days and 14 days of treatments. At the end of experiment, they were sacrificed and hippocampi were isolated for the determination of oxidative damage markers including the level of malondialdehyde (MDA) and the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). In addition, the activity of acetylcholinesterase (AChE) in hippocampus, the crucial area for learning and memory, was also determined.

2.5. Determination of Spatial Memory. Spatial memory was evaluated using Morris water maze test. According to this test, a circular pool (160 cm in diameter × 60 cm height) filled with water (approximately 42 cm deep, temperature of 23-24°C) was divided into 4 quadrants. The removable platform was placed in the center on one quadrant under the water surface. The immersed platform was invisible due to the masking effect of nontoxic milk powder which covered the water surface. The animal must memorize the location of the immersed platform by forming the association memory between its location and the location of the platform via the environmental cues. The time which the animal spent to find the hidden platform and climb onto the platform was recorded as escape latency. The platform was removed 24 hr later and the animal was reexposed to the test again to evaluate the memory retention and retrieval capacity. The time which the animals spent swimming in the quadrant which the platform was previously located was recorded as the retention time [20, 21].

2.6. Determination of Oxidative Stress Markers. Rats were perfused with cold saline solution to get rid of the blood from the brain tissue; then, hippocampi were rapidly removed and stored at −80°C until used. To determine the oxidative stress markers, brains were prepared as homogenate and we determined the level of malondialdehyde (MDA) using the thiobarbituric acid reaction [22] whereas glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) were determined using a spectrophotometric method [23].

2.6.1. Determination of Malondialdehyde (MDA) Level. Level of malondialdehyde (MDA), a relatively stable lipid peroxidation marker, was determined by using thiobarbituric acid reacting substances (TBARS) assay. MDA which occurred as the result of the breakdown of polyunsaturated fatty acid must react with thiobarbituric acid reacting substances and gives rise to the pink color product. This product could be measured at 532 nm. In this test, 1,3,3-tetra ethoxy propane (TEP) was used as the standard.

2.6.2. Catalase (CAT) Assay. Catalase activity was determined spectrophotometrically by measuring the decrease in H_2O_2 absorbance at 490 nm. A system devoid of the substrate (hydrogen peroxide) served as the control. The difference in absorbance per unit time was expressed as the activity. One unit was defined as the amount of enzyme required to decompose 1.0 M of hydrogen peroxide per minute at pH 7.0 and 25°C.

2.6.3. Superoxide Dismutase (SOD) Assay. The activity of SOD was performed using a xanthine/xanthine oxidase system for the production of superoxide radical and subsequent measurement of cytochrome c as a scavenger of the radicals. Optical density was determined using a spectrometer (UV-1601, Shimadzu) at 550 nm. A system devoid of enzyme served as control. One unit of enzyme activity was defined as the quantity of SOD required to inhibit the rate of reduction of cytochrome by 50%. SOD activity was presented as units per milligram of protein (U/mg protein).

2.6.4. Glutathione Peroxidase (GSH-Px) Assay. The determination of GSH-Px was performed using t-butyl hydroperoxide as a substrate. The optical density was recorded at 340 nm. One unit of the enzyme was defined as micromole (μmol) of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per minute. GSH-Px activity was expressed as U/mg protein.

2.7. Determination of Acetylcholinesterase (AChE) Activity. Acetylcholinesterase (AChE) was analyzed based on the basis that this enzyme catalyzed the hydrolysis of acetylthiocholine (ATCh) which in turn gave rise to the formation of acetate and thiocholine. In the presence of the highly reactive 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) ion, thiocholine generated a yellow color substance which was quantitatively monitored by spectrophotometric absorption at 405 nm [24].

2.8. Histological Study

2.8.1. Tissue Preparation. At the end of study, all rats were exposed to transcardial perfusion. In brief, the thorax was carefully cut open, the heart was exposed, and a needle

connected to the tubing from the fixative bottle was inserted into the left ventricle. The right atrium was cut open to drain out the blood and fixative. First 20–30 mL of saline was passed transcardially to flush out the blood and then perfuse with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Fixation was monitored by the gradual discolorations of the tongue and eyeball. After fixation has been established, the brain specimens were further kept in the fixative containing 30% sucrose for 72 h. Regions of hippocampus were then dissected out using Paxinos stereotaxic coordinate method [25]. Serial sections of tissues containing hippocampus area were prepared using a sliding microtome at 20 μm thick. Tissue samples were picked up on slides coated with a 0.01% aqueous solution poly-L-lysine.

2.8.2. Cresyl Violet Staining of Brain Tissue. The sections containing hippocampus of all groups were stained with 0.5% cresyl violet. Analysis of neuronal density was performed on coronal sections of the dorsal hippocampus stained with cresyl violet and corresponding to brain sections located between 3.14 and 4.16 mm posterior to bregma [25]. The densities of living neurons in CA1, CA2, CA3, and dentate gyrus were performed using Olympus light microscope model BH-2 at 40x magnification by an observer who was blind to the treatment at time of analysis.

2.9. Statistical Analysis. Data were presented as mean ± standard error of mean (SEM). Statistical analysis was analyzed using one-way analysis of variance (ANOVA), followed by LSD post hoc test. Probability levels less than 0.05 were accepted as significance.

3. Results

3.1. Sample Analysis. The data obtained from this study showed that the *T. triandra* leaves extract used in this study contained total phenolic compounds at concentration of 593.33 mg of gallic acid equivalent (GAE)/mg extract. The HPLC fingerprint of *T. triandra* was shown in Figure 1. We had identified gallic acid, cyanidin, and quercetin according to their retention times and spectral characteristics of their peaks compared with standard. The ultraviolet spectrum of chromatographic bands presented in the fingerprinting of the samples indicated the presence of gallic acid at a concentration of 4.81 ± 0.05 μg gallic/100 mg of extract whereas the concentrations of cyanidins and quercetin were presented at the concentrations of 307.22 ± 4.74 μg Cyn-3-glu/100 mg and 9028.86 ± 695.97 μg QE/100 mg extract, respectively.

3.2. Effect of T. triandra Extract on Spatial Memory. Figure 2 showed that ethanol dependence rats induced by 15-week alcohol consumption which received vehicle or water showed the enhanced escape latency since the first day of treatment until the end of 14-day study period (P value < 0.001 all, compared to control rats). Donepezil, vitamin C, and all doses of *T. triandra* failed to modulate the enhanced escape latency induced by ethanol at 1-day treatment period. However, when the treatments were prolonged to 7 and 14 days, ethanol

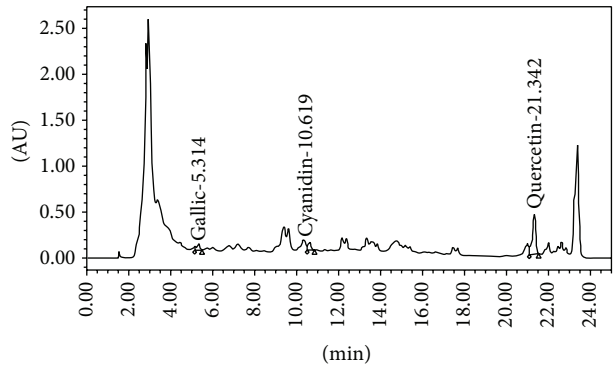

Figure 1: The chromatogram of water extract of *T. triandra*.

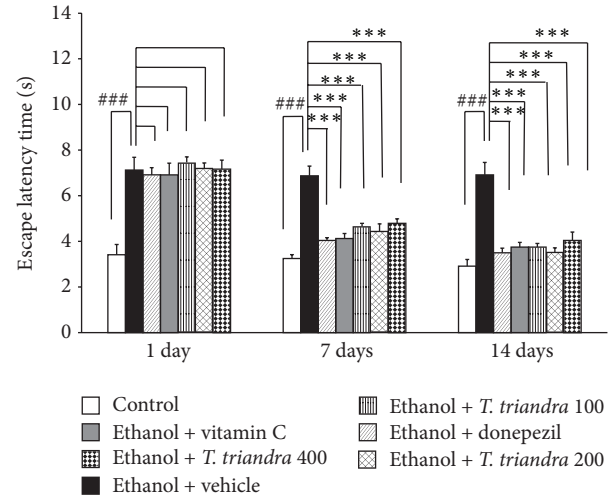

Figure 2: The effect of *T. triandra* on escape latency time in Morris water maze test. Data were presented as mean ± SEM, n = 6/group. $^{###}P$ value < 0.001 compared with control treated group. $^{***}P$ value < 0.001 compared with ethanol dependence treated group which received vehicle.

dependence rats which received donepezil or vitamin C or *T. triandra* extract at doses of 100, 200, and 400 mg·kg^{-1}BW significantly mitigated the elevation of escape latency induced by ethanol (P value < 0.001 all, compared with ethanol dependence rats which received vehicle).

The effect of *T. triandra* on retention time in Morris water maze test was also investigated and results were shown in Figure 3. It was found that ethanol dependence rats which received vehicle showed the decreased retention time throughout the 14-day study period (P value < 0.001 all, compared to control rats). Ethanol dependence rats which received either donepezil or vitamin C significantly mitigated the decreased retention time induced by alcohol consumption at 7 and 14 days of treatment (P value < 0.001 all, compared with ethanol dependence rats which received vehicle). In addition, ethanol dependence rats which received *T. triandra* at doses of 200 and 400 mg·kg^{-1}BW also showed the significant attenuation of the decreased retention time induced by ethanol consumption both at 7 (P value < 0.001 and 0.05, resp., compared with ethanol dependence rats

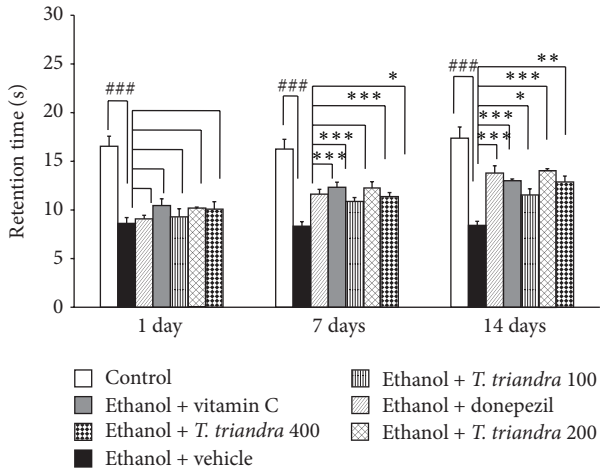

FIGURE 3: The effect of *T. triandra* on retention time in Morris water maze test. Data were presented as mean ± SEM, $n = 6$/group. $^{###}P$ value < 0.001 compared with control treated group. $^{*}P$ value < 0.05 compared with ethanol dependence treated group which received vehicle. $^{***}P$ value < 0.001 compared with ethanol dependence treated group which received vehicle.

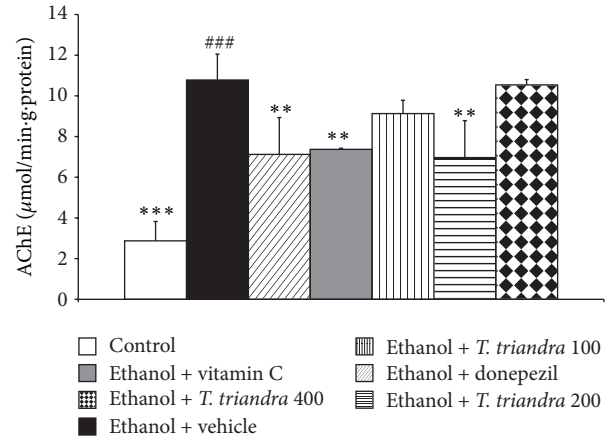

FIGURE 4: The effect of *T. triandra* on the activity of acetylcholinesterase (AChE) in hippocampus. Data were presented as mean ± SEM, $n = 6$/group $^{###}P$ value < 0.001 compared with control treated group. $^{**}P$ value < 0.01 compared with ethanol dependence treated group which received vehicle. $^{***}P$ value < 0.001 compared with ethanol dependence treated group which received vehicle.

which received vehicle) and 14 days of treatments (P value < 0.001 all, compared with ethanol dependence rats which received vehicle). However, ethanol dependence rats which received *T. triandra* at dose of 100 mg·kg^{-1}BW significantly attenuated the decreased retention time induced by ethanol consumption only at 14 days of treatment (P value < 0.05, compared with ethanol dependence rats which received vehicle).

3.3. Effect of T. triandra on AChE Activity in Hippocampus. Since cholinergic system played an important role on spatial memory, we also investigated the effect of *T. triandra* on AChE activity in this area. The results were shown in Figure 4. The significant elevation of AChE activity in hippocampus was observed in ethanol dependence rats which received vehicle (P value < 0.001, compared with control rats). Treatment with either donepezil or vitamin C could mitigate the elevation of AChE in hippocampus (P value < 0.01 all, compared with ethanol dependence rats which received vehicle). Ethanol dependence rats which received the extract at dose of 200 mg·kg^{-1}BW also significantly mitigated an elevation of AChE activity induced by ethanol consumption in hippocampus (P value < 0.01 all, compared with ethanol dependence rats which received vehicle) while no changes were observed in ethanol dependence rats which received low and high doses of extract.

3.4. Effect of T. triandra on Neuron Density in Hippocampus. Effect of *T. triandra* on neuron density in various subregions of hippocampus including CA1, CA2, CA3, and dentate gyrus was shown in Figures 5(a) and 5(b). Repetitive consumption of alcohol significantly decreased neuron density in CA1, CA2, CA3, and dentate gyrus of hippocampus (P value < 0.001 all, compared with control rats). Ethanol dependence rats which received either donepezil or vitamin C significantly attenuated the decreased neuron density induced by alcohol in all subregions mentioned earlier of hippocampus (P value < 0.01 all, compared with ethanol dependence rats which received vehicle). Interestingly, all doses of *T. triandra* extract treatment significantly counteracted the decreased neuron density induced by alcohol consumption in hippocampus (P value < 0.01 all, compared with ethanol dependence rats which received vehicle).

3.5. Effect of T. triandra on Oxidative Stress Markers. Table 2 demonstrated the effects of *T. triandra* on oxidative stress markers including malondialdehyde (MDA) level and the activities of main scavenger enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in hippocampus. The current data showed that alcohol consumption significantly enhanced MDA level (P value < 0.001, compared with control rats) but decreased SOD, CAT, and GSH-Px (P value < 0.001, 0.01, and 0.01, resp., compared with control rats) enzyme activities. Treatments with donepezil, vitamin C, and *T. triandra* at doses of 100, 200, and 400 mg·kg^{-1}BW produced the significant mitigation effect on the elevation of MDA level (P value < 0.01 all, compared with alcoholic rats which received vehicle) in hippocampus of ethanol dependence rats. The significant attenuation of SOD activity induced by ethanol in hippocampus was also observed in ethanol dependence rats which received donepezil or vitamin C or *T. triandra* at doses of 100, 200, and 400 mg·kg^{-1}BW (P value < 0.001, 0.001, 0.01, 0.001, and 0.01, resp., compared with ethanol dependence rats which received vehicle). In addition, ethanol dependence rats treated with donepezil showed a significant attenuation effect on the decreased CAT and GSH-Px activities induced by alcohol (P value < 0.01, all, compared with ethanol dependence rats which received vehicle) whereas those which received

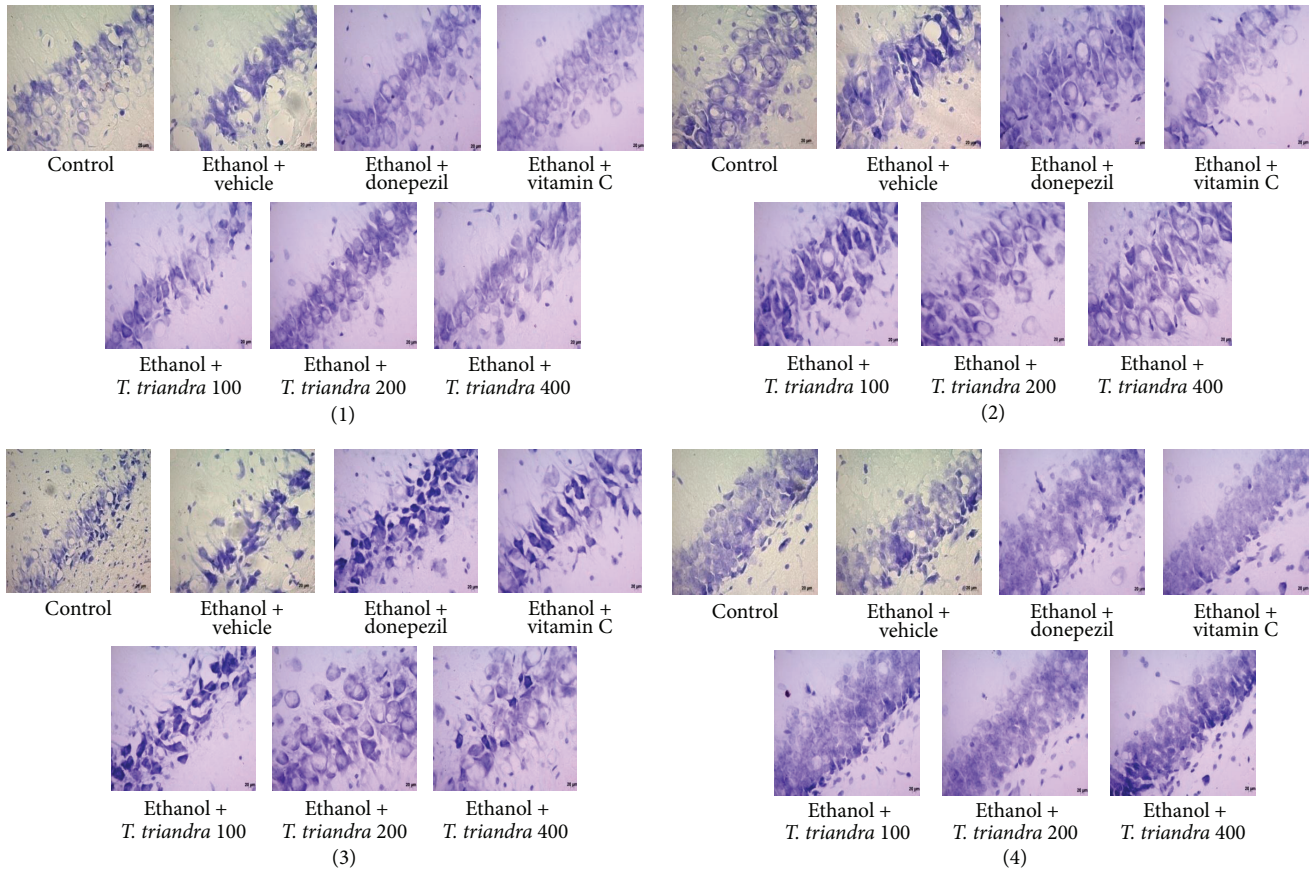

Figure 5: The effect of *T. triandra* extract on the neuron density in CA1, CA2, CA3, and dentate gyrus of hippocampus. (a) Photographs showing the density of the neurons stained with cresyl violet; (b) the bar graph showing density of neurons in various subregions of hippocampus. Data were presented as mean ± SEM, $n = 6$/group. $^{###}P$ value < 0.001 compared with control treated group. $^{***}P$ value < 0.001 compared with ethanol dependence treated group which received vehicle.

vitamin C showed only the significant change of CAT activity in hippocampus (P value < 0.05, compared with ethanol dependence rats which received vehicle). It was found that *T. triandra* extract at doses of 100, 200, and 400 mg·kg^{-1}BW significantly mitigated the enhanced MDA level induced by ethanol consumption (P value < 0.001 all, compared with ethanol dependence rats which received vehicle). The significant increased SOD activity in hippocampus was also observed in alcoholic rats which were treated with extract at all doses used in this study (P value < 0.01, 0.001, and 0.01, resp., compared with ethanol dependence rats which received vehicle). However, the significant increased CAT activity in

Table 2: The effect of *T. triandra* extract on oxidative stress markers including malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in hippocampus.

Groups/oxidative stress markers	MDA (nmol/mg·protein)	SOD (U/mg·protein)	CAT (U/mg·protein)	GSH-Px (U/mg·protein)
Control	$0.0017 \pm 0.0002^{***}$	$2.80 \pm 0.39^{***}$	$12.38 \pm 0.61^{**}$	$3.473 \pm 0.43^{**}$
Ethanol + vehicle	$0.012 \pm 0.0007^{\#\#\#}$	$0.98 \pm 0.31^{\#\#\#}$	$8.65 \pm 1.73^{\#\#}$	$2.473 \pm 0.18^{\#\#}$
Ethanol + donepezil	$0.005 \pm 0.0003^{***}$	$2.80 \pm 0.39^{***}$	$13.18 \pm 1.95^{**}$	$4.620 \pm 0.61^{**}$
Ethanol + vitamin C	$0.004 \pm 0.0007^{***}$	$2.80 \pm 0.39^{***}$	$10.91 \pm 0.64^{*}$	3.013 ± 0.10
Ethanol + *T. triandra* 100	$0.005 \pm 0.0005^{***}$	$1.62 \pm 0.43^{**}$	$12.877 \pm 0.72^{**}$	$4.30 \pm 0.53^{**}$
Ethanol + *T. triandra* 200	$0.004 \pm 0.0005^{***}$	$2.12 \pm 0.27^{***}$	$11.55 \pm 1.09^{**}$	$6.29 \pm 0.52^{***}$
Ethanol + *T. triandra* 400	$0.004 \pm 0.0004^{***}$	$1.56 \pm 0.18^{**}$	9.31 ± 1.63	$3.92 \pm 0.56^{**}$

Values are expressed as means ± SEM from 6 animals in each group.
$^{\#\#}P$ value < 0.01 and $^{\#\#\#}P$ value < 0.001 compared with control treated group.
$^{*}P$ value < 0.05, $^{**}P$ value < 0.01, and $^{***}P$ value < 0.001 compared with ethanol dependence rats which received vehicle.

hippocampus was observed only in ethanol dependence rats which were treated with low and medium doses of extract (P value < 0.01 all, compared with ethanol dependence rats which received vehicle). Treatment with *T. triandra* at the dosage range used in this study also mitigated the decrease of GSH-Px activity in hippocampus of ethanol dependence rats (P value < 0.01, 0.001, and 0.01, resp., compared with ethanol dependence rats which received vehicle).

4. Discussion

The data obtained from this study demonstrated that the ethanol dependence rats used in this study were hyperactive and increased responsiveness to environmental stimuli, such as startle, increased puffing and hair ruffling, irritability, and hypervigilance similar to that observed in the previous study [17]. The average ethanol concentration in the blood of all ethanol dependence rats prior to the intervention was 9.89 ± 0.86 mg/dL. According to this model, it was found that no serious signs of physical dependence such as spasticity and convulsion were observed. Therefore, the animal model used in this study successfully induced mild to moderate ethanol dependence.

It was found that ethanol dependence rats induced by repetitive consumption of alcohol showed the increased escape latency but decreased retention time together with the neurodegeneration of hippocampus and hypocholinergic function. This was in agreement with the previous studies [4–8, 26, 27]. It has been reported that chronic alcohol consumption induces hippocampal damage, spatial memory impairment [28], together with the decreased oxidative stress [19]. Therefore, the results obtained from this study suggested that the elevation of oxidative stress reflected by the increased MDA level in hippocampus was responsible for the hippocampal damage and memory impairment. In addition, the decreased cholinergic function induced by chronic ethanol consumption also plays the important role in the improved hippocampal damage and memory impairment. It was found that donepezil and vitamin C could attenuate an enhanced oxidative stress and a cholinergic impairment which in turn improved memory deficit [10, 29, 30]. In addition, *T. triandra* leaves extract at all doses used in this study could also mitigate both the enhanced escape latency and the decreased retention time. Moreover, it also increased neuron density and improved oxidative stress status and cholinergic function in hippocampus of ethanol dependence rats. Previous studies clearly demonstrated that the neurons in hippocampus play the crucial role in the spatial memory [31]. Therefore, we did suggest that the improved oxidative stress which in turn increased neuron density in hippocampus was responsible for the improved memory impairment in ethanol dependence rats which received *T. triandra* leaves extract. In addition, the improved cholinergic function also played a role in the improved memory deficit in ethanol dependence rats which received a medium dose of *T. triandra* leaves extract. It was found that high dose of the extract failed to show the suppression of AChE in hippocampus. The possible explanation might be associated with the masking effect of the other ingredients in the crude extract of *T. triandra* leaves. Since *T. triandra* contained high concentration of polyphenolic compounds and high concentration of quercetin and these compounds also exerted neuroprotective effect against various insults [32–36], we did suggest that the neuroprotective effect of *T. triandra* extract observed in this study might be partly associated with quercetin. However, this still required further investigation.

5. Conclusions

The current study clearly demonstrates that *Tiliacora triandra* provides neuroprotective and cognitive enhancing effects in ethanol dependence rats. Therefore, it should be used as functional food and nutraceutical product to improve memory impairment and brain damage induced by ethanol dependence. The possible mechanism of extract may be due to the antioxidant and the suppression of acetylcholinesterase activity in hippocampus. Further studies are required to investigate the possible active ingredients, detail mechanism, and signal pathways.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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References

[1] World Health Organization, "Country profile: South-East Asia region," in *WHO Global Status Report on Alcohol 2004*, pp. 1–5, World Health Organization, Geneva, Switzerland, 2004.

[2] I. Agartz, R. Momenan, R. R. Rawlings, M. J. Kerich, and D. W. Hommer, "Hippocampal volume in patients with alcohol dependence," *Archives of General Psychiatry*, vol. 56, no. 4, pp. 356–363, 1999.

[3] T. Arendt, "Impairment in memory function and neurodegenerative changes in the cholinergic basal forebrain system induced by chronic intake of ethanol," *Journal of Neural Transmission, Supplement*, no. 44, pp. 173–187, 1994.

[4] M. M. Paula-Barbosa, F. Brandao, M. D. Madeira, and A. Cadete-Leite, "Structural changes in the hippocampal formation after long-term alcohol consumption and withdrawal in the rat," *Addiction*, vol. 88, no. 2, pp. 237–247, 1993.

[5] A. Cadete-Leite, M. A. Tavares, and M. M. Paula-Barbosa, "Alcohol withdrawal does not impede hippocampal granule cell progressive loss in chronic alcohol-fed rats," *Neuroscience Letters*, vol. 86, no. 1, pp. 45–50, 1988.

[6] A. Cadete-Leite, M. A. Tavares, M. M. Pacheco, B. Volk, and M. M. Paula-Barbosa, "Hippocampal mossy fiber-CA3 synapses after chronic alcohol consumption and withdrawal," *Alcohol*, vol. 6, no. 4, pp. 303–310, 1989.

[7] N. V. Lukoyanov, F. Brandão, A. Cadete-Leite, M. D. Madeira, and M. M. Paula-Barbosa, "Synaptic reorganization in the hippocampal formation of alcohol-fed rats may compensate for functional deficits related to neuronal loss," *Alcohol*, vol. 20, no. 2, pp. 139–148, 2000.

[8] F. Melis, R. Stancampiano, A. Imperato, G. Carta, and F. Fadda, "Chronic ethanol consumption in rats: correlation between memory performance and hippocampal acetylcholine release in vivo," *Neuroscience*, vol. 74, no. 1, pp. 155–159, 1996.

[9] J. Haorah, S. H. Ramirez, N. Floreani, S. Gorantla, B. Morsey, and Y. Persidsky, "Mechanism of alcohol-induced oxidative stress and neuronal injury," *Free Radical Biology & Medicine*, vol. 45, no. 11, pp. 1542–1550, 2008.

[10] J. Wattanathorn, N. Phunchago, S. Muchimapura et al., "Mulberry fruit mitigates alcohol neurotoxicity and memory impairment induced by chronic alcohol intake," *American Journal of Applied Sciences*, vol. 9, no. 4, pp. 484–491, 2012.

[11] M. A. Collins and E. J. Neafsey, "Ethanol and adult CNS neurodamage: oxidative stress, but possibly not excitotoxicity," *Frontiers in Bioscience*, vol. 4, no. 4, pp. 1358–1367, 2012.

[12] E. Ernst, "Prevalence of use of complementary/alternative medicine: a systematic review," *Bulletin of the World Health Organization*, vol. 78, no. 2, pp. 252–257, 2000.

[13] S. Rattana, M. Padungkit, and B. Cushnie, "Phytochemical screening, flavonoid content, and antioxidant activity of *Tiliacora triandra* leaf extracts," in *Proceedings of the 2nd Annual International Conference of Northeast Pharmacy Research*, pp. 60–63, February 2010.

[14] S. Sireeratawong, N. Lertprasertsuke, U. Srisawat et al., "Acute and subchronic toxicity study of the water extract from *Tiliacora triandra* (Colebr.) Diels in rats," *Songklanakarin Journal of Science and Technology*, vol. 30, no. 5, pp. 611–619, 2008.

[15] M. M. Al-Dabbas, K. Al-Ismail, K. Kitahara et al., "The effects of different inorganic salts, buffer systems, and desalting of Varthemia crude water extract on DPPH radical scavenging activity," *Food Chemistry*, vol. 104, no. 2, pp. 734–739, 2007.

[16] Y. Y. Lim, T. T. Lim, and J. Jing, "Antioxidant properties of guava fruit: comparison with some local fruits," *Sunway Academic Journal*, vol. 3, pp. 9–20, 2006.

[17] M. S. Macieira, W. G. Almeida, E. A. Silva, L. C. Schenberg, and E. M. Nakamura-Palacios, "Alcohol dependence induced in rats by semivoluntary intermittent intake," *Brazilian Journal of Medical and Biological Research*, vol. 30, no. 9, pp. 1107–1111, 1997.

[18] M. Cochrane, A. Cochrane, P. Jauhar, and E. Ashton, "Acetylcholinesterase inhibitors for the treatment of Wernicke-Korsakoff syndrome—three further cases show response to donepezil," *Alcohol and Alcoholism*, vol. 40, no. 2, pp. 151–154, 2005.

[19] E. Macit, G. Ulusoy, T. Celik, H. Kayir, and T. Uzbay, "Comparative effects of antioxidants on chronic ethanol-induced oxidative stress in rat hippocampus," *Journal of Neurological Sciences*, vol. 29, no. 2, pp. 329–339, 2012.

[20] M. L. Byrnes, D. P. Richardson, J. F. Brien, J. N. Reynolds, and H. C. Dringenberg, "Spatial acquisition in the Morris water maze and hippocampal long-term potentiation in the adult guinea pig following brain growth spurt-prenatal ethanol exposure," *Neurotoxicology and Teratology*, vol. 26, no. 4, pp. 543–551, 2004.

[21] R. Morris, "Developments of a water-maze procedure for studying spatial learning in the rat," *Journal of Neuroscience Methods*, vol. 11, no. 1, pp. 47–60, 1984.

[22] H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction," *Analytical Biochemistry*, vol. 95, no. 2, pp. 351–358, 1979.

[23] C. J. Weydert and J. J. Cullen, "Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue," *Nature Protocols*, vol. 5, no. 1, pp. 51–66, 2010.

[24] G. L. Ellman, K. D. Courtney, V. Andres Jr., and R. M. Featherstone, "A new and rapid colorimetric determination of acetylcholinesterase activity," *Biochemical Pharmacology*, vol. 7, no. 2, pp. 88–95, 1961.

[25] G. Paxinos and C. Waston, *The Rats Brain in Stereotaxic Coordinates*, Academic Press, San Diego, Calif, USA, 4th edition, 1998.

[26] R. Khalil, M. A. King, and M. R. I. Soliman, "Testosterone reverses ethanol-induced deficit in spatial reference memory in castrated rats," *Pharmacology*, vol. 75, no. 2, pp. 87–92, 2005.

[27] V. Tiwari and K. Chopra, "Protective effect of curcumin against chronic alcohol-induced cognitive deficits and neuroinflammation in the adult rat brain," *Neuroscience*, vol. 244, pp. 147–158, 2013.

[28] A. M. White, P. E. Simson, and P. J. Best, "Comparison between the effects of ethanol and diazepam on spatial working memory in the rat," *Psychopharmacology*, vol. 133, no. 3, pp. 256–261, 1997.

[29] K. Nixon, "Alcohol and adult neurogenesis: roles in Neurodegeneration and recovery in chronic alcoholism," *Hippocampus*, vol. 16, no. 3, pp. 287–295, 2006.

[30] M. Jamal, K. Ameno, S. Ameno et al., "Changes in cholinergic function in the frontal cortex and hippocampus of rat exposed to ethanol and acetaldehyde," *Neuroscience*, vol. 144, no. 1, pp. 232–238, 2007.

[31] M.-B. Moser and E. I. Moser, "Distributed encoding and retrieval of spatial memory in the hippocampus," *The Journal of Neuroscience*, vol. 18, no. 18, pp. 7535–7542, 1998.

[32] K. B. Pandey and S. I. Rizvi, "Plant polyphenols as dietary antioxidants in human health and disease," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 5, pp. 270–278, 2009.

[33] S. Schaffer and B. Halliwell, "Do polyphenols enter the brain and does it matter? Some theoretical and practical considerations," *Genes and Nutrition*, vol. 7, no. 2, pp. 99–109, 2012.

[34] N. Sriraksa, J. Wattanathorn, S. Muchimapura, S. Tiamkao, K. Brown, and K. Chaisiwamongkol, "Cognitive-enhancing effect of quercetin in a rat model of Parkinson's disease induced by 6-hydroxydopamine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 823206, 9 pages, 2012.

[35] K. Selvakumar, S. Bavithra, G. Krishnamoorthy, P. Venkataraman, and J. Arunakaran, "Polychlorinated biphenyls-induced oxidative stress on rat hippocampus: a neuroprotective role of quercetin," *The Scientific World Journal*, vol. 2012, Article ID 980314, 10 pages, 2012.

[36] N. A. Kelsey, H. M. Wilkins, and D. A. Linseman, "Nutraceutical antioxidants as novel neuroprotective agents," *Molecules*, vol. 15, no. 11, pp. 7792–7814, 2010.

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Review Article

Polyphenol Stilbenes: Molecular Mechanisms of Defence against Oxidative Stress and Aging-Related Diseases

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Numerous studies have highlighted the key roles of oxidative stress and inflammation in aging-related diseases such as obesity, type 2 diabetes, age-related macular degeneration (AMD), and Alzheimer's disease (AD). In aging cells, the natural antioxidant capacity decreases and the overall efficiency of reparative systems against cell damage becomes impaired. There is convincing data that stilbene compounds, a diverse group of natural defence phenolics, abundant in grapes, berries, and conifer bark waste, may confer a protective effect against aging-related diseases. This review highlights recent data helping to clarify the molecular mechanisms involved in the stilbene-mediated protection against oxidative stress. The impact of stilbenes on the nuclear factor-erythroid-2-related factor-2 (Nrf2) mediated cellular defence against oxidative stress as well as the potential roles of SQSTM1/p62 protein in Nrf2/Keap1 signaling and autophagy will be summarized. The therapeutic potential of stilbene compounds against the most common aging-related diseases is discussed.

1. Introduction

Aging is one of the major risk factors for a wide range of chronic, metabolic, and neurodegenerative diseases. During the course of aging, reactive oxygen species (ROS) cause increasing oxidative stress in cells leading to oxidative damage and to the induction of inflammatory cascades (Figure 1). Furthermore, antioxidant defence systems against oxidative stress deteriorate during aging and many antioxidant phase II genes are upregulated only marginally or may even be downregulated. Moreover, the innate and adaptive immune defence systems tend to deteriorate during aging and this may contribute to the degeneration process [1].

Activation of antioxidant defence and phase II enzymes is a key endogenous mechanism protecting cells from the oxidative damage associated with many common diseases such as obesity, type 2 diabetes (T2D), age-related macular degeneration (AMD), and Alzheimer's disease (AD). The activation of cellular defence mechanisms by plant-derived bioactive compounds is believed to attenuate cellular oxidative stress and thus this seems to represent a feasible therapeutic approach against age-related diseases. There is increasing epidemiological and experimental data suggesting that the regular intake of berries, vegetables, and fruits containing significant amounts of polyphenols is a potential way to improve the quality of life as an individual grows older [2–4]. Polyphenols or polyphenol metabolites produced in the body mediate their protective action via several mechanisms and different pathways that are currently under intensive study. For example, the beneficial effects of anthocyanin-rich bilberry and blackcurrant diets, proanthocyanidin (later referred to as PAC) metabolites, and grape-derived polyphenolic preparations have been extensively studied not only in animal models but also in patients with T2D and AD [5–7].

Stilbene compounds are part of a vast group of natural defence polyphenols occurring in many plant species. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a well-known polyphenol phytoalexin which is found mainly in the skin of

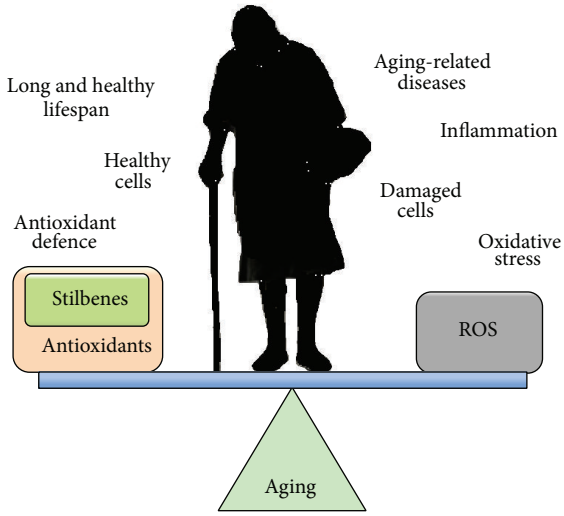


Figure 1: As an individual ages, the balance between a long, healthy lifespan and suffering age-related diseases is believed to be related to the interplay between the cellular antioxidant defence system and adverse effects related to oxidative stress. In aging cells, oxidative stress increases due to a progressive decline in the efficiency of antioxidant defence systems. There is convincing evidence to indicate that supplementation with polyphenols, such as stilbenes, anthocyanins, and catechins, can increase cellular antioxidant defence and promote health of the individual.

grapes; it has attracted extensive scientific attention due to its potential health benefits related with its cardiovascular (French paradox), chemopreventive, antiobesity, antidiabetic, and neuroprotective properties. However, recent data have highlighted that also other stilbene compounds such as pterostilbene (3,5-dimethyl ether derivative of resveratrol) may have higher bioavailability and possess better neuroprotective activity against AD than resveratrol itself [8]. Another interesting stilbene compound with potential health-beneficial properties is pinosylvin (3,5-dihydroxy-trans-stilbene), a naturally occurring trans-stilbenoid which is mainly found in the heartwood of *Pinus* species and occurs in high concentrations in bark waste and thus this stilbene compound may represent an inexpensive polyphenol with considerable potential for diverse health-promoting applications [9–11].

There are several comprehensive reviews available focusing on resveratrol, but very few reports have analyzed the bioactivity of other stilbenes. Here, we review shortly the most recent data to clarify the molecular mechanisms involved in stilbene-mediated protection against oxidative stress, emphasizing the potential roles of transcription factor nuclear factor-erythroid-2-related factor-2 (Nrf2) and SQSTM1/p62 protein (later referred to as p62) in the regulation of antioxidant enzymes and autophagy. The therapeutic potential of stilbene compounds to target the molecular pathways behind many common aging-related diseases will be reviewed.

2. Stilbene Compounds in Plants

Phenolic compounds are important mediators of adaptation and survival responses of plants in acute and chronic challenges, but polyphenols also act in plants regulating cell growth, differentiation, pollen fertility, and nodulation and thus seem to be essential for plant health. For example, stilbenes are natural phenolic defence compounds occurring in a number of different plant species that possess antimicrobial and antioxidant activities against phytopathogens and ozone or UV stress [12].

Stilbene compounds occur in many plant species (Table 1) including grape wine (*Vitis vinifera*), peanut (*Arachis hypogaea*), sorghum (*Sorghum bicolor*) and many tree species (*Pinus* and *Picea*) [13]. Moreover, commercial sources of stilbenes include many plants cultivated in Asia as folk medicines such as *Polygonum cuspidatum, Rhodomyrtus tomentosa, Rheum undulatum, Melaleuca leucadendron*, and *Euphorbia lagascae*, while pterostilbene is found predominantly in bilberries (*Vaccinium myrtillus*), blueberries (several *Vaccinium* species), and some other berries as well as in grapes and juice residues which are important source of this stilbene when it is used in nutraceutical applications [14]. Grape pomaces, residues produced during wine making, and other grape juice solids contain high polyphenol concentrations and are also attractive sources of many stilbene compounds not only resveratrol [15]. The bark waste of conifer trees contains substantial amount of stilbene compounds such as pinosylvin, piceatannol, and *trans*-resveratrol (*t*-Res). Thus, this enormous amount of industrial byproducts represents a very attractive and inexpensive source of stilbenes with commercial applications [16, 17]. Genetic tools are a very promising means to produce specific stilbenes such as pterostilbene via stilbene synthase and *O*-methyltransferase coexpression in plants [18]. These types of stilbenes may be especially suitable for pharmacological applications. The major stilbenes and their structures are described in Table 1 and a more complete list can be found in reviews [12, 19].

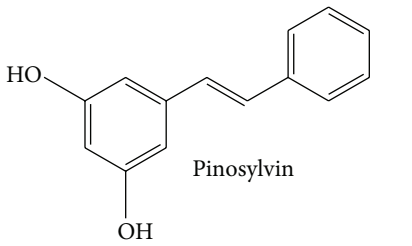
Stilbene synthase (STS) is the key enzyme that catalyzes the biosynthesis of stilbenic compounds. STS has evolved from the chalcone synthases (CHSs) apparently several times independently in stilbene-producing plants [13, 19]. Interestingly, different STS genes display different tissue and developmental specific expression. Thus, it has been reported that STS genes exhibited lower expression levels in young grape leaves than in old leaves while the transcript levels of eight STS genes increased dramatically in the berry skin of grape cultivars Cabernet Sauvignon and Norton post véraison reaching the highest level at the time of harvest [2]. The heartwood of pine trees contains high level of pinosylvin, but as a response to stress induction (fungal or UV light), young seedlings also accumulate high amounts of pinosylvin [20].

3. Bioavailability

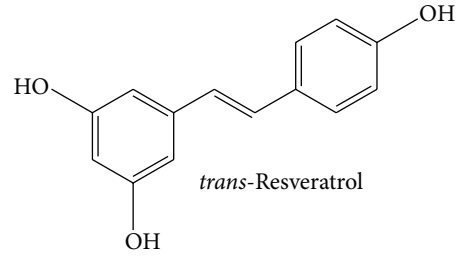
At least a proportion of the stilbene compounds or their metabolites present in extracts may be sufficiently bioavailable to reach even brain target cells and thus exert beneficial

Table 1: Major stilbenes and their structures.

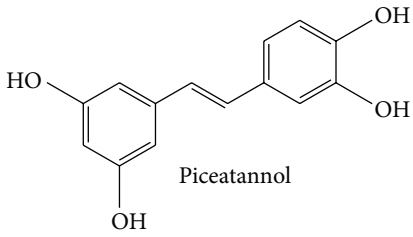
Plant	Compounds [reference]
Cocoa (*Theobroma cacao* L.)	Resveratrol [21]
Grape (*Vitis vinifera* L.)	Piceatannol, resveratrol [22]
Hop (*Humulus lupulus* L.)	Resveratrol [23]
Peanut (*Arachis hypogaea* L.)	Resveratrol [24]
Pinaceae trees (pines)	
Picea Mill.	Isorhapontigenin, piceatannol [25]
Pinus L.	Pinosylvin [16]
Rhubarbs (*Rheum* L.)	Piceatannol, rhapontigenin, resveratrol [26]
Strawberry (*Fragaria x ananassa* Duch.)	Resveratrol [27]
Sugar cane (*Saccharum* spp.)	Piceatannol, resveratrol [28]
Tomato (*Lycopersicon esculentum* Mill.)	Resveratrol [29]
Vaccinium berries	
Bilberry (*V. myrtillus*)	Resveratrol [30]
Cranberry (*V. macrocarpon*)	Resveratrol [30]
Highbush blueberry (*V. corymbosum*)	Piceatannol, resveratrol [30], pterostilbene [31]
Wines	
Red	Piceatannol, resveratrol [32]
White	Resveratrol [33]



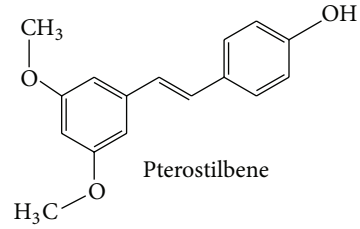
Pinosylvin



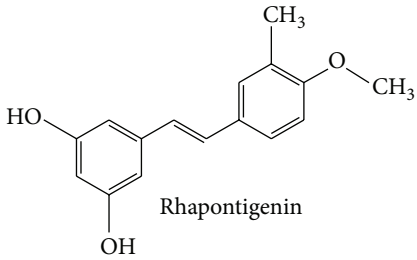
trans-Resveratrol



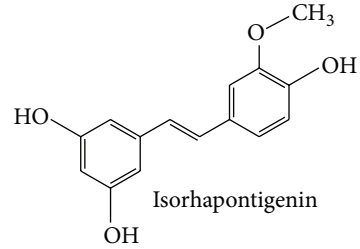
Piceatannol



Pterostilbene



Rhapontigenin



Isorhapontigenin

actions [7, 34]. Although the oral absorption of resveratrol in humans has been claimed to be as high as 75% [35], it is well known that stilbenes, however, are poorly bioavailable phenolic compounds after oral intake. Due to extensive metabolism in the major sites in intestine and liver (glucuronides and sulfates are the major metabolites of resveratrol), the oral bioavailability appears to be less than 1% in a rat model. The oral bioavailability of rhaponticin was calculated to be 0.03% [36]. In another rat study, following oral dosing, plasma levels of pterostilbene and pterostilbene sulfate were markedly greater than the levels of resveratrol and resveratrol sulfate indicating that the *in vivo* biological activity of equimolar doses of pterostilbene may be greater than that of resveratrol [37]. Moreover, the absolute oral bioavailability of pterostilbene was found to be around 12% in rat plasma with the values of terminal elimination half-life

and clearance of pterostilbene being 96.6 ± 23.7 min and 37.0 ± 2.5 mL/min/kg suggesting that bioabsorption is very rapid with peak concentration achieved at 0.5–2 h after the oral dose and excretion being complete a few hours after ingestion [38].

In a human trial, 10 healthy volunteers received single doses of 0.5, 1, 2.5, or 5 g resveratrol; the peak levels of resveratrol and six metabolites at the highest dose were 539 ± 384 ng/mL (2.4 micromol/L, mean ± SD; $n = 10$) analyzed 1.5 h after dose [39]. Interestingly, the area under the plasma levels curve (AUC) values for resveratrol-3-sulfate and resveratrol monoglucuronides was up to 23 times greater than that of resveratrol. In the other human trial performed in 40 healthy volunteers, repeated doses of resveratrol were tested with the volunteers ingesting 29 daily resveratrol doses of 0.5, 1.0, 2.5, or 5.0 g [40]. The data revealed that resveratrol-3-*O*-sulfate, resveratrol-4′-*O*-glucuronide, and resveratrol-3-*O*-glucuronide were the major plasma metabolites. Maximal plasma levels and areas under the concentration versus time curve for the metabolites exceeded the levels of resveratrol by about 20-fold. When resveratrol at doses of 0.5 or 1.0 g was given to 20 patients suffering from colorectal cancer, both resveratrol and resveratrol-3-*O*-glucuronide were recovered from tissues at maximal mean concentrations of 674 and 86.0 nmol/g, respectively [41]. Interestingly, it was claimed by the authors that these daily doses of 0.5 or 1.0 g produced levels in the human gastrointestinal tract at order of magnitude sufficient to elicit anticarcinogenic effects.

Low bioavailability, poor solubility, limited stability, high rate of metabolic breakdown, and low target specificity have been considered as major obstacles to the use of resveratrol and its natural analogies in major pharmacological applications. However, several research lines are currently underway to improve these properties [42]. A wide range of synthetic derivatives of resveratrol have been generated, and some of the best derivatives have improved the target specificity down to the nanomolar range [43]. There is a remarkable finding that inhibition of aromatase activity was enhanced by over 6000-fold when the central ring of resveratrol was substituted with 1,3-thiazole; this suggests that this modified resveratrol may be a potential drug for treating breast cancer.

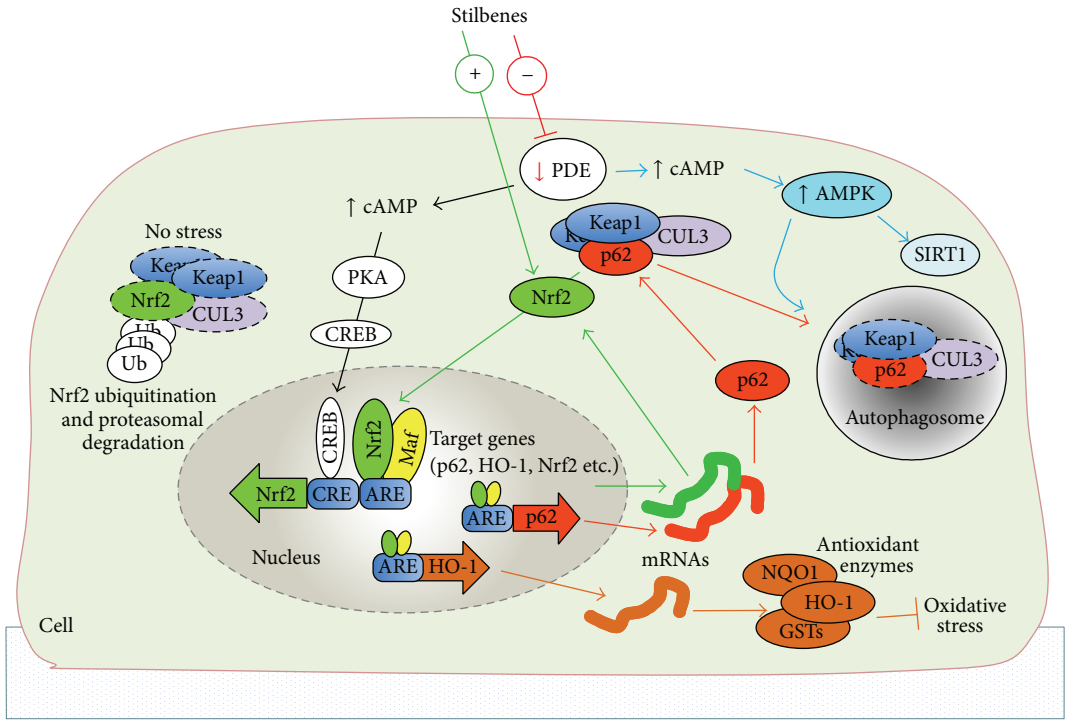
Recently it was reported that soluble galenic form improved low absorption of *t*-Res as a dry powder [44]. The efficacy of the new formulation was tested in 15 healthy volunteers receiving 40 mg of *t*-Res. The single dose (40 mg) of the soluble *t*-Res was found to be well absorbed and elicited biologically efficient blood levels (0.1–6 μM) for several hours; the new soluble galenic-based formulation led to 8.8-fold higher resveratrol levels in plasma versus the control powder. Interestingly, this new formulation elicited an intense anti-inflammatory response in various tissues of mice fed a high-fat diet (HFD) while the control diet exhibited only a weak response suggesting that improved plasma bioavailability confers significant enhancement of biological activity in the target cells. In another recent work, a modification of the liposome with polyethylene glycol (PEG) was used to improve the bioavailability of rhaponticin (RA) and its plasma protein binding ability [45]. This experiment revealed that the maximum plasma concentration (T_{max}) of PEGL-RA was about 4.5 times higher than that of free RA after oral administration due to the lower distribution into the gastrointestinal tract. Addition of piperine alkaloid (100 mg/kg; oral gavage) + piperine (10 mg/kg; oral gavage) in a mouse trial lead to a substantial increase (1544%) in the maximum serum concentration (C_{max}) as compared with the standard resveratrol dose [46]. Recently, it was reported that a higher concentration of resveratrol could be achieved in the brain tissue by administrating the compound inside lipid-core nanocapsules [47].

The safety of food ingredients (dietary supplements, functional foods, etc.) containing substantial amounts of polyphenols is an important issue. In the majority of studies, stilbene compounds such as resveratrol have appeared to be well tolerated and no marked toxicity has been reported [48]. In a human trial, 25, 50, 100, or 150 mg of *t*-Res was given six times a day, but adverse events were mild in severity and similar between groups [49]. Only at very high doses used in some human studies such as repeated doses at 2.5 and 5 g levels have there been reports of mild to moderate gastrointestinal symptoms [48], but even such high levels given as single doses did not cause any adverse events [39, 40]. However, stilbene-drug interactions have not been clarified and remain to be determined.

Gastrointestinal symptoms at a 1 g daily dose were also observed and it has been suggested that 1 g of daily resveratrol dose should not be exceeded in clinical trials [40]. Moreover, administration of 2 g *t*-Res twice a day was recently found to achieve adequate biological exposure and it was well tolerated in healthy subjects, although diarrhea was frequently observed; thus, it was proposed that to maximize *t*-Res exposure and safety these supplements should be taken with a standard breakfast and not with a high-fat meal [50].

4. Molecular Basis of Oxidative Stress

4.1. The Nrf2/ARE Pathway in Cellular Defence. Nuclear factor-erythroid-2-related factor-2 (Nrf2), a member of the basic leucine zipper (bZIP) transcription factor family, is an essential transcription factor for cellular detoxification and defence against oxidative stress. In the cell nucleus, Nrf2 is able to recognize the antioxidant response element (ARE) with the specific nucleotide binding sequence (5′-TGACnnnGC-3′) positioned in regulatory region of target genes [51, 52]. Role of other members of the Nrf-family such as Nrf1 and Nrf3 has not been so thoroughly studied but current evidence suggests that these genes have partially different functions, target genes, and tissue-specificities although they recognize the same ARE sequence as Nrf2 [51, 53]. By acting through the ARE element, Nrf2 has a central role in the regulation of a large group of phase II metabolite conjugation and antioxidant genes (Figure 2) as well as in influencing some of the genes involved in proteasome pathway and inflammation [52, 54–57]. Until now, several Nrf2 target genes (see Table 2) such as heme oxygenase-1 (HO-1) [58] and NAD(P)H dehydrogenase, quinone 1 (NQO1) [59], have been verified. Under basal conditions, the Nrf2/ARE pathway is suppressed since Nrf2 is trapped in the cytosol as it forms a protein complex with Kelch-like ECH-associated



ARE: antioxidant response element
CRE: cAMP response element in gene regulatory region

Figure 2: The Nrf2/ARE pathway and cAMP second messenger system together are the key regulators of cellular antioxidant defence. These pathways can be modulated by stilbenes. Stilbenes can activate nuclear localization of Nrf2 and activation of Nrf2 target genes associated with antioxidant defence and autophagy. Autophagy related protein p62 and Nrf2 form a regulatory loop where p62 enables the release of Nrf2 from cytoplasmic Keap1 complex. When cells are not stressed, the excess of cytoplasmic Nrf2 is eliminated by proteasomal degradation. In addition, stilbenes are capable of activating cAMP response element-binding protein (CREB) target genes and the AMPK pathway by PDE inhibition mediated increase of cellular cAMP levels.

protein 1 (Keap1) [55]. Keap1 acts as a molecular switch sensing cellular electrophile and oxidant homeostasis [60]. With assistance of Cullin-3 (CUL3), the Nrf2-Keap1-CUL3 protein complexes are constantly exposed to ubiquitin conjugation and proteasomal degradation [55, 61]. In condition of stress or exposure to electrophiles, Nrf2 dissociates from the Keap1-CUL3 complex and translocates into the nucleus. The dissociation of Nrf2 is mediated via modification of specific Keap1 cysteine residues by electrophiles, oxidants, and dietary supplements such as stilbenes [62]. Alternatively, dissociation of Nrf2 from cytoplasmic Nrf2-Keap1-CUL3 complex is enabled by p62 involved in autophagy process (see Section 4.2). In the nucleus, Nrf2 heterodimerizes with small Maf (sMaf) proteins which seems to be indispensable partners required for ARE binding and subsequent transactivation of target genes [52]. In contrast, in the nucleus, the transcriptional repressor BACH1 seems to have an important role as an antagonist for Nrf2 mediated activation by binding ARE-like elements in Nrf2 target genes [63]. It should be noted that Keap1 has the capability to undergo nuclear localization and to shuttle back to cytoplasm; this suggests that Keap1 is also involved in the regulation of Nrf2 in the cell nucleus [64]. Interestingly, in different species, ARE elements are also found in the regulatory regions of Nrf2 itself as well as in several regulators of the Nrf2/ARE pathway such as Keap1, sMaf, and p62 [65, 66]. In addition, it has been shown that the acetylation-deacetylation status of Nrf2 in the nucleus is also important for Nrf2 binding and target gene activation [67]. Auxiliary mechanisms such as the cAMP/CREB pathway [68] and the aryl hydrocarbon receptor (AhR) pathway [69] interacting with the Nrf2/ARE pathway will be discussed later in this review (see Section 4.3).

Convincing evidence from knockout animal models has proved that Nrf2 and Keap1 regulate numerous cellular functions [70, 71]. For example, Nrf2 knockout mouse displays progressive degeneration of retina (see Section 5.3) characteristic for AMD [71]. In Keap1 knockout mouse excessive accumulation of Nrf2 into nucleus stimulates aberrant expression of Nrf2 target genes causing growth retardation and the death of pups soon after birth [54]. The dramatic changes in growth indicate that Keap1 has an essential role in Nrf2 regulation and expression of Nrf2 target genes. For instance, Keap1 knockout mice displayed a constant overexpression of cytoprotective proteins such as phase II enzymes NQO1 and glutathione S-transferases (GSTs). Moreover, recent genome-wide studies have revealed numerous putative Nrf2 target genes [52, 72] suggesting that the Nrf2/ARE pathway may possess several novel molecular targets for polyphenols still to be found.

Table 2: Selected Nrf2 target gene candidates in human associated with defence against oxidative stress and age-related diseases.

Target gene	Function/role in defence against oxidative stress	Reference
Nrf2	Transcription factor, activator of detoxifying enzymes (autoregulation)	[73]
AhR	Regulator of xenobiotic metabolizing enzymes	[74]
HO-1	Cytoprotection, catabolize heme	[75]
GSTP1	Antioxidant enzyme, xenobiotic metabolizing enzyme	[76]
NQO1	Antioxidant enzyme, xenobiotic metabolizing enzyme	[77–79]
CBR3	Metabolizing enzyme of carbonyl compounds	[80]
UGT1A8, A10	Glucuronidation of xenobiotics	[81]
GCS	Glutathione biosynthesis	[82]
TRX	Antioxidant enzyme, protein redox regulation	[83]
SLC7A11	Transports cysteine, a precursor of antioxidant glutathione	[52, 84]
SLC48A1	Heme transporter	[85]
AMBP	Heme binding, free radical scavenger	[72] [85]
ABCB6	Mitochondrial porphyrin (heme) transporter	[85]
FECH	Heme biosynthesis, chelates ferrous iron	[72, 85]
TBXAS1	Thromboxane A2 synthesis (cytochrome P450 family)	[85]
IL-6	Inflammation, proinflammatory cytokine	[52, 86]
Bcl-2	Antiapoptotic protein	[87]
p62	Adaptor protein, proteasomal clearance, autophagy	[52, 66]

ABCB6: ATP-binding cassette subfamily B member 6, AhR: aryl hydrocarbon receptor, AMBP: α1-microglobulin/bikunin, Bcl-2: B-cell lymphoma 2 protein, CBR3: carbonyl reductase 3, FECH: ferrochelatase, GCS: γ-glutamylcysteine synthetase, GSTP1: glutathione S-transferase pi, HO-1: heme oxygenase-1, IL-6: interleukin-6, NQO1: NAD(P)H dehydrogenase, quinone 1, Nrf2: nuclear factor-erythroid-2-related factor-2, p62: sequestosome 1, SLC7A11: solute carrier family 7, member 11, SLC48A1: solute carrier family 48, member 1, TBXAS1: thromboxane A synthase 1, TRX: thioredoxin, and UGTs: UDP-glucuronosyltransferases.

4.2. Role of p62 Protein in Nrf2/ARE Signaling and Autophagy. Current data indicates that Nrf2 is involved in autophagy, a catabolic process activated during starvation. For instance, in the retinal pigment epithelial (RPE) cells of the Nrf2 knockout mouse eye, the autophagy process and lysosome-dependent degradation were disturbed with accumulated autophagy intermediates, photoreceptor outer segments (POS), and an aging pigment, lipofuscin [70]. Autophagy supplies an energy resource of amino acids and other substrates via lysosomal degradation and recycling of unnecessary cellular components [88]. The impaired autophagy system has been shown to associate with aging-related neurodegenerative diseases such as Parkinson's disease (PD) [89], AMD [90], and AD [91]. One sign of this impairment is the accumulation of autophagy receptor p62 in AMD [90]. In addition, p62 is a multifunctional protein involved in other cellular functions such as bone metabolism, inflammation, and adipogenesis [92–94]. In order to eliminate cellular waste and protein aggregates during nutrient deprivation, the cell may trigger the complex autophagy process where the p62 protein has a central role [88]. In an experiment conducted in autophagy deficient mice it was found that p62 is involved in the formation of cellular protein aggregates which are normally eliminated by autophagy [95]. There is growing evidence that p62 is also capable of interacting with the Nrf2/ARE pathway (Figure 2) by disrupting the cytoplasmic Nrf2-Keap1 complex [66]. Moreover, it has been shown that the functional ARE element is located in regulatory region of p62 gene [52, 66]. Nrf2-p62 couple seems to form a regulatory loop where Nrf2 is able to activate p62 expression and consequently Nrf2 nuclear localization is facilitated by p62 [96, 97]. Interestingly, there is data that p62 is able to bind on specific Keap1 motif required for Nrf2 binding [66, 96] and that Keap1 elimination is processed by p62 dependent autophagy [97]. This was also shown in autophagy deficient mouse where Nrf2 became accumulated in cell nucleus [95]. However, the nuclear localization of Nrf2 was diminished when p62 was abolished.

It has been shown that AMP-activated protein kinase (AMPK) is the key regulator of autophagy [97] suggesting that the AMPK pathway is also likely involved in the regulation of p62 and the Nrf2/ARE pathway (Figure 2). AMPK is able to induce autophagosome formation by activating numerous downstream kinases and interacting proteins such as autophagy-related proteins, protein kinase ULK1, and microtubule-associated protein LC3 [93, 98] finally achieving the oligomerization of p62 within autophagosomes [99]. The activation of autophagy is concurrently aided by AMPK mediated inhibition of mammalian target of rapamycin (mTOR), a known suppressor of autophagy [100]. In contrast, mTOR is capable of inhibiting autophagy via phosphorylation of ULK1 [101]. It is known that both AMPK and autophagy are activated upon starvation. This pathway has been verified by utilizing 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside ribonucleoside (AICAR), an activator of AMPK, autophagy, and NAD-dependent deacetylase sirtuin 1 (SIRT1) [90, 102–104]. For instance, AICAR mediated activation of the AMPK pathway can increase HO-1

expression via Nrf2 [104]. Interestingly, also stilbenes such as resveratrol can facilitate AMPK activation and autophagy [105].

4.3. Modulation of Other Pathways Underlying the Antioxidant Defence Systems. There is growing data that polyphenols have alternative molecular targets and this complicates elucidation of their role in cellular physiology and pathophysiology. Recently, interesting information regarding novel molecular targets of stilbenes such as cellular cAMP second messenger signaling, the AMPK pathway regulating energy homeostasis, estrogen-related receptor alpha (ERRα), and estrogen receptors (ER) as well as the enzymatic cofactor tetrahydrobiopterin (BH4) has become a focus of attention. Apparently, some of these targets may interact with the Nrf2/ARE pathway and the autophagic process.

Stilbenes Can Restore the Cellular Bioavailability of the Enzymatic Cofactor Tetrahydrobiopterin (BH4) under ROS Exposure. There is evidence that ROS are able to decrease the bioavailability of BH4 and coupling to aromatic amino acid hydroxylases, enzymes essential in many of the metabolic pathways involved in vascular and neurotransmitter homeostasis [106]. It is noteworthy that BH4 is an essential cofactor of nitric oxide synthase enzymes (NOS) involved in nitrogen oxide (NO) synthesis in almost all tissues [107, 108]. It has been shown that in NO synthesis, the lack of BH4 initiates superoxide generation and further synthesis of the powerful oxidant, peroxynitrite [109]. Moreover, in the brain and the eye, BH4 is required for the synthesis of tyrosine, dihydroxyphenylalanine (L-DOPA), dopamine, and serotonin [110] by acting as an essential cofactor of the key enzymes phenylalanine hydroxylase, tyrosine hydroxylase, tyrosinase [111], and tryptophan hydroxylase [112], respectively. Interestingly, BH4 is also pivotal for synthesis of two important neurotransmitters, norepinephrine and epinephrine [113], and serotonin [114]. It is noteworthy that serotonin derived melatonin can also act as an efficient free radical scavenger in the eye [115]. Therefore, it can be concluded that by increasing cellular BH4 levels, stilbenes can also contribute to the production of endogenous antioxidants. It has been shown in cell and animal models that resveratrol can increase BH4 synthesis and decrease the oxidation of BH4 [116]. In condition of oxidative stress, cellular BH4 stores are diminished and this disturbs several physiological functions [110]. It has been proposed that BH4 deficiency due to oxidative stress is associated with PD and AD [110] whereas the role of BH4 in AMD is unclear. However, in the retina, BH4 may have an essential role in regulation of retinal neovascularization via L-DOPA, since L-DOPA is capable of controlling levels of vascular endothelial growth factor (VEGF) via secretion of antiangiogenic pigment epithelial derived factor (PEDF) [117, 118].

Stilbene Mediated Activation of the cAMP Pathway Contributes to Autophagy and Defence against Oxidative Stress. It is known that resveratrol can increase cAMP levels in cells and animal models by their ability to inhibit cAMP phosphodiesterase [105]. Phosphodiesterases (PDE) are the enzymes responsible for degradation of cAMP and cGMP [119], second messengers involved in the regulation of numerous genes and cellular functions.

Recent findings have verified that activation of the cAMP signaling targets by stilbenes such as resveratrol can influence important functions in aging cells associated with antioxidant defence. First, cAMP is able to induce Nrf2 expression in cells [120]. Similarly, activation of Nrf2 via cAMP can be induced by α-melanocyte stimulating hormone (α-MSH), a known hormonal activator of G protein-coupled melanocortin receptors and the cAMP pathway [68]. Apparently, activation of Nrf2 transcription by cAMP is mediated via protein kinase A (PKA) and cAMP response element-binding protein (CREB). In the presence of cAMP stimuli, CREB can transactivate Nrf2 as well as Nrf2 target genes such as glutathione S-transferase pi (GSTP1) and NOS [121, 122] by binding on specific cAMP response element (CRE) in promoter region [123]. Second, by inhibiting PDE activity resveratrol is able to increase cellular cAMP and Ca^{2+} levels, finally activating the AMPK pathway involved in the regulation of nutrient homeostasis [105, 124] such as autophagy as described in Section 4.2. The AMPK can increase cellular levels of NAD^+ as well as the activity of SIRT1 [105, 125]. It has been shown that SIRT1 can modulate the activity of metabolic regulators peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and ERRα [102, 126]. However, information regarding the interaction between the AMPK and Nrf2/ARE pathways is contradictory. It was shown recently that known AMPK pathway inducers AICAR and berberine can also induce the Nrf2/ARE pathway [104, 127], whereas in contrast it was shown that deacetylation of Nrf2 by SIRT1 decreases DNA binding activity of Nrf2 causing decreased promoter activity of Nrf2 targets such as HO-1 and NQO1 [67]. This was also verified with resveratrol which was shown to act as a SIRT1 activator.

Polyphenols Are Involved in Regulation of Nuclear Receptors (NRs). The large family of NRs including nuclear hormone receptors and orphan receptors with somewhat unknown ligands is associated with multiple functions in the human body, from development to hormonal regulation and metabolism. In particular, NRs are also involved in the metabolism of xenobiotics [128, 129] and therefore NRs and their targets are under intense investigation in drug industry. Interestingly, current data suggests that several polyphenolic compounds can activate different NRs. This seems reasonable since the Nrf2/ARE pathway and some NRs are activated by phytochemicals and xenobiotics and they are thought to participate with regulation of xenobiotic metabolizing enzymes such as CYP3A4 and NQO1 [69, 130, 131]. In such interactions, polyphenols can act as a ligand for nuclear receptor (resveratrol/ERs) or activation of NRs is mediated via Nrf2 as has been found with retinoid X receptor alpha (RXRa) [72]. For instance, Nrf2 has been shown to bind aryl hydrocarbon receptor (AhR) promoter, and vice versa, AhR element is found from Nrf2 promoter [69]. AhR is involved in cytokine and growth factor signaling and particularly it is a regulator of several xenobiotic metabolizing enzymes activated by exogenous ligands such as dioxins [132]. Furthermore, Nrf2 and AhR have shared target genes such

as NQO1, GSTs, and UDP-glucuronosyltransferases (UTGs) [78, 133]. It should be noted that activation of UTG1A8 and UTG1A10, phase-II enzymes involved in glucuronidation mediated elimination of xenobiotics, requires cooperative induction via both factors AhR and Nrf2 [81]. Moreover, activation of NRs can also be mediated via kinase pathways such as the AMPK-SIRT1 pathway in case of ERRα regulation [102].

Estrogenic Activity of Stilbenes. Interestingly, it seems that some beneficial effects of stilbene derivatives in neuronal and vascular cells are mediated via ERs, typically activated by estrogens (17β-estradiol) [134]. In addition to estrogens, ERs can also bind nonsteroidal compounds such as phytoestrogens [135]. For instance, the ER-specific agonist functions of resveratrol are thought to be attributable to its structural similarities with estrogens; thus resveratrol and some stilbene derivatives can be classified as selective ER modulators (SERMs) [135, 136]. Recently, it has been shown that resveratrol can act through estrogen receptors (ERα and ERβ) to exert neuroprotective activity [137] and it can increase the expression of the dopamine transporter (DAT) in dopaminergic cells [138]. Moreover, it seems that ERs can also mediate the beneficial effects of resveratrol in vascular cells such as vasodilatation by increasing cGMP synthesis, eNOS activity, and NO production [106].

5. Therapeutic Potential of Stilbenes against Oxidative Stress and Age-Related Diseases

5.1. Obesity. Obesity is a major global health problem; for example, it is one of the major risk factors for T2D. The western life style including high energy food intake and inadequate physical activity is the cause of adipocyte dysfunction leading to the storage of extra energy as triglycerides. The release of proinflammatory cytokines from visceral adipose tissue, liver insulin resistance, and inflammation lead to an increasing risk of several metabolic diseases [139]. Prevention of extra energy storage via caloric restriction is a well-documented means to reduce obesity, to increase the life span [140–142], and even to prevent the memory decline [143]. However, the current obesity problem indicates clearly that, although lifestyle changes are effective in practice, they are very difficult to achieve. The molecular mechanism of obesity has not been fully clarified, but an increase in the number and size of adipocytes differentiated from preadipocytes in mature adipocytes seems to be a key pathway in the route towards [144]. It has long been known that resveratrol can mimic some of the impacts of calorie restriction (CR), but stilbene compounds may mediate their antiobesity action also by reducing the synthesis of lipids in adipocytes, modulating of lipolysis, and reducing inflammation and oxidative stress in the target tissue [140]. Although there are abundant data suggesting that stilbene compounds such as resveratrol may increase lifespan through the modulation of insulin signaling even on a high-calorie diet, the practical outcomes of these findings is far from clear [145, 146]. Moreover, comparison of the effectiveness of CR and resveratrol to the HFD-induced obesity and fatty liver formation in C57Bl/6J mice lead to the finding that CR provided superior protection against diet-induced obesity and fatty liver formation compared with resveratrol [147].

Stilbene compounds presumably act on several molecular targets in adipocytes eventually leading to the decreasing levels in adipocyte number and size. With respect to the recently synthesized several stilbene analogues, 3-hydroxy-trans stilbene inhibited adipocyte differentiation and enhanced glucose uptake resulting in a reduction of obesity [148]. The impact of resveratrol on fat cell apoptosis has not been intensively studied, but it has been reported that resveratrol inhibited human preadipocyte proliferation and adipogenic differentiation in a SIRT1-dependent manner and *de novo* lipogenesis was inhibited in parallel with a down-regulation of lipogenic gene expression [149]. SIRT1 may widely regulate fatty acid oxidation in liver, fat mobilization in white adipose tissue, insulin secretion in pancreas, and sense nutrient availability in hypothalamus [150]. However, resveratrol reduced fat cell number also via SIRT1-independent mechanism [151]. Thus, the apoptotic effects of stilbenes in 3T3-L1 preadipocytes may be complex and involve several pathways such as AMPK, AKT, and survivin [152].

Peroxisome proliferator-activated receptor-γ (PPARγ) is an important regulator of lipid and energy metabolism as well as one of key factors in the differentiation of adipocytes. In a microarray analysis it was demonstrated that there were changes identified in 35 genes involved in the PPARγ signaling pathway, lipid metabolism, or adipogenesis in adipocytes treated with grape seed extract (GSE) [153]. Most of these genes involved in PPARγ signaling, Adipoq, Scd1, Nr1h3, Fabp5, Scd2, and Pparg decreased upon GSE treatment, whereas the expression of Ppargc1a was elevated [153, 154]. However, lipid metabolism-associated genes Mlxp1, Stat5a, Hsl, Plin1, and Vdr were downregulated. Thus, GSE containing resveratrol has been claimed to modulate key transcription factors including peroxisome proliferator-activated receptor, CCAAT/enhancer-binding proteins, and their target genes (FAS, aP2, SCD-1, and LPL). It remains to be determined whether a novel regulator of mammalian target of rapamycin complex 1 (mTORC1) plays an important role in the stilbene-mediated adipocyte differentiation of 3T3-L1 preadipocytes and potential prevention of obesity as found for other polyphenols [155].

Lipolysis regulates the key metabolic roles in the formation of adipose tissue size, weight, and obesity and two enzymes, adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), are involved in the lipolytic activity. HSL is active against diglycerines while ATGL selectively act on the first step in the triglycerine hydrolysis resulting in the formation of diglycerines and free fatty acids [156]. Stilbene compounds can modulate lipogenesis in many ways; for example, using knockout mice it was found that resveratrol regulates lipolytic activity in human and murine adipocytes, as well as in white adipose tissue from mice mainly via ATGL at transcriptional and posttranscriptional levels [156]. SIRT1 and FOXO1 (Forkhead box protein O1) involve the regulation of lipolysis so that SIRT1 probably affects the acetylation status and functional activity of FoxO1 so that it may directly bind to the ATGL promoter [157]; thus it apparently regulates ATGL gene transcription. Two other studies [158, 159]

provide support for the important role of SIRTI and FOXO1 in the regulation of transcriptional expression of ATGL in adipocytes. Gene expression patterns of two human tissue samples (subcutaneous abdominal adipose tissue SAT and visceral adipose tissue VAT) derived from nonobese and class III obese subjects were recently analyzed [160]. Interestingly, adiponectin expression was lower only in VAT of obese subjects while FOXO1 and PPARγ levels were decreased in VAT of both groups. However, there was no difference with regard to the SIRT1 levels in VAT or SAT in both groups.

AMPK is an important regulator of energy metabolism and thus it is a key component in obesity regulation. There is an abundance of data indicating that resveratrol can activate AMPK for example [145]. Resveratrol may activate AMPK via inhibition of ATP production but this action seems to be dependent on high doses of resveratrol [161]. Importantly, it was shown that resveratrol increased cAMP levels by competitively inhibiting a number of cAMP phosphodiesterases (PDEs) [105, 161], which degrade cAMP; this suggests that PDE4 inhibitors may be used to develop drugs or special food supplements for therapeutic options for obesity management.

Obesity is known to be related with chronic low-grade inflammation condition leading to the production of a number of inflammatory cytokines, chemokines, and prostaglandins which eventually can lead to the development of insulin resistance. Consequently, targeting specific stilbene compounds to prevent or inhibit the inflammation cascade may be attractive means to reduce obesity and T2D. There is an extensive literature that different stilbenes including pinosylvin, piceatannol, and resveratrol can reduce the development of inflammatory cytokines [9, 162, 163]. Stilbenes and GSEs appear to mediate the attenuation of inflammation and insulin resistance apparently by suppressing the activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) [164, 165], but the anti-inflammatory property may also involve the SIRT1 pathway [166]. In rats, resveratrol may mediate body-fat reduction also via the modulation of thermogenesis as UCP protein was increasingly expressed after resveratrol treatment in the important thermogenic levels [167].

Collectively, *in vitro* and animal studies suggest that stilbenes mediate their antiobesity action via several mechanisms including the inhibition of lipid synthesis in adipocytes, modulation of lipolysis, modulation of apoptosis or mTORC1, and activation of AMPK via inhibition of ATP production as well as reducing inflammation and oxidative stress in the target tissue. The development of specific weight management food products focusing at multiple molecular targets may be a promising avenue for enhancing the antiobesity effect, but this approach may benefit from the combination of distinct polyphenols in the product [168, 169].

5.2. Type 2 Diabetes. Type 2 diabetes mellitus (T2D) is a rapidly and globally increasing complex metabolic disorder associated with elevated insulin resistance, decreased insulin secretion, impaired insulin signaling, hepatic β-cell dysfunction, abnormal glucose and lipid metabolisms, elevated inflammatory burden, and increased oxidative stress. Drugs are widely used to maintain the normal blood glucose level to prevent the development of hyperglycemia which may lead to a number of diabetic complications. It is well documented that diet is one of major risk factors for the development of metabolic disorders leading to T2D, and increasing data suggests that a diet rich in polyphenols and fiber may lower the incidence of T2D by reducing the major predisposing metabolic risk factors.

A considerable amount of *in vitro* and preclinical data implicates that stilbene compounds may lower risk factors behind T2D via several mechanisms. There are recent animal trials suggesting that stilbene compounds, particularly resveratrol, may reduce blood glucose levels in mice, rats, and rodents with hyperglycemia and also modulate insulin levels. In a recent mice trial both low (0.005%) and high levels (0.02%) of resveratrol diet given for six weeks significantly decreased blood glucose, plasma free fatty acid, triglyceride, and apo B/apo AI levels and increased plasma adiponectin levels [170]. Decreased glucose levels were found to be associated with activated levels of AMPK and its downstream targets leading to decreased blood HbA1c levels, hepatic gluconeogenic enzyme activity, and hepatic glycogen. However, only after high dose resveratrol supplementation, there were increases in levels of insulin, pancreatic insulin protein, and skeletal muscle GLUT4 protein. Furthermore, there is a report that, although low dose (30 mg/kg daily for two weeks) treatment could lower fasting glucose level, the resveratrol treatment enhanced insulin action only under insulin-resistant conditions and the treatment efficacy was found to depend on the target tissue and its metabolic stage [171]. In an experiment in T2D model db/db mice another stilbene treatment (piceatannol) was noted to enhance glucose uptake, AMPK phosphorylation, and GLUT4 translocation to the plasma membrane in conditions of insulin absence [172]. Interestingly, they found that piceatannol suppressed the elevations in blood glucose levels in the early stages and improved the impaired glucose tolerance in the later stages in db/db mice. In a rat trial, it was shown that resveratrol treatment inhibited HFD-induced glucose intolerance and insulin resistance in ovariectomized rats [173]. Furthermore, increased insulin-stimulated glucose uptake was demonstrated in isolated soleus muscle *in vivo* and in C2C12 myotubes *in vitro* with mechanism attributed to enhancement of GLUT4 translocation to the plasma membrane rather than increasing GLUT4 protein expression. Interestingly, they were able to show that CAV-3 protein (caveolin family proteins) expression was increased after resveratrol treatment, which contributed to GLUT4 translocation.

α-Glucosidase and α-amylase are digestive enzymes participating in starch and disaccharide degradation. By inhibiting the action of these enzymes with drugs (e.g., acarbose and voglibose) it is possible to slow down glucose absorption from intestine to bloodstream and hence to reduce postprandial hyperglycemia. In addition, many polyphenols are capable of inhibiting α-glucosidase and α-amylase enzyme activity. Numerous vegetable, herbal, fruit, and berry extracts especially those rich in flavonols, ellagitannins, anthocyanins, phenolic acids, and their derivatives

have demonstrated *in vitro* inhibitory activity with respect to both α-glucosidase and α-amylase [174–176]. However, little is known about the impact of stilbenes on these molecular targets. Some stilbenoids and stilbene glycosides (e.g., 4′-*O*-methyl piceid, rhapontin, rhapontigenin, and desoxyrhapontigenin) from rhubarb (*Rheum palmatum* and *Rheum emodi Wall. ex Meissn.*) have been observed to inhibit and modulate α-glucosidase activity [177, 178]. In addition, *trans*-stilbenes resveratrol and rumexoid from the roots of buckwheat *Rumex bucephalophorous* and monomeric and dimeric stilbenoids (e.g., piceatannol, resveratrol, and scirpusin) from the seeds of palm *Syagrus romanzoffiana* have revealed inhibitory activity against α-glucosidase [179, 180]. For example, piceatannol dimers, *trans* double bond, tetrahydrofuran ring, and free adjacent phenolic dihydroxyls may be important features in the inhibitory properties [181]. Based on *in vitro* assay and docking studies, resveratrol-3-*O*-glucosidase from grape skin extracts has been speculated to bind to α-amylase in an inhibitory manner [182]. It has also been postulated that biotransformation, for example, dimerization, of stilbene compounds may be a way to enhance their efficacies as antihyperglycemic agents [181].

Insulin suppresses lipolysis in both transcriptional and posttranscriptional levels in adipose tissue. Apparently, insulin signaling acutely inhibits beta-adrenergic signaling by decreasing intracellular cyclic AMP (cAMP) levels and the rate of lipolysis [183]. Moreover, in the case of insulin resistance and T2D, attenuation of lipolysis by insulin action is impaired leading to an increased rate of lipolysis and enhanced release of free fatty acids (FFA) in the circulation [183]. A very interesting novel protective mechanism of resveratrol against aging-related metabolic degeneration was described by Park et al. [105]. They hypothesized that the metabolic impact of resveratrol results from competitive inhibition of cAMP-degrading phosphodiesterases. Apparently elevated cAMP levels can activate a cAMP effector protein (Epac1), leading to higher concentrations of intracellular Ca^2; eventually this will lead to the increasing uptake of resveratrol and elevated NAD^+ levels and increased activity of SIRT1. It has therefore been postulated that the inhibition of PDE4 activity via bioactive compounds may protect from or ameliorate the symptoms of metabolic diseases associated with aging such as T2D.

There is considerable data highlighting the vital role of oxidative stress as an important risk factor in development of T2D. Activation of antioxidant defence and phase II enzymes is a key mechanism to protect cells from the oxidative damage involved in age-related diseases such as T2D. By using methylglyoxal (MG) as a tool to induce insulin resistance in HepG2 cells, Cheng et al. [80] demonstrated that resveratrol activated ERK pathway but not the p38 or JNK pathways, and this eventually led to Nrf2 nuclear translocation and elevation of HO-1 and glyoxalase expression levels. Furthermore, they found that resveratrol significantly elevated glucose uptake and protected HepG2 cells against MG-induced insulin resistance. Recently, when a 20 mg/kg daily dose of resveratrol was administrated for 12 weeks to db/db mice, improved glucose tolerance, attenuated β-cell loss, and reduced oxidative stress were documented [184]. The protective function of resveratrol against cellular oxidative stress through the SIRT1-FOXO pathway under high-glucose (HG) conditions was recently demonstrated [185]. Under HG conditions *in vitro*, SIRT1 and FOXO3a were significantly decreased compared with normal glucose conditions and this was reversed by resveratrol treatment concomitant with the reduction in HG-induced superoxide production and p47phox. Thus, the data suggests that resveratrol decreases HG-induced superoxide production via upregulation of SIRT1, induction of FOXO3a, and inhibition of p47phox in monocytes. Although a vast number of *in vitro* and animal studies hint at the vital role of oxidative stress in T2D, more clinical data, however, are needed to confirm this hypothesis.

T2D is also an inflammation-related disease: expanded visceral adipose tissue may disturb insulin signaling pathways by excreting inflammatory factors. It has long been known that anti-inflammatory agents may be one therapeutic means to reduce the risk of developing this disease. A wide body of data indicates that stilbene compounds demonstrate anti-inflammatory properties *in vitro*.

Overall, the accumulated data suggest that stilbene-like polyphenols can modulate blood glucose and insulin levels and reduce oxidative stress and inflammation meaning that this represents a rational molecular target for novel target-specific food product development.

Beneficial Role of Stilbenes on Diabetic Vascular Diseases. Diabetes has been shown to associate with the development of cardiovascular diseases (CVD) such as atherosclerosis [186]. In CVD, vascular inflammation, increased platelet aggregation, and decreased levels of vascular nitrogen oxide (NO) production disturb the functions of the vascular endothelium [187]. In vascular endothelium, oxidative stress decreases NO bioavailability and in the presence of superoxide anion (O^{2-}) it increases the formation of peroxynitrite (ONO_2^-), a powerful oxidant [106]. Although NO is a free radical, it is also an important cellular signaling molecule and a major regulator of vascular functions such as vascular tone, platelet aggregation, and vascular proliferation [106, 187]. In the vascular endothelium, NO is synthesized by endothelial nitric oxide synthase enzyme (eNOS) with the assistance of tetrahydrobiopterin (BH4), an essential cofactor of eNOS [116]. Importantly, it has been shown that in CVD and diabetes, decreased NO levels are a result of ROS induced elimination of BH4 stores in vascular endothelium [106, 108, 188].

The cardioprotective functions of stilbene compounds such as piceatannol and resveratrol have been intensively studied in animals [145, 189] and in humans [112, 190–192]. Several molecular targets for stilbenes with cardioprotective activity such as cyclooxygenases (COX-1 and COX-2), eNOS, Nrf2, ERs, and SIRT1 have been proposed [188]. Resveratrol and piceatannol can support endothelial functions such as vasorelaxation by increasing NO production and by reducing ROS via eNOS and NADPH enzymes, respectively [193–196]. The beneficial effects of stilbenes in CVD are also mediated via regulation of cellular BH4 homeostasis. It has been shown that resveratrol decreases BH4 degradation in parallel with the induction of BH4 synthesis via GTP cyclohydrolase 1

(GCH1) [116]. Apparently, the anti-inflammatory functions of stilbenes can also be mediated via the COX enzymes [197] as well as the Toll-like receptor 4 (TLR4) [198].

5.3. Neurodegenerative Diseases of the Aging Eye and Brain. AMD and AD share common features with the neurodegenerative aging diseases, that is, abnormal accumulation of insoluble protein aggregates (lipofuscin, drusen, and AD plaques), perturbation of autophagy clearance system, and increased cellular status of oxidative stress and inflammation [199–203]. Moreover, increased levels of labile cellular iron, a powerful generator of ROS involved in oxidative stress, have been observed in both diseases [204, 205]. It is noteworthy that, although these diseases have similarities, the genetic component of AMD and AD seems to be specific for the disease. In this section, the characteristics of AMD and AD, the beneficial functions of stilbenes, and associated cellular mechanisms are discussed.

5.3.1. Age-Related Macular Degeneration (AMD). Age-related macular degeneration (AMD) is the leading cause of blindness in an aging population affecting the life of 30–50 million individuals [206]. AMD is a multifactorial, progressive degeneration of the central retina with two distinct subforms [207]. The atrophic form (dry AMD) with a prevalence of 85–90% represents a major healthcare burden since no effective cure is available. The wet form of AMD (prevalence 10–15%) with choroidal neovascularization and leaky blood vessels under the macula is more severe and has faster progression. AMD initiates from the RPE, eventually leading to degeneration of photoreceptors.

In AMD patients, retinal changes such as the formation of extracellular deposits (drusens) [208], accumulation of RPE lipofuscin [209], chronic inflammation [210], impaired autophagy [211], and neovascularization [212] are frequently observed. In addition to aging, genetic component, smoking, extensive light exposure, and decreased RPE pigmentation are known to be risk factors for AMD [213, 214]. In particular, the probability of increased chronic oxidative stress triggered by several factors unique for the eye seems to play central role in development of AMD [215]. First, RPE is located in exceptionally oxygen rich environment next to the choroidal vasculature network [215]. Second, due to continuous phagocytosis of photoreceptor outer segments (POS), RPE cells are repeatedly exposed to lipid peroxidation products [216] and to the phototoxic lipofuscin intermediate, bisretinoid A2E [217, 218]. Third, during its lifespan, RPE is exposed to intense stress and photobleaching of RPE melanin caused by sunlight and UV-radiation [214, 219]. In healthy RPE tissue, melanin is a potent scavenger of free radicals, which also inhibits lipid peroxidation, absorbs UV-radiation, and chelates metals such as labile iron [220, 221]. Labile iron is capable of inducing a Fenton reaction in cells, which is a powerful generator of free radicals and oxidative stress [205, 222, 223]. Reduced levels of RPE melanin pigment [219, 224–226] are commonly observed in AMD patients with concurrent increase of cytotoxic levels of labile iron in the RPE [204]. In addition to the devastating general actions of ROS in cells, increased levels of oxidative stress may specifically disturb fundamental functions of the RPE such as POS phagocytosis [227], visual cycle [93], and the integrity of the RPE barrier functions [228].

The reduced antioxidant capacity in the RPE is known to associate with age. For instance, data from mouse models indicates that the Nrf2 system declines with age subjecting RPE cells to oxidative stress [229]. It seems that Nrf2 is involved in the maintenance of retinal functions in general as revealed by current data obtained from Nrf2 knockout mouse model showing that perturbation of the Nrf2/ARE pathway has a remarkable role in development of age-related signs in retina AMD [71, 230]. Nrf2 knockout mouse seems to display all of the typical hallmark retinal changes encountered in AMD such as drusens, lipofuscin, choroidal neovascularization (CNV), and changes in RPE pigmentation. Experiments with Nrf2 deficient mice indicate that Nrf2 is also involved in reducing the chronic inflammation in the eye [231]. After inflammation, induced by lipopolysaccharide (LPS), Nrf2 deficient mice displayed increased levels of inflammation markers (ICAM, IL-6, TNFa, MCP-1, COX-2, and iNOS) in the retina in comparison to their wild-type counterparts.

It is claimed that polyphenolic compounds can exert a protective effect against the stress associated with aging of retinal cells. In particular, the induction of phase II enzymes via the Nrf2 pathway seems to play a key role in this defence system. For instance, pinosylvin was revealed to protect ARPE-19 cells (human RPE cell line) against oxidative stress mediated via the Nrf2 pathway by inducing HO-1 expression [232] and quercetin reduced the levels of inflammation markers IL-6 and IL-1β after oxidative stress induction in ARPE-19 cell line [233]. Similarly, hydroxytyrosol, a phenolic compound present in olive oil and red wine, has been demonstrated to activate Nrf2, HO-1, NQO-1, GCL, GSH, and p62 expression in ARPE-19 cells, and interestingly, GSH production was partially mediated via induced p62 expression [234]. Convincing evidence indicates that accumulation of p62 due to an impairment of the autophagy process is associated with degeneration of RPE cells [90]. Impaired autophagy clearance has been shown to associate with AMD [90, 212]. There is growing data that polyphenols can also modulate autophagy clearance mediated via the cAMP and AMPK pathways (see Section 4.3) [100, 235].

There are interesting results indicating that polyphenols can also influence the secretion of specific growth factors associated with AMD and other retinal diseases such as diabetic retinopathy. In a long-term trial of a small group of elderly AMD patients, daily administration of a polyphenol supplement containing 100 mg resveratrol, quercetin, apigenin, ferulic acid, and so forth exerted a beneficial effect on retinal integrity and anti-VEGF activity as well as an improvement of visual function [236]. In mice, resveratrol has been shown to suppress angiogenesis [237]. Similarly, resveratrol was able to decrease oxysterol induced VEGF secretion in ARPE-19 cells [238]. It is noteworthy that cigarette smoke is a major risk factor in AMD [230]. Cigarette smoke contains abundance free radicals such as hydroquinone (HQ) and these can decrease the levels of antiangiogenic PEDF accompanied by a simultaneous increase in the VEGF levels in RPE cells of smoking AMD patients [239]. This is feasible based on evidence detailing the role of PEDF as an inhibitor of

choroidal neovascularization (CNV) in the retina [117, 240]. A recent study conducted in ARPE-19 cells revealed that 10 μM resveratrol was able to prevent platelet-derived growth factor (PDGF) induced RPE cell proliferation and migration which are common phenomena in AMD, diabetic retinopathy, and proliferative vitreoretinopathy [241]. The beneficial effects of resveratrol have also been demonstrated in animal models where the animals were subjected to different retinal injuries such as retinal detachment [240], retinal ischemic injury [242, 243], light-induced retinal degeneration [244], endoplasmic reticulum stress related vascular degeneration [245], and retinal ganglion cell degeneration associated optic nerve injury [246]. Moreover, the beneficial effects of resveratrol against inflammation have been observed in mouse models. For instance, the activation of SIRT1 and the decreased nuclear localization of NF-κB achieved by resveratrol were associated with reduced oxidative stress and a decrease in inflammation in the mouse retina [247]. A similar type of action was found with the resveratrol analog, piceatannol in the retina. In rodents, this compound has been shown to suppress endotoxin induced ocular inflammation [248] as well as Toll-like receptor 4 (TLR4) mediated inflammatory response and retinal damage occurring after retinal ischemia [249].

Taken together, polyphenols seem to exert numerous beneficial effects in retinal cells and thus they display a potential for the prevention of retinal diseases such as AMD. In addition to their direct antioxidant activity, polyphenols seem to display beneficial effects in the eye through anti-inflammatory activity and activation of autophagy as well as by induction of phase II enzymes via the Nrf2 pathway. Thus one can speculate that polyphenols may support integrity of the retina by controlling the expression and secretion of many of the growth factors such as VEGF, PEDF, and PDGF involved in neovascularization and cell proliferation.

5.3.2. Alzheimer's Disease (AD). Alzheimer's disease (AD) is a devastating neurodegenerative disorder exhibiting synaptic changes and neuronal loss in the hippocampus and cerebral cortex in the central areas of the brain involved in memory and cognition. Accumulation of extracellular plaques of amyloid β (Aβ) peptide and aggregation of microtubule-associated protein tau into insoluble intracellular neurofibrillary tangles are the characteristic hallmarks of AD. About 2.5% of the US population carries two genetic risk genes for ApoE4, the cholesterol-carrying protein, which increases the risk of developing AD by about 10-fold [250]. Despite intensive research and drug development, there is still no effective therapy against AD, and at present preventive approaches are thought to be the best way to address this growing public health problem. There are epidemiological studies indicating that the consumption of phenolic-containing berries, fruit, and vegetables can lower the risk of AD [3, 251]. For example, individuals drinking three or more glasses of fruit or vegetable juice per week have been shown to lower by over 50% their risk of AD in comparison to individuals who consumed less than one serving per week [251]. The protective activity conferred by the bioactive polyphenols in the juice is likely to be a result of multiple-target properties such as impaired insulin and insulin-like growth factor (IGF) signaling, Aβ and tau-protein accumulation, synaptic disconnection, and impaired energy in addition to limiting damage due to of oxidative stress and inflammation.

Since polyphenols have difficulties passing through the blood-brain barrier following oral administration, the resulting low concentration of polyphenols in the brain tissue has been thought as limiting their use against Alzheimer's disease, but it was recently shown that resveratrol, and particularly resveratrol metabolites, can reach such concentrations in the brain capable of achieving beneficial physiological changes [7, 47, 252]. For example, a higher concentration of resveratrol was achieved in the rat brain tissue when the compound was dispensed in lipid-core nanocapsules [47]. Importantly, the enhanced penetration of resveratrol into the brain was found to protect tissue from the deleterious effect of Aβ1-42 and the subsequent impairment of memory functions more effectively than resveratrol treatment without lipid-core nanocapsulation. Furthermore, the polyphenol metabolite, quercetin-3-*O*-glucuronide, was found to significantly reduce the generation of Aβ peptides by primary neuron cultures obtained from the Tg2576 AD mouse model [252]. Interestingly, quercetin-3-*O*-glucuronide was also capable of interfering with the initial protein-protein interaction of Aβ (1–40) and Aβ (1–42) necessary for the formation of the neurotoxic oligomeric Aβ species [252]. These are important findings since it is known that Aβ, which is released after sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases, is a key participant in AD pathogenesis [253]. Tau protein is known to be abnormally hyperphosphorylated in AD and aberrant tau phosphorylation contributes to the neuropathology of AD. Administration of polyphenol-rich GSE has been shown to interfere with the assembly of tau peptides into neurotoxic aggregates suggesting that polyphenols such as stilbenes can directly modulate the aggregation process of tau [254]. Interestingly, feeding mice for four months with a protein restriction (nonessential amino acid) based diet achieved a cognitive improvement and reduced pathological changes, associated with altered tau phosphorylation and disturbed levels of IGF-1 [255]. This observation suggests that provision of polyphenols and a protein restriction diet may mediate their neuroprotective action via common molecular mechanisms. There are recent findings indicating that only the apolipoprotein ApoE4 allele significantly decreases the ratio of soluble amyloid precursor protein alpha (sAPPα) to Aβ and is able to reduce SIRT1 expression resulting in markedly differing ratios of the levels of neuroprotective SirT1 to the neurotoxic SIRT2 as well as also triggering Tau and APP phosphorylation [250]. Stimulation of innate immunity via the Toll-like receptors such as TLR9 has been reported to effectively reduce the amyloid burden [256], but it remains to be determined whether stilbene compounds have sufficient efficacy to activate innate immunity.

Brain-derived neurotrophic factor (BDNF) plays a key role in brain cell development, growth, and survival since this growth factor promotes synaptic plasticity in the hippocampus. For example, BDNF mediates neuroprotective and cognitive function via inhibiting food intake and increasing energy expenditure in the hypothalamus [257]. There is some

data indicating that stilbenes may directly modulate brain synaptic plasticity. Treatment of rats for 3, 10, and 30 days with resveratrol significantly and dose-dependently elevated the levels of BDNF mRNA expression in hippocampal tissue suggesting that the resveratrol mediated neuroprotective impact may be related with activation of the BDNF pathway [258]. Recently, grape powder extract was found to prevent oxidative stress-induced anxiety, memory impairment, and hypertension in rats by regulating also brain CREB and BDNF levels [259]. Furthermore, blueberry-fed animals exhibited a faster rate of learning and better spatial memory performance as compared to those on the control diet providing further evidence that polyphenols may mediate their neuroprotective action via BDNF [260]. Importantly, it was also observed that the improved behavioral performance was associated with increases in total CREB and elevated levels of pro- and mature BDNF in the hippocampus. Recent important findings have indicated that the monomeric proanthocyanidin metabolites seem to be the key PAC metabolite that is able to attain concentrations of ~400 nM in brain and improve cognitive function [7]. They further revealed that one of the epicatechin metabolites, 3′-*O*-methyl-epicatechin-5-*O*-β-glucuronide, could enhance synaptic plasticity through mechanisms associated with CREB signaling.

Autophagy, the lysosomal mediated degradative pathway for proteins and organelles, may be essential for the survival of mature neurons, but the underlying mechanisms remain to be elucidated. However, Marambaud and coworkers reported that resveratrol could not inhibit Aβ production, since it had no apparent effect on the Aβ-producing enzymes beta- and gamma-secretases, but instead it promoted intracellular degradation of Aβ via a mechanism involving the proteasome [261]. Subsequently considerable data have accumulated that autophagy is involved in the resveratrol mediated protection against oxidative stress, inflammation, and associated cardiovascular, cancer, and neurological diseases [262]. Resveratrol-mediated autophagy mechanisms may be dose-dependent [263], but their importance in neuroprotection, however, still remains to be clarified.

There is a growing body of experimental data strongly supporting the hypothesis that CR is a significant way to extend longevity and delay many age-related diseases review by [143, 264, 265]. The protective action of CR is potentially mediated via a reduction of inflammation and oxidative stress, but recent data have highlighted the possibility that CR may modulate also critical signaling pathways such as IGF-1/insulin signaling, sirtuin, AMPK, and mTOR pathways [255]. These metabolic pathways are considered as key risk factors affecting brain health and AD. However, one recent report suggested that part of the beneficial metabolic impacts may not simply be due to general CR, but also the food ingredients in the CR diet may play a role since those individuals consuming a CR diet had high intakes of vegetables, berries, and fruits which contain substantial amounts of bioactive compounds [265]. Stilbenes such as resveratrol are considered to exhibit to some degree of CR mimetic properties via the action on sirtuin. By using SAMP8 (Senescence-accelerated mouse mice), it was revealed that long-term resveratrol treatment could reduce the cognitive impairment by reducing the amyloid burden and tau hyperphosphorylation [266]. It was further demonstrated that resveratrol activated AMPK pathways and prosurvival routes such as SIRT1 *in vivo*. However, in the same SAMP8 mice model, resveratrol and another stilbene, pterostilbene, did not increase SIRT1 expression although markers of cellular stress and inflammation and reduced AD pathology were positively modulated by pterostilbene but not resveratrol [8]; hence, pterostilbene's higher bioavailability (i.e., better than resveratrol) may have important protective implications.

There is emerging evidence suggesting that AD is fundamentally a metabolic disease and brain glucose utilization and responsiveness to IGF stimulation may play key roles behind neuronal loss, loss of synaptic connections, tau hyperphosphorylation, and Aβ accumulation [267]. Thus, suppression of energy expenditure by modulation AMPK, glucose transport, and the insulin pathway via stilbene-like polyphenols may represent a promising avenue to delay the onset of AD and slow disease development. Importantly, resveratrol was able to activate AMPK in neuronal cells *in vitro* as well as in the brain and it enhanced activation of mitochondrial biogenesis in an AMPK-dependent manner [268]. There are several animal experiments indicating that supplementation with stilbenes (resveratrol and piceatannol) can enhance glucose uptake, AMPK phosphorylation, and GLUT4 translocation (see diabetes section), but very few human trials have been performed. Interestingly, when mice (C57BL/6 Jl) were fed with HFD (high-fat diet) supplemented with resveratrol for 20 weeks, there were signs of reduced insulin resistance, lower levels of tumor necrosis factor-α, and Iba-1 in hippocampus as well as improvements in the normal memory deficits in HFD-fed mice [269].

Taken together, there is an impressive body of *in vitro* and animal data to suggest that stilbenes mediate their neuroprotective action via several mechanisms, that is, through the modulation of generation of Aβ and tau peptides, modulation of brain synaptic plasticity via BDNF, modulation of brain energy expenditure via AMPK, glucose transport, and insulin pathway, and reducing inflammation and oxidative stress burden. Experiments in mice models of ADs have indicated that polyphenol based diets can alleviate the spatial working memory deficit and some other cognitive traits. These studies provide new ideas for the development of novel target-specific medicinal foods and dietary supplements for the ever increasing elderly population.

6. Lessons from Preclinical and Clinical Trials

There is convincing epidemiological data emphasizing that a diet rich in vegetables and fruits confers many health benefits. There is also increasing preclinical data obtained in various animal models indicating that polyphenols such as stilbenes can target critical sites involved in the disease process and ultimately alleviate disease outcomes. However, very few well-planned clinical trials are available which would have provided solid evidence for the health benefits of stilbenes for humans.

Recently, a very interesting long-term trial determining the impact of resveratrol supplementation on adipose tissue,

insulin signaling, and inflammatory response was reported using rhesus monkeys as the model [270]. Two-year supplementation with 80 and 480 mg/day for the first and second year, respectively, was found to decrease adipocyte size, increase SIRT1 expression, decrease NF-κB activation, and improve insulin sensitivity in visceral, but not subcutaneous WAT (white adipose tissue) in the HFS-fed animals. Furthermore, the impact of resveratrol supplementation on obesity has been evaluated in a few clinical trials. For example, 11 healthy obese men were supplemented with 150 mg/day resveratrol in a randomized double-blind crossover study for 30 days [271]. Resveratrol treatment activated AMPK, increased SIRT1 and PGC-1α protein levels, improved muscle mitochondrial respiration on a fatty acid-derived substrate, elevated intramyocellular lipid levels and decreased intrahepatic lipid content, reduced levels of circulating glucose, triglycerides, alanine-aminotransferase, and decreased the levels of inflammation markers. Furthermore, declines were observed in the systolic blood pressure, adipose tissue lipolysis, and plasma fatty acid in the postprandial stage. Interestingly, resveratrol supplementation for 30 days in obese men modulated postprandial glucagon responses [105]; it is known that glucagon and related hormones may stimulate adenylate cyclases (AC), resulting in increased cAMP production [161].

In a long-term human trial, molecular changes in peripheral blood mononuclear cells (PBMCs) associated with the one-year daily intake of a resveratrol grape extract (GE) and GE enriched 8 mg of resveratrol (GE-RES) in hypertensive male patients was studied [272]. This data revealed that GE or GE-RES did not affect body weight, blood pressure, glucose, HbA1c, or lipids, but a significant reduction in the levels of ALP (alkaline phosphatase) and IL-6 was recorded. The expression of the proinflammatory cytokines CCL3, IL-1β, and TNF-α was significantly reduced and that of the transcriptional repressor LRRFIP-1 increased in PBMCs from patients consuming the GE-RES extract. Furthermore, a group of miRs (microRNA expression) involved in the regulation of the inflammatory response, that is, miR-21, miR-181b, miR-663, miR-30c2, miR-155, and miR-34a, were found to be highly correlated and altered in the group consuming the GE-RES for 12 months. Thus this human trial provided the first indication that long-term supplementation with a GE-RES could downregulate the expression of key proinflammatory cytokines suggesting that this polyphenol treatment may have a true beneficial immunomodulatory impact in humans suffering from hypertension [272]. A recent meta-analysis based on eleven human clinical trials (involving a total of 388 subjects) indicated that resveratrol supplementation could significantly reduce fasting glucose, insulin, hemoglobin A_{1c}, and insulin resistance levels in participants with diabetes, but no significant impact on glycemic parameters was recorded in nondiabetic participants [4].

Epidemiological studies have indicated that greater intakes of fruit and vegetable juices [251] as well as higher intakes of blueberries or its component anthocyanidins [3] were associated with slower rates of cognitive decline. Consumption of the Mediterranean-type diet, which consists of foods such as vegetables, nuts, and fish and combines micro- and macronutrients with substantial amounts of bioactive

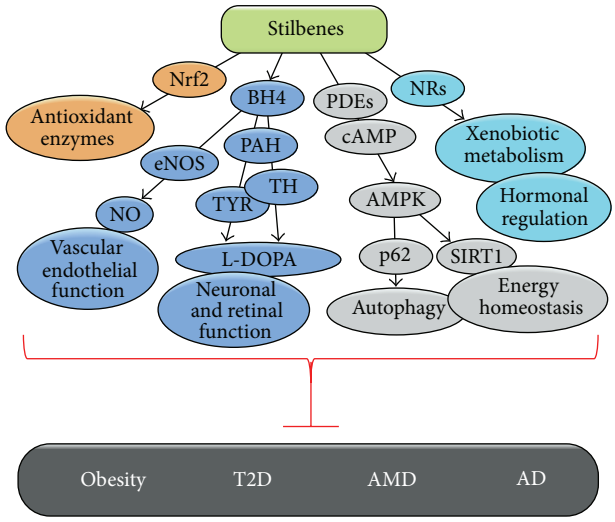


Figure 3: Schematic diagram showing the potential beneficial actions of stilbenes in prevention of age-related diseases.

polyphenols, has been also associated with decreased cognitive decline [273]. New prospective studies performed with the Mediterranean diet also provide evidence that not only can this diet slow down the progression of AD but it also reduces the risk of CVDs and other metabolic disorders [274]. However, the therapeutic value of stilbene compounds in the prevention of progression of AD remains to be demonstrated in long-term clinical trials with different stilbenes and variable doses. There are animal trials suggesting that different stilbene compounds together may provide enhanced protection above that obtained with the single compounds [275] perhaps through affecting different molecular targets. Thus, long-term human trials with different combinations of stilbenes may be a promising avenue for revealing the potential therapeutic value of stilbenes in AD management. Souvenaid, a currently marketed medicinal food product, has achieved some improvements in cognitive function in patients with AD in small human trials [276].

7. Conclusions

There is emerging *in vitro* and preclinical data to indicate that stilbene compounds are capable of suppressing oxidative stress, inflammation, and energy expenditure as well as modulating the secretion of neurotropic factors. However, most of the available experimental data has concentrated on resveratrol and only limited research has been carried out with the other stilbene compounds. These other stilbenes such as pinosylvin, piceatannol, and pterostilbene may have higher biological activity than resveratrol and deserve to receive more scientific attention. The limited bioavailability, the low target specificity, and the rapid metabolism of stilbenes represent obstacles to achieving high enough concentrations of these compounds in plasma and the target tissue in order that they can exert their beneficial actions. However, recent investigations into the biological activity of polyphenol metabolites have been promising. Unlike drugs, stilbene-like polyphenols affect several key metabolic pathways (Figure 3),

and promising therapeutic approaches may require focusing on multiple targets. This may lead to the development of next-generation functional type foods and supplements for slowing down the increasingly common diseases such as obesity, T2D, AMD, and AD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] L. Müller, T. Fülöp, and G. Pawelec, "Immunosenescence in vertebrates and invertebrates," *Immunity & Ageing*, vol. 10, no. 1, article 12, 2013.

[2] R. Dai, H. Ge, S. Howard, and W. Qiu, "Transcriptional expression of Stilbene synthase genes are regulated developmentally and differentially in response to powdery mildew in Norton and Cabernet Sauvignon grapevine," *Plant Science*, vol. 197, pp. 70–76, 2012.

[3] E. E. Devore, J. H. Kang, M. M. B. Breteler, and F. Grodstein, "Dietary intakes of berries and flavonoids in relation to cognitive decline," *Annals of Neurology*, vol. 72, no. 1, pp. 135–143, 2012.

[4] K. Liu, R. Zhou, B. Wang, and M.-T. Mi, "Effect of resveratrol on glucose control and insulin sensitivity: a meta-analysis of 11 randomized controlled trials," *The American Journal of Clinical Nutrition*, vol. 99, no. 6, pp. 1510–1519, 2014.

[5] M. Takikawa, S. Inoue, F. Horio, and T. Tsuda, "Dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity via activation of amp-activated protein kinase in diabetic mice," *The Journal of Nutrition*, vol. 140, no. 3, pp. 527–533, 2010.

[6] S. Vepsäläinen, H. Koivisto, E. Pekkarinen et al., "Anthocyanin-enriched bilberry and blackcurrant extracts modulate amyloid precursor protein processing and alleviate behavioral abnormalities in the APP/PS1 mouse model of Alzheimer's disease," *The Journal of Nutritional Biochemistry*, vol. 24, no. 1, pp. 360–370, 2013.

[7] J. Wang, M. G. Ferruzzi, L. Ho et al., "Brain-targeted proanthocyanidin metabolites for Alzheimer's disease treatment," *The Journal of Neuroscience*, vol. 32, no. 15, pp. 5144–5150, 2012.

[8] J. Chang, A. Rimando, M. Pallas et al., "Low-dose pterostilbene, but not resveratrol, is a potent neuromodulator in aging and Alzheimer's disease," *Neurobiology of Aging*, vol. 33, no. 9, pp. 2062–2071, 2012.

[9] E. Jeong, H.-R. Lee, J. Pyee, and H. Park, "Pinosylvin induces cell survival, migration and anti-adhesiveness of endothelial cells via nitric oxide production," *Phytotherapy Research*, vol. 27, no. 4, pp. 610–617, 2013.

[10] T. Mačičková, K. Drábiková, R. Nosál' et al., "In vivo effect of pinosylvin and pterostilbene in the animal model of adjuvant arthritis," *Neuroendocrinology Letters*, vol. 31, no. 2, pp. 91–95, 2010.

[11] K. Bauerova, A. Acquaviva, S. Ponist et al., "Markers of inflammation and oxidative stress studied in adjuvant-induced arthritis in the rat on systemic and local level affected by pinosylvin and methotrexate and their combination," *Autoimmunity*, vol. 48, no. 1, pp. 46–56, 2015.

[12] J. Chong, A. Poutaraud, and P. Hugueney, "Metabolism and roles of stilbenes in plants," *Plant Science*, vol. 177, no. 3, pp. 143–155, 2009.

[13] C. Parage, R. Tavares, S. Réty et al., "Structural, functional, and evolutionary analysis of the unusually large stilbene synthase gene family in grapevine," *Plant Physiology*, vol. 160, no. 3, pp. 1407–1419, 2012.

[14] C. Rivière, A. D. Pawlus, and J.-M. Mérillon, "Natural stilbenoids: distribution in the plant kingdom and chemotaxonomic interest in Vitaceae," *Natural Product Reports*, vol. 29, no. 11, pp. 1317–1333, 2012.

[15] A. R. Fontana, A. Antoniolli, and R. Bottini, "Grape pomace as a sustainable source of bioactive compounds: extraction, characterization, and biotechnological applications of phenolics," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 38, pp. 8987–9003, 2013.

[16] S. P. Pietarinen, S. M. Willför, M. O. Ahotupa, J. E. Hemming, and B. R. Holmbom, "Knotwood and bark extracts: strong antioxidants from waste materials," *Journal of Wood Science*, vol. 52, no. 5, pp. 436–444, 2006.

[17] M.-E. García-Pérez, M. Royer, G. Herbette, Y. Desjardins, R. Pouliot, and T. Stevanovic, "Picea mariana bark: a new source of trans-resveratrol and other bioactive polyphenols," *Food Chemistry*, vol. 135, no. 3, pp. 1173–1182, 2012.

[18] A. M. Rimando, Z. Pan, J. J. Polashock et al., "In planta production of the highly potent resveratrol analogue pterostilbene via stilbene synthase and O-methyltransferase co-expression," *Plant Biotechnology Journal*, vol. 10, no. 3, pp. 269–283, 2012.

[19] P. Jeandet, B. Delaunois, A. Conreux et al., "Biosynthesis, metabolism, molecular engineering, and biological functions of stilbene phytoalexins in plants," *Biofactors*, vol. 36, no. 5, pp. 331–341, 2010.

[20] R. Preisig-Müller, A. Schwekendiek, I. Brehm, H.-J. Reif, and H. Kindl, "Characterization of a pine multigene family containing elicitor-responsive stilbene synthase genes," *Plant Molecular Biology*, vol. 39, no. 2, pp. 221–229, 1999.

[21] W. J. Hurst, J. A. Glinski, K. B. Miller, J. Apgar, M. H. Davey, and D. A. Stuart, "Survey of the trans-resveratrol and trans-piceid content of cocoa-containing and chocolate products," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 18, pp. 8374–8378, 2008.

[22] R. Flamini, M. de Rosso, F. de Marchi et al., "An innovative approach to grape metabolomics: stilbene profiling by suspect screening analysis," *Metabolomics*, vol. 9, no. 6, pp. 1243–1253, 2013.

[23] V. Jerkovic, D. Callemien, and S. Collin, "Determination of stilbenes in hop pellets from different cultivars," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 10, pp. 4202–4206, 2005.

[24] Ö. Tokuşoğlu, M. K. Ünal, and F. Yemiş, "Determination of the phytoalexin resveratrol (3,5,4′-Trihydroxystilbene) in peanuts and pistachios by High-Performance Liquid Chromatographic Diode Array (HPLC-DAD) and Gas Chromatography-Mass Spectrometry (GC-MS)," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 12, pp. 5003–5009, 2005.

[25] H. Latva-Mäenpää, T. Laakso, T. Sarjala, K. Wähälä, and P. Saranpää, "Variation of stilbene glucosides in bark extracts obtained from roots and stumps of Norway spruce (*Picea abies* L. Karst.)," *Trees: Structure and Function*, vol. 27, no. 1, pp. 131–139, 2013.

[26] N. Krafczyk, M. Kötke, N. Lehnert, and M. A. Glomb, "Phenolic composition of rhubarb," *European Food Research and Technology*, vol. 228, no. 2, pp. 187–196, 2008.

[27] S. Y. Wang, C.-T. Chen, C. Y. Wang, and P. Chen, "Resveratrol content in strawberry fruit is affected by preharvest conditions," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 20, pp. 8269–8274, 2007.

[28] S. M. Boue, B. Y. Shih, M. E. Burow et al., "Postharvest accumulation of resveratrol and piceatannol in sugarcane with enhanced antioxidant activity," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 35, pp. 8412–8419, 2013.

[29] A. S. Ragab, J. van Fleet, B. Jankowski, J.-H. Park, and S. C. Bobzin, "Detection and quantitation of resveratrol in tomato fruit (*Lycopersicon esculentum* Mill.)," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 19, pp. 7175–7179, 2006.

[30] A. M. Rimando, W. Kalt, J. B. Magee, J. Dewey, and J. R. Ballington, "Resveratrol, pterostilbene, and piceatannol in *Vaccinium* berries," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 15, pp. 4713–4719, 2004.

[31] P. Rodríguez-Bonilla, J. M. López-Nicolás, L. Méndez-Cazorla, and F. García-Carmona, "Development of a reversed phase high performance liquid chromatography method based on the use of cyclodextrins as mobile phase additives to determine pterostilbene in blueberries," *Journal of Chromatography B*, vol. 879, no. 15-16, pp. 1091–1097, 2011.

[32] R. Moss, Q. Mao, D. Taylor, and C. Saucier, "Investigation of monomeric and oligomeric wine stilbenoids in red wines by ultra-high-performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry," *Rapid Communications in Mass Spectrometry*, vol. 27, no. 16, pp. 1815–1827, 2013.

[33] N. Landrault, F. Larronde, J.-C. Delaunay et al., "Levels of stilbene oligomers and astilbin in French varietal wines and in grapes during noble rot development," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 7, pp. 2046–2052, 2002.

[34] S. Doré, "Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity," *NeuroSignals*, vol. 14, no. 1-2, pp. 61–70, 2005.

[35] T. Walle, "Bioavailability of resveratrol," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 9–15, 2011.

[36] Y.-Y. Zhao, Q. Su, X.-L. Cheng, X.-J. Tan, X. Bai, and R.-C. Lin, "Pharmacokinetics, bioavailability and metabolism of rhaponticin in rat plasma by UHPLC-Q-TOF/MS and UHPLC-DAD-MSn," *Bioanalysis*, vol. 4, no. 6, pp. 713–723, 2012.

[37] I. M. Kapetanovic, M. Muzzio, Z. Huang, T. N. Thompson, and D. L. McCormick, "Pharmacokinetics, oral bioavailability, and metabolic profile of resveratrol and its dimethylether analog, pterostilbene, in rats," *Cancer Chemotherapy and Pharmacology*, vol. 68, no. 3, pp. 593–601, 2011.

[38] H.-S. Lin, B.-D. Yue, and P. C. Ho, "Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study," *Biomedical Chromatography*, vol. 23, no. 12, pp. 1308–1315, 2009.

[39] D. J. Boocock, K. R. Patel, G. E. S. Faust et al., "Quantitation of trans-resveratrol and detection of its metabolites in human plasma and urine by high performance liquid chromatography," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 848, no. 2, pp. 182–187, 2007.

[40] V. A. Brown, K. R. Patel, M. Viskaduraki et al., "Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics, and effect on the insulin-like growth factor axis," *Cancer Research*, vol. 70, no. 22, pp. 9003–9011, 2010.

[41] K. R. Patel, V. A. Brown, D. J. L. Jones et al., "Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients," *Cancer Research*, vol. 70, no. 19, pp. 7392–7399, 2010.

[42] A. Amri, J. C. Chaumeil, S. Sfar, and C. Charrueau, "Administration of resveratrol: what formulation solutions to bioavailability limitations?" *Journal of Controlled Release*, vol. 158, no. 2, pp. 182–193, 2012.

[43] T. Ogas, T. P. Kondratyuk, and J. M. Pezzuto, "Resveratrol analogs: promising chemopreventive agents," *Annals of the New York Academy of Sciences*, vol. 1290, no. 1, pp. 21–29, 2013.

[44] M. J. Amiot, B. Romier, T.-M. Anh Dao et al., "Optimization of trans-resveratrol bioavailability for human therapy," *Biochimie*, vol. 95, no. 6, pp. 1233–1238, 2013.

[45] Y. Sun and Y. Zhao, "Enhanced pharmacokinetics and antitumor efficacy of PEGylated liposomal rhaponticin and plasma protein binding ability of rhaponticin," *Journal of Nanoscience and Nanotechnology*, vol. 12, no. 10, pp. 7677–7684, 2012.

[46] J. J. Johnson, M. Nihal, I. A. Siddiqui et al., "Enhancing the bioavailability of resveratrol by combining it with piperine," *Molecular Nutrition & Food Research*, vol. 55, no. 8, pp. 1169–1176, 2011.

[47] R. L. Frozza, A. Bernardi, J. B. Hoppe et al., "Neuroprotective effects of resveratrol against Aβ administration in rats are improved by lipid-core nanocapsules," *Molecular Neurobiology*, vol. 47, no. 3, pp. 1066–1080, 2013.

[48] C.-H. Cottart, V. Nivet-Antoine, C. Laguillier-Morizot, and J.-L. Beaudeux, "Resveratrol bioavailability and toxicity in humans," *Molecular Nutrition & Food Research*, vol. 54, no. 1, pp. 7–16, 2010.

[49] L. Almeida, M. Vaz-da-Silva, A. Falcão et al., "Pharmacokinetic and safety profile of trans-resveratrol in a rising multiple-dose study in healthy volunteers," *Molecular Nutrition & Food Research*, vol. 53, no. 1, pp. S7–S15, 2009.

[50] C. La Porte, N. Voduc, G. Zhang et al., "Steady-state pharmacokinetics and tolerability of trans-resveratrol 2000mg twice daily with food, quercetin and alcohol (Ethanol) in healthy human subjects," *Clinical Pharmacokinetics*, vol. 49, no. 7, pp. 449–454, 2010.

[51] M. Ohtsuji, F. Katsuoka, A. Kobayashi, H. Aburatani, J. D. Hayes, and M. Yamamoto, "Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes," *Journal of Biological Chemistry*, vol. 283, no. 48, pp. 33554–33562, 2008.

[52] Y. Hirotsu, F. Katsuoka, R. Funayama et al., "Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks," *Nucleic Acids Research*, vol. 40, no. 20, pp. 10228–10239, 2012.

[53] M. Biswas and J. Y. Chan, "Role of Nrf1 in antioxidant response element-mediated gene expression and beyond," *Toxicology and Applied Pharmacology*, vol. 244, no. 1, pp. 16–20, 2010.

[54] N. Wakabayashi, K. Itoh, J. Wakabayashi et al., "*Keap1*-null mutation leads to postnatal lethality due to constitutive Nrf2 activation," *Nature Genetics*, vol. 35, no. 3, pp. 238–245, 2003.

[55] M. Kobayashi and M. Yamamoto, "Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species," *Advances in Enzyme Regulation*, vol. 46, no. 1, pp. 113–140, 2006.

[56] T. Nguyen, P. Nioi, and C. B. Pickett, "The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress," *The Journal of Biological Chemistry*, vol. 284, no. 20, pp. 13291–13295, 2009.

[57] M. Zhang, C. An, Y. Gao, R. K. Leak, J. Chen, and F. Zhang, "Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection," *Progress in Neurobiology*, vol. 100, no. 1, pp. 30–47, 2013.

[58] R. Gozzelino, V. Jeney, and M. P. Soares, "Mechanisms of cell protection by heme oxygenase-1," *Annual Review of Pharmacology and Toxicology*, vol. 50, pp. 323–354, 2010.

[59] A. T. Dinkova-Kostova and P. Talalay, "NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 116–123, 2010.

[60] E. Kansanen, H.-K. Jyrkkänen, and A.-L. Levonen, "Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids," *Free Radical Biology and Medicine*, vol. 52, no. 6, pp. 973–982, 2012.

[61] S. B. Cullinan, J. D. Gordan, J. Jin, J. W. Harper, and J. A. Diehl, "The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase," *Molecular and Cellular Biology*, vol. 24, no. 19, pp. 8477–8486, 2004.

[62] E. Kansanen, S. M. Kuosmanen, H. Leinonen, and A.-L. Levonenn, "The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer," *Redox Biology*, vol. 1, no. 1, pp. 45–49, 2013.

[63] J. F. Reichard, G. T. Motz, and A. Puga, "Heme oxygenase-1 induction by NRF2 requires inactivation of the transcriptional repressor BACH1," *Nucleic Acids Research*, vol. 35, no. 21, pp. 7074–7086, 2007.

[64] Z. Sun, T. Wu, F. Zhao, A. Lau, C. M. Birch, and D. D. Zhang, "KPNA6 (Importin α7)-mediated nuclear import of Keap1 represses the Nrf2-dependent antioxidant response," *Molecular and Cellular Biology*, vol. 31, no. 9, pp. 1800–1811, 2011.

[65] M.-K. Kwak, K. Itoh, M. Yamamoto, and T. W. Kensler, "Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter," *Molecular and Cellular Biology*, vol. 22, no. 9, pp. 2883–2892, 2002.

[66] A. Jain, T. Lamark, E. Sjøttem et al., "p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription," *The Journal of Biological Chemistry*, vol. 285, no. 29, pp. 22576–22591, 2010.

[67] Y. Kawai, L. Garduño, M. Theodore, J. Yang, and I. J. Arinze, "Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization," *The Journal of Biological Chemistry*, vol. 286, no. 9, pp. 7629–7640, 2011.

[68] A. Kokot, D. Metze, N. Mouchet et al., "Alpha-melanocyte-stimulating hormone counteracts the suppressive effect of UVB on Nrf2 and Nrf-dependent gene expression in human skin," *Endocrinology*, vol. 150, no. 7, pp. 3197–3206, 2009.

[69] N. Wakabayashi, S. Shin, S. L. Slocum et al., "Regulation of Notch1 signaling by Nrf2: implications for tissue regeneration," *Science Signaling*, vol. 3, no. 130, p. ra52, 2010.

[70] W. O. Osburn, M. S. Yates, P. D. Dolan et al., "Genetic or pharmacologic amplification of Nrf2 signaling inhibits acute inflammatory liver injury in mice," *Toxicological Sciences*, vol. 104, no. 1, pp. 218–227, 2008.

[71] Z. Zhao, Y. Chen, J. Wang et al., "Age-related retinopathy in NRF2-deficient mice," *PLoS ONE*, vol. 6, no. 4, Article ID e19456, 2011.

[72] B. N. Chorley, M. R. Campbell, X. Wang et al., "Identification of novel NRF2-regulated genes by ChiP-Seq: influence on retinoid X receptor alpha," *Nucleic Acids Research*, vol. 40, no. 15, pp. 7416–7429, 2012.

[73] J. M. Marzec, J. D. Christie, S. P. Reddy et al., "Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury," *FASEB Journal*, vol. 21, no. 9, pp. 2237–2246, 2007.

[74] S. Shin, N. Wakabayashi, V. Misra et al., "NRF2 modulates aryl hydrocarbon receptor signaling: influence on adipogenesis," *Molecular and Cellular Biology*, vol. 27, no. 20, pp. 7188–7197, 2007.

[75] K. L. Cheung, S. Yu, Z. Pan, J. Ma, T. Y. Wu, and A.-N. T. Kong, "TBHQ-induced HO-1 expression is mediated by calcium through regulation of Nrf2 binding to enhancer and polymerase II to promoter region of HO-1," *Chemical Research in Toxicology*, vol. 24, no. 5, pp. 670–676, 2011.

[76] T. Nishinaka, Y. Ichijo, M. Ito et al., "Curcumin activates human glutathione S-transferase P1 expression through antioxidant response element," *Toxicology Letters*, vol. 170, no. 3, pp. 238–247, 2007.

[77] P. Nioi, M. McMahon, K. Itoh, M. Yamamoto, and J. D. Hayes, "Identification of a novel NRF2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence," *The Biochemical Journal*, vol. 374, no. 2, pp. 337–348, 2003.

[78] P. Nioi and J. D. Hayes, "Contribution of NAD(P)H: quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors," *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 555, no. 1-2, pp. 149–171, 2004.

[79] T.-C. Hsieh, X. Lu, Z. Wang, and J. M. Wu, "Induction of quinone reductase NQO1 by resveratrol in human K562 cells involves the antioxidant response element ARE and is accompanied by nuclear translocation of transcription factor Nrf2," *Medicinal Chemistry*, vol. 2, no. 3, pp. 275–285, 2006.

[80] Q. Cheng, J. L. Kalabus, J. Zhang, and J. G. Blanco, "A conserved antioxidant response element (ARE) in the promoter of human carbonyl reductase 3 (CBR3) mediates induction by the master redox switch Nrf2," *Biochemical Pharmacology*, vol. 83, no. 1, pp. 139–148, 2012.

[81] S. Kalthoff, U. Ehmer, N. Freiberg, M. P. Manns, and C. P. Strassburg, "Interaction between oxidative stress sensor Nrf2 and xenobiotic-activated aryl hydrocarbon receptor in the regulation of the human phase II detoxifying UDP-glucuronosyltransferase 1A10," *The Journal of Biological Chemistry*, vol. 285, no. 9, pp. 5993–6002, 2010.

[82] A. C. Wild, H. R. Moinova, and R. T. Mulcahy, "Regulation of γ-glutamylcysteine synthetase subunit gene expression by the

transcription factor Nrf2," *The Journal of Biological Chemistry*, vol. 274, no. 47, pp. 33627–33636, 1999.

[83] J.-Y. Im, K.-W. Lee, J.-M. Woo, E. Junn, and M. M. Mouradian, "Dj-1 induces thioredoxin 1 expression through the Nrf2 pathway," *Human Molecular Genetics*, vol. 21, no. 13, pp. 3013–3024, 2012.

[84] X. Lin, H. Yang, H. Zhang, L. Zhou, and Z. Guo, "A novel transcription mechanism activated by ethanol: induction of Slc7a11 gene expression via inhibition of the DNA-binding activity of transcriptional repressor octamer-binding transcription factor 1 (OCT-1)," *The Journal of Biological Chemistry*, vol. 288, no. 21, pp. 14815–14823, 2013.

[85] M. R. Campbell, M. Karaca, K. N. Adamski, B. N. Chorley, X. Wang, and D. A. Bell, "Novel hematopoietic target genes in the NRF2-Mediated transcriptional pathway," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 120305, 12 pages, 2013.

[86] C. J. Wruck, K. Streetz, G. Pavic et al., "Nrf2 induces interleukin-6 (IL-6) expression via an antioxidant response element within the IL-6 promoter," *The Journal of Biological Chemistry*, vol. 286, no. 6, pp. 4493–4499, 2011.

[87] S. K. Niture and A. K. Jaiswal, "Nrf2 protein up-regulates antiapoptotic protein bcl-2 and prevents cellular apoptosis," *The Journal of Biological Chemistry*, vol. 287, no. 13, pp. 9873–9886, 2012.

[88] M. Komatsu, S. Kageyama, and Y. Ichimura, "P62/SQSTM1/A170: physiology and pathology," *Pharmacological Research*, vol. 66, no. 6, pp. 457–462, 2012.

[89] A. M. Cuervo, L. Stafanis, R. Fredenburg, P. T. Lansbury, and D. Sulzer, "Impaired degradation of mutant α-synuclein by chaperone-mediated autophagy," *Science*, vol. 305, no. 5688, pp. 1292–1295, 2004.

[90] J. Viiri, M. Amadio, N. Marchesi et al., "Autophagy activation clears ELAVL1/HuR-mediated accumulation of SQSTM1/p62 during proteasomal inhibition in human retinal pigment epithelial cells," *PLoS ONE*, vol. 8, no. 7, Article ID e69563, 2013.

[91] A. Salminen, K. Kaarniranta, A. Kauppinen et al., "Impaired autophagy and APP processing in Alzheimer's disease: the potential role of Beclin 1 interactome," *Progress in Neurobiology*, vol. 106-107, pp. 33–54, 2013.

[92] R. Layfield, J. R. Cavey, D. Najat et al., "p62 mutations, ubiquitin recognition and Paget's disease of bone," *Biochemical Society Transactions*, vol. 34, no. 5, pp. 735–737, 2006.

[93] J.-H. Lee, W. H. Yu, A. Kumar et al., "Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations," *Cell*, vol. 141, no. 7, pp. 1146–1158, 2010.

[94] J. Moscat, M. T. Diaz-Meco, and M. W. Wooten, "Signal integration and diversification through the p62 scaffold protein," *Trends in Biochemical Sciences*, vol. 32, no. 2, pp. 95–100, 2007.

[95] M. Komatsu, S. Waguri, M. Koike et al., "Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice," *Cell*, vol. 131, no. 6, pp. 1149–1163, 2007.

[96] A. Lau, X.-J. Wang, F. Zhao et al., "A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between keap1 and p62," *Molecular and Cellular Biology*, vol. 30, no. 13, pp. 3275–3285, 2010.

[97] K. Taguchi, N. Fujikawa, M. Komatsu et al., "Keap1 degradation by autophagy for the maintenance of redox homeostasis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 34, pp. 13561–13566, 2012.

[98] E. Itakura and N. Mizushima, "p62 targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding," *Journal of Cell Biology*, vol. 192, no. 1, pp. 17–27, 2011.

[99] Y. Ichimura and M. Komatsu, "Selective degradation of p62 by autophagy," *Seminars in Immunopathology*, vol. 32, no. 4, pp. 431–436, 2010.

[100] J. M. T. Hyttinen, G. Petrovski, A. Salminen, and K. Kaarniranta, "5*ı*-adenosine monophosphate-activated protein kinase-mammalian target of rapamycin axis as therapeutic target for age-related macular degeneration," *Rejuvenation Research*, vol. 14, no. 6, pp. 651–660, 2011.

[101] J. Kim, M. Kundu, B. Viollet, and K.-L. Guan, "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1," *Nature Cell Biology*, vol. 13, no. 2, pp. 132–141, 2011.

[102] C. Cantó, Z. Gerhart-Hines, J. N. Feige et al., "AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity," *Nature*, vol. 458, no. 7241, pp. 1056–1060, 2009.

[103] A. Gormand, E. Henriksson, K. Ström, T. E. Jensen, K. Sakamoto, and O. Göransson, "Regulation of AMP-activated protein kinase by LKB1 and CaMKK in adipocytes," *The Journal of Cellular Biochemistry*, vol. 112, no. 5, pp. 1364–1375, 2011.

[104] X.-M. Liu, K. J. Peyton, A. R. Shebib, H. Wang, R. J. Korthuis, and W. Durante, "Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 1, pp. H84–H93, 2011.

[105] S.-J. Park, F. Ahmad, A. Philp et al., "Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases," *Cell*, vol. 148, no. 3, pp. 421–433, 2012.

[106] M. Frombaum, S. Le Clanche, D. Bonnefont-Rousselot, and D. Borderie, "Antioxidant effects of resveratrol and other stilbene derivatives on oxidative stress and NO bioavailability: potential benefits to cardiovascular diseases," *Biochimie*, vol. 94, no. 2, pp. 269–276, 2012.

[107] F. X. Guix, I. Uribesalgo, M. Coma, and F. J. Muñoz, "The physiology and pathophysiology of nitric oxide in the brain," *Progress in Neurobiology*, vol. 76, no. 2, pp. 126–152, 2005.

[108] M. J. Crabtree and K. M. Channon, "Synthesis and recycling of tetrahydrobiopterin in endothelial function and vascular disease," *Nitric Oxide: Biology and Chemistry*, vol. 25, no. 2, pp. 81–88, 2011.

[109] J. Vásquez-Vivar, N. Hogg, P. Martásek, H. Karoui, K. A. Pritchard Jr., and B. Kalyanaraman, "Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase," *The Journal of Biological Chemistry*, vol. 274, no. 38, pp. 26736–26742, 1999.

[110] R. H. Foxton, J. M. Land, and S. J. R. Heales, "Tetrahydrobiopterin availability in Parkinson's and Alzheimer's disease; potential pathogenic mechanisms," *Neurochemical Research*, vol. 32, no. 4-5, pp. 751–756, 2007.

[111] K. U. Schallreuter, J. Moore, D. J. Tobin et al., "α-MSH can control the essential cofactor 6-tetrahydrobiopterin in melanogenesis," *Annals of the New York Academy of Sciences*, vol. 885, pp. 329–341, 1999.

[112] L. Wang, H. Erlandsen, J. Haavik, P. M. Knappskog, and R. C. Stevens, "Three-dimensional structure of human tryptophan hydroxylase and its implications for the biosynthesis of the neurotransmitters serotonin and melatonin," *Biochemistry*, vol. 41, no. 42, pp. 12569–12574, 2002.

[113] H. K. Kim, S. H. Ha, and J. Han, "Potential therapeutic applications of tetrahydrobiopterin: from inherited hyperphenylalaninemia to mitochondrial diseases," *Annals of the New York Academy of Sciences*, vol. 1201, pp. 177–182, 2010.

[114] J. Z. Nowak and J. B. Zawilska, "Melatonin and its physiological and therapeutic properties," *Pharmacy World & Science*, vol. 20, no. 1, pp. 18–27, 1998.

[115] P. O. Lundmark, S. R. Pandi-Perumal, V. Srinivasan, D. P. Cardinali, and R. E. Rosenstein, "Melatonin in the eye: implications for glaucoma," *Experimental Eye Research*, vol. 84, no. 6, pp. 1021–1030, 2007.

[116] N. Xia, A. Daiber, A. Habermeier et al., "Resveratrol reverses endothelial nitric-oxide synthase uncoupling in apolipoprotein E knockout mice," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 335, no. 1, pp. 149–154, 2010.

[117] J. Cai, W. G. Jiang, M. B. Grant, and M. Boulton, "Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1," *The Journal of Biological Chemistry*, vol. 281, no. 6, pp. 3604–3613, 2006.

[118] V. M. Lopez, C. L. Decatur, W. D. Stamer, R. M. Lynch, and B. S. McKay, "L-DOPA is an endogenous ligand for OA1," *PLoS Biology*, vol. 6, no. 9, article e236, 2008.

[119] B. Omar, E. Zmuda-Trzebiatowska, V. Manganiello, O. Göransson, and E. Degerman, "Regulation of AMP-activated protein kinase by cAMP in adipocytes: roles for phosphodiesterases, protein kinase B, protein kinase A, Epac and lipolysis," *Cellular Signalling*, vol. 21, no. 5, pp. 760–766, 2009.

[120] S. R. Kulkarni, A. C. Donepudi, J. Xu et al., "Fasting induces nuclear factor E2-related factor 2 and ATP-binding cassette transporters *via* protein kinase a and sirtuin-1 in mouse and human," *Antioxidants & Redox Signaling*, vol. 20, no. 1, pp. 15–30, 2014.

[121] H.-W. Lo and F. Ali-Osman, "Cyclic AMP mediated GSTP1 gene activation in tumor cells involves the interaction of activated CREB-1 with the GSTP1 CRE: a novel mechanism of cellular GSTP1 gene regulation," *Journal of Cellular Biochemistry*, vol. 87, no. 1, pp. 103–116, 2002.

[122] H. Si, J. Yu, H. Jiang, H. Lum, and D. Liu, "Phytoestrogen genistein up-regulates endothelial nitric oxide synthase expression via activation of cAMP response element-binding protein in human aortic endothelial cells," *Endocrinology*, vol. 153, no. 7, pp. 3190–3198, 2012.

[123] D. de Rasmo, A. Signorile, F. Papa, E. Roca, and S. Papa, "cAMP/Ca^{2+} response element-binding protein plays a central role in the biogenesis of respiratory chain proteins in mammalian cells," *IUBMB Life*, vol. 62, no. 6, pp. 447–452, 2010.

[124] D. G. Hardie, F. A. Ross, and S. A. Hawley, "AMPK: a nutrient and energy sensor that maintains energy homeostasis," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 4, pp. 251–262, 2012.

[125] M. Lagouge, C. Argmann, Z. Gerhart-Hines et al., "Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 alpha," *Cell*, vol. 127, no. 6, pp. 1109–1122, 2006.

[126] B. J. Wilson, A. M. Tremblay, G. Deblois, G. Sylvain-Drolet, and V. Giguère, "An acetylation switch modulates the transcriptional activity of estrogen-related receptor α," *Molecular Endocrinology*, vol. 24, no. 7, pp. 1349–1358, 2010.

[127] C. Mo, L. Wang, J. Zhang et al., "The crosstalk between Nrf2 and AMPK signal pathways is important for the anti-inflammatory effect of Berberine in LPS-stimulated macrophages and endotoxin-shocked mice," *Antioxidants & Redox Signaling*, vol. 20, no. 4, pp. 574–588, 2014.

[128] C. Handschin and U. A. Meyer, "Induction of drug metabolism: the role of nuclear receptors," *Pharmacological Reviews*, vol. 55, no. 4, pp. 649–673, 2003.

[129] J. T. Moore, J. L. Collins, and K. H. Pearce, "The nuclear receptor superfamily and drug discovery," *ChemMedChem*, vol. 1, no. 5, pp. 504–523, 2006.

[130] D. Kluth, A. Banning, I. Paur, R. Blomhoff, and R. Brigelius-Flohé, "Modulation of pregnane X receptor-and electrophile responsive element-mediated gene expression by dietary polyphenolic compounds," *Free Radical Biology and Medicine*, vol. 42, no. 3, pp. 315–325, 2007.

[131] K. C. Wu, J. Y. Cui, and C. D. Klaassen, "Effect of graded nrf2 activation on phase-I and -II drug metabolizing enzymes and transporters in mouse liver," *PLoS ONE*, vol. 7, no. 7, Article ID e39006, 2012.

[132] T. Haarmann-Stemmann, H. Bothe, and J. Abel, "Growth factors, cytokines and their receptors as downstream targets of arylhydrocarbon receptor (AhR) signaling pathways," *Biochemical Pharmacology*, vol. 77, no. 4, pp. 508–520, 2009.

[133] R. L. Yeager, S. A. Reisman, L. M. Aleksunes, and C. D. Klaassen, "Introducing the 'TCDD-inducible AhR-Nrf2 gene battery'," *Toxicological Sciences*, vol. 111, no. 2, pp. 238–246, 2009.

[134] E. R. Levin, "Integration of the extranuclear and nuclear actions of estrogen," *Molecular Endocrinology*, vol. 19, no. 8, pp. 1951–1959, 2005.

[135] A. L. Ososki and E. J. Kennelly, "Phytoestrogens: a review of the present state of research," *Phytotherapy Research*, vol. 17, no. 8, pp. 845–869, 2003.

[136] R. Lappano, C. Rosano, A. Madeo et al., "Structure-activity relationships of resveratrol and derivatives in breast cancer cells," *Molecular Nutrition and Food Research*, vol. 53, no. 7, pp. 845–858, 2009.

[137] M. C. Saleh, B. J. Connell, and T. M. Saleh, "Resveratrol induced neuroprotection is mediated via both estrogen receptor subtypes, ERα and ERβ," *Neuroscience Letters*, vol. 548, pp. 217–221, 2013.

[138] V. Di Liberto, J. Mäkelä, L. Korhonen et al., "Involvement of estrogen receptors in the resveratrol-mediated increase in dopamine transporter in human dopaminergic neurons and in striatum of female mice," *Neuropharmacology*, vol. 62, no. 2, pp. 1011–1018, 2012.

[139] A. Tchernof and J.-P. Després, "Pathophysiology of human visceral obesity: an update," *Physiological Reviews*, vol. 93, no. 1, pp. 359–404, 2013.

[140] D. A. Sinclair, "Toward a unified theory of caloric restriction and longevity regulation," *Mechanisms of Ageing and Development*, vol. 126, no. 9, pp. 987–1002, 2005.

[141] L. Fontana, L. Partridge, and V. D. Longo, "Extending healthy life span-from yeast to humans," *Science*, vol. 328, no. 5976, pp. 321–326, 2010.

[142] A. Soare, R. Cangemi, D. Omodei, J. O. Holloszy, and L. Fontana, "Long-term calorie restriction, but not endurance exercise, lowers core body temperature in humans," *Aging*, vol. 3, no. 4, pp. 374–379, 2011.

[143] A. V. Witte, M. Fobker, R. Gellner, S. Knecht, and A. Flöel, "Caloric restriction improves memory in elderly humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 4, pp. 1255–1260, 2009.

[144] C.-L. Hsu and G.-C. Yen, "Phenolic compounds: evidence for inhibitory effects against obesity and their underlying molecular signaling mechanism," *Molecular Nutrition & Food Research*, vol. 52, no. 1, pp. 53–61, 2008.

[145] J. A. Baur and D. A. Sinclair, "Therapeutic potential of resveratrol: the in vivo evidence," *Nature Reviews Drug Discovery*, vol. 5, no. 6, pp. 493–506, 2006.

[146] A.-S. Cheng, Y.-H. Cheng, C.-H. Chiou, and T.-L. Chang, "Resveratrol upregulates Nrf2 expression to attenuate methylglyoxal-induced insulin resistance in hep G2 cells," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 36, pp. 9180–9187, 2012.

[147] E. Tauriainen, M. Luostarinen, E. Martonen et al., "Distinct effects of calorie restriction and resveratrol on diet-induced obesity and fatty liver formation," *Journal of Nutrition and Metabolism*, vol. 2011, Article ID 525094, 10 pages, 2011.

[148] T. Ito-Nagahata, C. Kurihara, M. Hasebe et al., "Stilbene analogs of resveratrol improve insulin resistance through activation of AMPK," *Bioscience, Biotechnology and Biochemistry*, vol. 77, no. 6, pp. 1229–1235, 2013.

[149] P. Fischer-Posovszky, V. Kukulus, D. Tews et al., "Resveratrol regulates human adipocyte number and function in a Sirt1-dependent manner," *The American Journal of Clinical Nutrition*, vol. 92, no. 1, pp. 5–15, 2010.

[150] T. T. Schug and X. Li, "Sirtuin 1 in lipid metabolism and obesity," *Annals of Medicine*, vol. 43, no. 3, pp. 198–211, 2011.

[151] I. Mader, M. Wabitsch, K.-M. Debatin, P. Fischer-Posovszky, and S. Fulda, "Identification of a novel proapoptotic function of resveratrol in fat cells: SIRT1-independent sensitization to TRAIL-induced apoptosis," *The FASEB Journal*, vol. 24, no. 6, pp. 1997–2009, 2010.

[152] S. Chen, X. Xiao, X. Feng et al., "Resveratrol induces Sirt1-dependent apoptosis in 3T3-L1 preadipocytes by activating AMPK and suppressing AKT activity and survivin expression," *The Journal of Nutritional Biochemistry*, vol. 23, no. 9, pp. 1100–1112, 2012.

[153] Y. S. Jeong, J. H. Hong, K. H. Cho, and H. K. Jung, "Grape skin extract reduces adipogenesis- and lipogenesis-related gene expression in 3T3-L1 adipocytes through the peroxisome proliferator-activated receptor-γ signaling pathway," *Nutrition Research*, vol. 32, no. 7, pp. 514–521, 2012.

[154] X.-H. Zhang, B. Huang, S.-K. Choi, and J.-S. Seo, "Anti-obesity effect of resveratrol-amplified grape skin extracts on 3T3-L1 adipocytes differentiation," *Nutrition Research and Practice*, vol. 6, no. 4, pp. 286–293, 2012.

[155] D. Y. Jung, U. Chalasani, N. Pan et al., "KLF15 is a molecular link between endoplasmic reticulum stress and insulin resistance," *PLoS ONE*, vol. 8, no. 10, Article ID e77851, 2013.

[156] A. Lasa, M. Schweiger, P. Kotzbeck et al., "Resveratrol regulates lipolysis via adipose triglyceride lipase," *The Journal of Nutritional Biochemistry*, vol. 23, no. 4, pp. 379–384, 2012.

[157] P. Chakrabarti, T. English, S. Karki et al., "SIRT1 controls lipolysis in adipocytes via FOXO1-mediated expression of ATGL," *Journal of Lipid Research*, vol. 52, no. 9, pp. 1693–1701, 2011.

[158] C. D. S. Costa, F. Rohden, T. O. Hammes et al., "Resveratrol upregulated SIRT1, FOXO1, and adiponectin and downregulated PPARγ1-3 mRNA expression in human visceral adipocytes," *Obesity Surgery*, vol. 21, no. 3, pp. 356–361, 2011.

[159] T. Shan, Y. Ren, and Y. Wang, "Sirtuin 1 affects the transcriptional expression of adipose triglyceride lipase in porcine adipocytes," *Journal of Animal Science*, vol. 91, no. 3, pp. 1247–1254, 2013.

[160] T. O. Hammes, C. D. S. Costa, F. Rohden et al., "Parallel downregulation of FOXO1, PPARγ and adiponectin mRNA expression in visceral adipose tissue of class III obese individuals," *Obesity Facts*, vol. 5, no. 3, pp. 452–459, 2012.

[161] J. H. Chung, V. Manganiello, and J. R. B. Dyck, "Resveratrol as a calorie restriction mimetic: therapeutic implications," *Trends in Cell Biology*, vol. 22, no. 10, pp. 546–554, 2012.

[162] J. Lee, E. Jung, J. Lim et al., "Involvement of nuclear factor-κB in the inhibition of pro-inflammatory mediators by pinosylvin," *Planta Medica*, vol. 72, no. 9, pp. 801–806, 2006.

[163] M. Frombaum, P. Therond, R. Djelidi, J.-L. Beaudeux, D. Bonnefont-Rousselot, and D. Borderie, "Piceatannol is more effective than resveratrol in restoring endothelial cell dimethylarginine dimethylaminohydrolase expression and activity after high-glucose oxidative stress," *Free Radical Research*, vol. 45, no. 3, pp. 293–302, 2011.

[164] A. Overman, A. Bumrungpert, A. Kennedy et al., "Polyphenol-rich grape powder extract (GPE) attenuates inflammation in human macrophages and in human adipocytes exposed to macrophage-conditioned media," *International Journal of Obesity*, vol. 34, no. 5, pp. 800–808, 2010.

[165] C.-C. Chuang, A. Bumrungpert, A. Kennedy et al., "Grape powder extract attenuates tumor necrosis factor α-mediated inflammation and insulin resistance in primary cultures of human adipocytes," *Journal of Nutritional Biochemistry*, vol. 22, no. 1, pp. 89–94, 2011.

[166] J. Ołlholm, S. K. Paulsen, K. B. Cullberg, B. Richelsen, and S. B. Pedersen, "Anti-inflammatory effect of resveratrol on adipokine expression and secretion in human adipose tissue explants," *International Journal of Obesity*, vol. 34, no. 10, pp. 1546–1553, 2010.

[167] G. Alberdi, V. M. Rodríguez, J. Miranda, M. T. Macarulla, I. Churruca, and M. P. Portillo, "Thermogenesis is involved in the body-fat lowering effects of resveratrol in rats," *Food Chemistry*, vol. 141, no. 2, pp. 1530–1535, 2013.

[168] H. J. Park, J.-Y. Yang, S. Ambati et al., "Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes," *Journal of Medicinal Food*, vol. 11, no. 4, pp. 773–783, 2008.

[169] M. Herranz-López, S. Fernández-Arroyo, A. Pérez-Sanchez et al., "Synergism of plant-derived polyphenols in adipogenesis: perspectives and implications," *Phytomedicine*, vol. 19, no. 3-4, pp. 253–261, 2012.

[170] G.-M. Do, U. J. Jung, H.-J. Park et al., "Resveratrol ameliorates diabetes-related metabolic changes via activation of AMP-activated protein kinase and its downstream targets in db/db mice," *Molecular Nutrition & Food Research*, vol. 56, no. 8, pp. 1282–1291, 2012.

[171] W. Kang, H. J. Hong, J. Guan et al., "Resveratrol improves insulin signaling in a tissue-specific manner under insulin-resistant conditions only: in vitro and in vivo experiments in rodents," *Metabolism: Clinical and Experimental*, vol. 61, no. 3, pp. 424–433, 2012.

[172] M. Minakawa, Y. Miura, and K. Yagasaki, "Piceatannol, a resveratrol derivative, promotes glucose uptake through glucose transporter 4 translocation to plasma membrane in L6 myocytes and suppresses blood glucose levels in type 2 diabetic model db/db mice," *Biochemical and Biophysical Research Communications*, vol. 422, no. 3, pp. 469–475, 2012.

[173] Z. Tan, L.-J. Zhou, P.-W. Mu et al., "Caveolin-3 is involved in the protection of resveratrol against high-fat-diet-induced insulin resistance by promoting GLUT4 translocation to the

plasma membrane in skeletal muscle of ovariectomized rats," *The Journal of Nutritional Biochemistry*, vol. 23, no. 12, pp. 1716–1724, 2012.

[174] T. Matsui, T. Ueda, T. Oki, K. Sugita, N. Terahara, and K. Matsumoto, "α-glucosidase inhibitory action of natural acylated anthocyanins. 1. Survey of natural pigments with potent inhibitory activity," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 4, pp. 1948–1951, 2001.

[175] A. C. L. Barbosa, M. D. S. Pinto, D. Sarkar, C. Ankolekar, D. Greene, and K. Shetty, "Varietal influences on antihyperglycemia properties of freshly harvested apples using *in vitro* assay models," *Journal of Medicinal Food*, vol. 13, no. 6, pp. 1313–1323, 2010.

[176] D. Grussu, D. Stewart, and G. J. McDougall, "Berry polyphenols inhibit α-amylase in vitro: identifying active components in rowanberry and raspberry," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 6, pp. 2324–2331, 2011.

[177] I. Kubo, Y. Murai, I. Soediro, S. Soetarno, and S. Sastrodihardjo, "Efficient isolation of glycosidase inhibitory stilbene glycosides *from Rheum palmatum*," *Journal of Natural Products*, vol. 54, no. 4, pp. 1115–1118, 1991.

[178] K. S. Babu, A. K. Tiwari, P. V. Srinivas, A. Z. Ali, B. C. Raju, and J. M. Rao, "Yeast and mammalian α-glucosidase inhibitory constituents from Himalayan rhubarb *Rheum emodi* Wall.ex Meisson," *Bioorganic and Medicinal Chemistry Letters*, vol. 14, no. 14, pp. 3841–3845, 2004.

[179] Z. Kerem, I. Bilkis, M. A. Flaishman, and L. Sivan, "Antioxidant activity and inhibition of α-glucosidase by *trans*-resveratrol, piceid, and a novel *trans*-stilbene from the roots of Israeli *Rumex bucephalophorus* L.," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 4, pp. 1243–1247, 2006.

[180] S.-H. Lam, J.-M. Chen, C.-J. Kang, C.-H. Chen, and S.-S. Lee, "Alpha-glucosidase inhibitors from the seeds of syagrus romanzoffiana," *Phytochemistry*, vol. 69, no. 5, pp. 1173–1178, 2008.

[181] X. Wan, X.-B. Wang, M.-H. Yang, J.-S. Wang, and L.-Y. Kong, "Dimerization of piceatannol by Momordica charantia peroxidase and α-glucosidase inhibitory activity of the biotransformation products," *Bioorganic and Medicinal Chemistry*, vol. 19, no. 17, pp. 5085–5092, 2011.

[182] M. Miao, H. Jiang, B. Jiang, T. Zhang, S. W. Cui, and Z. Jin, "Phytonutrients for controlling starch digestion: evaluation of grape skin extract," *Food Chemistry*, vol. 145, pp. 205–211, 2014.

[183] P. Chakrabarti and K. V. Kandror, "FoxO1 controls insulin-dependent adipose triglyceride lipase (ATGL) expression and lipolysis in adipocytes," *The Journal of Biological Chemistry*, vol. 284, no. 20, pp. 13296–13300, 2009.

[184] Y.-E. Lee, J.-W. Kim, E.-M. Lee et al., "Chronic resveratrol treatment protects pancreatic islets against oxidative stress in db/db mice," *PLoS ONE*, vol. 7, no. 11, Article ID e50412, 2012.

[185] J.-M. Yun, A. Chien, I. Jialal, and S. Devaraj, "Resveratrol up-regulates SIRT1 and inhibits cellular oxidative stress in the diabetic milieu: mechanistic insights," *Journal of Nutritional Biochemistry*, vol. 23, no. 7, pp. 699–705, 2012.

[186] F. Paneni, J. A. Beckman, M. A. Creager, and F. Cosentino, "Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy. Part I," *European Heart Journal*, vol. 34, no. 31, pp. 2436–2446, 2013.

[187] B. B. Dokken, "The pathophysiology of cardiovascular disease and diabetes: beyond blood pressure and lipids," *Diabetes Spectrum*, vol. 21, no. 3, pp. 160–165, 2008.

[188] N. J. Alp, S. Mussa, J. Khoo et al., "Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression," *Journal of Clinical Investigation*, vol. 112, no. 5, pp. 725–735, 2003.

[189] H. Li, N. Xia, and U. Förstermann, "Cardiovascular effects and molecular targets of resveratrol," *Nitric Oxide—Biology and Chemistry*, vol. 26, no. 2, pp. 102–110, 2012.

[190] A. A. E. Bertelli, L. Giovannini, D. Giannessi, M. Migliori, W. Bernini, and M. Fregoni, "Antiplatelet activity of synthetic and natural resveratrol in red wine," *International Journal of Tissue Reactions*, vol. 17, no. 1, pp. 1–3, 1995.

[191] K. R. Patel, E. Scott, V. A. Brown, A. J. Gescher, W. P. Steward, and K. Brown, "Clinical trials of resveratrol," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 161–169, 2011.

[192] B. Agarwal, M. J. Campen, M. M. Channell et al., "Resveratrol for primary prevention of atherosclerosis: clinical trial evidence for improved gene expression in vascular endothelium," *International Journal of Cardiology*, vol. 166, no. 1, pp. 246–248, 2013.

[193] T. Wallerath, G. Deckert, T. Ternes et al., "Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase," *Circulation*, vol. 106, no. 13, pp. 1652–1658, 2002.

[194] P. Gresele, P. Pignatelli, G. Guglielmini et al., "Resveratrol, at concentrations attainable with moderate wine consumption, stimulates human platelet nitric oxide production," *The Journal of Nutrition*, vol. 138, no. 9, pp. 1602–1608, 2008.

[195] G. Spanier, H. Xu, N. Xia et al., "Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1) and NADPH oxidase subunit (Nox4)," *Journal of Physiology and Pharmacology*, vol. 60, pp. 111–116, 2009.

[196] H. Wang, Y.-J. Yang, H.-Y. Qian, Q. Zhang, H. Xu, and J.-J. Li, "Resveratrol in cardiovascular disease: what is known from current research?" *Heart Failure Reviews*, vol. 17, no. 3, pp. 437–448, 2012.

[197] M. Jang, L. Cai, G. O. Udeani et al., "Cancer chemopreventive activity of resveratrol, a natural product derived from grapes," *Science*, vol. 275, no. 5297, pp. 218–220, 1997.

[198] H. S. Youn, J. Y. Lee, K. A. Fitzgerald, H. A. Young, S. Akira, and D. H. Hwang, "Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex," *The Journal of Immunology*, vol. 175, no. 5, pp. 3339–3346, 2005.

[199] L. V. Johnson, W. P. Leitner, A. J. Rivest, M. K. Staples, M. J. Radeke, and D. H. Anderson, "The Alzheimer's Aβ-peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 18, pp. 11830–11835, 2002.

[200] D. Sulzer, E. Mosharov, Z. Talloczy, F. A. Zucca, J. D. Simon, and L. Zecca, "Neuronal pigmented autophagic vacuoles: lipofuscin, neuromelanin, and ceroid as macroautophagic responses during aging and disease," *Journal of Neurochemistry*, vol. 106, no. 1, pp. 24–36, 2008.

[201] J. M. Isas, V. Luibl, L. V. Johnson et al., "Soluble and mature amyloid fibrils in drusen deposits," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 3, pp. 1304–1310, 2010.

[202] K. Kaarniranta, A. Salminen, A. Haapasalo, H. Soininen, and M. Hiltunen, "Age-related macular degeneration (AMD): alzheimer's disease in the eye?" *Journal of Alzheimer's Disease*, vol. 24, no. 4, pp. 615–631, 2011.

[203] R. A. Nixon and D.-S. Yang, "Autophagy failure in Alzheimer's disease-locating the primary defect," *Neurobiology of Disease*, vol. 43, no. 1, pp. 38–45, 2011.

[204] P. Hahn, A. H. Milam, and J. L. Dunaief, "Maculas affected by age-related macular degeneration contain increased chelatable iron in the retinal pigment epithelium and Bruch's membrane," *Archives of Ophthalmology*, vol. 121, no. 8, pp. 1099–1105, 2003.

[205] S. Oshiro, M. S. Morioka, and M. Kikuchi, "Dysregulation of iron metabolism in Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis," *Advances in Pharmacological Sciences*, vol. 2011, Article ID 378278, 8 pages, 2011.

[206] K. M. Gehrs, D. H. Anderson, L. V. Johnson, and G. S. Hageman, "Age-related macular degeneration—emerging pathogenetic and therapeutic concepts," *Annals of Medicine*, vol. 38, no. 7, pp. 450–471, 2006.

[207] J. Z. Nowak, "Age-related macular degeneration (AMD): pathogenesis and therapy," *Pharmacological Reports*, vol. 58, no. 3, pp. 353–363, 2006.

[208] M. A. Williams, D. Craig, P. Passmore, and G. Silvestri, "Retinal drusen: harbingers of age, safe havens for trouble," *Age and Ageing*, vol. 38, no. 6, pp. 648–654, 2009.

[209] K. P. Ng, B. Gugiu, K. Renganathan et al., "Retinal pigment epithelium lipofuscin proteomics," *Molecular and Cellular Proteomics*, vol. 7, no. 7, pp. 1397–1405, 2008.

[210] H. Xu, M. Chen, and J. V. Forrester, "Para-inflammation in the aging retina," *Progress in Retinal and Eye Research*, vol. 28, no. 5, pp. 348–368, 2009.

[211] K. Kaarniranta, D. Sinha, J. Blasiak et al., "Autophagy and heterophagy dysregulation leads to retinal pigment epithelium dysfunction and development of age-related macular degeneration," *Autophagy*, vol. 9, no. 7, pp. 973–984, 2013.

[212] A. Klettner, A. Kauppinen, J. Blasiak, J. Roider, A. Salminen, and K. Kaarniranta, "Cellular and molecular mechanisms of age-related macular degeneration: from impaired autophagy to neovascularization," *The International Journal of Biochemistry & Cell Biology*, vol. 45, no. 7, pp. 1457–1497, 2013.

[213] S. Binder, B. V. Stanzel, I. Krebs, and C. Glittenberg, "Transplantation of the RPE in AMD," *Progress in Retinal and Eye Research*, vol. 26, no. 5, pp. 516–554, 2007.

[214] E. Arnault, C. Barrau, C. Nanteau et al., "Phototoxic action spectrum on a retinal pigment epithelium model of age-related macular degeneration exposed to sunlight normalized conditions," *PLoS ONE*, vol. 8, no. 8, Article ID e71398, 2013.

[215] S. G. Jarrett and M. E. Boulton, "Consequences of oxidative stress in age-related macular degeneration," *Molecular Aspects of Medicine*, vol. 33, no. 4, pp. 399–417, 2012.

[216] J. Kopitz, F. G. Holz, E. Kaemmerer, and F. Schutt, "Lipids and lipid peroxidation products in the pathogenesis of age-related macular degeneration," *Biochimie*, vol. 86, no. 11, pp. 825–831, 2004.

[217] J. R. Sparrow and M. Boulton, "RPE lipofuscin and its role in retinal pathobiology," *Experimental Eye Research*, vol. 80, no. 5, pp. 595–606, 2005.

[218] Y. Wu, E. Yanase, X. Feng, M. M. Siegel, and J. R. Sparrow, "Structural characterization of bisretinoid A2E photocleavage products and implications for age-related macular degeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 16, pp. 7275–7280, 2010.

[219] A. Zadlo, M. B. Rozanowska, J. M. Burke, and T. J. Sarna, "Photobleaching of retinal pigment epithelium melanosomes reduces their ability to inhibit iron-induced peroxidation of lipids," *Pigment Cell Research*, vol. 20, no. 1, pp. 52–60, 2007.

[220] S. Memoli, A. Napolitano, M. D'Ischia, A. Palumbo, and G. Prota, "Diffusible melanin-related metabolites are potent inhibitors of lipid peroxidation," *Biochimica et Biophysica Acta*, vol. 1346, no. 1, pp. 61–68, 1997.

[221] L. Hong and J. D. Simon, "Current understanding of the binding sites, capacity, affinity, and biological significance of metals in melanin," *The Journal of Physical Chemistry B*, vol. 111, no. 28, pp. 7938–7947, 2007.

[222] P. Kaczara, M. Zareba, A. Herrnreiter et al., "Melanosome-iron interactions within retinal pigment epithelium-derived cells," *Pigment Cell and Melanoma Research*, vol. 25, no. 6, pp. 804–814, 2012.

[223] K. Jomova, D. Vondrakova, M. Lawson, and M. Valko, "Metals, oxidative stress and neurodegenerative disorders," *Molecular and Cellular Biochemistry*, vol. 345, no. 1-2, pp. 91–104, 2010.

[224] T. Sarna, J. M. Burke, W. Korytowski et al., "Loss of melanin from human RPE with aging: possible role of melanin photooxidation," *Experimental Eye Research*, vol. 76, no. 1, pp. 89–98, 2003.

[225] R. Klein, B. E. K. Klein, E. K. Marino et al., "Early age-related maculopathy in the cardiovascular health study," *Ophthalmology*, vol. 110, no. 1, pp. 25–33, 2003.

[226] C. L. Thompson, G. Jun, B. E. K. Klein et al., "Genetics of pigment changes and geographic atrophy," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 7, pp. 3005–3013, 2007.

[227] S. Qin and G. A. Rodrigues, "Roles of $\alpha v\beta 5$, FAK and MerTK in oxidative stress inhibition of RPE cell phagocytosis," *Experimental Eye Research*, vol. 94, no. 1, pp. 63–70, 2012.

[228] T. A. Bailey, N. Kanuga, I. A. Romero, J. Greenwood, P. J. Luthert, and M. E. Cheetham, "Oxidative stress affects the junctional integrity of retinal pigment epithelial cells," *Investigative Ophthalmology and Visual Science*, vol. 45, no. 2, pp. 675–684, 2004.

[229] M. M. Sachdeva, M. Cano, and J. T. Handa, "Nrf2 signaling is impaired in the aging RPE given an oxidative insult," *Experimental Eye Research*, vol. 119, pp. 111–114, 2014.

[230] M. Cano, R. Thimmalappula, M. Fujihara et al., "Cigarette smoking, oxidative stress, the anti-oxidant response through Nrf2 signaling, and age-related macular degeneration," *Vision Research*, vol. 50, no. 7, pp. 652–664, 2010.

[231] N. Nagai, R. K. Thimmulappa, M. Cano et al., "Nrf2 is a critical modulator of the innate immune response in a model of uveitis," *Free Radical Biology & Medicine*, vol. 47, no. 3, pp. 300–306, 2009.

[232] A. Koskela, M. Reinisalo, J. M. Hyttinen, K. Kaarniranta, and R. O. Karjalainen, "Pinosylvin-mediated protection against oxidative stress in human retinal pigment epithelial cells," *Molecular Vision*, vol. 20, pp. 760–769, 2014.

[233] N. M. M. Saviranta, L. Veeroos, L. J. Granlund, V. H. Hassinen, K. Kaarniranta, and R. O. Karjalainen, "Plant flavonol quercetin and isoflavone biochanin A differentially induce protection against oxidative stress and inflammation in ARPE-19 cells," *Food Research International*, vol. 44, no. 1, pp. 109–113, 2011.

[234] X. Zou, Z. Feng, Y. Li et al., "Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: activation of Nrf2 and JNK-p62/SQSTM1 pathways," *The Journal of Nutritional Biochemistry*, vol. 23, no. 8, pp. 994–1006, 2012.

[235] E.-J. Park, H. J. Park, H.-J. Chung et al., "Antimetastatic activity of pinosylvin, a natural stilbenoid, is associated with the suppression of matrix metalloproteinases," *Journal of Nutritional Biochemistry*, vol. 23, no. 8, pp. 946–952, 2012.

[236] S. Richer, W. Stiles, L. Ulanski, D. Carroll, and C. Podella, "Observation of human retinal remodeling in octogenarians with a resveratrol based nutritional supplement," *Nutrients*, vol. 5, no. 6, pp. 1989–2005, 2013.

[237] E. Bråkenhielm, R. Cao, and Y. Cao, "Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes.," *The FASEB Journal*, vol. 15, no. 10, pp. 1798–1800, 2001.

[238] B. Dugas, S. Charbonnier, M. Baarine et al., "Effects of oxysterols on cell viability, inflammatory cytokines, VEGF, and reactive oxygen species production on human retinal cells: cytoprotective effects and prevention of VEGF secretion by resveratrol," *European Journal of Nutrition*, vol. 49, no. 7, pp. 435–446, 2010.

[239] M. Pons and M. E. Marin-Castaño, "Cigarette smoke-related hydroquinone dysregulates MCP-1, VEGF and PEDF expression in retinal pigment epithelium *in vitro* and *in vivo*," *PLoS ONE*, vol. 6, no. 2, Article ID e16722, 2011.

[240] Z. Ablonczy, A. Prakasam, J. Fant, A. Fauq, C. Crosson, and K. Sambamurti, "Pigment epithelium-derived factor maintains retinal pigment epithelium function by inhibiting vascular endothelial growth factor-R2 signaling through gamma-secretase," *The Journal of Biological Chemistry*, vol. 284, no. 44, pp. 30177–30186, 2009.

[241] C.-M. Chan, H.-H. Chang, V.-C. Wang, C.-L. Huang, and C.-F. Hung, "Inhibitory effects of resveratrol on PDGF-BB-induced retinal pigment epithelial cell migration via PDGFRβ, PI3K/Akt and MAPK pathways," *PLoS ONE*, vol. 8, no. 2, Article ID e56819, 2013.

[242] X.-Q. Liu, B.-J. Wu, W. H. T. Pan et al., "Resveratrol mitigates rat retinal ischemic injury: the roles of matrix metalloproteinase-9, inducible nitric oxide, and heme oxygenase-1," *Journal of Ocular Pharmacology and Therapeutics*, vol. 29, no. 1, pp. 33–40, 2013.

[243] A. P. Vin, H. Hu, Y. Zhai et al., "Neuroprotective effect of resveratrol prophylaxis on experimental retinal ischemic injury," *Experimental Eye Research*, vol. 108, pp. 72–75, 2013.

[244] S. Kubota, T. Kurihara, M. Ebinuma et al., "Resveratrol prevents light-induced retinal degeneration via suppressing activator protein-1 activation," *The American Journal of Pathology*, vol. 177, no. 4, pp. 1725–1731, 2010.

[245] C. Li, L. Wang, K. Huang, and L. Zheng, "Endoplasmic reticulum stress in retinal vascular degeneration: protective role of resveratrol," *Investigative Ophthalmology & Visual Science*, vol. 53, no. 6, pp. 3241–3249, 2012.

[246] S. H. Kim, J. H. Park, Y. J. Kim, and K. H. Park, "The neuroprotective effect of resveratrol on retinal ganglion cells after optic nerve transection," *Molecular Vision*, vol. 19, pp. 1667–1676, 2013.

[247] S. Kubota, T. Kurihara, H. Mochimar et al., "Prevention of ocular inflammation in endotoxin-induced uveitis with resveratrol by inhibiting oxidative damage and nuclear factor-kappaB activation," *Investigative Ophthalmology & Visual Science*, vol. 50, no. 7, pp. 3512–3519, 2009.

[248] N. M. Kalariya, M. Shoeb, A. B. M. Reddy, R. Sawhney, and K. V. Ramana, "Piceatannol suppresses endotoxin-induced ocular inflammation in rats," *International Immunopharmacology*, vol. 17, no. 2, pp. 439–446, 2013.

[249] F. Ishizuka, M. Shimazawa, Y. Inoue et al., "Toll-like receptor 4 mediates retinal ischemia/reperfusion injury through nuclear factor-κB and spleen tyrosine kinase activation," *Investigative Ophthalmology and Visual Science*, vol. 54, no. 8, pp. 5807–5816, 2013.

[250] V. Theendakara, A. Patent, C. A. P. Libeu et al., "Neuroprotective Sirtuin ratio reversed by ApoE4," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 45, pp. 18303–18308, 2013.

[251] Q. Dai, A. R. Borenstein, Y. Wu, J. C. Jackson, and E. B. Larson, "Fruit and vegetable juices and alzheimer's disease: the kame project," *The American Journal of Medicine*, vol. 119, no. 9, pp. 751–759, 2006.

[252] L. Ho, M. G. Ferruzzi, E. M. Janle et al., "Identification of brain-targeted bioactive dietary quercetin-3-O-glucuronide as a novel intervention for Alzheimer's disease," *The FASEB Journal*, vol. 27, no. 2, pp. 769–781, 2013.

[253] R. E. Tanzi, R. D. Moir, and S. L. Wagner, "Clearance of Alzheimer's Aβ peptide: the many roads to perdition," *Neuron*, vol. 43, no. 5, pp. 605–608, 2004.

[254] J. Wang, I. Santa-Maria, L. Ho et al., "Grape derived polyphenols attenuate Tau neuropathology in a mouse model of alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 22, no. 2, pp. 653–661, 2010.

[255] E. Parrella, T. Maxim, F. Maialetti et al., "Protein restriction cycles reduce IGF-1 and phosphorylated tau, and improve behavioral performance in an Alzheimer's disease mouse model," *Aging Cell*, vol. 12, no. 2, pp. 257–268, 2013.

[256] H. Scholtzova, R. J. Kascsak, K. A. Bates et al., "Induction of toll-like receptor 9 signaling as a method for ameliorating alzheimer's disease-related pathology," *Journal of Neuroscience*, vol. 29, no. 6, pp. 1846–1854, 2009.

[257] S. M. Rothman and M. P. Mattson, "Activity-dependent, stress-responsive BDNF signaling and the quest for optimal brain health and resilience throughout the lifespan," *Neuroscience*, vol. 239, pp. 228–240, 2013.

[258] M. Rahvar, M. Nikseresht, S. M. Shafiee et al., "Effect of oral resveratrol on the BDNF gene expression in the hippocampus of the rat brain," *Neurochemical Research*, vol. 36, no. 5, pp. 761–765, 2011.

[259] F. Allam, A. T. Dao, G. Chugh et al., "Grape powder supplementation prevents oxidative stress-induced anxiety-like behavior, memory impairment, and high blood pressure in rats," *The Journal of Nutrition*, vol. 143, no. 6, pp. 835–842, 2013.

[260] C. Rendeiro, D. Vauzour, R. J. Kean et al., "Blueberry supplementation induces spatial memory improvements and region-specific regulation of hippocampal BDNF mRNA expression in young rats," *Psychopharmacology*, vol. 223, no. 3, pp. 319–330, 2012.

[261] P. Marambaud, H. Zhao, and P. Davies, "Resveratrol promotes clearance of Alzheimer's disease amyloid-β peptides," *The Journal of Biological Chemistry*, vol. 280, no. 45, pp. 37377–37382, 2005.

[262] K. Pallauf and G. Rimbach, "Autophagy, polyphenols and healthy ageing," *Ageing Research Reviews*, vol. 12, no. 1, pp. 237–252, 2013.

[263] N. Gurusamy, I. Lekli, S. Mukherjee et al., "Cardioprotection by resveratrol: a novel mechanism via autophagy involving the mTORC2 pathway," *Cardiovascular Research*, vol. 86, no. 1, pp. 103–112, 2010.

[264] L. Fontana, "The scientific basis of caloric restriction leading to longer life," *Current Opinion in Gastroenterology*, vol. 25, no. 2, pp. 144–150, 2009.

[265] W. Rizza, N. Veronese, and L. Fontana, "What are the roles of calorie restriction and diet quality in promoting healthy longevity?" *Ageing Research Reviews*, vol. 13, no. 1, pp. 38–45, 2014.

[266] D. Porquet, G. Casadesús, S. Bayod et al., "Dietary resveratrol prevents alzheimer's markers and increases life span in SAMP8," *Age*, vol. 35, no. 5, pp. 1851–1865, 2013.

[267] S. M. de la Monte, "Brain insulin resistance and deficiency as therapeutic targets in Alzheimer's disease," *Current Alzheimer Research*, vol. 9, no. 1, pp. 35–66, 2012.

[268] B. Dasgupta and J. Milbrandt, "Resveratrol stimulates AMP kinase activity in neurons," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 17, pp. 7217–7222, 2007.

[269] B. T. Jeon, E. A. Jeong, H. J. Shin et al., "Resveratrol attenuates obesity-associated peripheral and central inflammation and improves memory deficit in mice fed a high-fat diet," *Diabetes*, vol. 61, no. 6, pp. 1444–1454, 2012.

[270] Y. Jimenez-Gomez, J. A. Mattison, K. J. Pearson et al., "Resveratrol improves adipose insulin signaling and reduces the inflammatory response in adipose tissue of rhesus monkeys on high-fat, high-sugar diet," *Cell Metabolism*, vol. 18, no. 4, pp. 533–545, 2013.

[271] S. Timmers, E. Konings, L. Bilet et al., "Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans," *Cell Metabolism*, vol. 14, no. 5, pp. 612–622, 2011.

[272] J. Tomé-Carneiro, M. Gonzálvez, M. Larrosa et al., "Grape resveratrol increases serum adiponectin and downregulates inflammatory genes in peripheral blood mononuclear cells: a triple-blind, placebo-controlled, one-year clinical trial in patients with stable coronary artery disease," *Cardiovascular Drugs and Therapy*, vol. 27, no. 1, pp. 37–48, 2013.

[273] V. Solfrizzi, V. Frisardi, D. Seripa et al., "Mediterranean diet in predementia and dementia syndromes," *Current Alzheimer Research*, vol. 8, no. 5, pp. 520–542, 2011.

[274] V. Solfrizzi, F. Panza, V. Frisardi et al., "Diet and alzheimer's disease risk factors or prevention: the current evidence," *Expert Review of Neurotherapeutics*, vol. 11, no. 5, pp. 677–708, 2011.

[275] L. Ho, L. H. Chen, J. Wang et al., "Heterogeneity in red wine polyphenolic contents differentially influences Alzheimer's disease-type neuropathology and cognitive deterioration," *Journal of Alzheimer's Disease*, vol. 16, no. 1, pp. 59–72, 2009.

[276] P. Scheltens, P. J. G. H. Kamphuis, F. R. J. Verhey et al., "Efficacy of a medical food in mild Alzheimer's disease: a randomized, controlled trial," *Alzheimer's and Dementia*, vol. 6, no. 1, pp. 1.e1–10.e1, 2010.

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Research Article

Doses of Quercetin in the Range of Serum Concentrations Exert Delipidating Effects in 3T3-L1 Preadipocytes by Acting on Different Stages of Adipogenesis, but Not in Mature Adipocytes

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Scope. To determine whether doses of quercetin in the range of serum concentrations exert any effect on triacylglycerol accumulation in maturing preadipocytes and mature adipocytes. The influence on the expression of adipogenic markers as well as on gene expression and activity of enzymes involved in triacylglycerol metabolism were assessed. *Methods and Results*. 3T3-L1 preadipocytes were treated during differentiation and mature adipocytes for 24 hours with low doses (0.1–10 μM) of quercetin. Triacylglycerol content in both cell types and free fatty acid and glycerol in the incubation medium of mature adipocytes were measured spectrophotometrically. Gene and protein expression were assessed by RT-PCR and Western blot. LPL and FAS activities were quantified. During differentiation quercetin reduced triacylglycerol content at doses from 0.5 to 10 μM. 1 μM of quercetin reduced C/EBPβ gene expression, SREBP1 mature protein levels, and PPARγ gene expression. 10 μM of quercetin reduced LPL gene expression and PPARγ and SREBP1c expression. In mature adipocytes, only 10 μM of quercetin reduced triacylglycerol content. Lipogenic FAS expression and activity were reduced at this dose. *Conclusion*. Quercetin, in the range of serum concentrations, is able to inhibit adipogenesis, but higher doses, at least 10 μM, are needed to reduce fat accumulation in mature adipocytes.

1. Introduction

Overweight and obesity have become a public health problem in developed societies due to their high prevalence [1–3]. They cause numerous metabolic alterations and comorbidities such as insulin resistance, diabetes, dyslipidemia, and hypertension [4, 5]. Scientific research is constantly looking for new molecules which could be used as effective functional biomolecules in the fight against this disease and its comorbidities.

Among these molecules, flavonoids, a group of natural substances which have a variable phenolic structure and are found in fruits, vegetables, tea, and wine, have received a great deal of interest in recent years because they have been reported to have beneficial effects on health [6–9]. Quercetin, the most abundant flavonoid [10], is present in onions, broccoli, tomatoes, apples, and berries [11]. Its intake in the diet is higher than that of other polyphenols [12]. A wide range of biological effects, such as prevention of oxidation, inflammation, and cancer, have been attributed to this compound [13–16]. It has also been reported to improve diabetic status in animal models of either type 1 or type 2 diabetes [17]. With regard to obesity, data concerning its delipidating effect, as well as its mechanisms of action, are scarce.

In this context, the present study aimed to analyze the effect of low doses of quercetin on triacylglycerol accumulation in both maturing preadipocytes and mature adipocytes. In order to gain insight into the mechanisms underlying this

action, the influence on the expression of adipogenic markers, as well as on gene expression, and activity of enzymes involved in triacylglycerol metabolism were assessed.

It is important to underline that our main interest focused on concentrations of this polyphenol which were lower than those used in the reported studies (10 to 500 μM) [18–24], which are far from those reached by this molecule in plasma after oral ingestion [25–27].

2. Materials and Methods

2.1. Reagents. Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (BRL Life Technologies, Grand Island, NY). Quercetin was purchased from Sigma (St. Louis, MO, USA). Triacylglycerols (TG) were determined by Infinity Triglycerides reagent (Thermo Electron Corporation, Rockford, IL, USA) and protein concentrations of cell extracts were measured with BCA reagent (Thermo Scientific, Rockford, IL, USA). Commercial kits for analyzing free fatty acids and free glycerol were supplied by Roche and Sigma, respectively (Free Fatty Acids, Half Micro Test, Roche, Basilea, Sweden, and F6428, Sigma, St. Louis, MO, USA).

2.2. Experimental Design. 3T3-L1 preadipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10% foetal calf serum (FCS). Two days after confluence (day 0), the cells were stimulated to differentiate with DMEM containing 10% FCS, 10 μg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 μM of dexamethasone for 2 days. From day 4 onward, the differentiation medium was replaced by FBS/DMEM medium (10%) containing 0.2 μg/mL insulin. This medium was changed every two days until cells were harvested. All media contained 1% penicillin/streptomycin (10,000 U/mL), and the media for differentiation and maturation contained 1% (v/v) of biotin and pantothenic acid. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.3. Cell Treatment. For the treatment of maturing preadipocytes, cells grown in 6-well plates were incubated with quercetin, at 0.1, 0.5, 1, 2, 5, and 10 μM (diluted in 95% ethanol), during differentiation. In the case of the control group the same volume of the vehicle (ethanol 95%) was used. Media containing or not containing quercetin were changed every two days: on day 0, day 2, day 4, and day 6. On day 8, supernatant was collected and cells were used for TG determination and RNA extraction. Each experiment was performed 3 times.

In order to distinguish the effects of quercetin in early and late stages of adipogenesis additional cultures were carried out. To assess the effects on early stages, cells grown in 6-well plates were incubated with quercetin at doses of 1, 2, 5, and 10 μM (diluted in 95% ethanol) from day 0 to 60 hours after the induction of differentiation. The effects on late stages were assessed by incubating cells grown in 6-well plates, with the same doses of quercetin, from 60 hours after differentiation to day 8, as reported by Tang et al. [28]. In the case of the control group the same volume of the vehicle (ethanol 95%) was used. Incubation media containing or not containing quercetin were changed every two days. Supernatants were collected and cells were used for TG determination. Each experiment was performed 3 times.

For the treatment of mature adipocytes, cells grown in 6-well plates were incubated with quercetin, at 1, 2, 5, and 10 μM (diluted in 95% ethanol), on day 12 after differentiation (>90% of cells showed visible lipid droplets). In the case of the control group the same volume of the vehicle (ethanol 95%) was used. After 24 hours, supernatant was collected and cells were used for TG determination and RNA extraction. Each experiment was performed 3 times.

2.4. Measurement of Triacylglycerol Content in Adipocytes. After treatment, the medium was removed and cell extracts were used for TG determination. Maturing preadipocytes and mature adipocytes were washed extensively with phosphate-buffered saline (PBS) and incubated 3 times with 800 μL of hexane/isopropanol (2 : 1). The total volume was then evaporated by vacuumed centrifugation and the pellet was resuspended in 200 μL Tritón X-100 in 1% distilled water. Afterwards, TGs were disrupted by sonication and the content was measured by means of a commercial kit. For protein determinations, cells were lysed in 0.3 N NaOH, 0.1% SDS. Protein measurements were performed using the BCA reagent. TG content values were obtained as mg triacylglycerols/mg protein and converted into arbitrary units.

2.5. RNA Preparation and Quantitative Real Time PCR. RNA samples from cells treated were extracted using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using a RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with DNase I kit (Applied Biosystems, California, USA) to remove any contamination with genomic DNA.

1.5 μg of total RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Relative CCAAT-enhancer-binding proteins α and β (C/EBPα and C/EBPβ), peroxisome proliferator-activated receptor γ (PPARγ), lipoprotein lipase (LPL), and sterol regulatory element-binding factor 1c (SREBF1C) mRNA levels in maturing preadipocytes and relative adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), lipoprotein lipase (LPL), fatty acid synthase (FASN), acetyl CoA carboxylase (ACC), deacetylase sirtuin 1 (SIRT1), leptin, adiponectin, visfatin, and apelin mRNA levels in mature adipocytes were quantified using real time PCR with an iCycler-MyiQ Real Time PCR Detection System (BioRad, Hercules, CA, USA). β-actin mRNA levels were similarly measured and served as the reference gene. The PCR reagent mixture consisted of 4.75 μL aliquot of each diluted cDNA, SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), and the upstream and downstream primers (300 nM each, except in the case of CEBPα and apelin, whose primer concentration was 600 nM, and ATGL whose primer concentration was 900 nM). In the case of CEBP β, SIRT-1, and visfatin the reagent mixture consisted of 1 μL of each cDNA, Premix Ex

TaqTM (Takara, USA), and the upstream and downstream primers (600 nM for CEBP β, visfatin, and β-actin and 900 nM for SIRT-1). Specific primers and probes were synthesized commercially (Tib Molbiol, Berlin, Germany, and Eurogentec, Liège, Belgium) (Table 1).

RT-PCR parameters were as follows: initial 2 min at 50°C, denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s (except in the case of LPL, PPARγ, and CEBPα in maturing preadipocytes where the annealing was at 62.1°C, 63.9°C, and 66.4°C, resp., and leptin in mature adipocytes where the annealing was at 63.9°C and 66.4°C, resp.), and extension at 60°C for 30 s. All sample mRNA levels were normalized to the values of β-actin and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [29].

2.6. Western Blot Analysis. Total proteins were isolated from maturing 3T3-L1 adipocytes using the modified Trizol (Invitrogen, Carlsbad, CA, USA) method [30], and the protein concentration was determined by BCA assay (Pierce, USA). Total protein (70 μg) was subjected to 7.5% SDS-polyacrylamide gel, electroblotted onto PVDF membranes (Millipore, Bradford, MA, USA), and immunodetected using mouse anti-SREBP1 (1 : 1000), mouse anti-PPARγ (1 : 1000), mouse anti-β-actin (1 : 5000) (Santa-Cruz Biotech, CA, USA), and goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (1 : 5000) (Santa-Cruz Biotech, CA, USA) with Chemi-Doc MP imaging system (BioRad, CA, USA).

2.7. Enzymatic Activity. Lipoprotein lipase enzyme activity was assessed following the method described by del Prado et al. [31] with modifications. For total lipoprotein lipase (LPL) activity determination, 130 μg of protein homogenate (0.3 N NaOH, 0.1% SDS) was incubated (15 min, 37°C) with a 2.9 mL of a buffer containing 1.5 mL of dibutyryl fluorescein (20 μM), 150 μL of 2-methoxyethanol, and 1.25 mL of phosphate buffer (3 mM NaH_2PO_4 and 50 mM Na_2HPO_4, with or without 2.5 M NaCl). Subsequently the reaction was halted in ice. Finally, fluorescence was measured. Total LPL activity was calculated by subtracting non-LPL lipolytic activity in the presence of NaCl from the total lipolytic activity, determined without NaCl, and expressed as *p*mol oleate released per minute per μg of protein.

FAS activity was measured by a spectrophotometer at 340 nm of NADPH absorption. The overall reaction system contained 100 μL of 1 M KH_2PO_4, 50 μL of 50 mM EDTA, 100 μL of cysteine 100 mM, 50 μL of bovine serum albumin 6 mg/mL, 50 μL of 1.2 mM acetyl-CoA, 20 μL of 10 μM malonyl-CoA, 65 μL of 2.4 mM NADPH, and 150 μg of protein homogenate (0.3 N NaOH, 0.1% SDS pH 7.4) in a total volume of 1.75 mL as previously described [32].

2.8. Measurements of Glycerol and Free Fatty Acids in the Media. After treatment in mature adipocytes, aliquots of the medium treated with 10 μM of quercetin were removed and analyzed for glycerol and free fatty acid (FFA) quantification by using commercial kits. Results were expressed as μg glycerol/mg protein and nmol FFA/mg protein.

2.9. Statistical Analysis. Results are presented as mean ± standard error of the mean. Statistical analysis was performed using SPSS 20.0 (SPSS Inc. Chicago, IL, USA). Comparisons between each treatment and the controls were analyzed by Student's t-test. Statistical significance was set up at the $P < 0.05$ level.

3. Results

3.1. Effect of Quercetin on Triacylglycerol Content in Maturing Preadipocytes. When cells were treated with quercetin from day 0 to day 8 (whole adipogenesis), triacylglycerol content was significantly reduced by all the doses used, with the exception of 0.1 μM which only showed a tendency towards lower values (Table 2). The greatest percentages of reduction in triacylglycerol content were obtained with 1 and 10 μM (−42% and −37%, resp.). When cultures were carried out at early and late stages by using these doses, it was observed that 1 μM of quercetin tended to reduce TG accumulation during the early stages of adipogenesis, but not during the late stages (Table 2). By contrast, 10 μM of quercetin was only able to decrease TG content in the late stage of differentiation (Table 2).

3.2. Effect of Quercetin on Adipogenic Gene Expression. For this purpose, 1 and 10 μM of quercetin were selected because they induced the greatest delipidating effects. The dose of 1 μM significantly reduced C/EBPβ and PPARγ expression (Figure 1(a)). The dose of 10 μM significantly reduced the expression of PPARγ, SREBF1c, and LPL (Figure 1(a)).

3.3. Effect of Quercetin on Adipogenic Protein Expression. The dose of 1 μM of quercetin did not modify the protein levels of PPARγ_1 and PPARγ_2, but it did induce a decrease in protein expression of mature SREBP1 (68 kDa) (Figure 1(b)). The dose of 10 μM significantly reduced protein expression of mature SREBP1 and PPARγ_2, but not that of PPARγ_1 (Figure 1(b)).

3.4. Effect of Quercetin on Triacylglycerol Content in Mature Adipocytes. When mature adipocytes were treated with 1 μM of quercetin no effects on TG content were observed. The doses of 2 and 5 μM induced reductions that did not reach statistical significance, and only the highest dose (10 μM) showed a significant effect (Table 2).

3.5. Effect of Quercetin on the Expression of Genes Involved in Triacylglycerol Metabolism in Mature Adipocytes. In mature adipocytes, gene expression was analyzed only in cells treated with 10 μM of quercetin because this dose was the only effective one. With regard to lipolytic enzymes, HSL expression was significantly reduced and ATGL remained unchanged (Figure 2). FASN expression was significantly reduced, but no changes were observed in ACC and LPL (Figure 3(a)).

Table 1: Primers for PCR amplification of each studied gene.

	Sense primer	Antisense primer	Probe
SYBR Green RT-PCR:			
LPL	5′-CAG CTG GGC CTAACT TTG AG-3′	5′-CCT CTC TGC AAT CAC ACG AA-3′	
PPARγ	5′-ATT CTG GCC CAC CAA CTT CGG-3′	5′-TGG AAG CCT GAT GCT TTA TCC CCA-3′	
SREBP1c	5′-AAA TCT TGC TGC CAT TCG-3′	5′-TTG ATC CCG GAA GCT CTG TG-3′	
CEBPα	5′-TTC CTC CGG CTA AGA CTT AGG C-3′	5′-CAG GGG TGT GTG TAT GAA CTG G-3′	
ATGL	5′-CAC TTT AGC TCC AAG GAT GA-3′	5′-TGG TTC AGT AGG CCA TTC CT-3′	
HSL	5′-GGT GAC ACT CGC AGA AGA CAA TA-3′	5′-GCC GCC GTG CTG TCT CT-3′	
FASN	5′-AGC CCC TCA AGT GCA CAG TG-3′	5′-TGC CAA TGT GTT TTC CCT GA-3′	
ACC	5′-GGA CCA CTG CAT GGA ATG TTA A-3′	5′-TGA GTG ACT GCC GAA ACA TCT C-3′	
PGC1α	5′-CCA AAG CTG AAG CCC TCT TGC-3′	5′-GTT TAG TCT TCC TTT CCT CGT GTC C-3′	
Leptin	5′-TGG ACC AGA CTC TGG CAG TC-3′	5′-AGG ACA CCA TCC AGG CTC TC-3′	
Adiponectin	5′-TG TAG GAT TGT CAG TGG ATC TG-3′	5′-GCT CTT CAG TTG TAG TAA CGT CAT C-3′	
Apelin	5′-ATT TAA GGA CAC GCT GAT CAA AGG-3′	5′-AGT CCC GAA AGT ATT CAA AAG CAG-3′	
β-actin	5′-ACG AGG CCC AGA GCA AGA G-3′	5′-GGT GTG GTG CCA GAT CTT CTC-3′	
TaqMan RT-PCR:			
CEBPβ	5′-GAG CGA CGA GTA CAA GAT GCG-3′	5′-GCT GCT CCA CCT TCT TCT GC-3′	5′-FAM-TCG TTC TCC GCC GTC AGC TCC AGC-TAMRA-3′
SIRT-1	5′-GAC GAC GAG GGC GAG GAG-3′	5′-ACA GGA GGT TGT CTC GGT AGC-3′	5′-FAM-CTG CCG CCG CCG CTG CCG-TAMRA-3′
Visfatin	5′-CCG GCC CGA GAT GAA TGC-3′	5′-GGA ATA AAC TTT GCT TGT GTT GGG-3′	5′-FAM-AGC CGA GTT CAA CAT CCT GCT GGC-TAMRA-3′
β-actin	5′-TCT ATG AGG GCT ACG CTC TCC-3′	5′-CAC GCT CGG TCA GGA TCT TC-3′	5′-FAM-CCT GCG TCT GGA CCT GGC TGG C-TAMRA-3′

LPL = lipoprotein lipase, PPARγ = peroxisome proliferator-activated receptor, SREBP = sterol regulatory element-binding protein, C/EBPα and C/EBPβ = CCAAT-enhancer-binding proteins alpha and beta, ATGL = adipose triglyceride lipase, HSL = hormone sensitive lipase, FASN = fatty acid synthase, ACC = acetyl-CoA carboxylase, PGC-1 α = peroxisome proliferator-activated receptor gamma coactivator, and SIRT1 = deacetylase sirtuin 1.

Table 2: Relative triacylglycerol content (arbitrary units) after quercetin treatment in maturing preadipocytes and mature adipocytes.

	C	Q0.1	*P*	Q0.5	*P*	Q1	*P*	Q2	*P*	Q5	*P*	Q10	*P*
Stage of adipogenesis													
Whole adipogenesis (d0–d8)	100 ± 5.1	77.95 ± 12.8	0.09	75.57 ± 8.6	<0.01	58.4 ± 8.6	<0.001	81.3 ± 7.4	<0.05	79.6 ± 7.2	<0.05	62.9 ± 10.2	<0.01
Early stage (day 0–60 hours)	100 ± 6.9					86.0 ± 5.7	0.09					95.3 ± 7.3	ND
Late stage (60 hours–d8)						92.2 ± 5.6	ND					75.9 ± 10.5	<0.05
Mature adipocytes (d12 for 24 h)	100 ± 6.3					109.3 ± 34.0	ND	86.2 ± 3.4	0.07	79.3 ± 12.8	0.10	76.3 ± 4.8	<0.01

C = control; ND = no difference, Q0.1 = 0.1 μM of quercetin, Q0.5 = 0.5 μM of quercetin, Q1 = 1 μM of quercetin, Q2 = 2 μM of quercetin, Q5 = 5 μM of quercetin, and Q10 = 10 μM of quercetin.
Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The difference was established at $P \leq 0.10$.

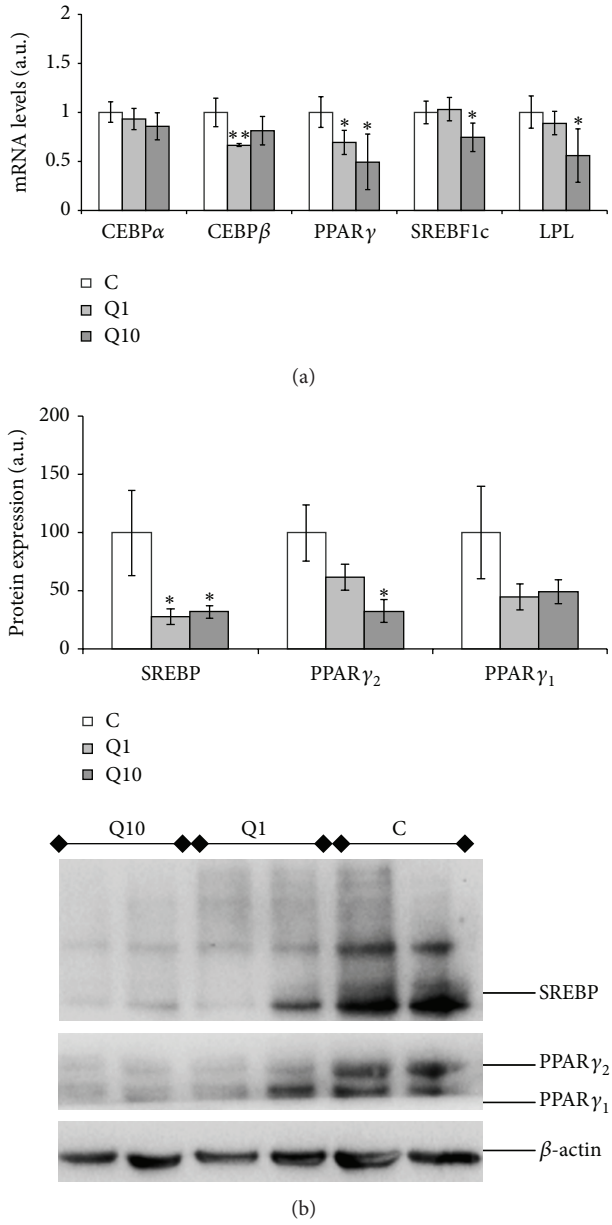

Figure 1: Effects of 1 and 10 μM of quercetin (Q1 and Q10) on gene expression of CEBPβ, CEBPα, PPARγ, SREBF1c, and LPL (a) and on protein expression of PPARγ_1, PPARγ_2, and SREBP1 (b) in 3T3-L1 maturing preadipocytes treated from day 0 to day 8. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's t-test. The asterisks represent differences versus the controls ($^{*}P < 0.05$; $^{**}P < 0.01$).

When SIRT1 expression was measured, 10 μM of quercetin increased SIRT1 mRNA levels (Figure 4).

3.6. Effect of Quercetin on Lipoprotein Lipase and Fatty Acid Synthase Activities in Mature Adipocytes. In order to find potential changes in LPL at a posttranscriptional level, the activity of this enzyme was measured in mature cells treated with 10 μM of quercetin and no changes were observed (0.90 ± 0.14 $p$mol fluorescein/μg protein/min in the case of the control group and 1.15 ± 0.23 $p$mol oleate/μg protein/min in the case of the group treated with quercetin at 10 μM; $P = 0.38$).

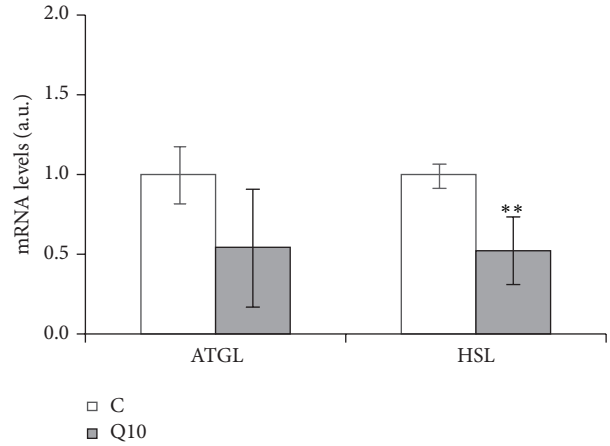

Figure 2: Effects of 10 μM of quercetin (Q10) on the gene expression of lipases, ATGL and HSL, in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's t-test. The asterisks represent differences versus the controls ($^{**}P < 0.01$).

The dose of 10 μM of quercetin had an inhibitory effect on FAS activity. This result is in line with the reduced gene expression of this enzyme (Figure 3(b)).

3.7. Effect of Quercetin on Glycerol and Free Fatty Acid Release in Mature Adipocytes. In order to know if the decrease in HSL expression was accompanied by changes in the lipolytic pathway, glycerol and free fatty acids were quantified in the incubation media, as markers of lipolysis. Both parameters remained unchanged after treatment of cells with 10 μM of quercetin (65.3 ± 7.5 versus 60.6 ± 14.9 nmol FFA/mg protein and 393.05 ± 10.1 versus 429.16 ± 31.6 μg glycerol/mg protein for control and 10 μM quercetin-treated cells, resp.).

3.8. Effect of Quercetin on Adipokine Gene Expression in Mature Adipocytes. No changes were observed in gene expression of adiponectin, leptin, visfatin, and apelin in mature adipocytes treated with 10 μM of quercetin (Figure 5).

4. Discussion

In recent years, a great number of studies have been conducted in the field of natural compounds in order to find new tools to combat obesity. In this line, one of the most studied molecules has been resveratrol, a polyphenol (stilbene) present in grapes and wine, which has shown a clear antiobesity effect in cultured cells [33–41] and in animal models [40, 42–48]. The effects of flavonoids, which show interesting biological effects on cancer and diabetes [7, 9], on obesity prevention or treatment have not been so widely analyzed [49]. The present study focuses on quercetin, the most abundant flavonoid in food stuffs [11].

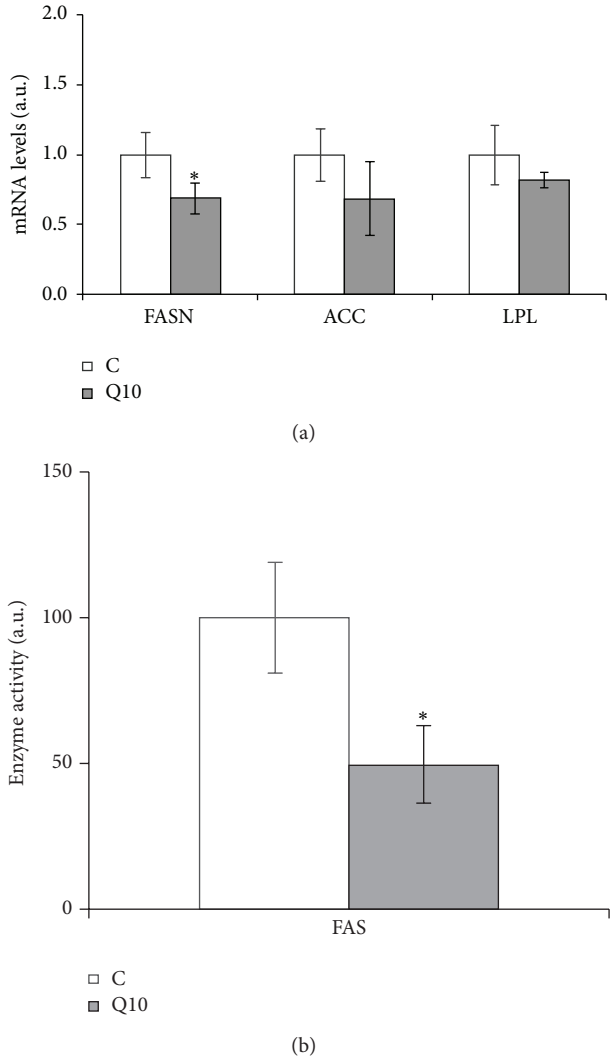

Figure 3: Effects of 10 μM of quercetin (Q10) on the gene expression of FASN, ACC, and LPL (a) and on the activity of FAS enzyme (b), in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's t-test. The asterisks represent differences versus the controls ($^*P < 0.05$).

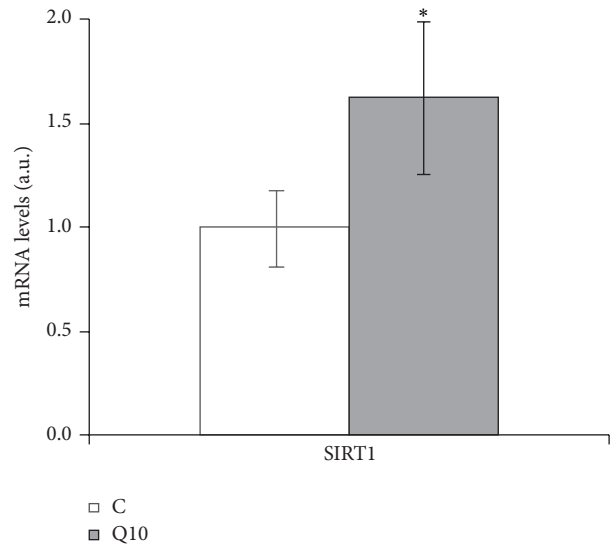

Figure 4: Effects of 10 μM of quercetin (Q10) on the gene expression of SIRT1 in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's t-test. The asterisk represents differences versus the controls ($^*P < 0.05$).

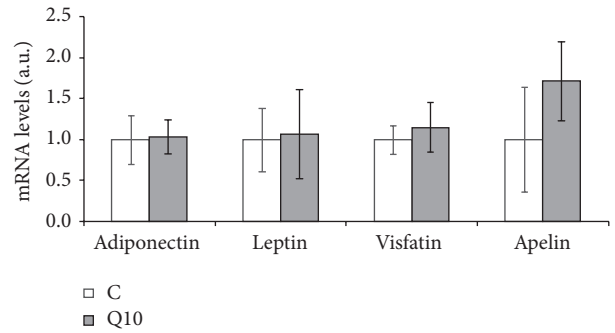

Figure 5: Effects of 10 μM of quercetin (Q10) on the gene expression of adiponectin, leptin, visfatin, and apelin in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's t-test.

To date, there are only few studies in the literature demonstrating that quercetin reduces triacylglycerol accumulation in cultured adipocytes and animal models, and very little has been reported concerning the mechanisms of action [18–23, 50]. One advantage of *in vitro* studies is that they allow one to clearly differentiate between the effects of a molecule on preadipocytes (and thus on adipogenesis) and on mature adipocytes. This is the reason for choosing this experimental model for our study.

It is important to point out that previously reported *in vitro* studies have been performed by using high doses of quercetin (10 to 500 μM). These doses are far from those achieved in plasma in humans and animals. Thus, in the present study we aimed to complete the information provided in the abovementioned studies by analyzing the effects of quercetin on cultured adipocytes at lower doses. In fact doses from 0.1 to 2 μM are in the range of quercetin concentrations found in plasma in several studies performed either in rodents or in humans [26, 27, 51, 52].

Quercetin has been demonstrated to exert genotoxicity and mutagenicity in *in vitro* experiments. Nevertheless, according to the critical examination of quercetin safety carried out by Harwood et al. [27], 34 μM was the lowest dose to show this effect in mouse cells, specifically in mouse lymphoma L5178Y cells [53]. Thus, we assumed that the doses used in the present study did not exert this toxic effect.

All the quercetin doses tested in the present study (0.1–10 μM) significantly reduced TG content in 3T3-L1 maturing preadipocytes, with the exception of 0.1 μM which only showed a tendency towards lower values (8 days of treatment after the confluence). In the experiment reported by

Ahn et al. [20] in 3T3-L1 preadipocytes, cells were treated with 10, 50, and 100 μM of quercetin during differentiation. The three doses of this flavonoid reduced TG content. Data concerning 10 μM of quercetin are in good accordance with the present study. Yang et al. [21] used 12.5 μM and 25 μM of quercetin for 3T3-L1 preadipocyte incubation, and only the highest dose was effective in TG reduction. The difference between the present study and that reported by Yang et al. could be the treatment duration (from day 0 to day 8 in the present study and from day 0 to day 6 in the study reported by Yang et al.). The effect of 5 μM of quercetin is also in the same line as that reported by Bae et al. (2014) [54] by using low doses of quercetin (3.3–6.6 μM). With regard to the lowest doses used in the present study (0.1, 0.5, 1, and 2 μM), as far as we know, this is the first time that they have been used and thus comparisons with the literature cannot be made.

To compare the delipidating effect of quercetin during adipogenesis with that observed with other polyphenols is a matter of interest. In a recent study performed in our laboratory we analyzed the delipidating effect of resveratrol at 1, 10, and 25 μM, in maturing preadipocytes by using the same experimental conditions [37]. No changes were observed in TG content at 1 and 10 μM, but the polyphenol was effective at 25 μM. These results show that quercetin is more efficient in reducing adipogenesis than resveratrol is. There are very few studies where low concentrations of other flavonoids have been tested in 3T3-L1 maturing preadipocytes. Yang et al. showed that 3.125, 6.25, and 12.5 μM of xanthohumol and isoxanthohumol, flavonoids present in hops, reduced TG accumulation during adipocyte differentiation [55]. Nevertheless, other authors have not found this antiadipogenic effect in the case of other flavonoids (naringenin) or have stated that higher doses are needed to reach this effect (genistein) [22, 56, 57].

Two phases can be distinguished in adipogenesis, the premitotic phase (early stage of differentiation; 60 hours after confluence) [28] and the postmitotic phase (late stage). During the early phase C/EBPβ and SREBP1c expressions are increased. This change, in turn, triggers high level expression of PPARγ, which is considered the master coordinator of adipocyte differentiation. C/EBPα and LPL are induced during later stage of differentiation [58]. Interestingly, when the influence of quercetin on the expression of these genes was assessed it was observed that, depending on the dose, the mechanisms of action of this flavonoid to reduce adipogenesis were different (Figure 6). Quercetin, at a dose of 1 μM, significantly reduced C/EBPβ and PPARγ gene expressions, but PPARγ_1 and PPARγ_2 protein levels remained unchanged. Moreover, a reduction in protein expression of mature SREBP1c was observed. As stated before, C/EBPβ and SREBP1c are adipogenic markers expressed in the early stage of the process [28]. Taken together these results suggest that adipogenesis was stopped at this phase. This fact was confirmed by the reduction in TG content (tendency) observed after 60 hours of treatment, but not when preadipocytes were treated from 60 hours to 8 days.

Treatment with 10 μM of quercetin reduced both gene and protein expressions of PPARγ and SREBP1c, as well as LPL gene expression. These results are in good accordance

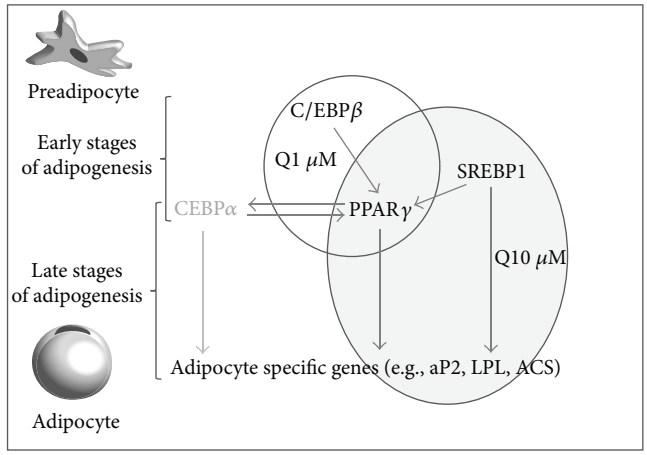

Figure 6: Effects of different doses of quercetin in the pathways of the adipogenic process.

with those reported by other authors [20, 21] who showed that 10 and 25 μM of quercetin reduced PPARγ and SREBP1c expression. Although SREBP1c expression starts at early stages of differentiation, protein expression of mature SREBP reaches its peak at the 5th-6th day after the confluence [59]. For this reason SREBP1c is also considered an important factor in the late stages of adipogenesis, as is the case of PPARγ. The reduction in PPARγ and SREBP1c expression after treatment with 10 μM of quercetin, together with the strong decrease in TG content observed when maturing adipocytes were treated with this dose of quercetin from 60 hours to 8th day after the confluence, suggests that this dose acts primarily at the late stage of adipogenesis.

When the effects on mature adipocytes were assessed, quercetin significantly reduced TGs at the dose of 10 μM. These results are in the same line as those obtained by Park et al. by incubating murine adipocytes, but with a higher dose (25 μM) of this flavonoid [22]. In our previous study, where mature adipocytes were treated with 1, 10, and 25 μM of resveratrol during 24 hours [37], the lowest dose of this polyphenol resulted in a significant reduction in TG content. By comparing the present data with those of this study, it can be suggested that quercetin is less efficient than resveratrol when delipidating mature adipocytes. The effect of other flavonoids at low doses on mature adipocytes has not been widely analyzed. Reported studies show that doses around 100 μM are needed in the case of naringenin and genistein to reach a reduction in TG content [56].

The expression of genes involved in TG metabolism in mature adipocytes was analyzed by real time RT-PCR after 24 hours of treatment. Quercetin reduced HSL expression but did not change that of ATGL. Taking into account that HSL is an enzyme regulated mainly at posttranscriptional level and considering that a reduction in the expression of a lipolytic enzyme is not an expected result for a delipidating molecule, the release of glycerol and free fatty acids to the incubation medium was quantified as an index of lipolytic activity. No changes were observed in treated cells when compared with

the controls, and thus a lack of effect of quercetin in HSL activity could be suggested, despite its downregulation.

Gene expression of fatty acid synthase, as well as the activity of this enzyme, was lower after quercetin treatment, suggesting that the delipidating effect of this molecule could be due to its inhibitory effect on *de novo* lipogenesis. When the expression of LPL, the enzyme which allows adipose tissue to uptake free fatty acids from TGs circulating in lipoproteins, was measured, no changes were observed in quercetin-treated mature adipocytes. This is a surprising result because as quercetin reduced LPL mRNA expression in maturing preadipocytes, a similar effect could be expected in mature adipocytes. Nevertheless, it is important to highlight that LPL is controlled not only transcriptionally but also posttranscriptionally [60]. Given this, in order to find potential changes in LPL at a posttranscriptional level the activity of this enzyme was measured in mature adipocytes treated with 10 μM of quercetin. No changes were observed and thus it may be suggested that the metabolic process controlled by this enzyme was not affected by the flavonoid.

In previous studies performed in our laboratory, incubation with 10 μM resveratrol increased ATGL expression and reduced FASN gene expression [37, 41]. These data suggest that while 10 μM of quercetin exerts its delipidating effect via inhibition of adipogenesis and lipogenesis, resveratrol acts by increasing lipolysis and inhibiting lipogenesis.

It has been reported that several beneficial effects of polyphenols are mediated by the deacetylase SIRT1 [61]. In the present study the incubation of mature adipocytes with 10 μM of quercetin led to increased SIRT1 expression. SIRT1 can induce SREBP1c deacetylation, which leads to the inactivation of this transcription factor and thus to decreased expression of lipogenic enzymes [62]. Taking this into account, it can be proposed that the increased expression of SIRT1 observed in mature adipocytes in the present study could be related to the reduced expression of FASN.

In relation to glycaemic control, flavonoids have been reported to improve insulin sensitivity by modifying adipokines secretion. Specifically, quercetin has been demonstrated to inhibit visfatin secretion in SGBS human adipocytes or to increase adiponectin secretion in rats fed a high fat diet [23, 63]. As far as we know, the effect of low doses of quercetin on adipokine expression and secretion has not been analyzed to date. In the present study no changes were observed in adipokine gene expression, suggesting that low doses of this flavonoid do not affect these mediators of insulin signalling and glycaemic control.

It has been reported that quercetin is able to reduce resveratrol metabolism; more specifically it can decrease the formation of sulfate metabolites [64]. Thus, the combination of quercetin and resveratrol has been proposed as a tool to increase the low bioavailability of resveratrol and consequently its effectiveness. In view of the results obtained in the present study, it can be proposed that the combination of quercetin and resveratrol could be more effective, in terms of body fat reduction, than the administration of these molecules separately. This proposal is justified not only by the effects on resveratrol bioavailability, as proposed in the literature, but also by the fact that at 1 μM (a dose close to quercetin and resveratrol serum concentrations found in *in vivo* studies) quercetin is more effective in reducing adipogenesis in preadipocytes whereas resveratrol is more effective in inhibiting lipid metabolism in mature adipocytes. Thus, the combination of these two molecules can induce body fat reduction more effectively by targeting both cell types, preadipocytes and adipocytes, at the same time.

Taking all the data presented into account, it can be concluded that doses of quercetin in the range of serum concentrations are able to inhibit adipogenesis, but higher doses, at least 10 μM, are needed to reduce fat accumulation in mature adipocytes. In the case of maturing preadipocytes, 1 μM of quercetin exerts its antiadipogenic effect at the early stages of adipogenesis, whereas 10 μM of quercetin acts at the later stages.

Disclaimer

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Conflict of Interests

The authors have declared no conflict of interests.

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References

[1] H.-R. Berthoud, "Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance," *Obesity*, vol. 14, supplement 8, pp. 197S–200S, 2006.

[2] C. Pérez-Rodrigo, J. A. Bartrina, L. S. Majem, B. Moreno, and A. D. Rubio, "Epidemiology of obesity in Spain. Dietary guidelines and strategies for prevention," *International Journal for Vitamin and Nutrition Research*, vol. 76, no. 4, pp. 163–171, 2006.

[3] B. Rokholm, J. L. Baker, and T. I. A. Sørensen, "The levelling off of the obesity epidemic since the year 1999—a review of evidence and perspectives," *Obesity Reviews*, vol. 11, no. 12, pp. 835–846, 2010.

[4] A. H. Mokdad, E. S. Ford, B. A. Bowman et al., "Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001," *The Journal of the American Medical Association*, vol. 289, no. 1, pp. 76–79, 2003.

[5] S. Allender and M. Rayner, "The burden of overweight and obesity-related ill health in the UK," *Obesity Reviews*, vol. 8, no. 5, pp. 467–473, 2007.

[6] D. Grassi, G. Desideri, P. D. Giosia et al., "Tea, flavonoids, and cardiovascular health: endothelial protection," *The American Journal of Clinical Nutrition*, vol. 98, no. 6, 2013.

[7] P. V. A. Babu, D. Liu, and E. R. Gilbert, "Recent advances in understanding the anti-diabetic actions of dietary flavonoids," *Journal of Nutritional Biochemistry*, vol. 24, no. 11, pp. 1777–1789, 2013.

[8] D. Vauzour, K. Vafeiadou, A. Rodriguez-Mateos, C. Rendeiro, and J. P. E. Spencer, "The neuroprotective potential of flavonoids: a multiplicity of effects," *Genes and Nutrition*, vol. 3, no. 3-4, pp. 115–126, 2008.

[9] D. F. Romagnolo and O. I. Selmin, "Flavonoids and cancer prevention: a review of the evidence," *Journal of Nutrition in Gerontology and Geriatrics*, vol. 31, no. 3, pp. 206–238, 2012.

[10] S. M. Somerset and L. Johannot, "Dietary flavonoid sources in Australian adults," *Nutrition and Cancer*, vol. 60, no. 4, pp. 442–449, 2008.

[11] A. R. Mangels, J. M. Holden, G. R. Beecher et al., "Carotenoid content of fruits and vegetables: an evaluation of analytic data," *Journal of the American Dietetic Association*, vol. 93, no. 3, pp. 284–296, 1993.

[12] J. Cao, Y. Zhang, W. Chen, and X. Zhao, "The relationship between fasting plasma concentrations of selected flavonoids and their ordinary dietary intake," *British Journal of Nutrition*, vol. 103, no. 2, pp. 249–255, 2010.

[13] S. C. Bischoff, "Quercetin: potentials in the prevention and therapy of disease," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 11, no. 6, pp. 733–740, 2008.

[14] M. Zhang, S. G. Swarts, L. Yin et al., "Antioxidant properties of quercetin," *Advances in Experimental Medicine and Biology*, vol. 701, pp. 283–289, 2011.

[15] S. Chirumbolo, "The role of quercetin, flavonols and flavones in modulating inflammatory cell function," *Inflammation and Allergy—Drug Targets*, vol. 9, no. 4, pp. 263–285, 2010.

[16] C. H. Jung, I. Cho, J. Ahn, T.-I. Jeon, and T.-Y. Ha, "Quercetin reduces high-fat diet-induced fat accumulation in the liver by regulating lipid metabolism genes," *Phytotherapy Research*, vol. 27, no. 1, pp. 139–143, 2013.

[17] L. Aguirre, N. Arias, M. T. Macarulla, A. Gracia, and M. P. Portillo, "Beneficial effects of quercetin on obesity and diabetes," *The Open Nutraceuticals Journal*, vol. 4, pp. 189–198, 2011.

[18] T. Motoyashiki, T. Morita, and H. Ueki, "Involvement of the rapid increase in cAMP content in the vanadate-stimulated release of lipoprotein lipase activity from rat fat pads," *Biological and Pharmaceutical Bulletin*, vol. 19, no. 11, pp. 1412–1416, 1996.

[19] C. L. Hsu and G. C. Yen, "Induction of cell apoptosis in 3T3-L1 pre-adipocytes by flavonoids is associated with their antioxidant activity," *Molecular Nutrition and Food Research*, vol. 50, no. 11, pp. 1072–1079, 2006.

[20] J. Ahn, H. Lee, S. Kim, J. Park, and T. Ha, "The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways," *Biochemical and Biophysical Research Communications*, vol. 373, no. 4, pp. 545–549, 2008.

[21] J.-Y. Yang, M. A. Della-Fera, S. Rayalam et al., "Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin," *Life Sciences*, vol. 82, no. 19-20, pp. 1032–1039, 2008.

[22] H. J. Park, J. Y. Yang, S. Ambati et al., "Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes," *Journal of Medicinal Food*, vol. 11, no. 4, pp. 773–783, 2008.

[23] C. S. Derdemezis, D. N. Kiortsis, V. Tsimihodimos et al., "Effect of plant polyphenols on adipokine secretion from human SGBS adipocytes," *Biochemistry Research International*, vol. 2011, Article ID 285618, 5 pages, 2011.

[24] S. L. Huang, C. L. Hsu, and G. C. Yen, "Growth inhibitory effect of quercetin on SW 872 human liposarcoma cells," *Life Sciences*, vol. 79, no. 2, pp. 203–209, 2006.

[25] M. Reinboth, S. Wolffram, G. Abraham, F. R. Ungemach, and R. Cermak, "Oral bioavailability of quercetin from different quercetin glycosides in dogs," *British Journal of Nutrition*, vol. 104, no. 2, pp. 198–203, 2010.

[26] M. Tamura, H. Suzuki, and K. Shinohara, "Effects of intestinal microflora on the bioavailability of dietary quercetin in adult mice," *Food Science and Technology Research*, vol. 6, no. 4, pp. 291–293, 2000.

[27] M. Harwood, B. Danielewska-Nikiel, J. F. Borzelleca, G. W. Flamm, G. M. Williams, and T. C. Lines, "A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties," *Food and Chemical Toxicology*, vol. 45, no. 11, pp. 2179–2205, 2007.

[28] Q.-Q. Tang, T. C. Otto, and M. D. Lane, "Mitotic clonal expansion: a synchronous process required for adipogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 1, pp. 44–49, 2003.

[29] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[30] A. E. S. Simões, D. M. Pereira, J. D. Amaral et al., "Efficient recovery of proteins from multiple source samples after TRIzol or TRIzol LS RNA extraction and long-term storage," *BMC Genomics*, vol. 14, no. 1, article 181, 2013.

[31] M. del Prado, H. Hernández-Montes, and S. Villalpando, "Characterization of a fluorometric method for lipoprotein lipase," *Archives of Medical Research*, vol. 25, no. 3, pp. 331–335, 1994.

[32] F. Lynen, "[3] Yeast fatty acid synthase," *Methods in Enzymology*, vol. 14, pp. 17–33, 1969.

[33] F. Picard, M. Kurtev, N. Chung et al., "Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ," *Nature*, vol. 429, no. 6993, pp. 771–776, 2004.

[34] S. Rayalam, J.-Y. Yang, S. Ambati, M. A. Della-Fera, and C. A. Baile, "Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes," *Phytotherapy Research*, vol. 22, no. 10, pp. 1367–1371, 2008.

[35] L. Bai, W.-J. Pang, Y.-J. Yang, and G.-S. Yang, "Modulation of Sirt1 by resveratrol and nicotinamide alters proliferation and differentiation of pig preadipocytes," *Molecular and Cellular Biochemistry*, vol. 307, no. 1-2, pp. 129–140, 2008.

[36] S. Chen, Z. Li, W. Li, Z. Shan, and W. Zhu, "Resveratrol inhibits cell differentiation in 3T3-L1 adipocytes via activation of AMPK," *Canadian Journal of Physiology and Pharmacology*, vol. 89, no. 11, pp. 793–799, 2011.

[37] A. Lasa, I. Churruca, I. Eseberri, C. Andrés-Lacueva, and M. P. Portillo, "Delipidating effect of resveratrol metabolites in 3T3-L1 adipocytes," *Molecular Nutrition and Food Research*, vol. 56, no. 10, pp. 1559–1568, 2012.

[38] I. Eseberri, A. Lasa, I. Churruca, and M. P. Portillo, "Resveratrol metabolites modify adipokine expression and secretion in 3T3-L1 pre-adipocytes and mature adipocytes," *PLoS ONE*, vol. 8, no. 5, Article ID e63918, 2013.

[39] P. Hu, L. Zhao, and J. Chen, "Physiologically achievable doses of resveratrol enhance 3T3-L1 adipocyte differentiation," *European Journal of Nutrition*, 2014.

[40] L. Aguirre, A. Fernández-Quintela, N. Arias, and M. Portillo, "Resveratrol: anti-obesity mechanisms of action," *Molecules*, vol. 19, no. 11, pp. 18632–18655, 2014.

[41] A. Lasa, M. Schweiger, P. Kotzbeck et al., "Resveratrol regulates lipolysis via adipose triglyceride lipase," *Journal of Nutritional Biochemistry*, vol. 23, no. 4, pp. 379–384, 2012.

[42] M. Lagouge, C. Argmann, Z. Gerhart-Hines et al., "Resveratrol Improves Mitochondrial Function and Protects against Metabolic Disease by Activating SIRT1 and PGC-1α," *Cell*, vol. 127, no. 6, pp. 1109–1122, 2006.

[43] J. Shang, L. L. Chen, F. X. Xiao, H. Sun, H. C. Ding, and H. Xiao, "Resveratrol improves non-alcoholic fatty liver disease by activating AMP-activated protein kinase," *Acta Pharmacologica Sinica*, vol. 29, no. 6, pp. 698–706, 2008.

[44] L. Rivera, R. Morón, A. Zarzuelo, and M. Galisteo, "Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats," *Biochemical Pharmacology*, vol. 77, no. 6, pp. 1053–1063, 2009.

[45] M. T. Macarulla, G. Alberdi, S. Gómez et al., "Effects of different doses of resveratrol on body fat and serum parameters in rats fed a hypercaloric diet," *Journal of Physiology and Biochemistry*, vol. 65, no. 4, pp. 369–376, 2009.

[46] G. Alberdi, V. M. Rodríguez, J. Miranda et al., "Changes in white adipose tissue metabolism induced by resveratrol in rats," *Nutrition and Metabolism*, vol. 8, article 29, 2011.

[47] K. Nagao, T. Jinnouchi, S. Kai, and T. Yanagita, "Effect of dietary resveratrol on the metabolic profile of nutrients in obese OLETF rats," *Lipids in Health and Disease*, vol. 12, no. 1, article 8, 2013.

[48] S. J. Cho, U. J. Jung, and M. S. Choi, "Differential effects of low-dose resveratrol on adiposity and hepatic steatosis in diet-induced obese mice," *British Journal of Nutrition*, vol. 108, no. 12, pp. 2166–2175, 2012.

[49] S. Wang, N. Moustaid-Moussa, L. Chen et al., "Novel insights of dietary polyphenols and obesity," *Journal of Nutritional Biochemistry*, vol. 25, no. 1, pp. 1–18, 2014.

[50] U. R. Kuppusamy and N. P. Das, "Effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes," *Biochemical Pharmacology*, vol. 44, no. 7, pp. 1307–1315, 1992.

[51] M. Carbonaro and G. Grant, "Absorption of quercetin and rutin in rat small intestine," *Annals of Nutrition and Metabolism*, vol. 49, no. 3, pp. 178–182, 2005.

[52] J. A. Conquer, G. Maiani, E. Azzini, A. Raguzzini, and B. J. Holub, "Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects," *Journal of Nutrition*, vol. 128, no. 3, pp. 593–597, 1998.

[53] M. L. Meltz and J. T. MacGregor, "Activity of the plant flavanol quercetin in the mouse lymphoma L5178Y $TK^{+/-}$ mutation, DNA single-strand break, and Balb/c 3T3 chemical transformation assays," *Mutation Research/Genetic Toxicology*, vol. 88, no. 3, pp. 317–324, 1981.

[54] C.-R. Bae, Y.-K. Park, and Y.-S. Cha, "Quercetin-rich onion peel extract suppresses adipogenesis by down-regulating adipogenic transcription factors and gene expression in 3T3-L1 adipocytes," *Journal of the Science of Food and Agriculture*, vol. 94, no. 13, pp. 2655–2660, 2014.

[55] J. Y. Yang, M. A. Della-Fera, S. Rayalam, and C. A. Baile, "Effect of xanthohumol and isoxanthohumol on 3T3-L1 cell apoptosis and adipogenesis," *Apoptosis*, vol. 12, no. 11, pp. 1953–1963, 2007.

[56] A. W. Harmon and J. B. Harp, "Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis," *The American Journal of Physiology—Cell Physiology*, vol. 280, no. 4, pp. C807–C813, 2001.

[57] S. Rayalam, M. A. Della-Fera, J.-Y. Yang, J. P. Hea, S. Ambati, and C. A. Baile, "Resveratrol potentiates genistein's antiadipogenic and proapoptotic effects in 3T3-L1 adipocytes," *Journal of Nutrition*, vol. 137, no. 12, pp. 2668–2673, 2007.

[58] L. Fajas, J. C. Fruchart, and J. Auwerx, "Transcriptional control of adipogenesis," *Current Opinion in Cell Biology*, vol. 10, no. 2, pp. 165–173, 1998.

[59] P. Dowell, C. Flexner, P. O. Kwiterovich, and M. D. Lane, "Suppression of preadipocyte differentiation and promotion of adipocyte death by HIV protease inhibitors," *Journal of Biological Chemistry*, vol. 275, no. 52, pp. 41325–41332, 2000.

[60] H. Wang and R. H. Eckel, "Lipoprotein lipase: from gene to obesity," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 297, no. 2, pp. E271–E288, 2009.

[61] S. Chung, H. Yao, S. Caito, J.-W. Hwang, G. Arunachalam, and I. Rahman, "Regulation of SIRT1 in cellular functions: role of polyphenols," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 79–90, 2010.

[62] B. Ponugoti, D.-H. Kim, Z. Xiao et al., "SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism," *Journal of Biological Chemistry*, vol. 285, no. 44, pp. 33959–33970, 2010.

[63] S. Wein, N. Behm, R. K. Petersen, K. Kristiansen, and S. Wolffram, "Quercetin enhances adiponectin secretion by a PPAR-gamma independent mechanism," *European Journal of Pharmaceutical Sciences*, vol. 41, no. 1, pp. 16–22, 2010.

[64] C. de Santi, A. Pietrabissa, R. Spisni, F. Mosca, and G. M. Pacifici, "Sulphation of resveratrol, a natural compound present in wine, and its inhibition by natural flavonoids," *Xenobiotica*, vol. 30, no. 9, pp. 857–866, 2000.

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Research Article

Flaxseed Oil Containing α-Linolenic Acid Ester of Plant Sterol Improved Atherosclerosis in ApoE Deficient Mice

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Plant sterols (PS) have potential preventive function in atherosclerosis due to their cholesterol-lowering ability. Dietary α-linolenic acid in flaxseed oil is associated with a reduction in cardiovascular events through its hypolipidemic and anti-inflammation properties. This study was designed to evaluate the effects of flaxseed oil containing α-linolenic acid ester of PS (ALA-PS) on atherosclerosis and investigate the underlying mechanisms. C57BL/6 mice were administered a regular diet and apoE knockout (apoE-KO) mice were given a high fat diet alone or supplemented with 5% flaxseed oil with or without 3.3% ALA-PS for 18 weeks. Results demonstrated that flaxseed oil containing ALA-PS was synergistically interaction in ameliorating atherosclerosis as well as optimizing overall lipid levels, inhibiting inflammation and reducing oxidative stress. These data were associated with the modification effects on expression levels of genes involved in lipid metabolism (PPARα, HMGCR, and SREBPs), inflammation (IL-6, TNF, MCP-1, and VCAM-1), and oxidative stress (NADPH oxidase).

1. Introduction

Atherosclerosis is the primary cause of cardiovascular diseases (CVD), which is one of the leading causes of mortality and morbidity in the world [1]. Although the precise mechanisms for atherosclerosis have not been fully elucidated, an elevated concentration of low-density lipoprotein cholesterol (LDL-C) is a well-established independent risk factor for atherosclerosis [2]. In addition, LDL oxidation has been illustrated as one of the initial steps of atherogenesis [3]. Therefore, dietary and pharmacologic interventions targeted at lowering LDL-C are very important elements for atherosclerosis prevention.

The pathophysiological significance of plant sterols (PS), one kind of natural compounds presenting in plant oils, nuts, cereals, and legumes, has been intensively discussed in previous reports. It has been reported that consumption of 2 g/day PS could reduce LDL-C by 10–15% in about 90% of individuals [4]. Another previous publication also demonstrated that supplementation of PS in diet may have decreased LDL-C level (10%) for reducing risk of cardiovascular (10–20%) [5]. However, PS was not observed to have any beneficial effect on triacylglycerol (TG) and high-density lipoprotein cholesterol (HDL-C) [6], two important independent factors for cardiovascular disease [7, 8]. In addition, purified PS was shown to have low intestinal bioavailability and poor

solubility in diet. Because n-3 polyunsaturated fatty acids (PUFAs) have potent hypotriglyceridemic and HDL-C elevation effects, PS has been recently esterified by fish oil fatty acids to obtain final product plant sterols esters which have overall beneficial effects on lipids [9], well bioavailability, and extensive solubility in fat. Therefore, plant sterols esters can be incorporated into fatty foods, such as margarines, yoghurt, and spreads [10]. However, fish oil fatty acid is insufficient supply nutrient, and margarines, yoghurt, and spreads are not the main food in China. α-Linolenic acid (ALA, C18:3n-3), the major n-3 fatty acid, widely exists in flaxseed oil which is a traditional edible oil in China. As the precursor of longer chain n-3 PUFA (EPA and DHA), ALA had good effects on TG reduction and HDL-C elevation [11]. More importantly, a large number of epidemiological evidences showed that consumption of ALA could protect against CVD [12, 13]. These data suggest that ALA may be a perfect vehicle for PS esterification and the combination treatment may synergistically improve atherosclerosis. Meanwhile, as one of the high frequency and largely consumed oils in Chinese daily cooking, flaxseed oil is a good way for Chinese to get enough PS. However, the efficiency of this novel flaxseed oil fortified with α-linolenic acid ester of plant sterol (ALA-PS) has not been shown.

In the current study, we detected the effects of dietary flaxseed oil containing ALA-PS on atherosclerosis in apoE-KO mice and investigated the underlying molecular mechanisms.

2. Materials and Methods

2.1. Study Design. A total of 42 male apoE-KO mice and 14 wild type C57BL/6 mice (6 weeks of age, 15–19 g) were purchased from Peking University Resources Centre (Permission number: SCXK 2011-0012). After one-week acclimatization period, 14 wild type C57BL/6 mice were assigned to control group and given normal chow. 42 male apoE-KO mice were randomly divided into HFD-, FO-, and FO+ALA-PS-treated groups matched with their mean body weight and plasma total cholesterol levels as previously published methods [14]. HFD group was given a high fat diet containing of 21% fat and 0.15% cholesterol [15]. This high fat diet was further supplemented with 5% (w/w) flaxseed oil for FO group; a combination of 3.3% (w/w) ALA-PS (provided 2% PS) mixture with flaxseed oil which provided equivalent ALA to FO group was added to the high fat diet and used for ALA-PS group. The flaxseed oil was manufactured by Inner Mongolia Caoyuan Kanghen Food Co. Ltd. (Hohhot, China) and contained 57.82% ALA (% of total fatty acids). Plant sterol (β-sitosterol 77%, campesterol 17%, and stigmasterol 5%) was provided by BASF Co. Ltd. (Shanghai, China). ALA (α-linolenic acid 80%, linoleic acid 15%, and oleic acid 5%) was purchased from Henan Linuo Biochemical Co. Ltd. (Anyang, Henan, China). The ALA-PS was synthesized by Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan, China (Wuhan, China) using PS and ALA mentioned above [16]. The animals were maintained in constant temperature-controlled rooms (25 ± 2°C) with controlled lighting (12 h light-dark cycles). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by Tongji Medical College Council on Animal Care Committee.

After 18 weeks, three animals in each group were randomly selected for en face aorta and aortic sinus Oil-Red O staining to observe their pathological changes and lipid deposition. The remaining animals were feed-deprived for 8 h, anesthetized with ketamine HCl (50 mg/kg)/xylazine (10 mg/kg), and subsequently killed by cervical dislocation. Blood was collected and centrifuged for 20 min at 5000 g at 4°C and then stored at −80°C. Aorta and liver were excised, immediately frozen in liquid nitrogen, and stored at −80°C until use.

2.2. Histology and Morphometry Evaluations of Atherosclerotic Lesions. For en face analysis, the aorta from the ascending arch to the iliac bifurcation was cleaned of peripheral tissue, opened longitudinally, pinned flat, and stained with Oil-Red O. Images were captured with Canon IXUS 220 HS camera.

The heart samples were embedded in tissue freezing medium O.C.T. and sectioned into consecutive 8 μm thick sections at −20°C. The distal end of the aortic sinus was recognized by the disappearance of the three aortic valve cusps. Every sixth section was stained with Oil-Red O and digitally photographed under magnification (×40).

Images were analyzed using Image-Pro Plus (IPP) software. The lesion area index was calculated as the percentage of aortic lumen area covered by atherosclerotic lesions.

2.3. Immunohistochemistry Assay. Aortic arch cross-sections were immunostained with CD68 (1 : 400; ab955, Abcam Limited), Ly-6C (1 : 200; sc-52650, Santa Cruz Biotechnology), and α-smooth muscle actin antibodies (1 : 500, A 5228, Sigma-Aldrich) to detect macrophages, Ly-6C-positive macrophages, and smooth muscle cells. After washing, sections were incubated with anti-rat and anti-mouse horseradish peroxidase-conjugated secondary antibodies at 1 : 200 dilutions (CST). Immunocomplexes containing CD68, Ly-6C, and α-smooth muscle actin antibodies were detected using diaminobenzidine tetrahydrochloride dihydrate substrate (DAB; Sigma). The sections were digitally photographed under magnification (×200).

2.4. Determination of Lipid Parameters in Serum and Liver. The concentrations of TC, TG, LDL-C, and HDL-C in serum and liver were measured by enzymatic colorimetric assays using commercially available detection kits (Biosino Biotechnology Co., Ltd., Beijing, China).

2.5. Measurement of Inflammation Cytokines in Plasma. The levels of interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and soluble vascular cell adhesion molecule-1 (sVCAM-1) in plasma were measured by enzyme linked immunosorbent assays using commercially available detection kits (R&D Systems, USA.).

Table 1

Gene	Forward primer	Reverse primer
HMGCR	5′-AGCCGAAGCAGCACATGAT C-3′	5′-CTTGTGGAATGCCTTGTGATTG-3′
SREBP-2	5′-AAGTCTGGCGTTCTGAGGAA-3′	5′-GTTCTCCTGGCGAAGCT-3′
PPARα	5′-GGAGTGCAGCCTCAGCCAAGTT-3′	5′-AGGCCACAGAGCGCTAAGCTGT-3′
SREBP-1c	5′-TCCTTAACGTGGGCCTAGTCCGAAG-3′	5′-GCTCGAGTAACCCAGCACGGG-3′
VCAM-1	5′-GATAGACAGCCCACTAAACG-3′	5′-CAATGACGGGAGTAAAGGT-3′
TNF-α	5′-TCTCATTCCTGCTTGTGG-3′	5′-ACTTGGTGGTTTGCTACG-3′
MCP-1	5′-GCAGGTGTCCCAAAGAA-3′	5′-GGTGGTTGTGGAAAAGG-3′
IL-6	5′-ATTTCCTCTGGTCTTCTGG-3′	5′-TGGTCTTGGTCCTTAGCC-3′
IL-1β	5′-CTTTCCCGTGGACCTT-3′	5′-ATCTCGGAGCCTGTAGTG-3′
$p22^{phox}$	5′-GTGTGCGCAGGGTCCTCGTC-3′	5′-TCCAACCTGTGGCCGCTCCT-3′
$p47^{phox}$	5′-GGTCGACCATCCGCAACGCA-3′	5′-GCGGCGATAGGTGTCCTGGC-3′
$p67^{phox}$	5′-GTGGGTCGGCTGTTCCGTCC-3′	5′-GCGTCTCCGGCACAAAGCCA-3′
$gp91^{phox}$	5′-GGGGCCTGTATGTGGCCGTG-3′	5′-AGGCATGCGTGTCCCTGCAC-3′

2.6. Determination of Oxidative Stress Parameters in Serum and Liver. The concentrations of glutathione (GSH) were measured according to its reaction with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) into 2-nitro-5-thiobenzoic acid (TNB), following deproteinization by 5% trichloroacetic acid [17]. Malondialdehyde (MDA), an index of lipid peroxidation, was measured by using thiobarbituric acid colorimetry slightly modified by Ohkawa et al. [18]. The contents of GSH and MDA in liver were standardized by protein concentration measured by the Bio-Rad Protein Assay Kit (USA).

2.7. Evaluation of ROS Level in Aorta of Mice. Dihydroethidium (DHE) (Molecular Probes, Eugene, OR, USA) was used for in situ detection of reactive oxygen species (ROS) in the aorta of mice. Fresh cross-sections (5 μm) of unfixed but frozen aorta were immediately incubated with 5 M DHE at 37°C for 15 min in a humidified chamber. Fluorescence level was then visualized with a fluorescence microscope. Fluorescence intensities in randomly selected areas of the images were quantified by using the IPP image analysis software.

2.8. Real-Time RT-PCR Analysis. Total RNA was isolated from the stored frozen liver, aorta tissue, and fresh blood monocytes using the Trizol reagent (Invitrogen, 154 Carlsbad, CA, USA). Messenger RNA (mRNA) expression was quantified by using specific oligo primers and SYBRGreen-based qRT-PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) in 7900HT instrument (Applied Biosystems, Forster, CA, USA). The specificity of the product was assessed from melting curve analysis. Gene expressions were determined using the $2^{-\Delta\Delta Ct}$ method. The mRNA of β-actin was quantified as an endogenous control. Gene expressions are presented as fold change relative to control. Quantitative real-time PCR primers were as shown in Table 1.

2.9. Western Blot Analysis. Thoracic aorta, liver tissues, and blood monocytes were homogenized and lysed in RIPA lysis buffer (1% Triton X-100, 1% deoxycholate, and 0.1% SDS). Tissue lysates with equal protein amounts were subjected to western blotting. The protein was separated by 10% SDS-polyacrylamide gel and then transferred onto a PVDF membrane. The membranes were incubated with specific primary antibodies under overnight at 4°C after blocked with 5% nonfat milk solution. Then the target proteins were incubated with the species-specific secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were detected by means of an ECL plus Western Blotting Detection System (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. Quantitative analysis of the relative density of the bands in western blots was performed by Quantity One 4.62 software (Bio-Rad, Hercules, CA, USA). Data were corrected for background standardized to β-actin as optical density (OD/mm^2). Primary antibodies were as follows: monoclonal anti-β-actin (Sigma), rabbit anti-SREBP2 (Abcam Limited), rabbit antibody anti-SREBP1 (Abcam Limited), rabbit anti-PPARα (Abcam Limited), rabbit anti-HMGCr (Abcam Limited), rabbit anti-IL-6 (Cell Signaling Technology), mouse anti-TNF alpha (Abcam Limited), rabbit anti-VCAM1 (Abcam Limited), rabbit anti-MCP1 (Abcam Limited), mouse anti-IL-1β (Cell Signaling Technology), rabbit anti-p22-phox (Santa Cruz), rabbit anti-p47-phox (Merck Millipore Corporation), rabbit anti-p67-phox (Merck Millipore Corporation), rabbit anti-gp91-phox (Abcam Limited), anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology), and anti-mouse IgG HRP-linked antibody (Cell Signaling Technology).

2.10. Statistical Analysis. Results are expressed as means ± SD, and $P < 0.05$ was considered significant. Statistical analyses of data were performed using one-way analysis of variance with SPSS 12.0 software package (SN: 59245 46841 40655 89389 09859 21671 21957 29589 12).

3. Results

3.1. FO+ALA-PS Ameliorated Atherosclerosis Induced by HFD in Mice. En face analysis of aorta revealed atherosclerotic lesion formation with the aid of Oil-Red O staining. As

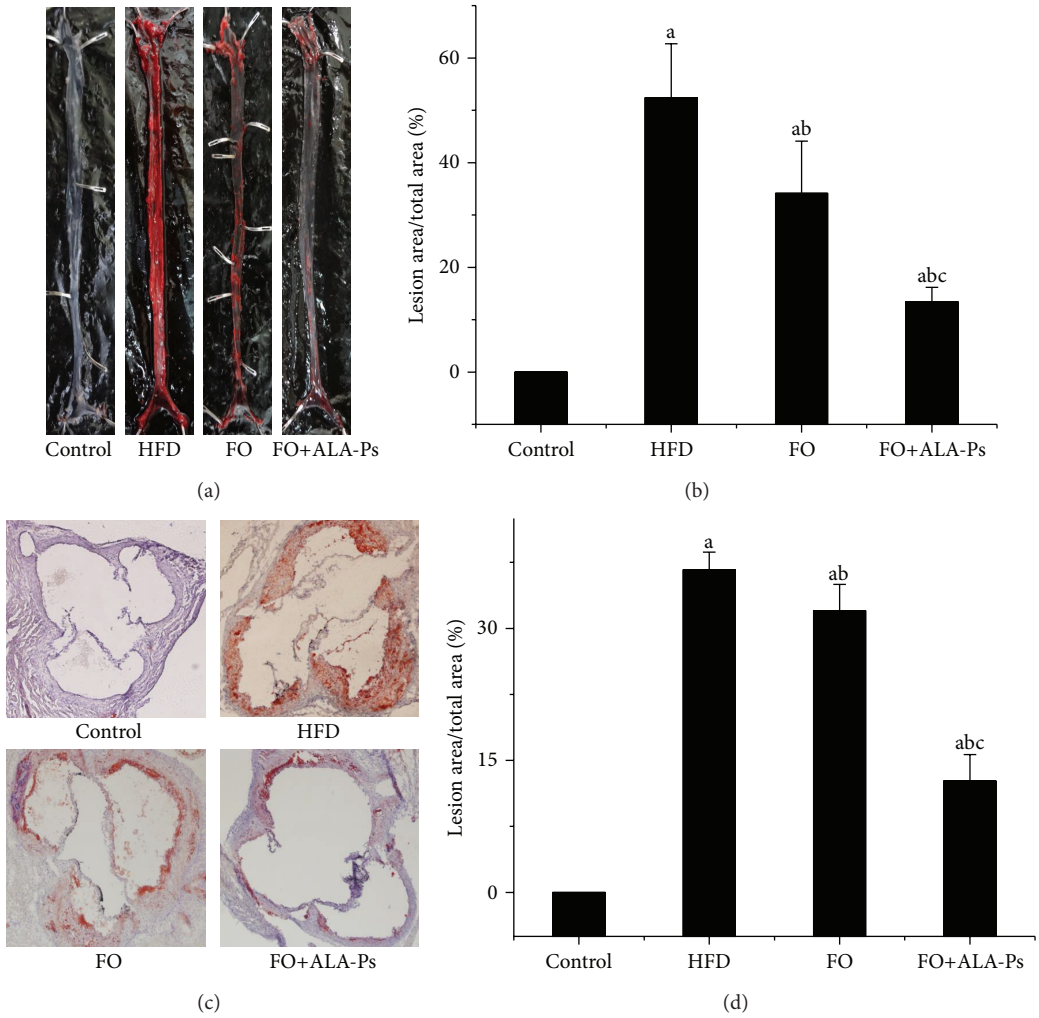


Figure 1: Intervention of FO+ALA-PS ameliorated atherosclerosis in HFD diet-fed mice. Photomicrographs of representative Oil-Red O stained aortas of mice in control, HFD, FO, and ALA-PS groups (a). Quantitative analysis of the atherosclerotic lesion aortas of mice. The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are given as mean ± standard deviation of the mean ($n = 3$). [a]$P < 0.05$ versus the control; [b]$P < 0.05$ versus the HFD group; [c]$P < 0.05$ versus the FO group (b). Representative images of cross-sections taken of the aortic sinuses obtained from mice in control, HFD, FO, and ALA-PS groups. The sections of aortic sinuses were stained with Oil-Red O (magnification ×40) for lipid deposition (red) and cross-stained with hematoxylin (blue) (c). Quantitative analysis of the atherosclerotic lesions in aortic sinuses of mice. The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are given as mean ± standard deviation of the mean ($n = 3$). [a]$P < 0.05$ versus the control; [b]$P < 0.05$ versus the HFD group; [c]$P < 0.05$ versus the FO group (d).

representative results showed in Figure 1(a), compared with control, a significantly atherogenic action was induced in apoE-KO mice given HFD while the lesions in the FO and FO+ALA-PS groups were obviously decreased. Quantitative analysis of the atherosclerotic lesions in the aortas of mice (Figure 1(b)) demonstrated statistically smaller lesion size in FO+ALA-PS-treated animals than those in FO-treated mice (13.43 ± 2.75% versus 34.13 ± 10.00%).

Cross-sectional analysis of atherosclerotic development at the aortic sinus revealed lipid deposition with the aid of Oil-Red O staining. Representative images were shown in Figure 1(c). The control group was shown to have little lipid deposition. Atherosclerotic lesions at the aortic sinus were extensive following HFD feeding, while FO and FO+ALA-PS treatment groups had much less lipid deposition. The extent of atherosclerotic development at the aortic sinus was quantified as a percentage of aortic cross-sectional luminal area occupied by Oil-Red O-stained lipid deposits. As illustrated in Figure 1(d), the addition of flaxseed oil to an atherogenic diet significantly inhibited the development of atherosclerotic lesions at the aortic sinus, and a further improvement effect was observed in FO+ALA-PS intervention group (12.67 ± 2.08% versus 32.00 ± 2.65%). These results indicated that the addition of ALA-PS markedly strengthened the antiatherogenic properties of flaxseed oil in apoE-KO mice.

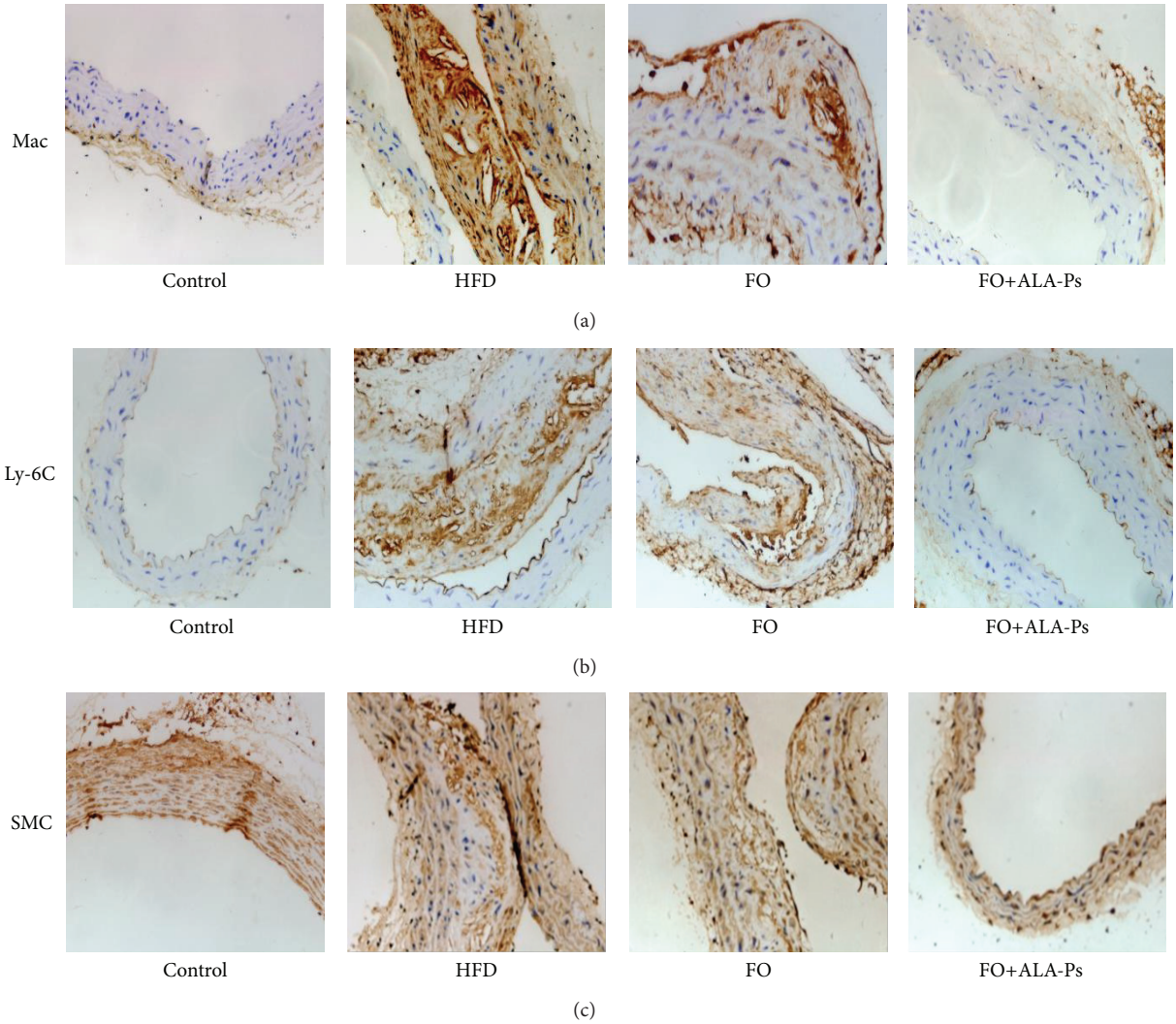
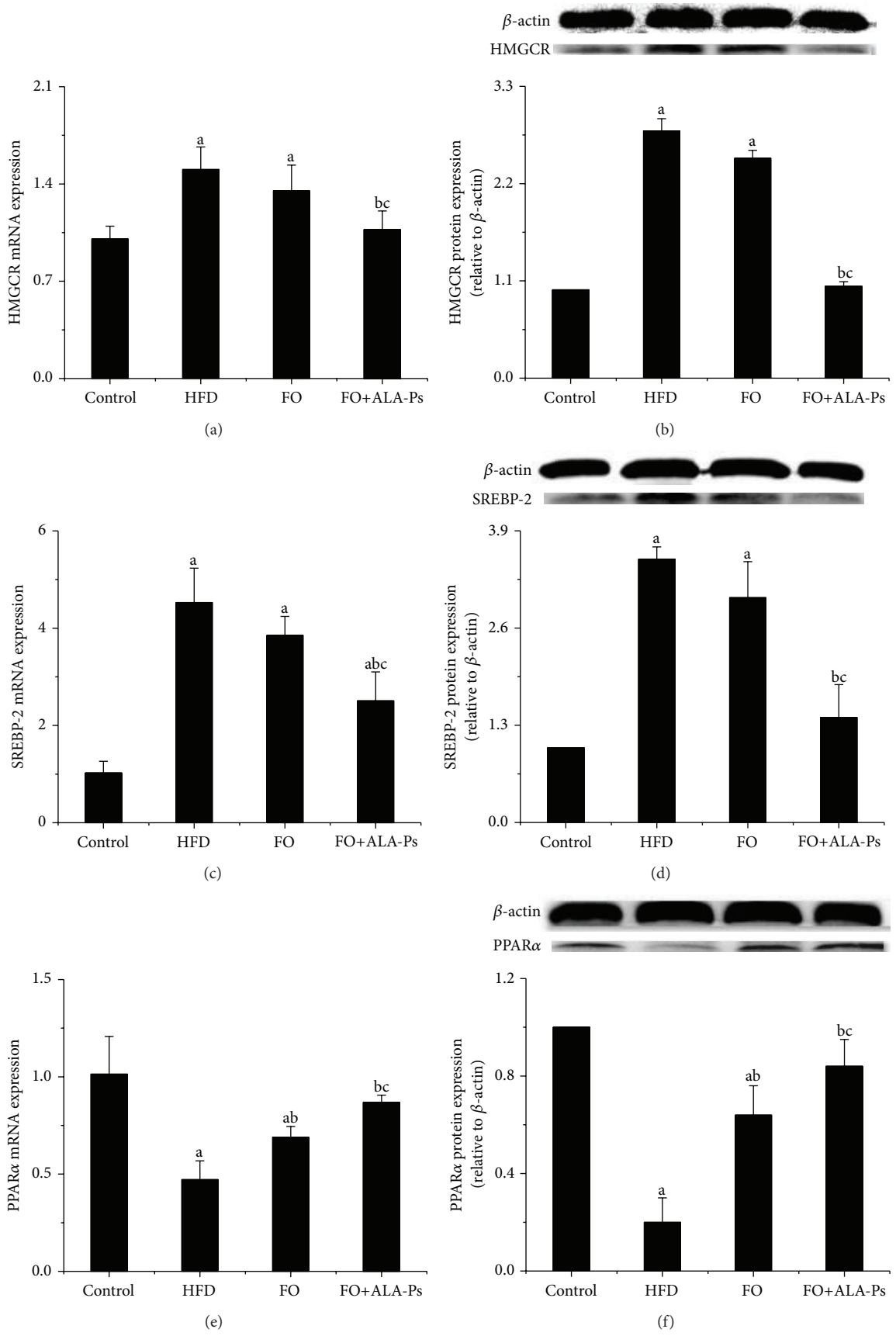


Figure 2: Treatment of FO+ALA-PS prevents atherosclerotic development via anti-inflammatory and antiproliferative actions. Representative images of aortic cross-sections immunostained with markers of macrophage infiltration, inflammation, and proliferation from mice fed a normal, HFD, FO, or ALA-PS diet for 18 wk. Immunoreactivity to MAC (a), Ly-6C (b), and SMC (c) antibodies are evident with brownish diaminobenzidine staining (magnification ×400).

3.2. Anti-Inflammatory and Antiproliferative Actions of FO+ALA-PS. Inflammation is now considered to be an important mechanistic process involved in atherosclerosis, and markers of inflammation are also detected as a function of the dietary interventions. The macrophage marker CD68 and Ly-6C have been used as an indicator of inflammatory reactions associated with atherosclerosis. As shown in Figures 2(a) and 2(b), mice fed HFD were characterized by increased infiltration of macrophages and Ly-6C monocyte compared with control. Flaxseed oil supplementation alone did not reduce macrophages, but when included with ALA-PS, flaxseed oil was capable of mitigating the accumulation of macrophages.

In addition, atherosclerotic plaque is generated partly by proliferation and migration of vascular smooth muscle cells (VSMC), and α-actin can be used as a marker of VSMC proliferation within the vessel wall. As shown in Figure 2(c), intimal VSMC contents were pronounced in mice of HFD and FO groups while dietary FO+ALA-PS inhibited proliferation and migration of VSMC.

3.3. Combined Treatment with FO+ALA-PS Improved Lipid Metabolism. The intervention of flaxseed oil alleviated the rise of TG induced by HFD in serum and liver while a much larger decrease in TG was observed in FO+ALA-PS group. Although flaxseed oil feeding alone elevated serum HDL-C, it had no effect on LDL-C and TC. Combination treatment not only apparently reduced LDL-C and TC but also increased the concentration of HDL-C (Table 2). To investigate the underlying molecular mechanism by which dietary FO+ALA-PS modulates lipid metabolism, gene and protein expressions of the major factors involved in hepatic cholesterol homoeostasis, fatty acid catabolism, and synthesis were detected. As illustrated in Figure 3, mRNA and protein



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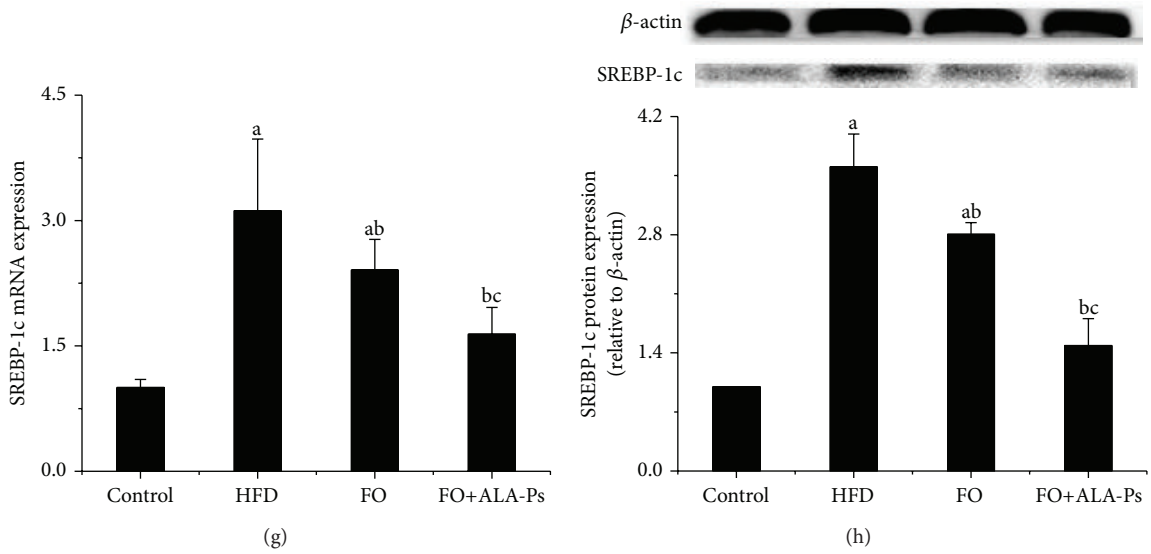


Figure 3: Effects of FO+ALA-PS on mRNA and protein expressions of hepatic HMGCR (a-b), SREBP-2 (c-d), PPARα (e-f), and SREBP-1c (g-h) in mice. After intervention by flaxseed oil with ALA-PS for 18 weeks, total RNA was extracted from liver of mice by Trizol. HMGCR, SREBP-2 PPARα, and SREBP-1c mRNA expressions were analyzed by real-time RT-PCR. The mRNA of β-actin was quantified as an endogenous control. Hepatic lysates were prepared and immunoblotted with corresponding antibody, respectively. Blotting with anti-β-actin was used as a protein loading control. HMGCR, SREBP-2, PPARα, and SREBP-1c are presented as fold change relative to control. Representative immunoblots are shown. Values are given as mean ± standard deviation of the mean ($n = 3$). $^{a}P < 0.05$ versus the control; $^{b}P < 0.05$ versus the HFD group; $^{c}P < 0.05$ versus the FO group.

Table 2: Effects of FO+ALA-PS on lipid profiles in mice.

	Groups			
	Control	HFD	FO	FO+ALA-PS
Serum TC (mmol/L)	1.72 ± 0.24	20.77 ± 5.00^{a}	16.04 ± 4.59^{a}	11.33 ± 3.15^{abc}
Serum TG (mmol/L)	0.91 ± 0.15	2.27 ± 0.40^{a}	1.83 ± 0.42^{ab}	1.39 ± 0.41^{abc}
Serum LDL-C (mmol/L)	0.31 ± 0.12	12.16 ± 3.11^{a}	10.24 ± 2.32^{a}	4.17 ± 1.35^{abc}
Serum HDL-C (mmol/L)	1.01 ± 0.14	0.26 ± 0.12^{a}	0.45 ± 0.23^{ab}	0.60 ± 0.16^{abc}
Liver TC (mg/g)	2.54 ± 0.19	4.09 ± 0.75^{a}	3.52 ± 0.38^{a}	2.55 ± 0.24^{bc}
Liver TG (mg/g)	26.47 ± 6.40	64.04 ± 18.49^{a}	40.81 ± 7.63^{ab}	30.80 ± 6.21^{bc}

Forty-two apoE KO mice were randomly divided into 3 groups on average, namely, HFD, FO, and FO + ALA-PS. Fourteen wild type C57BL/6 mice were divided into control group. Values are given as means ± SD ($n = 14$). $^{a}P < 0.05$ versus the control; $^{b}P < 0.05$ versus the HFD group; $^{c}P < 0.05$ versus the FO group. HFD: high fat diet; FO: HFD + 5% flaxseed oil; FO+ALA-PS: HFD + 2.7% flaxseed oil + 3.3% flaxseed oil ester of plant sterol.

expressions of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and sterol regulatory element binding protein 2 (SREBP-2) were found to be statistically decreased in FO+ALA-PS-treated but not FO-treated animals, as compared to those in HFD-fed animals. In addition, flaxseed oil intervention elevated peroxisome proliferator-activated receptor α (PPARα) and reduced sterol regulatory element-binding protein 1c (SREBP-1c), respectively. A more pronounced effect was achieved by FO+ALA-PS application.

3.4. FO+ALA-PS Attenuated Inflammation. Compared to HFD, mice administrated by flaxseed oil had 30.6% and 11.9% less plasma IL-6 and VCAM-1, while this decrease was 56.3% and 29.3% in mice given FO+ALA-PS. In addition, FO+ALA-PS had a significant decline effect on plasma sVCAM-1, TNF-α, and MCP-1 which were initially increased by HFD but not diminished by flaxseed oil intervention. However, there were no significant differences observed in plasma IL-10 in the four groups (Figure 4). On molecular level, we compared the ability of FO and FO+ALA-PS treatment in modulating aortic inflammation indicators. As shown in Figure 5, FO+ALA-PS was more effective than FO in decreasing mRNA and protein expressions of aortic VCAM-1, TNF-α, MCP-1, and IL-6 elevated by HFD. In an effort to further investigate molecular pathways involved in inflammation induction by HFD and the concomitant protection exerted by FO+ALA-PS on this process, we also examined the impact of FO+ALA-PS on the key inflammatory cytokines in circulating monocytes. The results clearly indicated that MCP-1, interleukin-1 beta (IL-1β), and IL-6 were upregulated in response to HFD, while such an effect was almost completely counteracted by the FO+ALA-PS supplement. However, flaxseed oil added alone

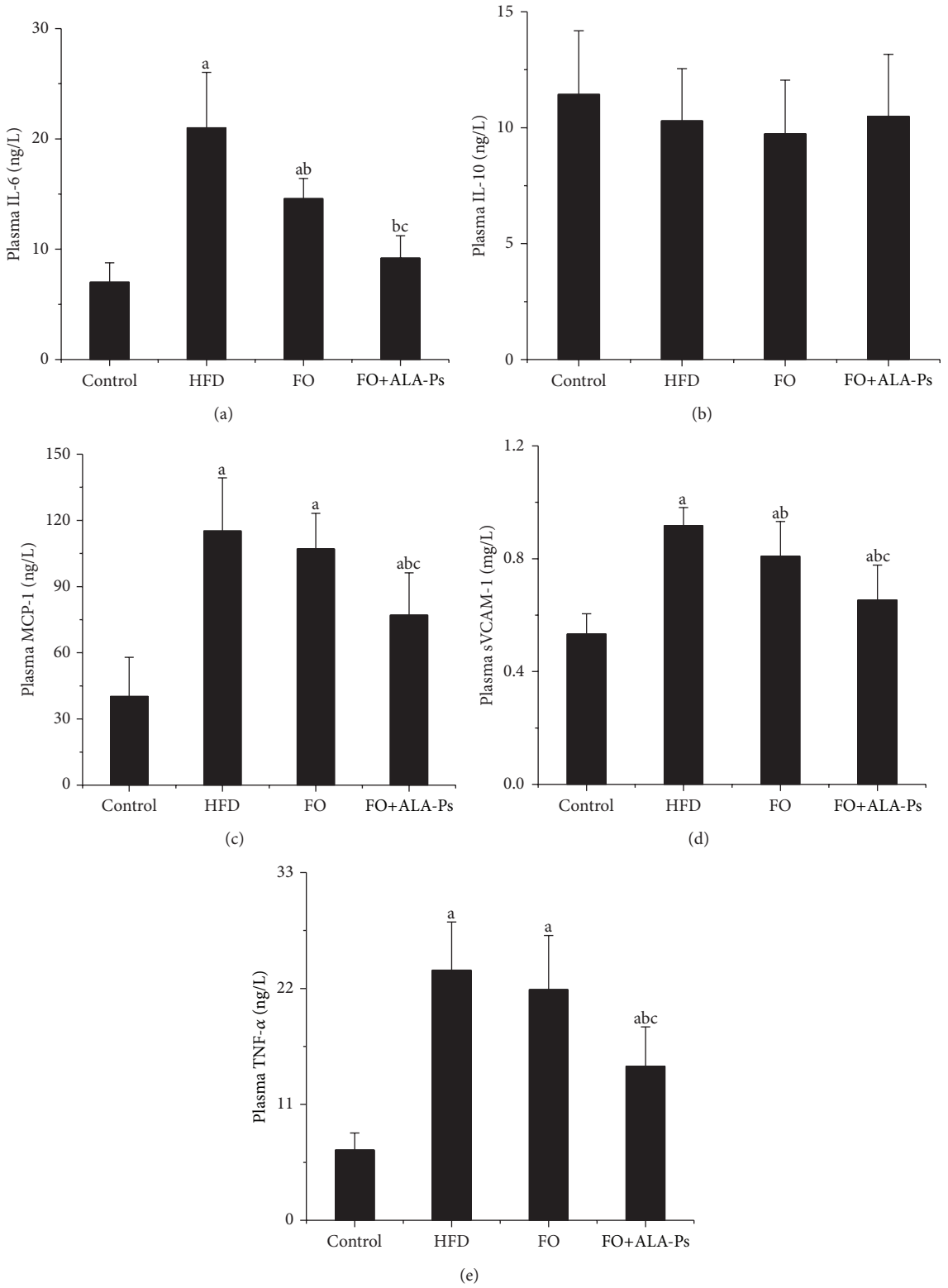
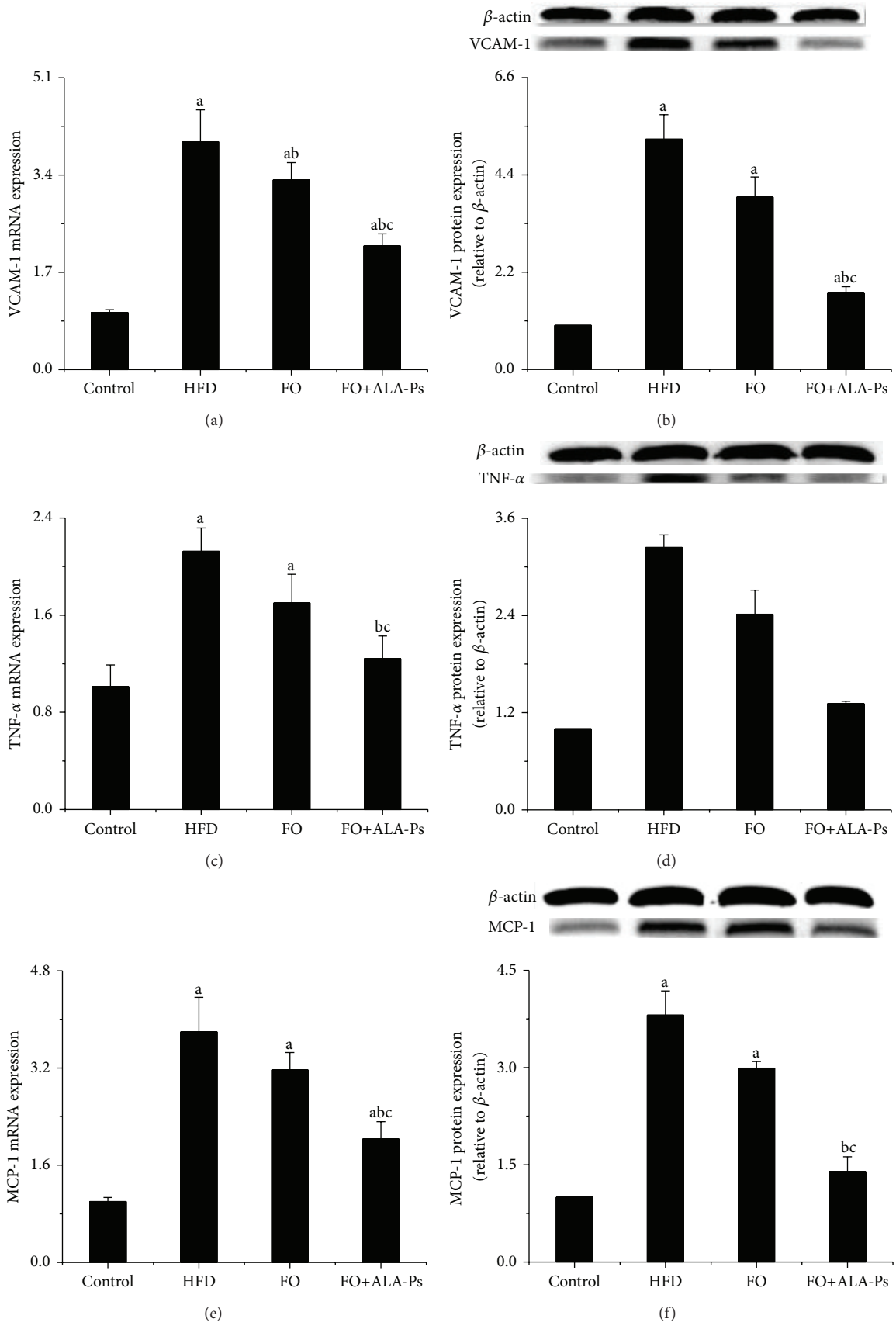


Figure 4: Supplementation of FO+ALA-PS reduced plasma inflammatory cytokines in mice. Values are means for plasma levels of IL-6 (a), IL-10 (b), MCP-1 (c), sVCAM-1 (d), and TNF-α (e). Values are given as mean ± standard deviation of the mean ($n = 14$). [a]$P < 0.05$ versus the control; [b]$P < 0.05$ versus the HFD group; [c]$P < 0.05$ versus the FO group.



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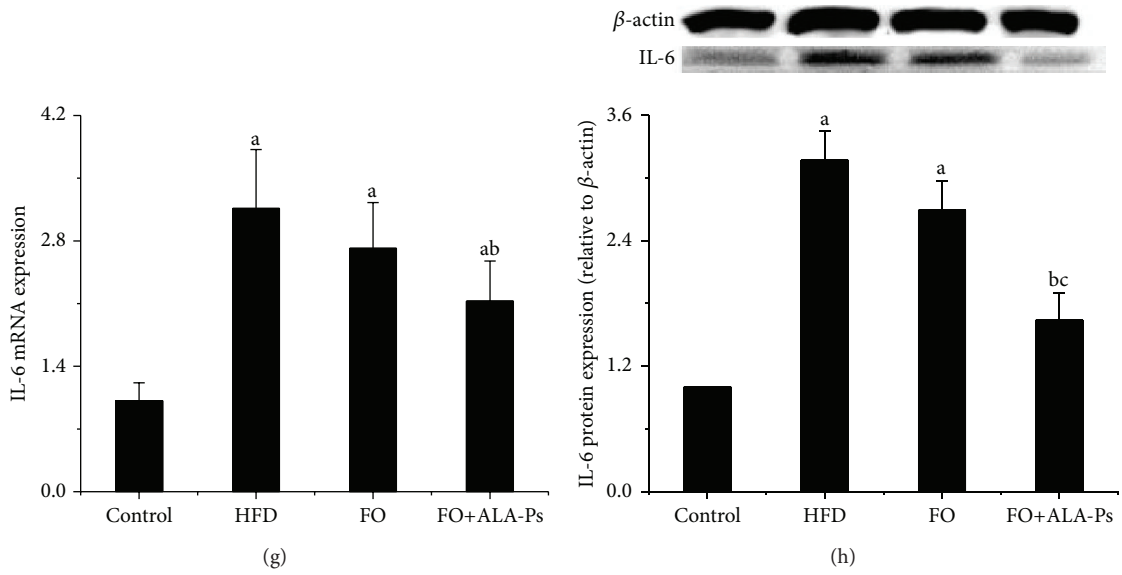

Figure 5: Effects of FO+ALA-PS on mRNA and protein expressions of aortic VCAM-1 (a-b), TNF-α (c-d), MCP-1 (e-f), and IL-6 (g-h) in mice. After combining treated flaxseed oil with ALA-PS for 18 weeks, total RNA was extracted from aorta of mice by Trizol. VCAM-1, TNF-α, MCP-1, and IL-6 mRNA expressions were analyzed by real-time RT-PCR. The mRNA of β-actin was quantified as an endogenous control. Aortic lysates were prepared and immunoblotted with corresponding antibody, respectively. Blotting with anti-β-actin was used as a protein loading control. VCAM-1, TNF-α, MCP-1, and IL-6 are presented as fold change relative to Control. Representative immunoblots are shown. Values are given as mean ± standard deviation of the mean ($n = 3$). $^{a}P < 0.05$ versus the control; $^{b}P < 0.05$ versus the HFD group; $^{c}P < 0.05$ versus the FO group.

only significantly modified the mRNA and protein expression of IL-1β (Figure 6).

3.5. Dietary FO+ALA-PS Inhibited Oxidative Stress. Recently acquired evidence pointed out that oxidative stress is a primary contributor to atherosclerosis. In this experiment, in situ ROS production of aorta was determined. As illustrated in Figures 7(a) and 7(b), high level of ROS in aorta induced by HFD was significantly reduced after exposed to flaxseed oil, and this depression effect was further improved by adding ALA-PS. Moreover, FO+ALA-PS but not FO consumption apparently decreased the concentrations of MDA and elevated the levels of GSH in serum and liver (Figures 7(c)–7(f)). To examine the potential molecular signaling pathways of FO+ALA-PS against oxidative stress, the main subunits of nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase contributing to atherosclerosis were detected. In mice given FO+ALA-PS supplemented diet, mRNA and protein expressions of aortic $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ were apparently less than those of HFD-fed mice (Figure 8).

4. Discussion

Atherosclerotic heart disease prevention is largely attributable to factors that can be altered or prevented by lifestyle modification such as nutritional interventions, cessation of smoking, and regular exercise [19]. Recently, dietary intake of naturally occurring PS has been demonstrated to associate with a lower risk of CVD [20]. Since cardiovascular risk factors such as dyslipidemia, inflammation, and oxidative stress rarely occur in isolation, combined nutritional interventions such as n-3 PUFA and PS may provide greater risk reduction compared to either of the supplements alone. In present study, we observed that FO+ALA-PS exerted better alleviative effect on atherosclerosis in apoE KO mice than single treatment of flaxseed oil, and the underlying molecular mechanisms were related to lipid metabolism, inflammation, and oxidative stress improvement.

Hyperlipidemia is a highly predisposing condition for arteriosclerosis and other cardiovascular diseases [21]. In this study, combined supplementation with flaxseed oil and ALA-PS exhibited synergistic and complementary lipid-lowering effects. These data are supported by other studies showing overall lipid improvement effects of flaxseed oil fortified with phytosterols in rats [22]. To investigate the possible mechanisms by which FO+ALA-PS exerts its hypocholesterolemic and hypotriglyceridemic effects, the key molecules involved in lipid metabolism pathways and atherosclerosis formation were detected in this study.

SREBP-2 and HMGCR play a pivotal role in cholesterol homoeostasis. In detail, transcriptional activity of SREBP-2, a transcription factors governed cholesterol synthesis, is primarily regulated at the posttranscriptional level involving INSIG and SREBP cleavage-activating protein (SCAP). SCAP/SREBP-2 precursor complex retains in the endoplasmic reticulum via sterol-induced interaction of SCAP with INSIG [23]. When cellular cholesterol is of low level, disassociation of SCAP from INSIG frees SCAP/SREBP-2 to the Golgi, where mature SREBP-2 is released by 2-step proteolytic cleavages [24]. Then, the mature SREBP-2 enters the nucleus

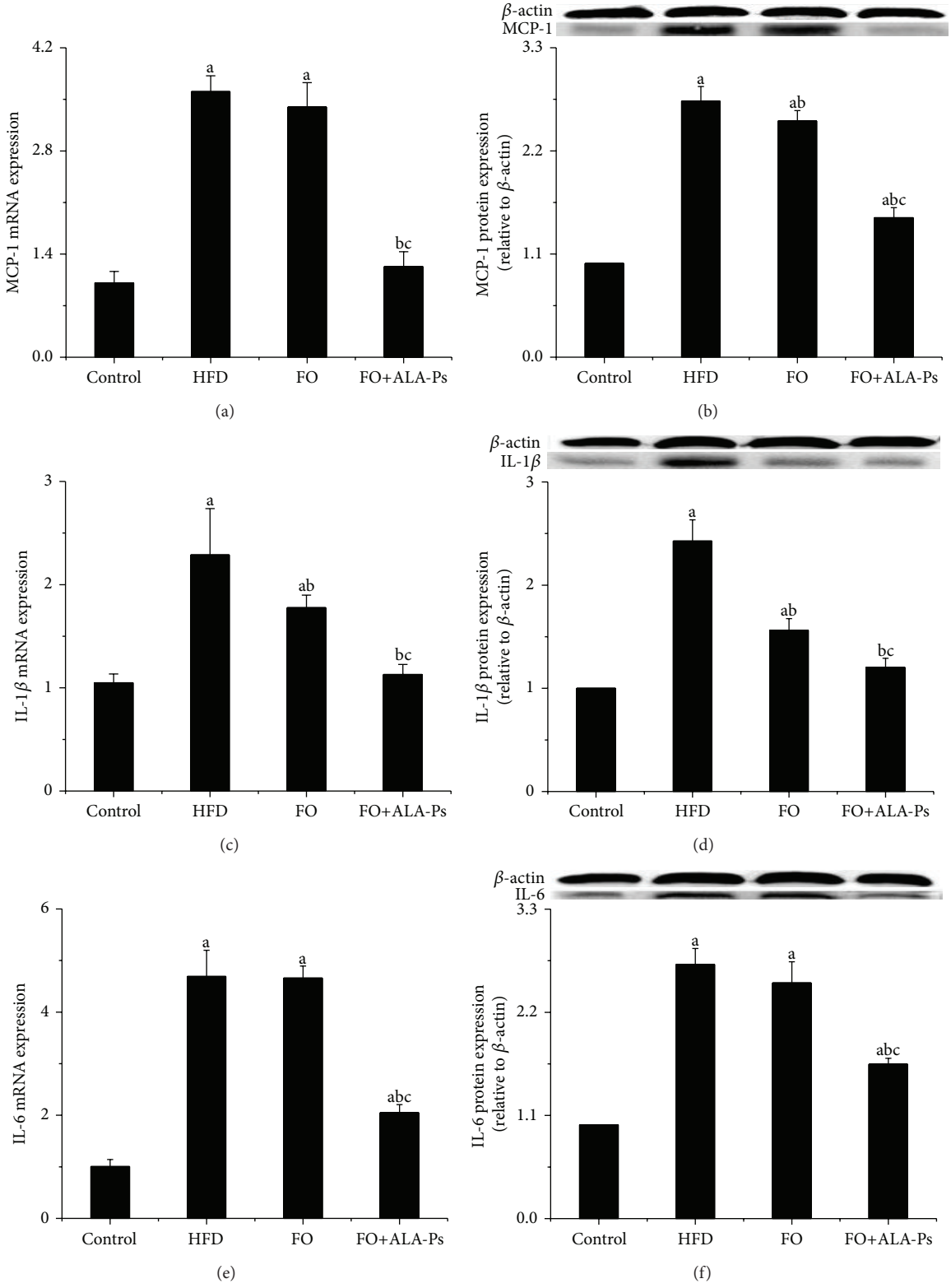

Figure 6: Effects of FO+ALA-PS on mRNA and protein expressions of MCP-1 (a-b), IL-1β (c-d), and IL-6 (e-f) in circulating monocytes of mice. After supplementing flaxseed oil with ALA-PS for 18 weeks, total RNA was extracted from circulating monocytes of mice by Trizol. MCP-1, IL-1β, and IL-6 mRNA expressions were analyzed by real-time RT-PCR. The mRNA of β-actin was quantified as an endogenous control. Monocytes lysates were prepared and immunoblotted with corresponding antibody, respectively. Blotting with anti-β-actin was used as a protein loading control. MCP-1, IL-1β, and IL-6 are presented as fold change relative to control. Representative immunoblots are shown. Values are given as mean ± standard deviation of the mean ($n = 3$). $^{a}P < 0.05$ versus the control; $^{b}P < 0.05$ versus the HFD group; $^{c}P < 0.05$ versus the FO group.

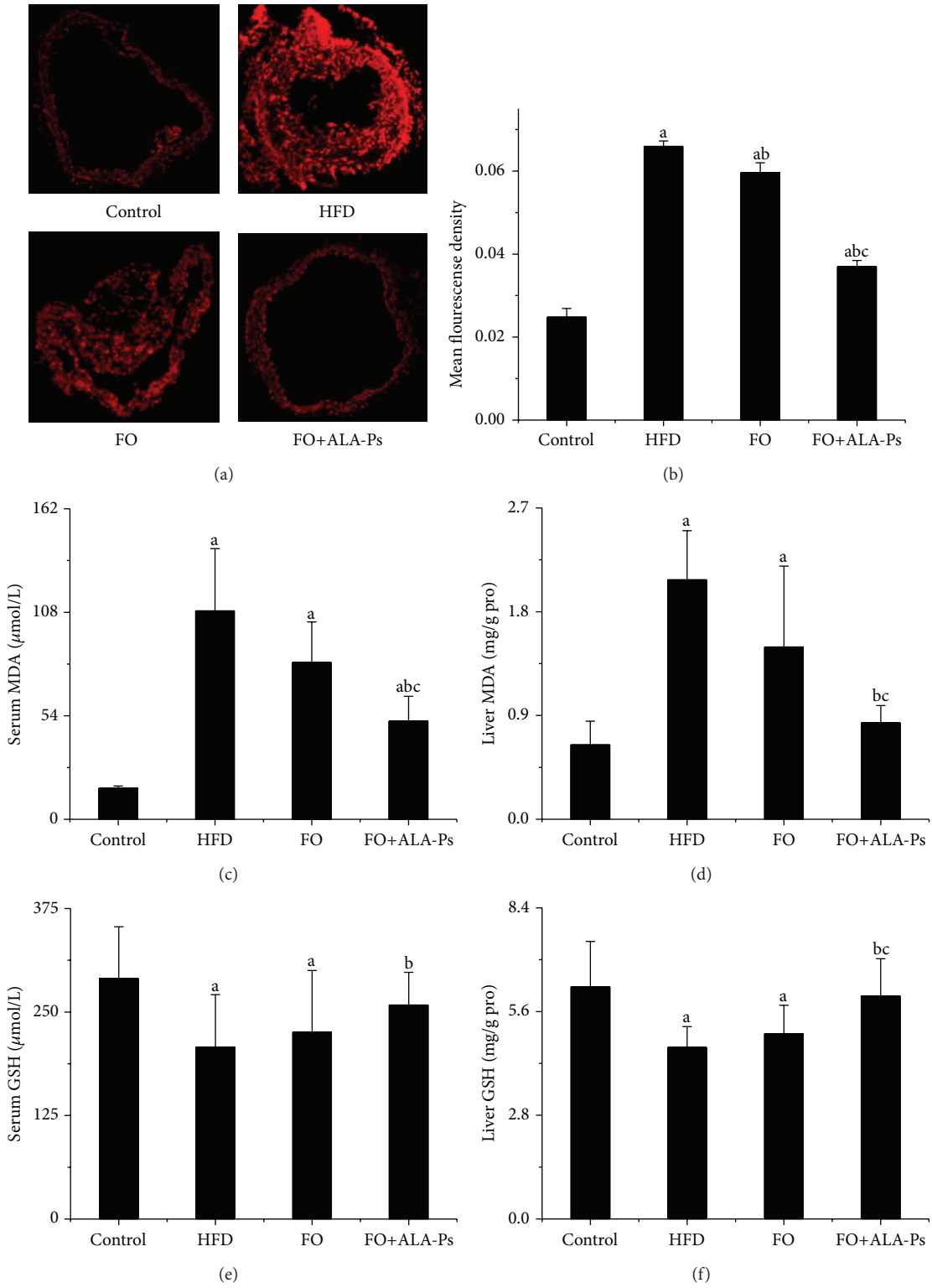

Figure 7: Effects of FO+ALA-PS on aortic ROS production and the levels of MDA and GSH in serum and liver of mice. ROS in the aorta of the mice was detected by using DHE which reacts with ROS and forms ETH that binds to DNA and produces red fluorescence signal, visualized with fluorescence microscope (×200) and quantified (a). Fluorescence intensities in randomly selected areas of the images were quantified by using the IPP image analysis software. Values are given as mean ± standard deviation of the mean ($n = 3$) (b). Values are means for serum MDA level (c), liver MDA level (d), serum GSH level (e), and liver GSH level (f) ($n = 14$) with standard deviations represented by vertical bars. $^{a}P < 0.05$ versus the control; $^{b}P < 0.05$ versus the HFD group; $^{c}P < 0.05$ versus the FO group.

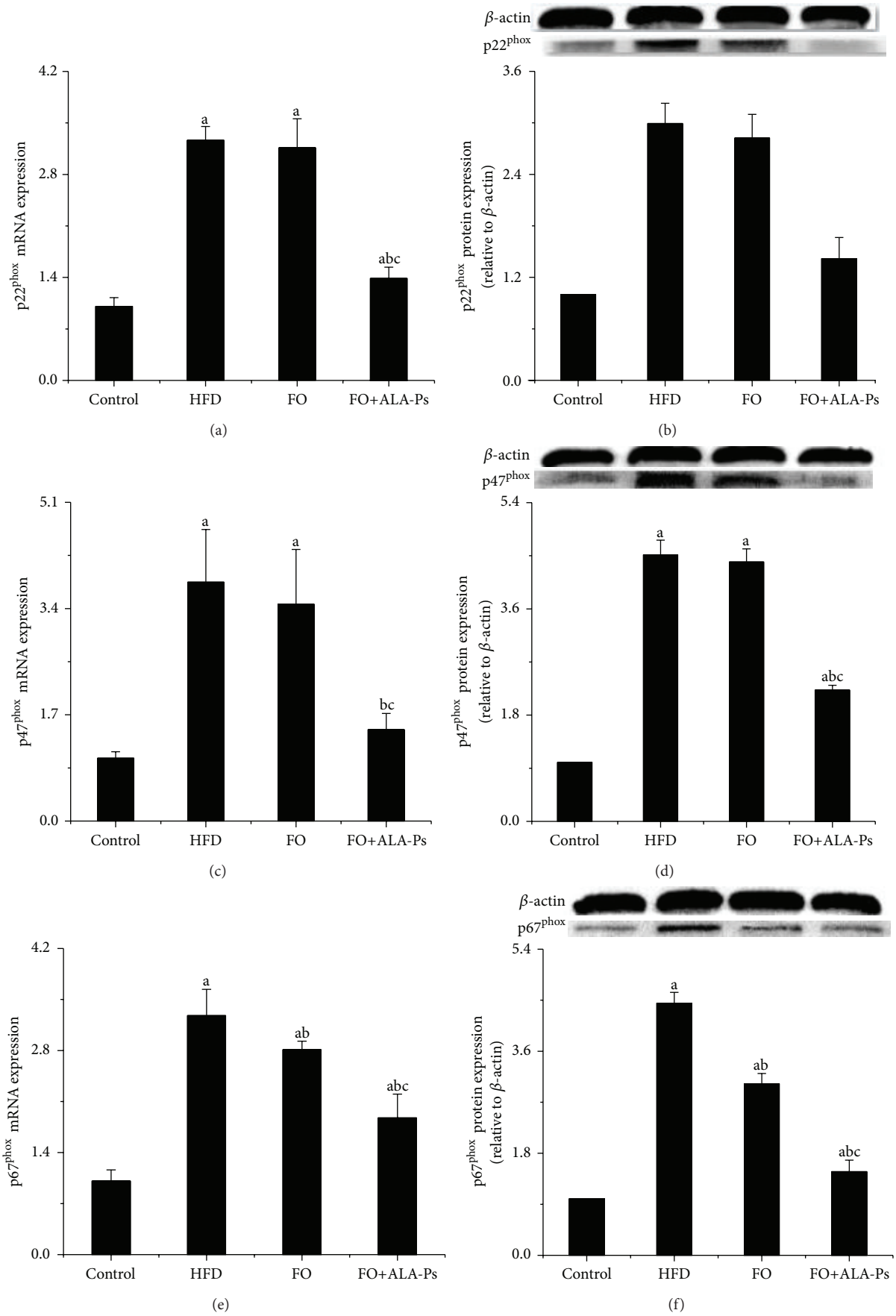

Figure 8: Continued.

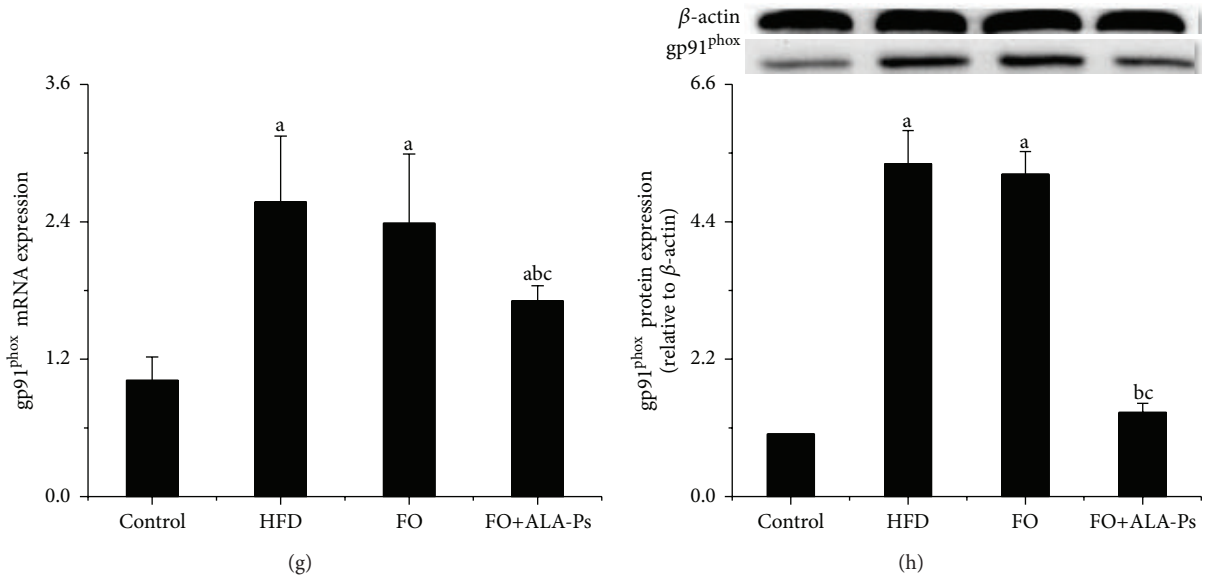

Figure 8: Effects of FO+ALA-PS on mRNA and protein expressions of $p22^{phox}$ (a-b), $p47^{phox}$ (c-d), $p67^{phox}$ (e-f), and $gp91^{phox}$ (g-h) in aorta of mice. Total RNA was extracted from aortas of mice by Trizol. $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ mRNA expressions were analyzed by real-time RT-PCR. The mRNA of β-actin was quantified as an endogenous control. Aortic lysates were prepared and immunoblotted with corresponding antibody, respectively. Blotting with anti-β-actin was used as a protein loading control. p22phox, p47phox, p67phox, and gp91phox are presented as fold change relative to control. Values are given as mean ± standard deviation of the mean ($n = 3$). $^{a}P < 0.05$ versus the control; $^{b}P < 0.05$ versus the HFD group; $^{c}P < 0.05$ versus the FO group.

to induce transcription of its target genes such as HMGCR which mediates the rate-limiting step in cholesterol synthesis [25]. It has been published that dietary β-sitosterol and its oxidation products (SiOP) decreased mRNA expressions of SREBP-2 and HMGCR in hamster given high cholesterol diet [26]. Consistent with this result, in present study, lower mRNA and mature protein expressions of HMGCR and SREBP-2 were observed in FO+ALA-PS group but not in FO group. The FO+ALA-PS supplementation may increase the regulatory cholesterol pool and inhibit the SREBP-2 pathway to downregulate HMGCR expression, which in turn reduce the cholesterol synthesis.

In addition, previous publications illustrated that PPARα and SREBP-1c were important regulators of lipogenic genes in the liver [27, 28]. It has been considered that n-3 PUFA exhibited TG-reducing effects through regulation of PPARα and SREBP-1c, which control hepatic fatty acid (FA) catabolism and synthesis, respectively [29, 30]. Recently Devarshi et al. demonstrated that flaxseed oil diet improved lipid metabolism through upregulating PPARα and downregulating SREBP-1 in s diabetic rats [31]. Meanwhile, aloe vera phytosterols were observed to improve the expression levels of PPAR target genes in the livers of mice with diet-induced obesity [32] and ameliorate obesity-associated metabolic disorders via significantly decreasing hepatic SREBP-1 expression in Zucker diabetic fatty (ZDF) rats [33]. Our study also showed similar data with previous reports showing that FO+ALA-PS elevated mRNA and protein expressions of PPARα and decreased SREBP-1, which may explain the lower concentrations of TG in FO+ALA-PS-treated group compared to HFD-treated group. Since the activations of PPARα and SREBP-1c have been shown to stimulate FA beta-oxidation and TG synthesis, respectively [34, 35], we speculate that increased beta-oxidation of FA and reduced TG synthesis are likely responsible for the TG-lowering effect of FO+ALA-PS. Based on the important lipid modulation role of molecules mentioned above and the results in present study, we suggest that improvement effect of FO+ALA-PS on lipid profiles and atherosclerosis may be linked to regulate HMGCR, SREBPs, and PPARα.

Besides hyperlipidemia, inflammation plays a crucial role in the etiology of atherosclerosis, from development of fatty streaks to plaque rupture and thrombosis. Once activated by stimuli such as oxidized lipoproteins, hypertension, or hyperglycemia, vascular endothelial cells express VCAM-1, which enhances the recruitment and adhesion of monocytes to the endothelium [36]. TNF-α, IL-6, and IL-1β increase VCAM-1 expression and mediate localization and recruitment of monocytes into the subendothelial space [37]. Chemoattractant, such as MCP-1, directly migrates leukocytes into the intima and recruits macrophage to the vessel wall [38]. Such endothelial injury ultimately leads to platelet aggregation and the release of platelet-derived growth factor, which initiates the proliferation of smooth muscle cells (SMCs) in the arterial intima, leading to the formation of atherosclerotic plaque [39]. Since these inflammatory markers have a key role in mediating inflammatory cascades and promoting atherosclerosis formation, the positive impact of dietary FO+ALA-PS on plasma levels, mRNA, and protein expressions of VCAM-1, TNF-α, IL-6, IL-1, and MCP-1 observed in this research supported that better function in atherosclerosis prevention of FO+ALA-PS is at least

partly through perfecting inflammatory response. However, previous researches showed that consumption of PS esters of sunflower oil and fish oil had no impact on plasma TNF-α, IL-6, and C-reactive protein in hypercholesterolemic persons [40]. One possibility to explain this discrepancy might be that flaxseed oil supplemented with ALA-PS offers a better anti-inflammatory approach due to the large content of ALA in flaxseed oil because dietary consumption of ALA has been demonstrated to reduce circulating levels of several atherogenic and inflammatory markers, including C-reactive protein, serum amyloid A, IL-6, and soluble VCAM-1 in dyslipidemic patients [41, 42]. On the other hand, previous report showed that high levels of plasma n-3 fatty acids were independently associated with lower levels of proinflammatory markers (IL-6, IL-1ra, TNFα, and C-reactive protein) [43]. Thus, anti-inflammatory effects of FO+ALA-PS described in this study may be also associated with its high ratio of n-3 to n-6 fatty acids.

Furthermore, previous publication also pointed out that chronic and acute overproduction of ROS under pathophysiologic conditions was integral in the development of atherosclerosis [44]. In present research, a pronounced reduction of aorta ROS along with lower concentrate of MDA and higher level of GSH was observed in FO+ALA-PS-fed animals than those in mice on HFD. These results suggested that FO+ALA-PS-mediated prevention of atherosclerosis is closely related with inhibition of oxidative stress. Our data is similar with previous reports that flaxseed oil or PS treatment has an antioxidative function [45, 46]. However, the effects of FO+ALA-PS on oxidative stress and the underlying molecular mechanisms for which are very limitedly reported. In the context of cardiovascular disease, oxidative stress leads to an increased ROS production largely determined by NADPH oxidase, a multicomponent enzyme consisting of four major units: $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ [47]. Some evidences showed that increased expression of $p22^{phox}$ contributed to the process of atherosclerosis and $apoE^{-/-}$ mice lacking $p47^{phox}$ had a marked reduction of atherosclerosis in the descending aorta [48, 49]. Here, we demonstrated that FO+ALA-PS treatment reversed mRNA and protein expressions of $p22^{phox}$, $p47^{phox}$ $p67^{phox}$, and $gp91^{phox}$ in aorta. These data indicate a mechanism that FO+ALA-PS inhibits aortic NADPH oxidase for ROS reduction and results in protection effects of atherosclerosis.

5. Conclusions

Our data is supportive of the synergistic and complementary effects of flaxseed oil containing ALA-PS on overall lipid, systemic inflammation, and oxidative stress which result in further amelioration in atherosclerosis. This study may provide new insights that dietary flaxseed oil containing ALA-PS therapy may be an ideal alternative or adjunct to pharmacological treatment for maximum cardioprotection in high risk individuals and flaxseed oil might be a good source for plant sterol ester supplementation as well as a good candidate to replace other fats in functional foods.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] A. S. Go, D. Mozaffarian, V. L. Roger et al., "Heart disease and stroke statistics—2014 update: a report from the American Heart Association," *Circulation*, vol. 129, no. 3, pp. e28–e292, 2014.

[2] A. Kitamura, H. Noda, M. Nakamura et al., "Association between non-high-density lipoprotein cholesterol levels and the incidence of coronary heart disease among Japanese: the Circulatory Risk in Communities Study (CIRCS)," *Journal of Atherosclerosis and Thrombosis*, vol. 18, no. 6, pp. 445–463, 2011.

[3] J. L. Witztum and D. Steinberg, "Role of oxidized low density lipoprotein in atherogenesis," *The Journal of Clinical Investigation*, vol. 88, no. 6, pp. 1785–1792, 1991.

[4] M. B. Madsen, A.-M. Jensen, and E. B. Schmidt, "The effect of a combination of plant sterol-enriched foods in mildly hypercholesterolemic subjects," *The American Journal of Clinical Nutrition*, vol. 26, no. 6, pp. 792–798, 2007.

[5] Expert Panel on Detection-Evaluation- and Treatment of High Blood Cholesterol in Adults, "Executive summary of the third report of The National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III)," *JAMA*, vol. 285, no. 19, pp. 2486–2497, 2001.

[6] European Atherosclerosis Society Consensus Panel on Phytosterols, "European Atherosclerosis Society Consensus Panel on Phytosterols, Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease," *Atherosclerosis*, vol. 232, no. 2, pp. 346–360, 2014.

[7] D. R. Chirovsky, V. Fedirko, Y. Cui, V. Sazonov, and P. Barter, "Prospective studies on the relationship between high-density lipoprotein cholesterol and cardiovascular risk: a systematic review," *European Journal of Cardiovascular Prevention and Rehabilitation*, vol. 16, no. 4, pp. 404–423, 2009.

[8] A. Morrison and J. E. Hokanson, "The independent relationship between triglycerides and coronary heart disease," *Vascular Health and Risk Management*, vol. 5, no. 1, pp. 89–95, 2009.

[9] I. Demonty, N. Ebine, X. Jia, and P. J. H. Jones, "Fish oil fatty acid esters of phytosterols alter plasma lipids but not red blood cell fragility in hamsters," *Lipids*, vol. 40, no. 7, pp. 695–702, 2005.

[10] M. Noakes, P. M. Clifton, A. M. E. Doornbos, and E. A. Trautwein, "Plant sterol ester-enriched milk and yoghurt effectively reduce serum cholesterol in modestly hypercholesterolemic subjects," *European Journal of Nutrition*, vol. 44, no. 4, pp. 214–222, 2005.

[11] D. Hanke, P. Zahradka, S. K. Mohankumar, J. L. Clark, and C. G. Taylor, "A diet high in α-linolenic acid and monounsaturated fatty acids attenuates hepatic steatosis and alters hepatic phospholipid fatty acid profile in diet-induced obese rats," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 89, no. 6, pp. 391–401, 2013.

[12] D. Rodriguez-Leyva, C. M. Dupasquier, R. McCullough, and G. N. Pierce, "The cardiovascular effects of flaxseed and its omega-3 fatty acid, alpha-linolenic acid," *The Canadian Journal of Cardiology*, vol. 26, no. 9, pp. 489–496, 2010.

[13] D. Kromhout, E. J. Giltay, and J. M. Geleijnse, "n-3 fatty acids and cardiovascular events after myocardial infarction," *The New England Journal of Medicine*, vol. 363, no. 21, pp. 2015–2026, 2010.

[14] M. H. Moghadasian, B. M. McManus, D. V. Godin, B. Rodrigues, and J. J. Frohlich, "Proatherogenic and antiatherogenic effects of probucol and phytosterols in apolipoprotein E-deficient mice: possible mechanisms of action," *Circulation*, vol. 99, no. 13, pp. 1733–1739, 1999.

[15] Y. Nakashima, A. S. Plump, E. W. Raines, J. L. Breslow, and R. Ross, "ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 14, no. 1, pp. 133–140, 1994.

[16] D. Qianchun, Z. Pin, H. Qingde et al., "Chemical synthesis of phytosterol esters of polyunsaturated fatty acids with ideal oxidative stability," *European Journal of Lipid Science and Technology*, vol. 113, no. 4, pp. 441–449, 2011.

[17] J. J. Eady, T. Orta, M. F. Dennis, M. R. L. Stratford, and J. H. Peacock, "Glutathione determination by the Tietze enzymatic recycling assay and its relationship to cellular radiation response," *British Journal of Cancer*, vol. 72, no. 5, pp. 1089–1095, 1995.

[18] H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction," *Analytical Biochemistry*, vol. 95, no. 2, pp. 351–358, 1979.

[19] S. Yusuf, S. Hawken, S. Ounpuu et al., "Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study," *The Lancet*, vol. 364, no. 9438, pp. 937–952, 2004.

[20] V. Escurriol, M. Cofán, C. Moreno-Iribas et al., "Phytosterol plasma concentrations and coronary heart disease in the prospective Spanish EPIC cohort," *The Journal of Lipid Research*, vol. 51, no. 3, pp. 618–624, 2010.

[21] J. G. Canto and A. E. Iskandrian, "Major risk factors for cardiovascular disease: debunking the 'only 50%' myth," *The Journal of the American Medical Association*, vol. 290, no. 7, pp. 947–949, 2003.

[22] Q. Deng, X. Yu, J. Xu et al., "Effect of flaxseed oil fortified with vitamin E and phytosterols on antioxidant defense capacities and lipids profile in rats," *Journal of Food Science*, vol. 77, no. 6, pp. H135–H140, 2012.

[23] T. Yang, P. J. Espenshade, M. E. Wright et al., "Crucial step in cholesterol homeostasis: Sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER," *Cell*, vol. 110, no. 4, pp. 489–500, 2002.

[24] J. L. Goldstein, R. B. Rawson, and M. S. Brown, "Mutant mammalian cells as tools to delineate the sterol regulatory element-binding protein pathway for feedback regulation of lipid synthesis," *Archives of Biochemistry and Biophysics*, vol. 397, no. 2, pp. 139–148, 2002.

[25] K. M. Boberg, J.-E. Akerlund, and I. Bjorkhem, "Effect of sitosterol on the rate-limiting enzymes in cholesterol synthesis and degradation," *Lipids*, vol. 24, no. 1, pp. 9–12, 1989.

[26] Y. T. Liang, W. T. Wong, L. Guan et al., "Effect of phytosterols and their oxidation products on lipoprotein profiles and vascular function in hamster fed a high cholesterol diet," *Atherosclerosis*, vol. 219, no. 1, pp. 124–133, 2011.

[27] A. Castrillo and P. Tontonoz, "Nuclear receptors in macrophage biology: at the crossroads of lipid metabolism and inflammation," *Annual Review of Cell and Developmental Biology*, vol. 20, pp. 455–480, 2004.

[28] K. N. Maxwell, R. E. Soccio, E. M. Duncan, E. Sehayek, and J. L. Breslow, "Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice," *Journal of Lipid Research*, vol. 44, no. 11, pp. 2109–2119, 2003.

[29] N. Tanaka, X. Zhang, E. Sugiyama et al., "Eicosapentaenoic acid improves hepatic steatosis independent of PPARα activation through inhibition of SREBP-1 maturation in mice," *Biochemical Pharmacology*, vol. 80, no. 10, pp. 1601–1612, 2010.

[30] S. D. Clarke, "Nonalcoholic steatosis and steatohepatitis. I. Molecular mechanism for polyunsaturated fatty acid regulation of gene transcription," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 281, no. 4, pp. G865–G869, 2001.

[31] P. P. Devarshi, N. M. Jangale, A. E. Ghule, S. L. Bodhankar, and A. M. Harsulkar, "Beneficial effects of flaxseed oil and fish oil diet are through modulation of different hepatic genes involved in lipid metabolism in streptozotocin-nicotinamide induced diabetic rats," *Genes and Nutrition*, vol. 8, no. 3, pp. 329–342, 2013.

[32] K. Nomaguchi, M. Tanaka, E. Misawa et al., "Aloe vera phytosterols act as ligands for PPAR and improve the expression levels of PPAR target genes in the livers of mice with diet-induced obesity," *Obesity Research and Clinical Practice*, vol. 5, no. 3, pp. e190–e201, 2011.

[33] E. Misawa, M. Tanaka, K. Nomaguchi et al., "Oral ingestion of Aloe vera phytosterols alters hepatic gene expression profiles and ameliorates obesity-associated metabolic disorders in Zucker diabetic fatty rats," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 11, pp. 2799–2806, 2012.

[34] J. D. Horton, J. L. Goldstein, and M. S. Brown, "SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver," *The Journal of Clinical Investigation*, vol. 109, no. 9, pp. 1125–1131, 2002.

[35] K. Schoonjans, B. Staels, and J. Auwerx, "The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation," *Biochimica et Biophysica Acta—Lipids and Lipid Metabolism*, vol. 1302, no. 2, pp. 93–109, 1996.

[36] P. Libby, "Inflammation in atherosclerosis," *Nature*, vol. 420, no. 6917, pp. 868–874, 2002.

[37] P. Libby, "Role of inflammation in atherosclerosis associated with rheumatoid arthritis," *The American Journal of Medicine*, vol. 121, no. 10, pp. S21–S31, 2008.

[38] A. Yadav, V. Saini, and S. Arora, "MCP-1: chemoattractant with a role beyond immunity: a review," *Clinica Chimica Acta*, vol. 411, no. 21-22, pp. 1570–1579, 2010.

[39] P. Libby, P. M. Ridker, and G. K. Hansson, "Inflammation in atherosclerosis: from pathophysiology to practice," *Journal of the American College of Cardiology*, vol. 54, no. 23, pp. 2129–2138, 2009.

[40] P. J. H. Jones, I. Demonty, Y. M. Chan, Y. Herzog, and D. Pelled, "Fish-oil esters of plant sterols differ from vegetable-oil sterol esters in triglycerides lowering, carotenoid bioavailability and impact on plasminogen activator inhibitor-1 (PAI-1) concentrations in hypercholesterolemic subjects," *Lipids in Health and Disease*, vol. 6, article 28, 2007.

[41] L. S. Rallidis, G. Paschos, G. K. Liakos, A. H. Velissaridou, G. Anastasiadis, and A. Zampelas, "Dietary alpha-linolenic acid

decreases C-reactive protein, serum amyloid A and interleukin-6 in dyslipidaemic patients," *Atherosclerosis*, vol. 167, no. 2, pp. 237–242, 2003.

[42] L. S. Rallidis, G. Paschos, M. L. Papaioannou et al., "The effect of diet enriched with α-linolenic acid on soluble cellular adhesion molecules in dyslipidaemic patients," *Atherosclerosis*, vol. 174, no. 1, pp. 127–132, 2004.

[43] L. Ferrucci, A. Cherubini, S. Bandinelli et al., "Relationship of plasma polyunsaturated fatty acids to circulating inflammatory markers," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 2, pp. 439–446, 2006.

[44] D. Pastori, R. Carnevale, and P. Pignatelli, "Is there a clinical role for oxidative stress biomarkers in atherosclerotic diseases?" *Internal and Emergency Medicine*, vol. 9, no. 2, pp. 123–131, 2014.

[45] A. A. Baskar, K. S. Al Numair, M. Gabriel Paulraj, M. A. Alsaif, M. A. Muamar, and S. Ignacimuthu, "β-sitosterol prevents lipid peroxidation and improves antioxidant status and histoarchitecture in rats with 1,2-dimethylhydrazine-induced colon cancer," *Journal of Medicinal Food*, vol. 15, no. 4, pp. 335–343, 2012.

[46] N. M. Jangale, P. P. Devarshi, A. A. Dubal et al., "Dietary flaxseed oil and fish oil modulates expression of antioxidant and inflammatory genes with alleviation of protein glycation status and inflammation in liver of streptozotocin-nicotinamide induced diabetic rats," *Food Chemistry*, vol. 141, no. 1, pp. 187–195, 2013.

[47] J. F. Keaney Jr., "Oxidative stress and the vascular wall: NADPH oxidases take center stage," *Circulation*, vol. 112, no. 17, pp. 2585–2588, 2005.

[48] H. Kaneto, N. Katakami, M. Matsuhisa, and T.-A. Matsuoka, "Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis," *Mediators of Inflammation*, vol. 2010, Article ID 453892, 11 pages, 2010.

[49] P. A. Barry-Lane, C. Patterson, M. Van Der Merwe et al., "p47phox is required for atherosclerotic lesion progression in ApoE$^{-/-}$ mice," *The Journal of Clinical Investigation*, vol. 108, no. 10, pp. 1513–1522, 2001.

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Research Article

Neuroprotective Effect of *Brassica oleracea* Sprouts Crude Juice in a Cellular Model of Alzheimer's Disease

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β-Amyloid peptide (Aβ) aberrant production and aggregation are major factors implicated in the pathogenesis of Alzheimer's disease (AD), causing neuronal death *via* oxidative stress. Several studies have highlighted the importance of polyphenolic antioxidant compounds in the treatment of AD, but complex food matrices, characterized by a different relative content of these phytochemicals, have been neglected. In the present study, we analyzed the protective effect on SH-SY5Y cells treated with the fragment Aβ_{25-35} by two crude juices of broccoli sprouts containing different amounts of phenolic compounds as a result of different growth conditions. Both juices protected against Aβ-induced cytotoxicity and apoptotic cell death as evidenced by cell viability, nuclear chromatin condensation, and apoptotic body formation measurements. These effects were mediated by the modulation of the mitochondrial function and of the *HSP70* gene transcription and expression. Furthermore, the juices upregulated the intracellular glutathione content and mRNA levels or activity of antioxidant enzymes such as heme oxygenase-1, thioredoxin, thioredoxin reductase, and NAD(P)H:quinone oxidoreductase 1 *via* activation of NF-E2-related factor 2 (Nrf2). Although the effects of the two juices were similar, the juice enriched in phenolic compounds showed a greater efficacy in inducing the activation of the Nrf2 signalling pathway.

1. Introduction

The incidence of neurodegenerative diseases, such as Alzheimer's disease (AD), increases as a function of age. Their aetiology may partially involve lifestyle determinants such as hypercaloric diets poor in fruit and vegetables and sedentary lifestyle, which may lead to obesity, decreased insulin sensitivity, and metabolic syndrome; all of these are characterized by a low-grade inflammation [1]. Due to the steady increase in life expectancy, the prevalence of neurodegenerative diseases is calculated to double within the next 30 years implying that unless ways are found to reduce age-related cognitive decline, healthcare costs will rise exponentially, with a deep social impact and serious implications in terms of economic burden [2]. Hence, therapeutic tools are actively sought which can counteract the neurodegenerative processes and many drugs have been developed and tested in various models and in humans. However, in spite of the promising effects observed in preclinical studies, in most cases clinical trials failed to show therapeutic effectiveness or to reverse the disease

course. One possible alternative strategy would be to operate on lifestyle determinants, in particular on physical activity level and nutrition.

Polyphenolic compounds contained in vegetables, fruits, nuts, and spices exhibit remarkable antioxidant and anti-inflammatory activities which may exert an important role in reducing age-related oxidative stress and inflammation thus hampering the neurodegenerative processes [3, 4]. In this perspective, functional foods and nutraceuticals enriched in polyphenols may represent a novel therapeutic approach in view of their ability to exert anti-inflammatory and antioxidant properties. A large amount of evidence has accumulated over the past few years which strongly implicates free radical-induced oxidative damage in the pathogenesis of several neurodegenerative diseases. The brain is particularly susceptible to oxidative stress due to its extremely high consumption of oxygen and glucose, high content in polyunsaturated fatty acids, and paucity of antioxidant defense systems [5]. *Post-mortem* studies on brain specimens collected from individuals affected by AD revealed an extensive oxidative stress compared to healthy controls, *that is*, increased levels of oxidative markers of lipid, protein, and DNA damage. Therefore, a role for the antioxidant polyphenols in the prevention and/or treatment of AD has been hypothesized [6].

Brassica vegetables are among the foods of increasing interest in nutrition science as a consequence of the beneficial effects of their different phytochemicals on human health [7]. The genus *Brassica* (family *Brassicaceae*, also known as *Cruciferae*) includes a high number of vegetables comprising amongst others broccoli, cauliflower, Brussels sprouts, kohlrabi, cabbage, and mustard. Several epidemiologic studies highlight that a regular intake of Brassica vegetables is associated with a reduced incidence of cancer, and further beneficial effects on health were demonstrated in cardiovascular diseases and in metabolic disorders such as diabetes [8, 9]. Indeed, the edible species within the *Brassicaceae* are a good source of many health promoting compounds including glucosinolates and their by-products, especially isothiocyanates (ITCs) such as sulforaphane (SFN), phenolics, vitamins, carotenoids, proteins, sugars, chlorophyll, minerals, fatty acids, and amino acids [10, 11]. Among phytochemicals contained in broccoli, the most studied are glucosinolates and related compounds together with phenolics [7]. SFN and phenolics (in particular flavonoids) exhibit anticarcinogenic, anti-inflammatory, antioxidant, chemopreventive, and cytoprotective properties [12–14]. Recently, it has been reported that SFN and flavonoids can penetrate the blood brain barrier and exert neuroprotective effects in animal models of neurological disorders [15–18]. In addition, SFN and polyphenols have been reported to attenuate microglia-induced inflammation in hippocampus of LPS-treated mice and microglia cell lines [19–21].

Although to date several evidences support the neuroprotective role of individual, purified broccoli phytochemicals, few studies have taken into account the potential protective effects of complex matrices of bioactive molecules contained in broccoli. Currently, nutrition research tends to emphasize the importance of assessing the interactions among the various phytochemicals in food or in their crude derivatives, with regard to their effects on the pathophysiology of the human body. In this work broccoli sprouts of *Brassica oleracea* L. var. *botrytis* subvar. *cymosa* were grown in two conditions, *that is*, in the dark or exposed to white light in the presence of sucrose, in order to obtain a clear difference in terms of the phenolics/anthocyanins content between the differently grown sprouts. Juices obtained by cold pressing of the sprouts were then assayed for their antioxidant capacity and for their ability to act as possible neuroprotective agents in a cellular model of AD, *that is*, SH-SY5Y human neuroblastoma cells treated with the 25–35 fragment of the β-amyloid peptide ($A\beta_{25-35}$), one of the most toxic fragments of the full length $A\beta_{1-42}$. In particular, we investigated whether broccoli sprouts juices can attenuate $A\beta_{25-35}$-induced cytotoxicity and cell death by stimulating the antioxidant defence capacity *via* activation of NF-E2-related factor 2 (Nrf2) and the subsequent expression of antioxidant and phase II detoxification enzymes which play key roles in counteracting oxidative damages.

2. Materials and Methods

2.1. Materials. Unless otherwise stated, reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). $A\beta_{25-35}$ was synthesized by conventional solid phase chemistry [22]. Other reagents were Trizol reagent from Invitrogen (Carlsberg, CA, USA); QuantiTect Reverse Transcription kit from QIAGEN (Hilden, Germany); primary antibody rabbit monoclonal anti-Nrf2 and secondary antibody goat polyclonal anti-rabbit IgG (Alexa Fluor 488) from Abcam (Cambridge, UK).

2.2. Plant Growth and Juice Preparation. Broccoli sprouts (*Brassica oleracea* L. var. *botrytis* subvar. *cymosa*) were grown essentially as described by Maldini et al. [23]. After sterilization the seeds were transferred into the Vitaseed (SUBA & UNICO, Longiano, FC, Italy) germinator filled with distilled water. Sprouts were grown for 5 days at 21°C and 70% humidity in a dedicated climatic chamber (Weiss Gallenkamp, Loughborough, UK) in the dark (sprouts of type A) or with 16 h of illumination and 8 h of darkness (sprouts of type B), respectively. After the first 3 days of growth the sprouts of type B were treated for 48 h with a solution of 176 mM sucrose, freshly prepared with sterile distilled water. At the end of the 5 days of growth, plantlets were weighed and cold-pressed with Angel 8500S Luxury juicer (Living Juice Ltd., Lecco, LC, Italy) for the production of juice. The juices obtained were centrifuged at 4,000 g for 30 min at 4°C. The supernatant was immediately frozen in liquid nitrogen and stored at −80°C until use. Before treating the cells, the juices were diluted in the culture medium and filtered on a 0.2 μm sterile cellulose acetate membrane.

2.3. Determination of Total Phenols, Flavonoids, Anthocyanins, and Sulforaphane

2.3.1. Total Phenols. Total phenols were determined by the Folin-Ciocalteu assay as described by Singleton and Rossi Jr. [24]. Briefly, the reaction solution was prepared by mixing

10 μL of blank, standard, or sample with 790 μL of distilled water. After addition of 50 μL of Folin-Ciocalteu reagent the reaction mixture was incubated for 3 min at room temperature (RT) and then 150 μL of a 20% (w/v) Na_2CO_3 aqueous solution was added. After 2 h of incubation, the absorbance at 760 nm was measured on a Hitachi U2000 spectrophotometer (Hitachi, Tokyo, Japan). The results were expressed as mg of gallic acid equivalents (GAE) per mL of juice.

2.3.2. Total Flavonoids. The total flavonoid content of the juices was determined using the aluminium trichloride assay as described by Dewanto et al. [25]. The assay was carried out in a 96-well plate and in each well the following solutions were added: 20 μL of rutin hydrate standard (half serial dilutions of 1 mg/mL) or 20 μL of a dilution of juices or blank, 20 μL of sodium nitrate solution (3%, w/v), 20 μL of aluminum trichloride solution (1%, w/v), and 100 μL of sodium hydroxide solution (0.5 M). Absorbance was measured at 450 nm (Appliskan microplate reader, Thermo Scientific, Vantaa, Finland). Results were expressed as mg of rutin equivalents (RE) per mL of juice.

2.3.3. Total Anthocyanins. Total anthocyanins quantification was performed by the pH-differential method as described by Giusti and Wrolstad [26]. The juice was diluted in a pH 1.0 solution (0.1 M HCl, 25 mM KCl) and in a pH 4.5 solution (0.4 M CH_3COONa). The absorbance of the mixtures was then measured at 535 and 700 nm against distilled water. The value $(Abs_{535}-Abs_{700})_{pH1.0}-(Abs_{535}-Abs_{700})_{pH4.5}$ corresponds to the absorbance due to the anthocyanins. Calculation of the anthocyanins concentration was based on a cyanidin-3-*O*-glucoside (Cy-3G) molar extinction coefficient of 25,965 $M^{-1} \times cm^{-1}$ and a molecular mass of 449.2 g $\times$ mol^{-1}. Results were expressed as μg cyanidin-3-*O*-glucoside equivalents (CGE) per mL of juice.

2.3.4. Sulforaphane. SFN determination was performed using an HPLC system (Perkin-Elmer, USA) interfaced to an Applied Biosystems (Foster City, CA, USA) API 3200 Q-Trap spectrometer. Quantitative on-line HPLC-ESI-MS/MS analyses were performed using mass spectrometer in Multiple Reaction Monitoring (MRM) mode. The API 3200 ES source was tuned by infusing a standard solution of SFN (1 μg/mL in methanol 50%, v/v) into the source at a flow rate of 10 μL/min. The optimized parameters were declustering potential 45 eV, entrance potential 5 eV; fragmentation reactions selected for SFN were 178 $\rightarrow$ 114 (CE = 18; CXP = 4; CEP = 14). The source temperature was held at 400°C and the voltage applied was −4500. The dwell time was 120 ms. Samples juices were opportunely diluted in H_2O with 0.1% (v/v) formic acid, filtered, injected (10 μL) into a Luna C18 column (Phenomenex, USA) (5 μm, 150 × 2.1 mm), and eluted at flow rate of 0.3 mL/min. Mobile phase A was H_2O containing 0.1% (v/v) formic acid while mobile phase B was acetonitrile containing 0.1% (v/v) formic acid. Elution gradient was from 100% A to 20 : 80 (A : B) in 20 min and from 20 : 80 (A : B) to 0 : 100 (A : B) in 1 min. The column was kept at 25°C, using a Peltier Column Oven Series 200 (Perkin Elmer). Data acquisition and processing were performed using Analyst software 1.5.1. SFN concentration was calculated over an external standard curve of SFN.

2.4. Antioxidant Activity

2.4.1. NBT Assay. Broccoli sprouts juices effect as superoxide anion scavenger was assayed by the inhibition of nitroblue tetrazolium chloride (NBT) reduction by β-nicotinamide adenine dinucleotide reduced form (βNADH) in the presence of phenazine methosulfate (PM) as described by Yuting et al. [27]. Reaction mixtures contained 73 μM βNADH, 15 μM PM, 50 μM NBT, and 0–20 μL/mL of A or B juice in 1 mL of 0.02 M Tris-HCl buffer, pH 8.0. Absorbance variations (ΔAbs/min) were determined at 560 nm, by measuring the initial rate of superoxide anion-induced NBT reduction. The percentage inhibition of $\Delta Abs_{560\,nm}$/min after 15 sec at 25°C was calculated and plotted as a function of concentration of antioxidants. Stock solutions (1 mM) were freshly prepared every day dissolving PM in ethanol, NBT in water, and βNADH in 0.05 M phosphate buffer, pH 7.4.

2.4.2. ABTS Assay. The 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was dissolved in water to a 7 mM final concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate for 16 h in the dark at RT [28]. $ABTS^{\bullet+}$ was diluted with ethanol, to obtain an absorbance of 0.70 (±0.02) at 734 nm. 10 μL of diluted juices in ethanol (0–10 μL/mL final concentration) was added to 1 mL of the working $ABTS^{\bullet+}$ solution. The percentage inhibition of absorbance at 734 nm after 6 min at RT was calculated and plotted as a function of antioxidant concentration.

2.4.3. DPPH Assay. An aliquot of 10 μL of diluted juices in ethanol (0–10 μL/mL final concentration) was added to 1 mL of 30 μM ethanolic solution of stable nitrogen centered free radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and the absorbance was monitored spectrophotometrically at 517 nm after 15 min at RT. Radical $DPPH^{\bullet}$ scavenging capacity was estimated from the difference in absorbance with or without antioxidants and expressed as percent $DPPH^{\bullet}$ disappearance as a function of the sample concentration.

2.4.4. Deoxyribose Assay. The Fe(II)-H_2O_2-induced degradation of 2-deoxy-D-ribose in the presence of EDTA was assayed as previously described by Halliwell et al. [29]. Briefly, 1 mL reaction mixture contained 2.8 mM 2-deoxy-D-ribose, 0.1 M phosphate buffer (pH 7.4), 0–20 μL juice A or juice B, 50 μM ammonium ferrous sulphate ($(NH_4)_2Fe(SO_4)_2$), 0.1 mM EDTA, and 1 mM hydrogen peroxide (H_2O_2). Fresh solution of Fe(II) was prepared in deaerated water immediately before each experiment and used to start the reactions which were carried out at 37°C for 30 min. The extent of 2-deoxy-D-ribose degradation was monitored by the formation of malondialdehyde determined by the addition of 1 mL of 1% (w/v) 2-thiobarbituric acid in 50 mM sodium hydroxide and 1 mL of 2.8% (w/v) trichloroacetic acid. After heating at 80°C for 20 min, the reaction solutions were cooled and the absorbance was read at 532 nm against appropriate blanks.

The scavenger activity towards $OH^{\bullet}$ radical was expressed as percentage inhibition of 2-deoxy-D-ribose degradation in the presence of antioxidants compared to the control.

2.4.5. ORAC Assay. For oxygen radical absorbance capacity (ORAC) assay, the methods of Cao and Prior [30] were used. The assay was carried out in black-walled 96-well plates and each well contained a final volume of 200 μL. Broccoli sprouts juices were diluted with 75 mM phosphate buffer (pH 7.0) to a final concentration of 0.5 μL/mL. The (±)6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a standard to a final concentration of 5 μM. The reaction solution also contained 3.4 μg/mL of R-phycoerythrin, and the plate was incubated at 37°C for 15 min. Then 2,2′-azobis(2-methylpropionamidine) dihydrochloride was added to each well to a final concentration of 16 mM, and fluorescence intensity was estimated every 5 min for a total of 120 min using an excitation filter of 492/10 nm and an emission filter of 570/10 nm. Results were calculated on the basis of the differences in area under the curve between the control and the sample.

2.5. Preparation of Aggregated Aβ_{25-35}. Aβ_{25-35} was dissolved in sterile phosphate buffered saline, pH 7.4 (PBS) at a concentration of 1 mM, and incubated in a Sonicator Bath at RT for 15–30 min to induce aggregation. After aggregation, the solution was stored at −20°C until use. Immediately before treating the cells, stock solution was diluted to 25 μM final concentration in culture medium.

2.6. Cell Culture. Human neuroblastoma SH-SY5Y cell line was obtained from the ICLC (Genova, Italy). Cells were grown in DMEM/F-12 medium containing 10% fetal bovine serum (Gibco BRL. Life Technologies Inc., Grand Island, NY, USA) and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO_2. Cells were plated at an appropriate density according to each experimental setting and treated with 25 μM aggregated Aβ_{25-35} in the presence or in the absence of 10 μL/mL of juice A or juice B. Appropriate controls in which the cells were treated only with juice A or juice B were run in parallel.

2.7. Cell Viability Assay. Cell viability was determined by using thiazolyl blue tetrazolium bromide (MTT) dye reduction assay. Briefly, cells were seeded in 96-well plates at a density of 15,000 cells/well. After treatment, 20 μL of a 5 mg/mL solution of MTT in PBS was added to the culture medium and cells were incubated at 37°C for 2 h. The supernatants were then aspirated off and formazan crystals were dissolved with 100 μL/well of dimethyl sulfoxide. The optical density of each well was determined at 570 nm with a reference at 690 nm using a microplate reader.

2.8. Assessment of Apoptosis by Nuclear Staining. Apoptosis was analyzed by using the fluorescent dye Hoechst 33258 [31]. Briefly, cells were seeded on 18 × 18 mm glass coverslips in 6-well plates at a density of 200,000 cells/well. After treatment, the cells were washed in PBS and fixed in 4% (w/v) formaldehyde for 20 min. The coverslips were permeabilized for 5 min in 0.5% (v/v) Triton X-100 and then stained with 0.1 μg/mL Hoechst 33258. Coverslips were mounted with 50% (v/v) glycerol in PBS and examined by fluorescence microscope (DM IL LED model, Leica Microsystems CMS Gmb H, Wetzlar, Germany) equipped with an 355 nm excitation filter, an 465 nm emission filter, and a digital camera (Leica DCF450 C). For each slide 5 images were collected at random position by means of the Leica Application Suite Advanced Fluorescence (LAS AF) software. Apoptotic cells were recognized by their characteristic nuclei condensation, fragmentation, and bright staining.

2.9. Flow Cytometric Detection of Apoptotic Cells. Apoptotic cells can be recognized after staining with a DNA-specific fluorochrome by flow cytometry as having less DNA than G1-cells. Cells were plated in 6-well plates at a density of 450,000 cells/well. After treatment cells were collected by centrifugation, suspended in ice-cold 70% (v/v) ethanol in PBS, and fixed at 4°C for 48 h, then the cells were washed and suspended in 600 μL of DNA staining reagent containing 180 μg/mL ribonuclease A and 50 μg/mL propidium iodide (PI). Red fluorescence (DNA) was detected by a flow cytometer (BD Accuri C6, BD Bioscences, Erembodegem, Belgium) equipped with a 488 nm excitation laser and a 585/40 nm band-pass filter (FL2 channel). 30,000 events were collected for each sample and the percentage of apoptotic cells accumulating in the sub-G1 peak was calculated.

2.10. Measurement of Mitochondrial Membrane Potential. The mitochondrial membrane potential ($\Delta\Psi$m) value was determined by measuring the change in red/green fluorescence emission of the mitochondrial probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Briefly, cells were plated in 6-well plates at a density of 450,000 cells/well and after treatment the cells were incubated with 2.5 μg/mL JC-1 in culture medium at 37°C for 20 min. After washing in PBS, cells were analyzed by flow cytometry with band-pass filters of 533/30 nm (FL1 channel) and 585/40 nm (FL2 channel), respectively. 30,000 events were collected for each sample. For detection of JC-1 dye, logarithmic signal amplification with typical green-orange electronic signal compensation near 4% and orange-green electronic signal compensation around 10% was used. $\Delta\Psi$m (red/green JC-1 fluorescence) was expressed as percentage of the control.

2.11. Determination of Intracellular GSH Levels. Intracellular reduced glutathione (GSH) levels were determined as described by Hissin and Hilf [32]. The cells were seeded in 25 cm^2 flask at a density of 1.2×10^6 cells/flask. After treatment, the cells were harvested by centrifugation at 700 g for 10 min at 4°C, washed twice with ice-cold PBS, then suspended in 300 μL ice-cold lysis buffer (1/2 (v/v) mixture of 1 mM hydrochloric acid/10% (v/v) phosphoric acid), and incubated on ice for 15 min. After centrifugation at 15,000 g for 15 min at 4°C, an aliquot of 12.5 μL supernatant was diluted with 250 μL of 0.1 M Na_2HPO_4 buffer (pH 8.0) containing 0.1% (w/v) EDTA and finally 20 μL of this solution was added to 320 μL derivatizing solution (1/15 (v/v) mixture of 0.1%

(w/v) ortho-phthalaldehyde in methanol/0.1 M Na_2HPO_4 buffer (pH 8.0) containing 0.1% (w/v) EDTA). After incubation at RT for 15 min in the dark, the reaction mixture was analyzed by HPLC with Waters 60F pumps and 600 pumps control unit system equipped with a X-Bridge C18, 5 μm, 4.6 × 150 mm column associated with a Symmetry C18, 3.9 × 20 mm guard column (Waters Corporation, Milford, Massachusetts, USA) and a Waters 71P autosampler. The mobile phase consisted of 15% (v/v) methanol in 25 mM Na_2HPO_4, pH 6.0. Isocratic elution was performed at 37°C at a flow rate of 0.5 mL/min. The excitation/emission wavelengths were set to 350/420 nm in a Shimadzu RF-551 spectrofluorometric detector. The instrument control and data acquisition were carried out using the Waters Millennium[32] software. GSH intracellular levels, normalized for cell number, were expressed as a percentage compared to the control cells.

2.12. Real Time PCR Analyses. The cells were plated in 6-well plates at a density of 450,000 cells/well and after treatment total RNA for reverse transcription-polymerase chain reaction (RT-PCR) was extracted using Trizol reagent according to manufacturer's instructions. Reverse transcription was performed from 1 μg of total RNA using the QuantiTect Reverse Transcription kit as recommended by the manufacturer. Real-time quantitative RT-PCR (qRT-PCR) measurements were performed using a Bio-Rad iCycler iQ (Bio-Rad, Milan, Italy) using ribosomal protein S27a (*RPS27A*) housekeeping gene as normalizing control. The genes of which we evaluated expression levels were heme oxygenase-1 (*HO-1*), heat shock protein 70 (*HSP70*) thioredoxin reductase (*TRXR*), and thioredoxin (*TRX*). For this purpose, we chose the following primers designed with GenScript Real-time PCR Primer Design Software: *HO-1*_for GTCAGAGGCCCTGAAGGAG and *HO-1*_rev GCCCTTCTGAAAGTTCCTCA, *HSP70*_for CAAGAAGAAGGTGCTGGACA and *HSP70*_rev TCCTCTTGTGCTCAAACTCG, *TRXR*_for GGTCCAAATGCTGGAGAAGT and *TRXR*_rev TGGATTCCAATTGTGCTGTC, *TRX*_for GCAGATCGAGAGCAAGACTG and *TRX*_rev CACGTGGCTGAGAAGTCAAC, *RPS27A*_for AAGCACCAAATTGATGGTCA and *RPS27A*_rev AACCACTGGGTCACATAATCC. The amplifications of qRT-PCR were monitored using the SYBR Green fluorescent stain and the presence of a single PCR product was verified by dissociation analysis in all amplifications. The comparative threshold cycle (ΔΔCT) method was used to calculate the relative amount of gene expression. Intracellular mRNAs levels were reported as fold induction compared to the 0 h control cells.

2.13. Western Blotting for Hsp70. After treatment, SH-SY5Y cells (4×10^6) were lysed in 100 μL RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 0,5% sodium deoxycholate) containing 1 mM PMSF, 0.2 mM Na_3VO_4, and SIGMAFAST Protease Inhibitor Cocktail. The lysates were incubated on ice for 30 min and centrifuged at 12,000 g for 20 min at 4°C. The supernatants were collected and proteins quantification was performed using a Bradford Assay (BIO-RAD). Equal amounts of proteins (20 μg) were separated on 12% SDS-PAGE and transferred to nitrocellulose membrane probed with the primary anti-Hsp70 mAb (1 : 1000) (Stressgen) or antiactin mAb (1 : 5000) (Alexis). The protein bands were visualized by ECL system (Millipore) according to the manufacturer's instructions. Densitometric analyses were performed with Image J software and normalized to the reference actin protein. The intracellular Hsp70 protein levels were expressed as a percentage compared to the control cells.

2.14. NAD(P)H:Quinone Oxidoreductase 1 (NQO1) Activity. Cells were seeded in 75 cm^2 flask at a density of 3.5×10^6 cells/flask. After treatment, the cells were washed with PBS and lysed in 250 μL of ice-cold 20 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose, 1 mM phenylmethanesulfonyl fluoride, and *cOmplete* Protease Inhibitor Cocktail by using the Potter-Elvejham homogenizing system. The homogenate was centrifuged at 15,000 g for 20 min at 4°C. NQO1 activity was carried out as previously described [33]. Briefly, the reaction mixture (1 mL) contained 50 mM sodium phosphate buffer (pH 7.4), 0.7 mg/mL bovine serum albumin (BSA), 40 μM 2,6-dichlorophenolindophenol (DCPIP), and 40 μg sample proteins. The reaction was started by adding nicotinamide adenine dinucleotide phosphate reduced form (βNADPH) at a final concentration of 0.3 mM. The decrease in absorbance due to reduction of DCPIP was monitored at 600 nm during the first 10 sec of the kinetics. Unspecific activity was determined by adding 10 μM dicumarol to the reaction mixture before addition of βNADPH and subtracted to total activity. NQO1 activity was calculated as nmol of reduced DCPIP per min per mg of total protein by using an extinction coefficient of 21×10^{-3} M × cm^{-1}. The intracellular NQO1 activity levels were expressed as a percentage compared to the control cells.

2.15. Nrf2 Immunostaining. Cells were seeded on 18 × 18 mm glass coverslips in 6-well plates at a density of 5×10^5 cells/well. After treatment the cells were fixed by incubating with 2% (w/v) formaldehyde in PBS and washed with PBS and permeabilized with 0.1% (v/v) Triton X-100 in PBS. After washing with PBS and finally with 0.05% (v/v) Tween 20 in PBS (PBST), the coverslips were exposed for 30 min to blocking buffer (5% (w/v) BSA in PBST) and then incubated overnight at 4°C with the rabbit anti-Nrf2 mAb (1 : 100 dilution) in 1% (w/v) BSA in PBST. The coverslips were washed with PBST and with PBS before incubation for 1 h with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1 : 500 dilution) in 1% (w/v) BSA in PBST. After washing with PBST and with PBS and incubating for 30 min with 5 μg/mL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in saline solution, the coverslips were washed again with PBS and mounted with 50% (v/v) glycerol in PBS. The samples were examined by fluorescence microscope (DM IL LED model, Leica Microsystems CMS Gmb H, Wetzlar, Germany) equipped with an 355 nm excitation filter, an 465 nm emission filter, and a digital camera (Leica DCF450 C). Microscopy imaging was performed using the LAS AF

software. Control samples were treated following identical protocol but omitting the primary antibody.

To analyze the Nrf2 nuclear translocation by immunostaining assay, Image J software was used and 3 fields for each sample were analyzed. The average nuclear signal quantification was obtained by integrated density measurement for each nucleus subtracted of background signal, according to

$$\sum_{i=1}^{N} \frac{\text{IntDen}\_i - (A\_i * \langle \text{bk} \rangle)}{N}, \quad (1)$$

where IntDen_i is the integrated density signal measured for each nucleus, A_i corresponds to single nucleus area, and ⟨bk⟩ features the mean of background signals. Densitometric analysis data were expressed as percentage of control.

2.16. Statistical Analysis. Experiments were repeated at least in triplicate and all the results are expressed as the mean value ± standard error of the mean (SEM). Statistical comparison between groups was made using unpaired Student's t-test. P values <0.05 were regarded as significant.

3. Results

3.1. Polyphenols and Sulforaphane Determination and Antioxidant Potential of Broccoli Sprouts Juices. The quantification of phenolics in the two broccoli sprouts juices shows a significant difference in bioactive phenols content between juice A (obtained from sprouts grown in the dark) and juice B (obtained from sprouts grown under light and sucrose stress). As shown in Table 1, juice B shows a 2-fold enrichment of total polyphenols compared to juice A (2.86 in B *versus* 1.40 in A mg GAE/mL, $P < 0.05$). Among polyphenols, flavonoid increase was found to be 2.7-fold (1.18 in B *versus* 0.44 in A mg RE/mL, $P < 0.001$), whereas anthocyanins, undetectable in juice A, were found to be 29.41 μg CGE/mL in juice B. Conversely, a comparable content of SFN was observed in both juices (20.40 in B *versus* 18.60 in A μg/mL, $P > 0.05$). To strengthen the evidence of the increase in polyphenols content, an evaluation of the intrinsic antioxidant activity of the juices was performed with several assays targeted versus different free radicals, *that is*, superoxide anion, ABTS radical cation, DPPH nitrogen radical, hydroxyl radical, and peroxyl radical (Table 2). Table 2 shows IC_{50} and ORAC values of the two juices, indicating that juice B shows a significantly higher antioxidant capability compared to juice A. The ~2-fold increase of antioxidant activity was in accordance with the enrichment in total phenolics amount.

3.2. Protective Effect of Broccoli Sprouts Juices on $A\beta_{25-35}$-Induced Cytotoxicity. Preliminary investigations were carried out to determine the effect of juice A and juice B on the viability of SH-SY5Y cells, in order to verify that these mixtures did not exert a direct cytotoxic effect and to optimize the amount of juice to be used in further experiments. Both juices showed no negative effects on cell viability up to a final concentration of 10 μL/mL and for 72 h of treatment. Juice B at higher concentrations (≥20 μL/mL) was found to reduce cell viability in the long term (data not shown). Based on these results, the possible protective effect of both broccoli juices on the viability of the $A\beta_{25-35}$-treated SH-SY5Y cells was studied at a final concentration of 10 μL/mL. The final concentration of the various compounds in culture medium was around 1 μM for sulforaphane for both juices and in the micromolar range for polyphenols, as inferred from Table 1.

Table 1: Phenolics and sulforaphane content of broccoli sprouts juices.

	Juice A	Juice B
Total polyphenols (mg GAE/mL)	1.40 ± 0.08	2.86 ± 0.33*
Total flavonoids (mg RE/mL)	0.44 ± 0.02	1.18 ± 0.06***
Total anthocyanins (μg CGE/mL)	ND	29.41 ± 4.02
Sulforaphane (μg/mL)	18.60 ± 1.20	20.40 ± 1.90

Mean ± SEM ($n = 3$) for total polyphenols, flavonoids, and anthocyanins.
Mean ± SEM ($n = 5$) for sulforaphane.
Student's t-test: $^{*}P < 0.05$; $^{***}P < 0.001$ versus juice A.
ND: not detectable.

Table 2: Antioxidant activity of broccoli sprouts juices.

Free radical scavenging activity	IC_{50} (μL/mL)	
	Juice A	Juice B
NBT assay ($^{\bullet}O_2^{-}$)	5.17 ± 0.54	2.37 ± 0.26*
ABTS assay ($ABTS^{\bullet+}$)	2.70 ± 0.06	1.63 ± 0.03**
DPPH assay ($DPPH^{\bullet}$)	8.73 ± 0.07	2.67 ± 0.03***
Deoxyribose assay ($^{\bullet}OH$)	1.85 ± 0.03	0.77 ± 0.03***
Oxygen radical absorbance capacity	ORAC value (mmol/L Trolox equivalents)	
	Juice A	Juice B
ORAC assay (R-O-$O^{\bullet}$)	7.06 ± 0.37	11.46 ± 0.46***

Mean ± SEM ($n = 3$) for free radical scavenging activity.
Mean ± SEM ($n = 5$) for oxygen radical absorbance capacity.
Student's t-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus juice A.

As shown in Figure 1, 25 μM $A\beta_{25-35}$ treatment significantly decreased SH-SY5Y cells viability as compared to untreated cells (~70% at 24–48 h and 63% at 72 h, $P < 0.001$ *versus* control). The cotreatment with 10 μL/mL juice A or juice B significantly reduced $A\beta_{25-35}$-induced cytotoxicity, restoring a cell viability comparable to that of the control (110% in (A + Aβ) and 117% in (B + Aβ) *versus* 71% in Aβ at 24 h, $P < 0.001$; 108% in (A + Aβ) and 113% in (B + Aβ) *versus* 69% in Aβ at 48 h, $P < 0.001$; 104% in (A + Aβ) and 102% in (B + Aβ) *versus* 63% in Aβ at 72 h, $P < 0.001$).

3.3. Protective Effect of Broccoli Sprouts Juices on $A\beta_{25-35}$-Induced Apoptosis. SH-SY5Y cells apoptosis was evaluated by fluorescence microscopy by using the nuclear dye Hoechst 33258 (Figure 2(a)). Treatment of cells with 10 μL/mL of juice A or juice B showed no proapoptotic effect compared to controls, whereas apoptosis increased from 7% in control cultures to about 18% in cells treated with 25 μM $A\beta_{25-35}$ for 24 ($P < 0.001$), 48 ($P < 0.05$), and 72 h ($P < 0.001$). Following treatment of the cells with $A\beta_{25-35}$ in the presence of 10 μL/mL of juice A or juice B, a significant reduction in apoptotic cells was observed compared to the treatment with

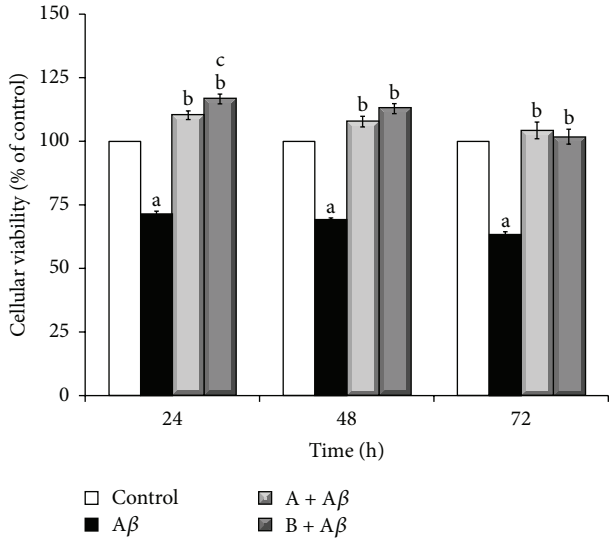

Figure 1: Protective effect of broccoli sprouts juices on Aβ_{25-35}-induced cytotoxicity. SH-SY5Y cells were incubated with 25 μM Aβ_{25-35} in the presence or in the absence of 10 μL/mL of juice A or juice B for 24, 48, and 72 h. Cell viability was determined by MTT reduction assay and expressed as percentage compared to control. Data are represented as mean ± SEM ($n = 5$), $^{a}P < 0.001$ *versus* control; $^{b}P < 0.001$ *versus* Aβ_{25-35}; $^{c}P < 0.05$ *versus* (A + Aβ).

Aβ_{25-35} alone (11% in the cotreatment with juice A or juice B and Aβ_{25-35} *versus* 18% in the exposure to Aβ_{25-35} alone at 24 ($P < 0.01$) and 48 h ($P < 0.05$); 9% in the cotreatment with juice A or juice B and Aβ_{25-35} *versus* 18% in exposure to Aβ_{25-35} alone at 72 h ($P < 0.001$) (Figure 2(b)). To confirm these data flow cytometric analyses were also performed by staining treated cells with PI (Figure 2(c)). Data obtained revealed that 25 μM Aβ_{25-35} treatment induced a marked increase in the sub-G1 (hypodiploid) fraction of the total cell population, compared to the control group with percentages raising from 1.2% to 6.4% at 24 h, from 0.6% to 6.3% at 48 h, and from 0.9% to 3.5% at 72 h. Flow cytometry analysis confirmed the absence of any proapoptotic effect of both juice A and juice B (data not shown). Treatment of cells with 25 μM Aβ_{25-35} in the presence of 10 μL/mL of juice A or juice B reduced apoptotic cells fraction from 6.4% in Aβ to 5.5% in (A + Aβ) and to 4.3% in (B + Aβ) at 24 h; from 6.3% in Aβ to 5.4% in (A + Aβ) and to 4.0% in (B + Aβ) at 48 h; finally, from 3.5% in Aβ to 2.0% in (A + Aβ) and to 2.1% in (B + Aβ) at 72 h.

3.4. Broccoli Sprouts Juices Counteract Aβ_{25-35}-Induced Reduction of the $\Delta\Psi$m. We also confirmed the protective effect of broccoli sprouts juices against Aβ_{25-35}-induced apoptotic cell death by examining a proapoptotic signal such as the reduction of the $\Delta\Psi$m. Cells exposed to 25 μM Aβ_{25-35} showed a rapid reduction in $\Delta\Psi$m (Figure 3), which at 1 and 4 h of incubation was found to be ~20% compared to control cells ($P < 0.01$), whereas starting from 7 h up to 72 h it was ~30% (statistical significance ranging from $P < 0.05$ to $P < 0.001$). During the first 7 h of treatment with 10 μL/mL of juice A, no significant changes in $\Delta\Psi$m were observed whereas a marked and progressive increase for longer exposure times was evidenced, with $\Delta\Psi$m values reaching 205% of control at 48 h ($P < 0.01$) and 194% of control at 72 h ($P < 0.05$). When the cells were incubated with 10 μL/mL of juice B, a decrease of $\Delta\Psi$m (73% *versus* control, $P < 0.05$) was observed after 4 h of exposure, but on the other hand the $\Delta\Psi$m readily returned to control values within 7 h of treatment. Similarly to what was observed for juice A, also juice B induced a significant increase of $\Delta\Psi$m, from 48 h of exposure (195%, $P < 0.05$) and up to 72 h (217%, $P < 0.01$), compared to control cells. When the cells were treated with Aβ_{25-35} in the presence of juice A or juice B, no protective effect on the $\Delta\Psi$m was observed within 48 h of treatment, whereas after 72 h of incubation, broccoli sprouts juices significantly improved Aβ_{25-35}-induced impairment in $\Delta\Psi$m, with juice B being more effective than juice A (75% *versus* 56%, resp., $P < 0.05$).

3.5. Broccoli Sprouts Juices Downregulate Aβ_{25-35}-Mediated Apoptotic Cell Death Signalling Pathways by Inducing the HSP70 Gene Overexpression. To elucidate the molecular mechanisms underlying the antiapoptotic effects of broccoli sprouts juices in SH-SY5Y cells, expression of the *HSP70* gene was examined. The heat shock protein Hsp70 plays a key role in the cellular response for the protection from oxidative stress [34–36]. The determination by qRT-PCR of Hsp70 mRNA levels in SH-SY5Y cells (Figure 4(a)) showed no significant changes upon exposure to 25 μM Aβ_{25-35}. Conversely, both broccoli sprouts juices, either in the absence or in the presence of Aβ_{25-35}, induced an increase of *HSP70* gene expression starting from 4 h of incubation and with a peak at 7 h (3.5–4 times *versus* control and Aβ_{25-35}, $P < 0.01$), still persistent up to 24 h.

The protein levels of Hsp70, as measured by western blot analysis (Figure 4(b)), were found to be significantly decreased after 7 h of treatment with Aβ_{25-35}, in line with literature data [37]. The cotreatment with both juice A and juice B restored Hsp70 protein levels, highlighting the importance of Hsp70 as an antiapoptotic factor.

3.6. Protective Effect of Broccoli Sprouts Juices on Aβ_{25-35}-Induced GSH Depletion. It is well known that Aβ can induce oxidative stress *via* several different mechanisms thus triggering cell death [38]. To further verify whether Aβ_{25-35} treatment induces oxidative stress in our model, we determined the intracellular content of GSH, one of the major antioxidant defence systems in the cell. As shown in Figure 5, the treatment of SH-SY5Y cells with 25 μM Aβ_{25-35} up to 72 h was responsible for a rapid and significant reduction of intracellular GSH levels compared to the control group, respectively, of ~65% in the first 7 h of incubation, 81% after 24 h, and finally 55% after 48 h, up to 72 h of exposure. Both juice A and juice B, also determined a fast and significant decrease in the intracellular GSH levels compared to control cells, with a *minimum* achieved within 4 h of treatment (40% and 23% for juice A and juice B, resp.). On the other hand, after 7 h of incubation, and even more clearly within 24 h of exposure to the juices, a cellular response was observed that led to

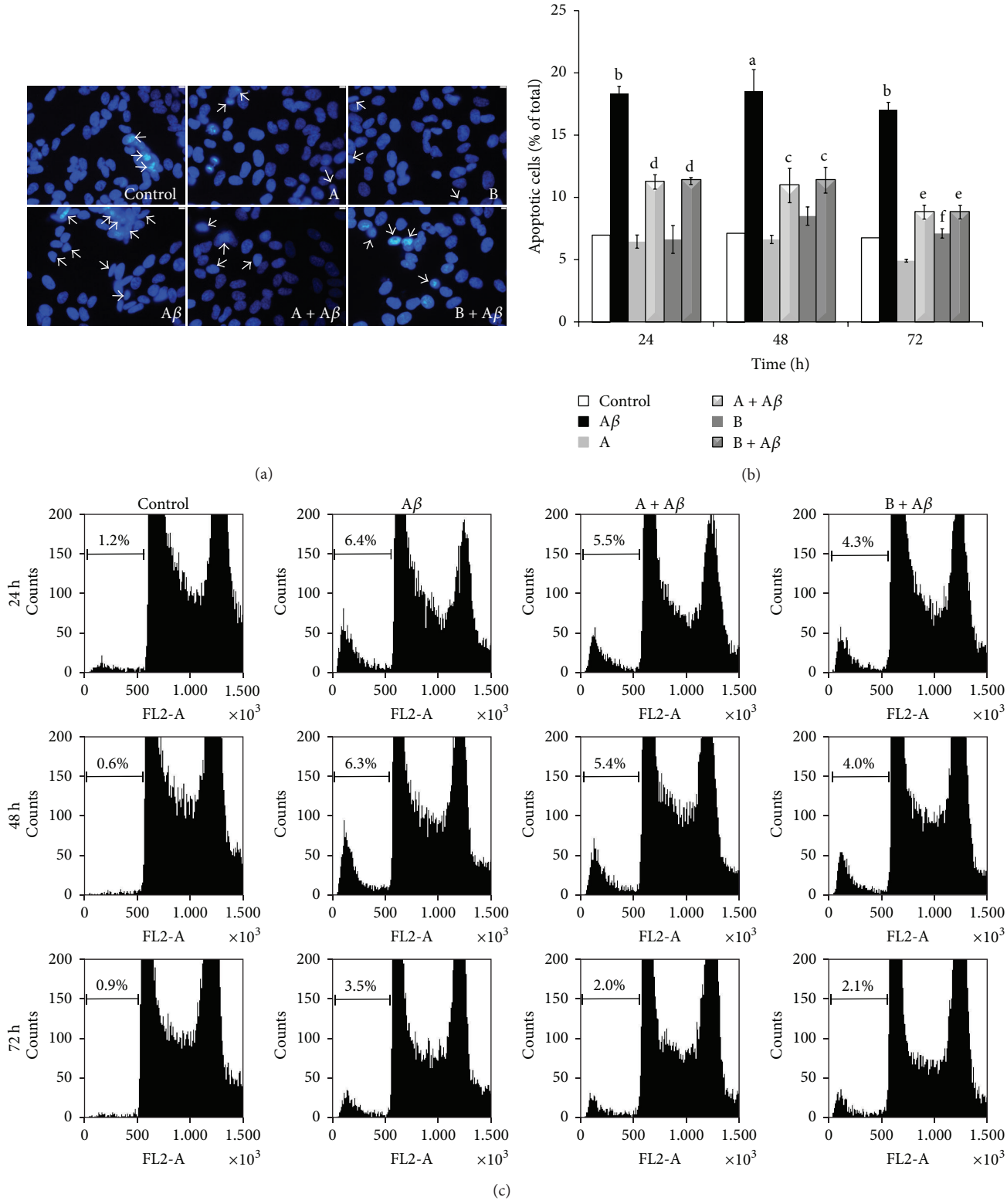

FIGURE 2: Protective effect of broccoli sprouts juices on $A\beta_{25-35}$-induced apoptosis. SH-SY5Y cells were incubated with 25 μM $A\beta_{25-35}$ in the presence or in the absence of 10 μL/mL of juice A or juice B for 24, 48, and 72 h. (a) Morphological analysis of apoptosis was achieved by staining of cell nuclei with Hoechst 33258. Arrows indicate the apoptotic cells. Images are representative of three different experiments. (b) Quantification of the percentage of apoptotic cells by fluorescence microscopy with the DNA intercalating Hoechst 33258. Data are expressed as mean ± SEM ($n = 3$), $^{a}P < 0.05$ and $^{b}P < 0.001$ *versus* control; $^{c}P < 0.05$, $^{d}P < 0.01$, and $^{e}P < 0.001$ *versus* $A\beta_{25-35}$; $^{f}P < 0.05$ *versus* juice A. (c) Analysis by flow cytometry of the sub-G1 peak in cells stained with propidium iodide (PI). The percentage of cells with hypodiploid DNA content is shown as function of the PI fluorescence signal (FL2-A). Histograms are representative of three independent experiments.

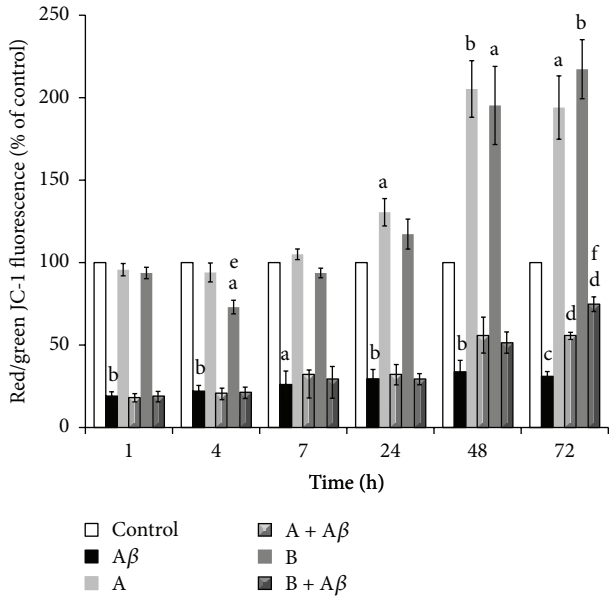

FIGURE 3: Effect of broccoli sprouts juices on $A\beta_{25-35}$-induced mitochondrial membrane potential ($\Delta\Psi m$) depletion. SH-SY5Y cells were incubated with 25 μM $A\beta_{25-35}$ in the presence or in the absence of 10 μL/mL of juice A or juice B for 1, 4, 7, 24, 48, and 72 h. After treatment the cells were stained with JC-1 and analyzed by flow cytometry. The $\Delta\Psi m$ is proportional to the ratio between red and green fluorescence of the mitochondrial probe JC-1 and is expressed as a percentage of the control. Data are represented as mean ± SEM ($n = 3$), $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ *versus* control; $^{d}P < 0.001$ *versus* $A\beta_{25-35}$; $^{e}P < 0.05$ *versus* juice A; $^{f}P < 0.05$ *versus* (A + Aβ).

the progressive recovery of GSH levels comparable to those of the control. Notably, treatment for 48 h with the juices induced a substantial increase of the cellular content of GSH, 196% for juice A ($P < 0.05$) and 255% for juice B ($P < 0.05$) compared to control, which returned to basal levels within 72 h. The treatment of SH-SY5Y cells with 25 μM $A\beta_{25-35}$ in the presence of 10 μL/mL of juice A or juice B showed a synergic effect on intracellular GSH depletion, reaching a *minimum* at 4 h of incubation (~20%, compared to the controls) and a recovery after 7 h of cotreatment. Within 48 h of exposure to $A\beta_{25-35}$ and the juices, the intracellular GSH content recovered to the level of the control group in the presence of juice A (97%) and even increased up to 144% with the juice B ($P < 0.05$ *versus* $A\beta_{25-35}$). In prolonged incubations up to 72 h, a protective effect on $A\beta_{25-35}$-mediated depletion of intracellular GSH content was still observed in the presence of juice A (83% *versus* 55% for $A\beta_{25-35}$, $P < 0.05$), while for the juice B such capacity was not appreciable (56% *versus* 55% for $A\beta_{25-35}$, $P > 0.05$).

3.7. Broccoli Sprouts Juices Upregulated Cellular Antioxidant Defence Capacity via Activation of Nrf2. To investigate the molecular mechanisms of neuroprotection exerted by broccoli sprouts juices against $A\beta_{25-35}$-induced cell death, the expression or activity of various cellular antioxidant enzymes was examined. For this purpose, we determined the mRNA levels of enzymes such as HO-1, Trx, and TrxR by qRT-PCR. Moreover, we tested the activity of the enzyme NQO1 by a specific spectrophotometric assay. As shown in Figure 6(a), *HO-1* gene expression in SH-SY5Y cells treated with 25 μM $A\beta_{25-35}$ for 1, 4, 7, 24, 48, and 72 h showed little fluctuation. In contrast, following exposure to 10 μL/mL of juices A or B, either alone or in the presence of 25 μM $A\beta_{25-35}$, HO-1 mRNA levels in the cells were strongly increased. Relative to control group, HO-1 mRNA started to increase after 4 h and up to 7 h treatment with broccoli sprouts juices. In this time frame, *HO-1* gene expression increased ~12 times ($P < 0.001$) with juice A and 25 times ($P < 0.001$) with juice B compared to control, respectively. At 24, 48, and 72 h of exposure, HO-1 mRNA levels were substantially lower than at 4 h, although an appreciable *HO-1* gene induction was still present compared to control cells. All through the time points, juice B was significantly more effective in upregulating *HO-1* gene expression compared to juice A, both in the absence and in the presence of $A\beta_{25-35}$. Trx mRNA levels were altered as a result of exposure to $A\beta_{25-35}$ only from 24 h, remaining constant up to 72 h (Figure 6(b)). In any case, the magnitude of induction was rather weak (1.2 times *versus* control, $P < 0.05$). Treatment with broccoli sprouts juices, alone or in presence of $A\beta_{25-35}$, significantly increased *TRX* gene expression starting from 1 h of incubation with juice B and at 4 h with juice A. Compared to control, during the first 7 h of exposure, the increase of Trx mRNA levels observed with juice B was higher than that caused by juice A, which only within 24 h, and up to 72 h, matched the inductive capacity of juice B. The *maximum* increase in *TRX* gene expression was observed at 72 h of treatment with broccoli sprouts juices, either in the absence or in the presence of $A\beta_{25-35}$, with levels reaching 1.7 and 2.0 times ($P < 0.01$) that of the control for juice A and juice B, respectively. The pattern of the kinetics of *TRXR* gene expression was comparable to that of the above described *TRX* gene (Figure 6(c)). After 7 h of incubation, and up to 72 h, the treatment with 25 μM $A\beta_{25-35}$ determined a modest (1.5 times) and stable increase in the level of TrxR mRNA compared to the control group ($P < 0.05$ at 7 and 24 h; $P < 0.01$ at 48 and 72 h). Already after 1 h of exposure, 10 μL/mL of juice B alone or in the presence of $A\beta_{25-35}$ induced a significant increase (1.5 times *versus* control, $P < 0.05$) of *TRXR* gene expression levels which reached a *maximum* (3.6 times *versus* control, $P < 0.05$) at 4 h and kept constant up to 72 h. The induction of *TRXR* gene by juice A was evidenced only after 4 h of treatment, both in the absence and in the presence of $A\beta_{25-35}$ (~2.6 times *versus* control, $P < 0.01$), reaching a plateau within 24 h (~3.3 times *versus* control, $P < 0.001$). At 7 h of exposure and for longer incubation periods, the effect of juice A was comparable to that of juice B. Measurement of NQO1 activity levels in SH-SY5Y cells did not show any effect of $A\beta_{25-35}$ compared to control (Figure 6(d)). Otherwise, starting from 7 h of treatment with broccoli sprouts juices, either alone or in the presence of $A\beta_{25-35}$, the NQO1 activity showed a time-dependent increase, with a significantly higher effect of juice B compared to juice A. The stimulating effect of juice B on NQO1

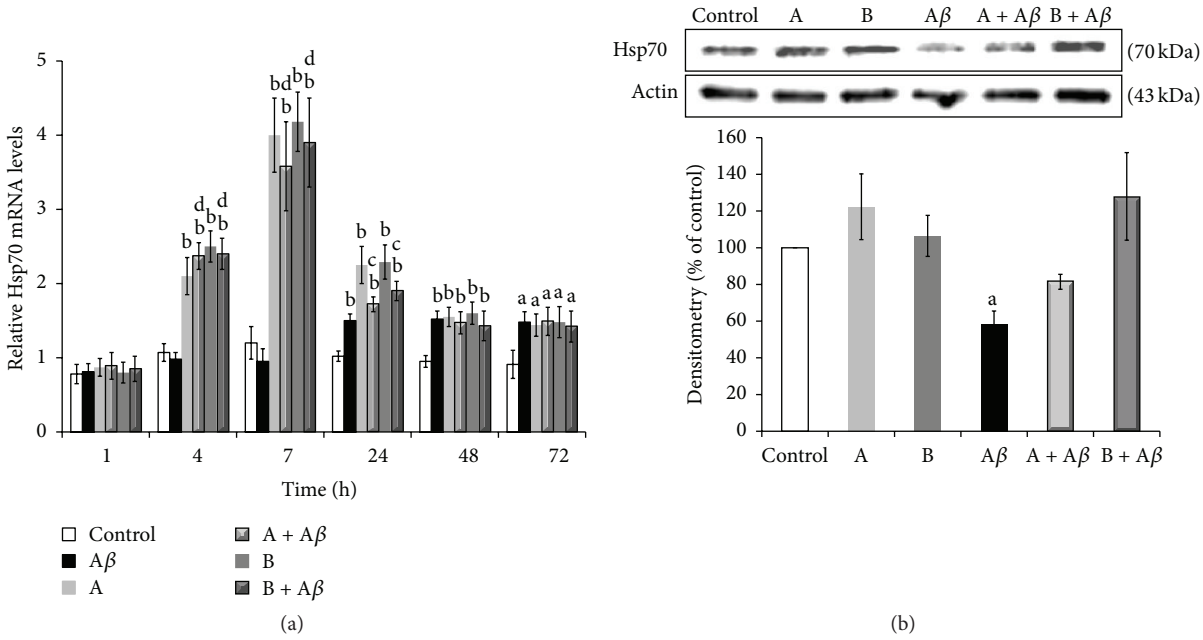

Figure 4: Broccoli sprouts juices protect SH-SY5Y cells from $A\beta_{25-35}$-mediated apoptosis through upregulation of *HSP70* gene expression. (a) SH-SY5Y cells were incubated with 25 μM $A\beta_{25-35}$ in the presence or in the absence of 10 μL/mL of juice A or juice B for 1, 4, 7, 24, 48, and 72 h. After treatment total RNA was extracted and used for qRT-PCR determination of *HSP70* gene induction. The *RSP27A* housekeeping gene was used as a control for normalization of the data. The relative amounts of gene expression were calculated by the comparative threshold cycle ($\Delta\Delta$CT) method. The intracellular levels of mRNA are expressed as fold induction compared to control cells at 0 h of incubation. (b) Cell lysates obtained after 7 h of treatment were subjected to western blot analysis with anti-Hsp70 antidoby. Actin levels were compared to ensure equal amount of protein loading. The intracellular Hsp70 protein levels in the samples were expressed as a percentage compared to the control. Data are represented as mean ± SEM ($n = 3$), $^{a}P < 0.05$ and $^{b}P < 0.01$ *versus* control; $^{c}P < 0.05$ and $^{d}P < 0.01$ *versus* $A\beta_{25-35}$.

activity was higher than that of juice A from 24 h up to 72 h of exposure (24 h: 3.1 times A and (A + Aβ) *versus* control, $P < 0.001$ and 3.9 times B and (B + Aβ) *versus* control, $P < 0.001$; 48 h: 5.2 times A and (A + Aβ) *versus* control, $P < 0.001$ and 6.8 times B and (B + Aβ) *versus* control, $P < 0.001$; 72 h: 6.7 times A and (A + Aβ) *versus* control, $P < 0.001$ e 13.9 times B and (B + Aβ) *versus* control, $P < 0.001$).

To elucidate the upstream signalling pathway involved in the broccoli sprouts juices-induced upregulation of the antioxidant enzymes, we focused on the activation of the redox-sensitive transcription factor Nrf2. When SH-SY5Y cells were treated with $A\beta_{25-35}$, or with juices A or B, alone or in the presence of $A\beta_{25-35}$, differences in Nrf2 nuclear translocation between samples after 3 h of incubation were observed (Figures 7(a) and 7(b)). In particular, densitometric analysis of the images collected by immunofluorescence microscopy showed that, compared to control cells, the nuclear content of Nrf2 was not altered by $A\beta_{25-35}$, while both broccoli sprouts juices were able to induce nuclear translocation of Nrf2, both alone (157% A *versus* control, $P < 0.001$; 246% B *versus* control, $P < 0.001$) and in the presence of $A\beta_{25-35}$ (152% (A + Aβ) *versus* control and $A\beta_{25-35}$, $P < 0.001$; 291% (B + Aβ) *versus* control and $A\beta_{25-35}$, $P < 0.001$), with juice B being significantly more effective than juice A.

4. Discussion

Epidemiological studies have highlighted the capacity of *Brassica* species to prevent cardiovascular diseases as well as to counteract the onset and progression of certain types of tumours [39, 40]. A number of clinical trials have shown favorable effects on oxidative stress and an improvement of insulin resistance in type 2 diabetes patients that consumed broccoli sprouts [41, 42]. Oxidative stress is recognized as a common factor in many neurodegenerative diseases [43], and the identification of novel antioxidants as potential therapeutics is a prolific area of neuroscience research [44].

Although several studies on biological systems, both *in vitro* and *in vivo*, have been devoted to the evaluation of the neuroprotective effects of single, purified bioactive components of plant foods, for example, polyphenolic compounds [16, 18] or organosulfur compounds such as SFN [14, 17], less investigated are food matrices as a whole, where many of these molecules interact in a complex mixture. The present study utilized broccoli sprouts crude juices with a different antioxidant phytochemicals profile to investigate their potential role as antioxidants and neuroprotective agents. Differences in phytochemical content in broccoli sprouts juices allowed us to investigate the relationship between phenolic content, antioxidant capacity, and protection of neuronal

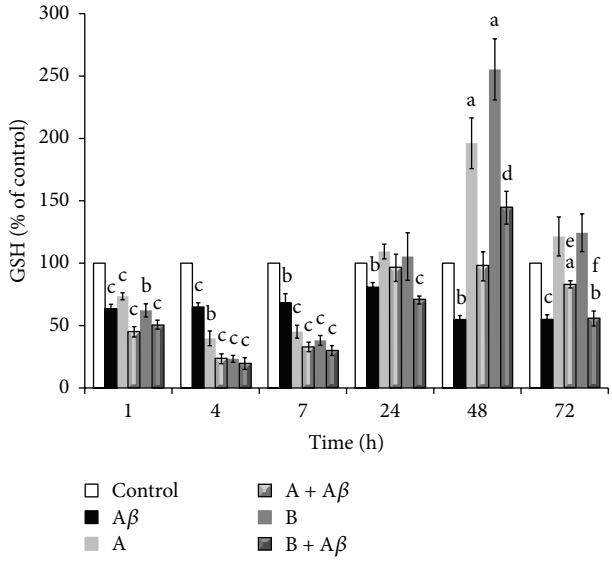

FIGURE 5: Protective effect of broccoli sprouts juices from $A\beta_{25-35}$-induced GSH depletion. SH-SY5Y cells were incubated with 25 μM $A\beta_{25-35}$ in the presence or in the absence of 10 μL/mL of juice A or juice B for 1, 4, 7, 24, 48, and 72 h. After treatment, total cell lysate was prepared for the determination of intracellular GSH levels by RP-HPLC analysis, using the fluorescence signal of the derivatized product with OPA. The concentration of GSH in the samples, normalized by the number of cells, was expressed as a percentage compared to the control. Data are represented as mean ± SEM (n = 3–5) $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ *versus* control; $^{d}P < 0.05$ and $^{e}P < 0.01$ *versus* $A\beta_{25-35}$; $^{f}P < 0.05$ *versus* (A + Aβ).

cells in culture from oxidative damage, in particular in the AD cell model represented by SH-SY5Y cell line exposed to $A\beta_{25-35}$, one of the most toxic fragments of the full length peptide $A\beta_{1-42}$. In addition, our findings contribute to the elucidation of the molecular mechanisms involved in the neuroprotective action of polyphenols, evidencing the relevance of the cell survival signalling pathways regulated by the transcription factor Nrf2.

The germination of broccoli seeds in different growth conditions (in the dark or under white light in the presence of sucrose) enabled us to obtain two groups of seedlings that provided, by cold pressing, raw juices with significantly different phenolics content. The juice B obtained from sprouts grown under stress conditions was enriched in total polyphenols, flavonoids, and in particular anthocyanins with respect to juice A. Our data are in line with previous investigations by Pérez-Balibrea et al. [45] and by Guo et al. [46] regarding the effect of light and sucrose stress on the modulation of phenolic content in broccoli sprouts.

As polyphenols are known to possess antioxidant activity, the intrinsic antioxidant potential of the two juices was tested by means of various assays specific for different species of free radicals. Results pointed to ~2-fold higher intrinsic antioxidant properties of juice B compared to juice A, in line with the different total polyphenol content of the two juices that were found to be ~2-fold higher in juice B than in juice A. These data indicate that the procedures adopted to alter the content of phenolic compounds in broccoli sprouts were successful in significantly increasing the levels of polyphenols, and in particular anthocyanins, leading to a proportional difference in the overall antioxidant capacity.

The extra- and intraneuronal aggregation and deposition of Aβ play a causal role in the pathogenetic cascade leading to AD [47, 48]. Aβ oligomers adhere to the plasma membrane of neurons and cause neuronal death *via* a combination of free radical damage and formation of ion-permeable pores [49]. Excessive production of reactive oxygen species (ROS) by Aβ peptides and exhaustion of the endogenous antioxidant defence systems including GSH, catalase, superoxide dismutase, and glutathione metabolizing enzymes can cause oxidative damages to critical cellular macromolecules, mitochondrial dysfunction, and altered cellular signal transduction cascades [50]. Given the antioxidant capability of juices A and B, we examined their protective effects against $A\beta_{25-35}$-induced neurotoxicity in SH-SY5Y cells. The cotreatment of SH-SY5Y cells with 25 μM $A\beta_{25-35}$ and 10 μL/mL juice A or juice B led to a significant decrease of Aβ toxicity. At the juice concentration utilized, the amounts of polyphenols and of sulforaphane were in the low micromolar range, in line with literature data indicating that these compounds reach a similar concentration in plasma after oral ingestion [51].

SH-SY5Y cells treated with $A\beta_{25-35}$ underwent apoptosis, as shown by alterations in nuclear morphology and hypodiploid cellular subpopulation levels and by perturbation of the mitochondrial transmembrane potential. However, cotreatment with broccoli sprouts juice effectively ameliorated the $A\beta_{25-35}$-induced proapoptotic signs. Apparently, the difference between juices A and B in terms of intrinsic antioxidant activity observed in cell-free chemical systems is not reflected in significant differences in the overall neuroprotective effect against $A\beta_{25-35}$-induced cytotoxicity. Hence the observed protective effect cannot be attributed to the direct antioxidant action of the extract, but most probably to other mechanisms still to be defined. For instance, Tarozzi et al. observed that the neuroprotective effect of the anthocyanin compound cyanidin-3-*O*-glucoside (Cy-3G) on $A\beta_{25-35}$-induced toxicity in SH-SY5Y cells was mainly due to the ability of Cy-3G to hinder the adsorption of $A\beta_{25-35}$ oligomers to the plasma membrane as well as to prevent cellular membrane and redox status impairment [52]. These authors also emphasize that the intrinsic antioxidant properties of Cy-3G may play a marginal role in its protective effects and showed the absence of Cy-3G antioxidant activity in the cytosol of SH-SY5Y cells, suggesting a low uptake of Cy-3G [53]. Actually, the anthocyanins content of juice A is undetectable and in juice B is such that the final concentration of these active compounds in culture medium reached under our experimental conditions was at least 100 times below those adopted in other studies on the neuroprotective activity of purified anthocyanins; hence the contribution of these class of compounds to the overall observed effect is doubtful.

Flavonoids exert cooperative effects by inhibiting distinct targets responsible for the generation of ROS and Aβ-induced toxicity, particularly at the mitochondrial level. Notably, complex I activity is reduced in patients with AD and PD, and excessive generation of ROS produced by neurotoxins such as

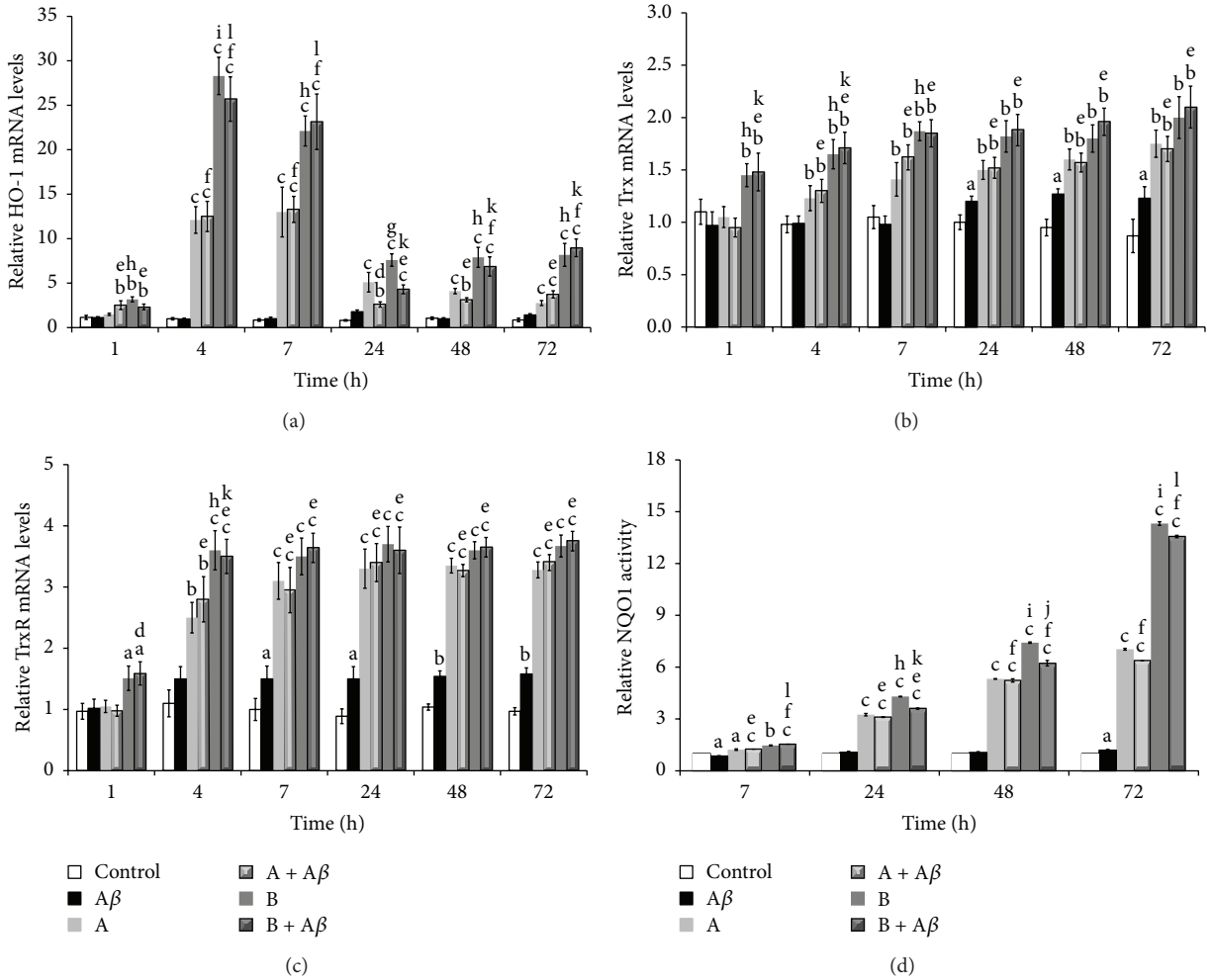

FIGURE 6: Broccoli sprouts juices upregulate the cellular antioxidant defence capacity. SH-SY5Y cells were incubated with 25 μM Aβ_{25-35} in the presence or in the absence of 10 μL/mL of juice A or juice B for 1, 4, 7, 24, 48, and 72 h. After treatment, total RNA was extracted and used for qRT-PCR determination of gene expression of (a) *HO-1*; (b) *TRX*; (c) *TrxR*. The *RSP27A* housekeeping gene was used as a control for normalization of the data. The relative amounts of gene expression were calculated by the comparative threshold cycle ($\Delta\Delta$CT) method. The intracellular levels of mRNA are expressed as fold induction compared to control cells at 0 h of incubation. (d) Total cell lysates were subjected to spectrophotometric assay of NQO1 activity which was expressed as a percentage compared to the control. Data are represented as mean ± SEM ($n = 3$), $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ *versus* control; $^{d}P < 0.05$, $^{e}P < 0.01$, and $^{f}P < 0.001$ *versus* Aβ_{25-35}; $^{g}P < 0.05$, $^{h}P < 0.01$, and $^{i}P < 0.001$ *versus* juice A; $^{j}P < 0.05$, $^{k}P < 0.01$, and $^{l}P < 0.001$ *versus* (A + Aβ).

rotenone and 1-methyl-4-phenylpyridinium (MPP+) is mediated by complex I inhibition [54]. It has been observed that quercetin binds to complex I preventing rotenone-induced production of superoxide anion with an $IC_{50} = 1.8\,\mu$M, without interfering with mitochondrial respiration up to a *maximum* of 10 μM [55]. This indicates that flavonoids can behave as "coenzyme Q-mimetic molecules" allowing normal electron flow along the entire electron transport chain (ETC) [56]. Since in our experimental settings the final concentrations of flavonoids in culture medium are in the micromolar range, we hypothesize that the observed functional recovery of the ETC could be ascribed to the flavonoid fraction of the juices through the activation of the pathways mentioned above. This could also explain the observed mitochondrial hyperpolarization in cells treated with both juices. However, in the presence of Aβ_{25-35}, broccoli sprouts juices slowly and only partially restore the $\Delta\Psi$m to control values. A possible explanation could be that, despite the positive effect of flavonoids on the activity of the ETC, the mitochondrion is seriously damaged by Aβ_{25-35}-induced mitochondrial permeability transition pore (PTP) opening responsible for a substantial dissipation of the protonic transmembrane gradient [50].

In addition to flavonoids, SFN is the component of the broccoli sprouts juices expected to exert a protective effect against Aβ_{25-35}-mediated mitochondrial damage. Indeed, intraperitoneal injection of rats with a nontoxic level of SFN resulted in resistance of isolated nonsynaptic brain mitochondria to peroxide-induced PTP opening [57]. Our data indicate that broccoli sprouts juices confer protection of

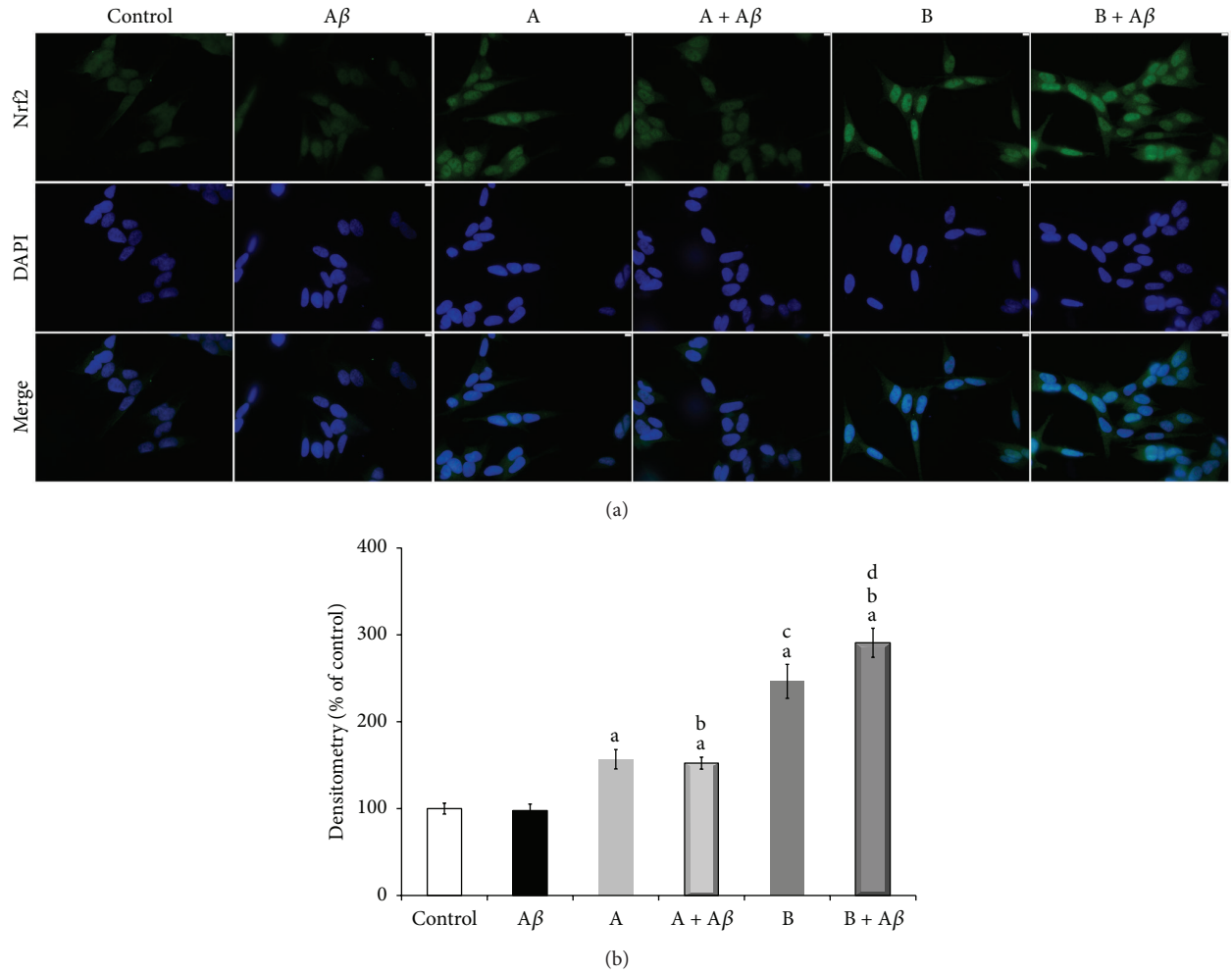

Figure 7: Broccoli sprouts juices activate nuclear translocation of Nrf2 transcription factor which controls the expression of cell survival genes involved in defence against oxidative stress-mediated damage. SH-SY5Y cells were incubated with 25 μM Aβ_{25-35} in the presence or in the absence of 10 μL/mL of juice A or juice B for 3 h. (a) Nrf2 nuclear translocation was verified by immunofluorescence microscopy by utilizing anti-Nrf2 antibody and DAPI staining. Representative images of three independent experiments show the stimulation of Nrf2 nuclear accumulation by broccoli sprouts juices A or B exposure, compared to both control cells and Aβ_{25-35} alone treated cells. (b) Densitometric analysis of the mean nuclear located-Nrf2 fluorescence signal as calculated analyzing immunofluorescence images by the Image J software. Data are expressed as percentage of control and represented as mean ± SEM ($n = 3$), [a]$P < 0.001$ *versus* control; [b]$P < 0.001$ *versus* Aβ_{25-35}; [c]$P < 0.001$ *versus* juice A; [d]$P < 0.001$ *versus* (A + Aβ).

cellular bioenergetics and counteract the $\Delta\Psi$m fall induced by Aβ_{25-35}. These results are in agreement with a recent study by Lee et al. who evaluated the protective action of 5 μM SFN against the $\Delta\Psi$m fall induced by treatment with 15 μM Aβ_{25-35} in SH-SY5Y cells [58].

We also investigated a possible molecular mechanism underlying the antiapoptotic effect of broccoli sprouts juices on SH-SY5Y cells treated with Aβ_{25-35}. Literature data indicate that in response to various types of central nervous system injuries, including stroke, trauma, or neurodegenerative disorders, the heat-shock proteins Hsp70s are expressed and appear to have neuroprotective actions [36]. Patel et al. [34] reported that Hsp70 overexpression attenuated neuronal apoptosis in an *in vitro* model of amyotrophic lateral sclerosis. It has recently been suggested that in PD the neuroprotective effect of Hsp70 is due to not only its role as a chaperone that attenuates protein aggregation and toxicity but also a more direct antiapoptotic effect [36]. In particular, examining primary cortical neurons and the SH-SY5Y neuronal cell line transfected with *HSP70*-expression plasmids and subjected to four independent models of apoptosis, including Aβ_{25-35}, Sabirzhanov et al. observed that Hsp70 overexpression corresponded to a significantly reduced induction of nuclear apoptotic markers and/or cell death [35]. Their results suggested that Hsp70 inhibits apoptosis both by attenuation of caspase-independent pathways by interacting with apoptosis-inducing factor (AIF) and preventing its translocation into the nucleus and by inhibiting caspase-dependent pathways by interacting with apoptotic protease-activating factor 1 (Apaf-1) thus blocking caspases activation. Our data demonstrate

that broccoli sprouts juices induce an early overexpression of Hsp70 mRNA in the SH-SY5Y cell line, both in the absence and in the presence of Aβ_{25-35}. Therefore, the broccoli sprouts juices may protect neurons from Aβ_{25-35}-induced apoptosis through upregulation of the *HSP70* gene thus maintaining Hsp70 protein levels, allowing the inhibition of both caspase-dependent and caspase-independent programmed cell death pathways.

Cells possess a complex network of nonenzymatic and enzymatic components to counteract oxidative stress that are regulated by a common mechanism that involves two proteins: Nrf2 and the Kelch-like-ECH-associated protein 1 (Keap1). Nrf2 is normally sequestered in the cytoplasm by Keap1 which contains several reactive cysteine residues serving as sensors of the intracellular redox state. Upon oxidative or covalent modification of Keap1 cysteine residues, Nrf2 is released from Keap1 and translocates to the nucleus where it heterodimerizes with small musculoaponeurotic fibrosarcoma (Maf) proteins before binding to the Nrf2-antioxidant response element (ARE) within the promoter regions of the abovementioned cytoprotective genes [59, 60]. Polyphenols and organosulfur compounds such as SFN share the ability to activate Nrf2-ARE transcriptional pathway thus modulating the expression of antioxidant enzymes (HO-1, glutathione peroxidase (GPx), γ-glutamylcysteine ligase (GCL), glutathione reductase (GR), Trx, TrxR, etc.) and phase II xenobiotic detoxification enzymes (glutathione S-transferase (GST), NQO1, etc.). Polyphenols and SFN have an electrophilic centre that serves as an attack site for nucleophiles, such as specific protein sulfhydryl groups present on Keap1 and critical for Nrf2-Keap1 interaction [14, 16, 17]. Our immunofluorescence data revealed that the juices treatment determined the translocation of Nrf2 into the nucleus, demonstrating a consistent modulation of the Nrf2-ARE-dependent cell survival response. Notably, the greater effect was shown by polyphenols-enriched juice B, indicating that the polyphenol fraction of the complex food matrix has a leading role in the induction of the antioxidant response.

Since the Nrf2 activation is able to regulate GSH through the modulation of GSH-related enzymes we evaluated GSH level following sprout juice treatment. In our study the Aβ_{25-35}-induced redox status of SH-SY5Y cells, in the presence or in the absence of broccoli sprouts juices, was evaluated by measuring cellular GSH levels. As expected, the oxidative stress associated with the Aβ peptide treatment, caused a significant reduction in the intracellular GSH content which persisted up to 72 h of incubation. During early stages of exposure, also broccoli sprouts juices, either alone or in combination with Aβ_{25-35}, determined a conspicuous depletion of cellular GSH. This phenomenon is most probably due to conjugation of GSH with ITCs, such as SFN, present in the broccoli juices, and to the subsequent transformation of the glutathione dithiocarbamate conjugate to mercapturic acid, the major route of metabolism of ITCs in eukaryotic cells [61]. The activation of Nrf2 by polyphenols or by free ITCs escaped to glutathionylation may explain the increase of intracellular GSH levels via the induction of GCL observed after 24 h of treatment. In addition, the higher effect of juice B compared to juice A in counteracting Aβ_{25-35}-mediated decrease of intracellular GSH in SH-SY5Y cells cotreated for 48 h can be attributed to the polyphenols enrichment of juice B compared to juice A. Actually, several studies indicate that polyphenols activate the transcription factor Nrf2 and increase the expression of the Nrf2 target gene *GCL* [62, 63]. However, after 72 h of treatment the increase in GSH intracellular content underwent a significant reduction, returning to basal levels in cells treated with the juices and lower than controls in cells treated with Aβ_{25-35} in the presence of the juices. It can be hypothesized that this phenomenon is the consequence of the overexpression of antioxidant enzymes such as GST, GPx, and NQO1, *via* the Nrf2-ARE transcriptional pathway activation mediated by SFN and polyphenols. Indeed, on the one hand GST and GPx determine a direct consumption of GSH for their enzymatic activity, and on the other hand, indirectly, NQO1, reducing quinones to semiquinones, subtracts NADPH necessary for the regeneration of GSH from GSSG by the enzyme GR. By investigating the mRNA expression or activity profiles of antioxidant enzymes known for their protective role against neurodegenerative disorders, we observed that broccoli sprouts juices, alone or in the presence of Aβ_{25-35}, markedly increased the mRNA levels or activity of these enzymes in a time-dependent manner, compared to control and Aβ_{25-35}-treated cells. In particular, between 4 and 7 h of incubation both juice A and juice B showed a *maximum* effect in the induction of the expression of *HO-1* gene, with juice B showing approximately twice the effect of juice A. Moreover, we observed a time-dependent increase of NQO1 activity caused by broccoli sprouts juices, compared to control and Aβ_{25-35}-treated cells, the effect juice B being two times higher than that of juice A within 72 h of treatment, in line with the polyphenol content.

HO-1 catalyzes the stepwise degradation of heme to release free iron, carbon monoxide, and biliverdin, which is converted to bilirubin by the enzyme biliverdin reductase. The biliverdin and bilirubin exhibit potent antioxidant properties responsible, at least in part, for the neuroprotective effects of HO-1 [64]. NQO1 is a ubiquitous FAD-dependent flavoprotein that promotes 2-electron reductions of quinones to semiquinones thereby minimizing the generation of reactive oxygen intermediates by redox cycling and the depletion of intracellular thiols pool [65]. Hence, studies that identify possible natural sources of factors that upregulate the expression and activity of enzymes such as HO-1 and NQO1 represent a relevant contribution to the identification of dietary components with a therapeutic potential against oxidative stress characterizing several degenerative diseases.

Trx is a multifunctional and ubiquitous protein characterized by the presence of a redox-active disulfide/dithiol within its conserved active site, which functions in the reduction of protein disulfides and as a scavenger of ROS [66]. *In vitro*, Trx has a protective effect against cytotoxicity mediated by ROS [67]. TrxR, a selenium-containing protein, reduces oxidized Trx. Together, Trx and TrxR operate as a powerful NADPH-dependent protein disulfide reductase system capable of repairing oxidized proteins or of maintaining levels of reduced Trx, which can directly interact with ROS. Our study demonstrates the involvement of the Trx/TrxR system

in protection against the Aβ-induced cytotoxicity. In SH-SY5Y cells, exposure to Aβ_{25-35} alone determined only a weak induction of Trx and TrxR genes. Conversely, the broccoli sprouts juices quickly showed a strong inductive effect on the Trx and TrxR mRNA expression, which could account for the increased ability to counteract oxidative stress-related cell death induced by Aβ_{25-35}. The involvement of Trx/TrxR in neurodegeneration has been demonstrated by Lovell and Xie [68] who showed a general reduction in the levels of Trx in various regions of human AD brains characterized by extensive oxidative damage and neuronal degeneration. Conversely, TrxR activity showed an opposite trend, pointing to a compensatory mechanism resulting from the increased oxidative stress due to Trx depletion. The same authors observed that sufficiently high levels of both components of the Trx/TrxR system, under oxidative stress conditions such as those present in AD, would ensure a neuroprotective effect. Indeed, the simultaneous exposure of primary hippocampal neurons to exogenous Trx and TrxR attenuated neuronal degeneration mediated by Aβ peptide, probably due to direct antioxidant activity of Trx. On the other hand, Venkateshappa et al. [69] observed that aging contributes to the vulnerability of those regions of the brain that are normally involved in AD. In the aging brain, the cerebral antioxidant response undergoes a significant depletion becoming increasingly inadequate to ensure the homeostasis of the redox state within neuronal cells. The age-dependent reduction of the availability of adequate levels of antioxidant enzymes, such as TrxR, and the consequent increase of oxidative stress would favor the gradual onset of neuronal dysfunction toward which a compensatory reaction that exploits the upregulation of enzymes related to cell survival (e.g., TrxR) may still not be adequately supported by a sufficient amount of critical cofactors (such as Trx).

Taken together, our findings suggest that beyond the benefits resulting from a direct action as scavengers of free radicals, antioxidant electrophilic compounds such as SFN and different classes of phenolic compounds can synergistically act as indirect stimulators of cellular endogenous antioxidant defences. By comparing two food matrices differing in polyphenols content but with a similar amount of SFN, we were able to point out consistent differences in their effectiveness in increasing intracellular levels of GSH—an essential factor for the action of antioxidant and detoxifying enzymes such as GST and GPx—and in upregulating mRNA expression or activity of other critical antioxidant enzymes such as HO-1 and NQO1 via Nrf2 activation.

5. Conclusions

Raw broccoli sprouts juice was shown here to protect against Aβ-induced cytotoxicity and apoptosis. This protection was exerted *via* the induction of antiapoptotic signals, such as increased Hsp70 mRNA levels, and the activation of Nrf2-ARE signalling pathway; this, through the upregulation of Nrf2-dependent antioxidant capacity, determined reduction in the Aβ-induced oxidative damages.

The results in the present study suggest that pharmacologic activation of the Nrf2 signalling pathway by broccoli sprouts juices might be a practical preventive and therapeutic strategy for AD patients. However, further studies are needed to elucidate the molecular basis of the neuroprotective effects of various chemical components of this dietary supplement and cross-talk between these and several cellular signalling cascades.

Conflict of Interests

The authors declare no conflict of interests.

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References

[1] J. Joseph, G. Cole, E. Head, and D. Ingram, "Nutrition, brain aging, and neurodegeneration," *The Journal of Neuroscience*, vol. 29, no. 41, pp. 12795–12801, 2009.

[2] A. Wimo, B. Winblad, H. Aguero-Torres, and E. von Strauss, "The magnitude of dementia occurrence in the world," *Alzheimer Disease and Associated Disorders*, vol. 17, no. 2, pp. 63–67, 2003.

[3] D.-Y. Choi, Y.-J. Lee, J. T. Hong, and H.-J. Lee, "Antioxidant properties of natural polyphenols and their therapeutic potentials for Alzheimer's disease," *Brain Research Bulletin*, vol. 87, no. 2-3, pp. 144–153, 2012.

[4] C. Ramassamy, "Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets," *European Journal of Pharmacology*, vol. 545, no. 1, pp. 51–64, 2006.

[5] B. Halliwell, "Free radicals and antioxidants: updating a personal view," *Nutrition Reviews*, vol. 70, no. 5, pp. 257–265, 2012.

[6] A. S. Darvesh, R. T. Carroll, A. Bishayee, W. J. Geldenhuys, and C. J. van der Schyf, "Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents," *Expert Review of Neurotherapeutics*, vol. 10, no. 5, pp. 729–745, 2010.

[7] A. M. Ares, M. J. Nozal, and J. Bernal, "Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds," *Journal of Chromatography A*, vol. 1313, pp. 78–95, 2013.

[8] S. Manchali, K. N. C. Murthy, and B. S. Patil, "Crucial facts about health benefits of popular cruciferous vegetables," *Journal of Functional Foods*, vol. 4, no. 1, pp. 94–106, 2012.

[9] S. Mohamed, "Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovasular disease," *Trends in Food Science & Technology*, vol. 35, no. 2, pp. 114–128, 2014.

[10] K. P. Latté, K.-E. Appel, and A. Lampen, "Health benefits and possible risks of broccoli—an overview," *Food and Chemical Toxicology*, vol. 49, no. 12, pp. 3287–3309, 2011.

[11] M. Björkman, I. Klingen, A. N. E. Birch et al., "Phytochemicals of Brassicaceae in plant protection and human health—influences of climate, environment and agronomic practice," *Phytochemistry*, vol. 72, no. 7, pp. 538–556, 2011.

[12] D. Vauzour, A. Rodriguez-Mateos, G. Corona, M. J. Oruna-Concha, and J. P. E. Spencer, "Polyphenols and human health: prevention of disease and mechanisms of action," *Nutrients*, vol. 2, no. 11, pp. 1106–1131, 2010.

[13] M. Lenzi, C. Fimognari, and P. Hrelia, "Sulforaphane as a promising molecule for fighting cancer," *Cancer Treatment and Research*, vol. 159, pp. 207–223, 2014.

[14] C. E. Guerrero-Beltrán, M. Calderón-Oliver, J. Pedraza-Chaverri, and Y. I. Chirino, "Protective effect of sulforaphane against oxidative stress: recent advances," *Experimental and Toxicologic Pathology*, vol. 64, no. 5, pp. 503–508, 2012.

[15] Q. R. D. Jones, J. Warford, H. P. V. Rupasinghe, and G. S. Robertson, "Target-based selection of flavonoids for neurodegenerative disorders," *Trends in Pharmacological Sciences*, vol. 33, no. 11, pp. 602–610, 2012.

[16] N. A. Kelsey, H. M. Wilkins, and D. A. Linseman, "Nutraceutical antioxidants as novel neuroprotective agents," *Molecules*, vol. 15, no. 11, pp. 7792–7814, 2010.

[17] A. Tarozzi, C. Angeloni, M. Malaguti, F. Morroni, S. Hrelia, and P. Hrelia, "Sulforaphane as a potential protective phytochemical against neurodegenerative diseases," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 415078, 10 pages, 2013.

[18] D. Vauzour, "Effect of flavonoids on learning, memory and neurocognitive performance: relevance and potential implications for Alzheimer's disease pathophysiology," *Journal of the Science of Food and Agriculture*, vol. 94, no. 6, pp. 1042–1056, 2014.

[19] F. C. Lau, D. F. Bielinski, and J. A. Joseph, "Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia," *Journal of Neuroscience Research*, vol. 85, no. 5, pp. 1010–1017, 2007.

[20] N. G. Innamorato, I. Lastres-Becker, and A. Cuadrado, "Role of microglial redox balance in modulation of neuroinflammation," *Current Opinion in Neurology*, vol. 22, no. 3, pp. 308–314, 2009.

[21] S. J. Hwang, Y.-W. Kim, Y. Park, H.-J. Lee, and K.-W. Kim, "Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide-stimulated RAW 264.7 cells," *Inflammation Research*, vol. 63, no. 1, pp. 81–90, 2014.

[22] E. Atherton and R. C. Sheppard, *Solid Phase Synthesis—A Practical Approach*, IRL Press at Oxford University Press, New York, NY, USA, 1989.

[23] M. Maldini, S. Baima, G. Morelli, C. Scaccini, and F. Natella, "A liquid chromatography-mass spectrometry approach to study 'glucosinoloma' in broccoli sprouts," *Journal of Mass Spectrometry*, vol. 47, no. 9, pp. 1198–1206, 2012.

[24] V. L. Singleton and J. A. Rossi Jr., "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents," *American Journal of Enology and Viticulture*, vol. 16, pp. 144–158, 1965.

[25] V. Dewanto, W. Xianzhong, K. K. Adom, and R. H. Liu, "Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 10, pp. 3010–3014, 2002.

[26] M. M. Giusti and R. E. Wrolstad, "Characterization and measurement of anthocyanins by UV-visible spectroscopy," in *Current Protocols in Food Analytical Chemistry*, R. E. Wrolstad and S. J. Schwartz, Eds., pp. F1.2.1–F.2.13, Wiley, New York, NY, USA, 2001.

[27] C. Yuting, Z. Rongliang, J. Zhongjian, and J. Yong, "Flavonoids as superoxide scavengers and antioxidants," *Free Radical Biology and Medicine*, vol. 9, no. 1, pp. 19–21, 1990.

[28] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radical Biology and Medicine*, vol. 26, no. 9-10, pp. 1231–1237, 1999.

[29] B. Halliwell, J. M. C. Gutteridge, and O. I. Aruoma, "The deoxyribose method: a simple 'test-tube' assay for determination of rate constants for reactions of hydroxyl radicals," *Analytical Biochemistry*, vol. 165, no. 1, pp. 215–219, 1987.

[30] G. Cao and R. L. Prior, "Measurement of oxygen radical absorbance capacity in biological samples," *Methods in Enzymology*, vol. 299, pp. 50–62, 1999.

[31] Z. Feng and J. T. Zhang, "Melatonin reduces amyloid beta-induced apoptosis in pheochromocytoma (PC12) cells," *Journal of Pineal Research*, vol. 37, no. 4, pp. 257–266, 2004.

[32] P. J. Hissin and R. Hilf, "A fluorometric method for determination of oxidized and reduced glutathione in tissues," *Analytical Biochemistry*, vol. 74, no. 1, pp. 214–226, 1976.

[33] A. Gaikwad, D. J. Long II, J. L. Stringer, and A. K. Jaiswal, "In vivo role of NAD(P)H:quinone oxidoreductase 1 (NQO1) in the regulation of intracellular redox state and accumulation of abdominal adipose tissue," *Journal of Biological Chemistry*, vol. 276, no. 25, pp. 22559–22564, 2001.

[34] Y. J. K. Patel, M. D. Payne Smith, J. de Belleroche, and D. S. Latchman, "Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant-induced cell death in mammalian neuronal cells," *Molecular Brain Research*, vol. 134, no. 2, pp. 256–274, 2005.

[35] B. Sabirzhanov, B. A. Stoica, M. Hanscom, C.-S. Piao, and A. I. Faden, "Over-expression of HSP70 attenuates caspase-dependent and caspase-independent pathways and inhibits neuronal apoptosis," *Journal of Neurochemistry*, vol. 123, no. 4, pp. 542–554, 2012.

[36] F. Geraci, G. Turturici, and G. Sconzo, "Hsp70 and its molecular role in nervous system diseases," *Biochemistry Research International*, vol. 2011, Article ID 618127, 18 pages, 2011.

[37] C.-F. Lau, Y.-S. Ho, C. H.-L. Hung et al., "Protective effects of testosterone on presynaptic terminals against oligomeric β-amyloid peptide in primary culture of hippocampal neurons," *BioMed Research International*, vol. 2014, Article ID 103906, 12 pages, 2014.

[38] D. G. Smith, R. Cappai, and K. J. Barnham, "The redox chemistry of the Alzheimer's disease amyloid β peptide," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1768, no. 8, pp. 1976–1990, 2007.

[39] X. Zhang, X.-O. Shu, Y.-B. Xiang et al., "Cruciferous vegetable consumption is associated with a reduced risk of total and cardiovascular disease mortality," *The American Journal of Clinical Nutrition*, vol. 94, no. 1, pp. 240–246, 2011.

[40] M. K. Kim and J. H. Y. Park, "Cruciferous vegetable intake and the risk of human cancer: epidemiological evidence," *Proceedings of the Nutrition Society*, vol. 68, no. 1, pp. 103–110, 2009.

[41] Z. Bahadoran, P. Mirmiran, F. Hosseinpanah, M. Hedayati, S. Hosseinpour-Niazi, and F. Azizi, "Broccoli sprouts reduce oxidative stress in type 2 diabetes: a randomized double-blind clinical trial," *European Journal of Clinical Nutrition*, vol. 65, no. 8, pp. 972–977, 2011.

[42] Z. Bahadoran, M. Tohidi, P. Nazeri, M. Mehran, F. Azizi, and P. Mirmiran, "Effect of broccoli sprouts on insulin resistance in type 2 diabetic patients: a randomized double-blind clinical trial," *International Journal of Food Sciences and Nutrition*, vol. 63, no. 7, pp. 767–771, 2012.

[43] M. T. Lin and M. F. Beal, "Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases," *Nature*, vol. 443, no. 7113, pp. 787–795, 2006.

[44] D. A. Linseman, "Targeting oxidative stress for neuroprotection," *Antioxidants and Redox Signaling*, vol. 11, no. 3, pp. 421–423, 2009.

[45] S. Pérez-Balibrea, D. A. Moreno, and C. García-Viguera, "Influence of light on health-promoting phytochemicals of broccoli sprouts," *Journal of the Science of Food and Agriculture*, vol. 88, no. 5, pp. 904–910, 2008.

[46] R. Guo, G. Yuan, and Q. Wang, "Sucrose enhances the accumulation of anthocyanins and glucosinolates in broccoli sprouts," *Food Chemistry*, vol. 129, no. 3, pp. 1080–1087, 2011.

[47] M. P. Mattson, "Pathways towards and away from Alzheimer's disease," *Nature*, vol. 430, no. 7000, pp. 631–639, 2004.

[48] F. M. LaFerla, K. N. Green, and S. Oddo, "Intracellular amyloid-beta in Alzheimer's disease," *Nature Reviews Neuroscience*, vol. 8, no. 7, pp. 499–509, 2007.

[49] A. Rauk, "Why is the amyloid beta peptide of Alzheimer's disease neurotoxic?" *Dalton Transactions*, no. 10, pp. 1273–1282, 2008.

[50] P. Mao and P. H. Reddy, "Aging and amyloid beta-induced oxidative DNA damage and mitochondrial dysfunction in Alzheimer's disease: implications for early intervention and therapeutics," *Biochimica et Biophysica Acta: Molecular Basis of Disease*, vol. 1812, no. 11, pp. 1359–1370, 2011.

[51] C. Manach, A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez, "Polyphenols: food sources and bioavailability," *The American Journal of Clinical Nutrition*, vol. 79, no. 5, pp. 727–747, 2004.

[52] A. Tarozzi, F. Morroni, A. Merlicco et al., "Neuroprotective effects of cyanidin 3-O-glucopyranoside on amyloid beta (25–35) oligomer-induced toxicity," *Neuroscience Letters*, vol. 473, no. 2, pp. 72–76, 2010.

[53] A. Tarozzi, F. Morroni, S. Hrelia et al., "Neuroprotective effects of anthocyanins and their in vivo metabolites in SH-SY5Y cells," *Neuroscience Letters*, vol. 424, no. 1, pp. 36–40, 2007.

[54] R. B. Mounsey and P. Teismann, "Mitochondrial dysfunction in Parkinson's disease: pathogenesis and neuroprotection," *Parkinson's Disease*, vol. 2011, Article ID 617472, 18 pages, 2011.

[55] R. Lagoa, I. Graziani, C. Lopez-Sanchez, V. Garcia-Martinez, and C. Gutierrez-Merino, "Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1807, no. 12, pp. 1562–1572, 2011.

[56] M. Fiorani, A. Guidarelli, M. Blasa et al., "Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid," *Journal of Nutritional Biochemistry*, vol. 21, no. 5, pp. 397–404, 2010.

[57] T. Greco and G. Fiskum, "Brain mitochondria from rats treated with sulforaphane are resistant to redox-regulated permeability transition," *Journal of Bioenergetics and Biomembranes*, vol. 42, no. 6, pp. 491–497, 2010.

[58] C. Lee, G. H. Park, S.-R. Lee, and J.-H. Jang, "Attenuation of β-amyloid-induced oxidative cell death by sulforaphane via activation of NF-E2-related factor 2," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 313510, 12 pages, 2013.

[59] L. Baird and A. T. Dinkova-Kostova, "The cytoprotective role of the Keap1-Nrf2 pathway," *Archives of Toxicology*, vol. 85, no. 4, pp. 241–272, 2011.

[60] K. Itoh, T. Chiba, S. Takahashi et al., "An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements," *Biochemical and Biophysical Research Communications*, vol. 236, no. 2, pp. 313–322, 1997.

[61] E. Lamy, C. Scholtes, C. Herz, and V. Mersch-Sundermann, "Pharmacokinetics and pharmacodynamics of isothiocyanates," *Drug Metabolism Reviews*, vol. 43, no. 3, pp. 387–407, 2011.

[62] T. Satoh, K. Kosaka, K. Itoh et al., "Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1," *Journal of Neurochemistry*, vol. 104, no. 4, pp. 1116–1131, 2008.

[63] A. Rajeswari, "Curcumin protects mouse brain from oxidative stress caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine," *European Review for Medical and Pharmacological Sciences*, vol. 10, no. 4, pp. 157–161, 2006.

[64] A. Jazwa and A. Cuadrado, "Targeting heme oxygenase-1 for neuroprotection and neuroinflammation in neurodegenerative diseases," *Current Drug Targets*, vol. 11, no. 12, pp. 1517–1531, 2010.

[65] A. T. Dinkova-Kostova and P. Talalay, "NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 116–123, 2010.

[66] S. Lee, S. M. Kim, and R. T. Lee, "Thioredoxin and thioredoxin target proteins: from molecular mechanisms to functional significance," *Antioxidants and Redox Signaling*, vol. 18, no. 10, pp. 1165–1207, 2013.

[67] A. Sobhakumari, L. Love-Homan, E. V. M. Fletcher et al., "Susceptibility of human head and neck cancer cells to combined inhibition of glutathione and thioredoxin metabolism," *PLoS ONE*, vol. 7, no. 10, Article ID e48175, 2012.

[68] M. A. Lovell, C. Xie, S. P. Gabbita, and W. R. Markesbery, "Decreased thioredoxin and increased thioredoxin reductase levels in Alzheimer's disease brain," *Free Radical Biology and Medicine*, vol. 28, no. 3, pp. 418–427, 2000.

[69] C. Venkateshappa, G. Harish, A. Mahadevan, M. M. Srinivas Bharath, and S. K. Shankar, "Elevated oxidative stress and decreased antioxidant function in the human hippocampus and frontal cortex with increasing age: implications for neurodegeneration in Alzheimer's disease," *Neurochemical Research*, vol. 37, no. 8, pp. 1601–1614, 2012.

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Research Article

Protocatechuic Acid Prevents oxLDL-Induced Apoptosis by Activating JNK/Nrf2 Survival Signals in Macrophages

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Protocatechuic acid (PCA), one of the main metabolites of complex polyphenols, exerts numerous biological activities including antiapoptotic, anti-inflammatory, and antiatherosclerotic effects. Oxidised LDL have atherogenic properties by damaging arterial wall cells and inducing p53-dependent apoptosis in macrophages. This study was aimed at defining the molecular mechanism responsible for the protective effects of PCA against oxidative and proapoptotic damage exerted by oxLDL in J774 A.1 macrophages. We found that the presence of PCA in cells treated with oxLDL completely inhibited the p53-dependent apoptosis induced by oxLDL. PCA decreased oxLDL-induced ROS overproduction and in particular prevented the early increase of ROS. This decrease seemed to be the main signal responsible for maintaining the intracellular redox homeostasis hindering the activation of p53 induced by ROS, p38MAPK, and PKCδ. Consequently the overexpression of the proapoptotic p53-target genes such as p66Shc protein did not occur. Finally, we demonstrated that PCA induced the activation of JNK, which, in turn, determined the increase of nuclear Nrf2, leading to inhibition of the early ROS overproduction. We concluded that the antiapoptotic mechanism of PCA was most likely related to the activation of the JNK-mediated survival signals that strengthen the cellular antioxidant defences rather than to the PCA antioxidant power.

1. Introduction

The adherence to a Mediterranean-type diet characterized by high intake of fruit, vegetables, fish, and extra virgin olive oil has been demonstrated to exert beneficial effects on human health most likely because of the high content in antioxidant compounds, for example, antioxidant vitamins and polyphenols [1]. Current evidence strongly supports a preventive role of polyphenols against several chronic degenerative processes, diseases, and syndromes, mostly thanks to their strong antioxidant power [2–4]. Oxidation-linked diseases or disorders, including cell transformation and cancer, atherosclerosis and cardiovascular diseases (CVD), central nervous system disorders, and a variety of age-related disorders [5–7], can be exacerbated and perhaps even initiated by numerous prooxidant agents to be found in the environment, drugs, and food.

Many of the biological actions of polyphenols have been attributed to their capacity to protect lipids, proteins, and DNA from oxidative damage. However, a variety of potential mechanisms of action of polyphenols may be independent of their conventional antioxidant activities [8, 9].

The 3,4-dihydroxybenzoic acid, protocatechuic acid (PCA), is a phenolic acid found in fruit, vegetables, and extra virgin olive oil but also in plant-derived beverages such as tea and red and white grape wine and in herbal medicine [10–12]. The PCA content varies considerably depending on the type of food; for example, raspberries contain up to 100 mg/kg fresh weight of PCA, while in extra virgin olive oil its concentration is about 0.22 mg/kg [13, 14].

Despite its low concentration in food, PCA is of great nutritional interest since it is one of the main metabolites of complex polyphenols [15, 16]. This is the case with anthocyanins normally present at high concentrations in vegetables and fruit. The daily intake of anthocyanins has been estimated to be much higher than of other polyphenols such as quercetin, kaempferol, or myricetin (180–250 mg/d versus 20–30 mg/d, resp.) [17]. Anthocyanins are absorbed by animals and humans, but their bioavailability has been demonstrated to be very poor. In fact, only a small portion of dietary anthocyanins are absorbed in the gastrointestinal tract, and in addition at physiological pH (e.g., in the bloodstream) anthocyanins easily convert to PCA [18, 19]. Most part of the ingested compounds enter the colon, where they undergo structural modifications by colonic microflora that hydrolyze glycosides and break down anthocyanins into simple phenolic acids, such as PCA and aldehydes [15, 16, 20], which are then absorbed by colonocytes. PCA has been demonstrated to have antiatherosclerosis properties in *in vitro* and *in vivo* studies mainly via its antioxidant and anti-inflammatory activities, by preventing macrophage-mediated oxidation of LDL, monocyte/macrophage endothelial infiltration, and expression of cytokines and adhesion/chemoattractant molecules, thus inhibiting the formation of the early vessel lesion and the severity of atherosclerosis [21–24].

Atherosclerosis, one of the prevalent causes of morbidity and mortality in Western countries, is an inflammatory process triggered by the presence of lipids in the vascular wall. Subendothelial retention of lipoproteins such as LDL is one of the key events that set off the atherosclerosis process. Oxidized LDL (oxLDL) contain various toxic oxidized lipids (e.g., lipid peroxides, oxysterols, and aldehydes) [25] and exhibit a wide range of biological effects, such as proinflammatory, anti-inflammatory, and proliferative or apoptotic effects on the endothelial cells and leukocytes in the atherosclerotic plaque microenvironment [25, 26].

The pathophysiology of atherosclerosis involves both apoptosis and proliferation at different stages of the vessel lesion. Apoptosis is likely involved in the progression or regression of lesions, vascular remodelling, and plaque instability. Indeed, apoptosis is frequently observed in endothelial cells, macrophages, and vascular smooth muscle cells (VSMC) in atherosclerotic plaques [27]. In advanced atherosclerotic plaques, up to 50% of the apoptotic cells are macrophages, which may promote core expansion and plaque instability. In advanced human atheromata the defective phagocytic clearance of dead macrophages leads to plaque necrosis, which triggers acute atherothrombotic vascular events [28, 29].

We have previously demonstrated that oxLDL induce p53-dependent apoptosis by activating p38MAPK and PKCδ signalling pathways in J774.A1 macrophage-like cell line [30].

The present study was aimed at evaluating the potential protective effect of PCA against oxLDL-induced cytotoxicity in macrophages. We demonstrated that, by activating JNK/Nrf2-mediated survival signals, PCA completely counteracted both ROS- and kinase-induced activation of p53 and the consequent p66Shc-mediated oxidative stress that eventually led to the apoptotic process.

2. Materials and Methods

2.1. Plasma LDL Isolation and Oxidation. LDL (1.019–1.063 g/mL), kindly provided by the Centro Trasfusionale, University of Rome La Sapienza, were isolated by density gradient ultracentrifugation in vertical rotor from fresh pooled plasma of healthy volunteers as described elsewhere [21]. Protein content was measured by Lowry's method using BSA as standard [31]. Native LDL (nLDL) were oxidized with 20 μM $CuSO_4$ for 18 h at 37°C. Oxidation was stopped by 1 mM EDTA. The degree of LDL oxidation was assessed by determining TBARS content according to Yagi [32] and the increase in electrophoretic mobility on agarose gel. The TBARS content of oxLDL was 45 ± 7 nmol malondialdehyde equivalent/mg LDL protein (versus 4±0.3 nmol in nLDL); the relative electrophoretic mobility of oxLDL versus nLDL was 1.9 ± 0.2.

2.2. Cell Cultures. J774A.1 cells, purchased from the American Tissue Culture Collection, were seeded (500,000) in 25 cm^2 flasks and grown in RPMI 1640 medium containing 0.2 mM glutamine, 10 U/mL antibiotics, and 10% FCS, at 37°C, 5% CO_2. J774A.1 is a useful tool to investigate in-depth the molecular mechanisms potentially involved in the atherosclerosis pathological processes, and it is widely used as an *in vitro* model [33, 34].

2.3. Experimental Procedure. Twenty-four hours before the experiments, cell cultures were washed twice with serum-free medium. The medium was then replaced with RPMI 1640 containing 20 mL/L Ultroser G (Flow), a lipoprotein-free serum substitute, and exposed to nLDL or oxLDL.

Preliminary experiments were performed to evaluate optimal cell treatment conditions regarding oxLDL exposure time, phenol incubation time, and the most effective antioxidant concentrations (different exposure times of treatments and different concentrations of PCA were assessed).

On the basis of these preliminary experiments (data not shown) we determined the following: (i) the lowest concentration of oxLDL still effective in causing a significant increase in cell apoptosis, but less than 10% cell necrosis (cells positive for both annexin-V and propidium iodide); (ii) the most effective PCA concentration, and (iii) the length of preincubation time.

All the experiments herein presented were time-course experiments (0–48 h) carried out with (i) untreated cells, (ii) cells treated with 0.1 mg/mL nLDL, (iii) cells treated with 0.1 mg/mL oxLDL, and (iv) cells treated with nLDL or oxLDL in the presence of 25 μM PCA. The phenolic acid was added 1 h before treatments with the lipoproteins and was present in the culture medium for the entire experimental period in all the experiments. Since the values obtained in untreated cells overlapped those obtained in cells treated with nLDL, the figures show only the results obtained in nLDL-treated cells, which were considered as controls and shown as CTR in all the figures.

2.4. Cytotoxicity. Cytotoxicity was measured by incorporation of the radiolabelled precursor of DNA synthesis ^{14}C Thymidine and by trypan blue exclusion method.

Cells were exposed to LDL or oxLDL 5 days after seeding in 24 multiwell plates in the presence or absence of PCA. For measuring proliferating activity, 1.85 kBq of [^{14}C] thymidine (Amersham, Buckinghamshire, UK; special activity: 2.09 GBq/mmol) was added to each well. After 4 h, [^{14}C] thymidine incorporation was stopped and cells were prepared as previously reported [35]. Radioactivity was evaluated in 0.2 mL aliquots of NaOH extracts by a liquid scintillation spectrometer. [^{14}C] thymidine incorporation was expressed as percentage of values observed in control cells. For assessing cell viability, an aliquot of the cell suspension was mixed with an equal volume of trypan blue. Nonviable cells stained with the dye were counted under light microscope. Data were reported as percent of the total number of cells displayed.

2.5. Evaluation of Apoptosis. Apoptosis was evaluated with the ApoAlert Annexin V apoptosis Kit (Clontech Laboratories, Palo Alto, CA, USA) following the manufacturer's instructions. The two-colour cytometric analysis (fluorescence-activated cell sorting (FACS)) was performed on a Coulter Epics Elite ESP cell Sorter with an argon-ion laser tuned to 488 nm.

2.6. ROS Assay. Intracellular ROS levels were determined using a fluorescence probe, 5-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), which is converted to highly fluorescent dichlorofluorescein (DCF) in the presence of intracellular ROS. The probe, as other DCFDA-based fluorophores, is widely used to indirectly detect ROS that lead to cell peroxide formation and is a suitable indicator of oxidative cell status. Cells were washed with PBS and incubated with freshly diluted CM-H2DCFDA (25 μM) in PBS for 1 h and then washed twice with PBS. Cell fluorescence intensity was measured on a spectrofluorometer with excitation wavelength of 485 nm and emission wavelength of 530 nm.

2.7. GSH and GSSG Determination. The levels of reduced glutathione (GSH) and oxidized GSH (GSSG) and the GSH/GSSG ratio were determined by the Bioxytech GSH/GSSG-412 assay kit (OXIS International, Inc., Portland, OR, USA) according to the manufacturer's instructions.

2.8. Protein Determination by Immunoblotting Analysis. Whole cell extracts were prepared from cells collected and washed twice in ice-cold PBS, suspended in 50 μL 1% TRITON X (Sigma) containing 2 mM Na_3VO_4 and 5 μL of a mixture of protease inhibitors (Sigma), and incubated on ice for 20 min. Cells were centrifuged at 18,000 g for 10 min at 4°C. Supernatants, assessed for protein concentration, were used for western blot analysis as described elsewhere [36]. Nuclear protein extracts were prepared by the Nuclear/Cytosol fractionation Kit (Medical & Biological Laboratories, Watertown, LA, USA) according to the manufacturer's instructions. For immunoprecipitation, whole-cell lysates containing 500 μg of protein were incubated for 2 h at 4°C with specific antibodies, namely, anti-p53, anti-p38, anti-PKCδ, or anti-JNK. Then the samples were incubated with protein G-Sepharose for 30 min and the beads washed thrice with the same lysis buffer. Immunoblotting analyses were carried out using specific antibodies for p53, PKCδ, caspase-3 active form (caspase-3 p11), Bax, p66Shc, Nrf2 (Santa Cruz), phospho-PKCδ, p38, phospho-p38, JNK, phospho-JNK (Cell Signaling), and serine-phosphorylated proteins (Sigma). Blots were treated with appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) followed by ECL detection (Amersham Bio-sciences, UK). Densitometric analysis was performed with a molecular imager FX (Bio-Rad, Hercules CA). Equal loading of proteins was verified by immunoblotting with anti-cyclophilin and anti-lamin B antibodies for whole cell and nuclear extracts, respectively.

2.9. Evaluation of Inhibitor Effects. In a set of experiments aimed at defining whether JNK plays the kinase responsible for the protective effects of PCA, the cells were treated with SP600125, a specific JNK1/2 inhibitor, one hour before PCA and oxLDL addition, at a concentration (50 μM) that effectively inhibited targeted pathways without any signs of cytotoxicity.

2.10. Gene Silencing and Small Interfering RNA. Nrf2 expression was inhibited with Nrf2-directed siRNA reagents (Nrf2 siRNA mouse; Santa Cruz). Briefly, J774A.1 cells were transfected with 100 nM Nrf2-siRNA mixed with Lipofectamine 2000 transfection reagent (Invitrogen) in the absence of antibiotics, according to the manufacturer's instructions. Scrambled nontargeting siRNA was introduced in the cells following the same protocol and used as negative control. At selected time points after transfection, proteins were extracted to assess phospho-p53, Bax, and the active form of caspase-3 expressions.

2.11. Statistical Analysis. Student's *t*-test and one-way analysis of variance on the Statview programme for Macintosh were used to analyse data. Values of P lower than 0.05 were considered statistically significant.

3. Results

3.1. Cytotoxicity. PCA completely counteracted the cytostatic/cytotoxic effects induced by oxLDL in A.1 cells. OxLDL reduced cell growth showing a dramatic cytostatic effect: about 30% and 50% decrease of relative incorporation of [^{14}C] thymidine after 18 and 24 h exposure, respectively, with respect to control cells. OxLDL exposure augmented the percentage of dead cells measured by counting trypan blue positive cells. PCA reversed oxLDL-induced cytotoxicity (Figures 1(a) and 1(b)).

3.2. Apoptosis and Oxidative Stress. To assess whether PCA was able to counteract oxLDL-induced apoptosis in macrophages, experiments were carried out to identify annexin V positive cells in (i) cells exposed to oxLDL, (ii) control cells, and (iii) cells exposed to oxLDL in the presence of PCA. Cells treated with oxLDL underwent apoptosis after

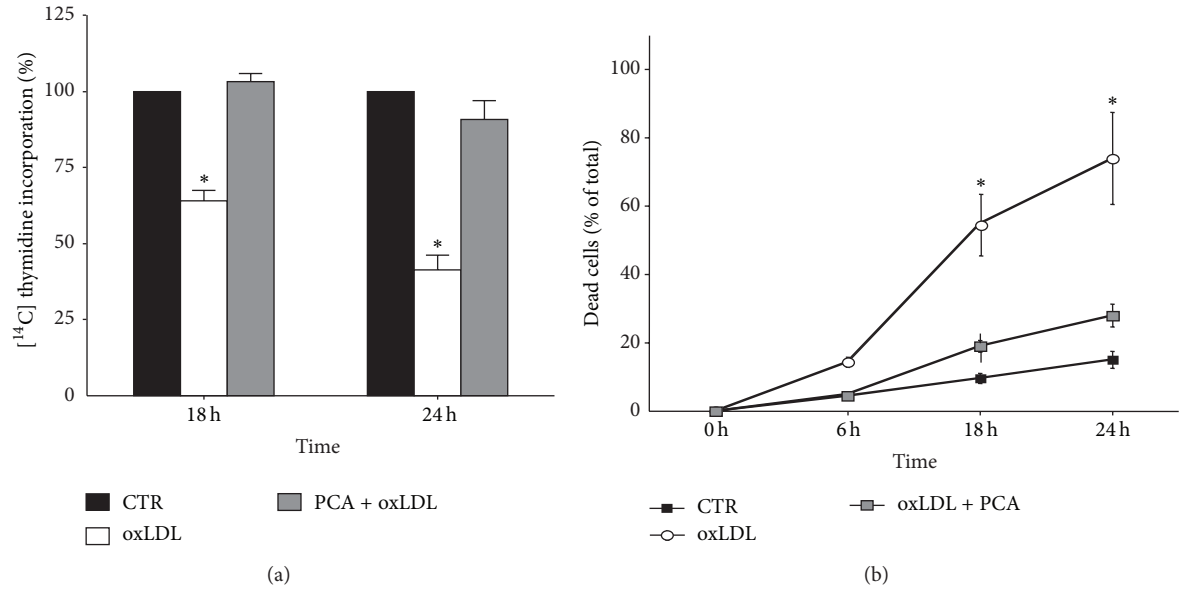

Figure 1: Protocatechuic acid completely protects macrophage cells from oxLDL-cytotoxic activity. J774A.1 cells were exposed to native LDL or oxLDL (0.2 mg/mL) in the presence or absence of 25 μM protocatechuic acid. (a) Relative incorporation of [^{14}C] thymidine. Each column represents the relative incorporation after 18 and 24 h treatment as compared with control values (100%). (b) Trypan blue staining. Percentages of dead cells (trypan blue positive cells) are reported at 6, 18, and 24 h. The data are expressed as mean ± S.E.M. of three independent experiments. $^{*}P < 0.001$ versus controls.

18 h exposure, while treatment with PCA prevented the death of the cells (Figure 2(a)).

Since the first toxic effect of oxLDL was an early and progressive increase in ROS production followed by a dramatic decrease in GSH content in macrophage-like cells [30], the effect of PCA on cellular oxidative stress was evaluated. The early increase in ROS production was completely abrogated in cells treated with PCA until hour 6 (Figure 2(b)). ROS production increased in PCA-treated cultures only after 6 h of oxLDL-exposure, and, although significantly higher with respect to control cultures, it was significantly lower than in oxLDL-treated cells. Notably, PCA treatment completely counteracted GSH depletion induced by oxLDL (Figure 2(c)), clearly indicating that it was able to prevent the onset of oxidative stress induced by oxLDL.

3.3. PCA Inhibited p53 Apoptotic Signal. Western blotting analyses were performed to elucidate if the antiapoptotic activity of PCA depended on the ability to modulate p53 expression and activation induced by oxLDL. For this purpose phosphorylated and nuclear p53 and protein expression of two of the main p53 target genes, p66Shc and BAX, both responsible for the execution of p53-mediated apoptosis, were evaluated in oxLDL-treated cells in the presence of PCA. The obtained results demonstrated that PCA was able to counteract all the changes induced by oxLDL in p53 pathway. In particular, PCA prevented (i) the significant overexpression of p53 protein observed throughout the time period considered (data not shown), (ii) the early (1 h) significant increase of the phosphorylated form, and (iii) the increase in p53 nuclear translocation that occurred after 3 h of oxLDL exposure (Figures 3(a) and 3(b)). Moreover, PCA completely counteracted the overexpression of both p66Shc, which was significantly higher at hour 6 in oxLDL exposed cells and then dropped to control level, and Bax, which significantly increased after 18 h in oxLDL-treated cells (Figures 3(c) and 3(d)).

3.4. PCA Reverted p38 MAPK and PKCδ Activation Induced by oxLDL. Since the p53 activation depends on the activation of both p38MAPK and PKCδ [30], we investigated PCA effect on the kinase activation/phosphorylation. While macrophage treated with oxLDL exhibited an early (30 minutes after cell exposure) activation/phosphorylation of both the kinases, the presence of PCA completely reverted the kinase activation to control levels (Figures 4(a) and 4(b)).

3.5. PCA Activated JNK/Nrf2 Pathway. PCA, *per se*, is able to potentiate the antioxidant cell system by activating JNK/Nrf2 pathway, which upregulates phase-2 detoxifying enzymes [37].

Thus, we investigated whether this was the mechanism responsible for PCA-mediated inhibition of oxLDL-induced apoptosis. Blotting experiments demonstrated an early (15 min after treatment), transient increase in phosphorylated-JNK and a significant and prolonged increase in nuclear Nrf2 expression (within 30′ after treatment) in oxLDL-treated cells incubated with PCA with respect to oxLDL-treated cells (Figures 5(a) and 5(b)). The increase of nuclear Nrf2 by PCA was inhibited when the cells were pretreated with SP600125, a specific inhibitor of JNK, strongly suggesting that the observed upregulation of Nrf2 was due to the activation of JNK (Figure 5(b)).

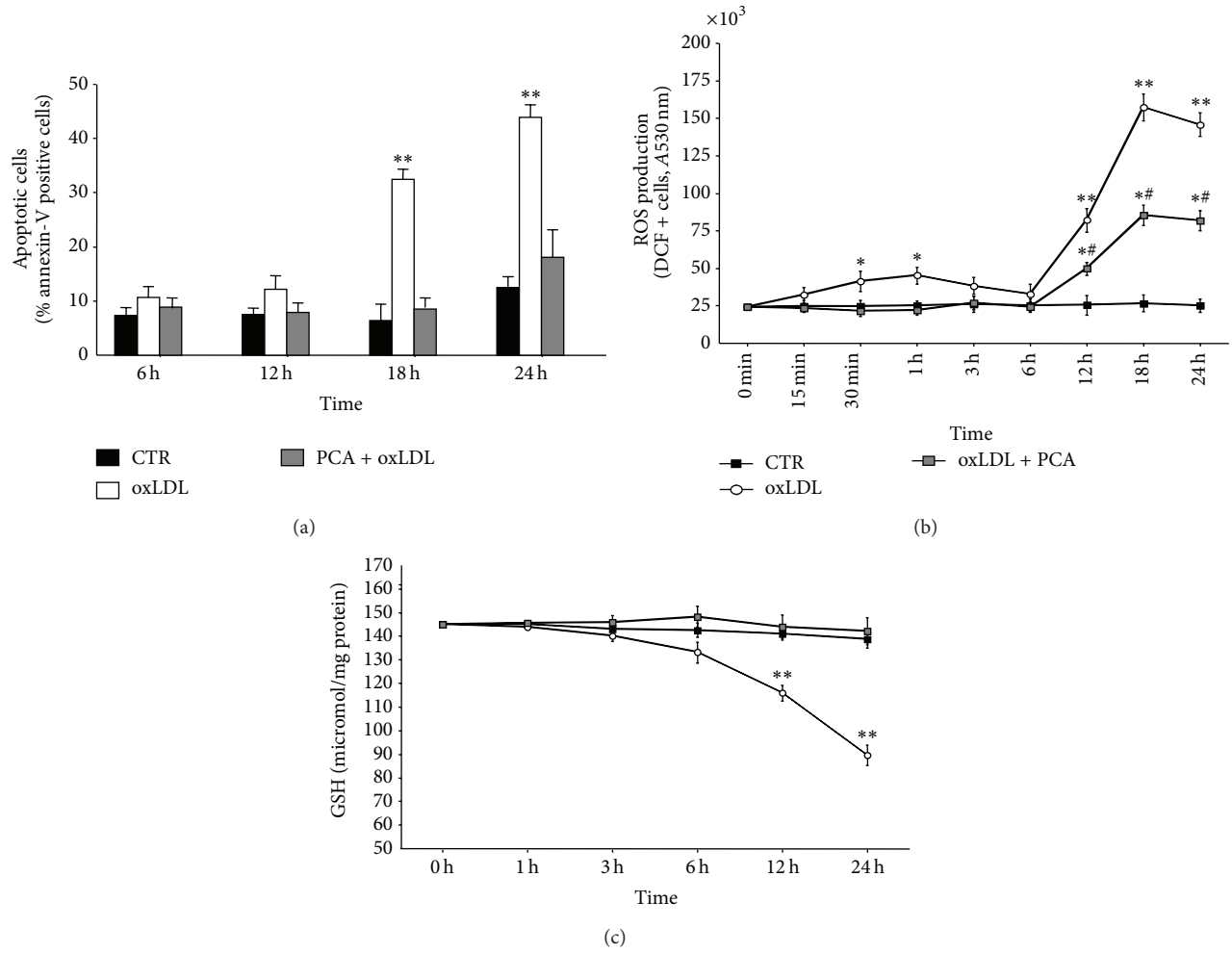

Figure 2: Protocatechuic acid inhibited OxLDL-induced apoptosis and the onset of oxidative stress in macrophage cells. Time-course experiments. (a) Percentage of apoptotic annexin-V positive cells, (b) intracellular ROS production, and (c) intracellular reduced glutathione (GSH) content in J774A.1 cells. Values are the mean ± S.E.M. of four independent experiments. Values obtained in control cells (CTR), oxLDL-treated cells (oxLDL), and oxLDL-treated cells preincubated with 25 μM PCA (PCA + oxLDL) were compared. $^{*}P < 0.05$ versus controls; $^{**}P < 0.001$ versus controls. $^{**\#}P < 0.05$ versus oxLDL-treated cells and versus controls, for each time point.

To definitively assess the crucial role of JNK and Nrf2 in counteracting oxLDL-induced apoptosis by PCA, we inhibited the activation of Nrf2 also by silencing Nrf2 (siNrf2). Both SP600125 and siNrf2 treatments determined expression levels of phosphorylated-p53, Bax, and the active form of caspase-3 completely comparable to those obtained in cells treated with oxLDL only. These findings supported the hypothesis that the inhibitory effects triggered by PCA were due to the activation of JNK and the consequent increase in nuclear Nrf2 (Figures 6(a), 6(b), and 6(c)).

3.6. Effect of the JNK Inhibitor on ROS Production. The JNK/Nrf2 signalling pathway was responsible for the anti-apoptotic effect of PCA. These results, together with those regarding the modulation of ROS production, suggested that PCA exerted its protective action mainly by maintaining cell redox balance rather than to directly inhibit cell macromolecule oxidation. To define the actual mechanism by which PCA impacted on the cellular redox status, we measured ROS in cells treated with oxLDL and PCA in the presence of the JNK inhibitor. We found that SP600125 completely hindered PCA protection against ROS production so that their levels were comparable to those found in cells treated with oxLDL only. Notably, JNK inhibitor mostly affected early ROS production, which was thus allowed to trigger the apoptotic signal (Figure 7).

4. Discussion

In this study we demonstrated the ability of PCA to counteract the cytotoxic activity of oxLDL in murine macrophages. In particular, the treatment of the cells with PCA inhibited apoptosis not only by counteracting oxidative stress occurrence but also through the modulation of intracellular signalling pathways responsible for caspase activation. This can have great relevance to atherosclerosis development

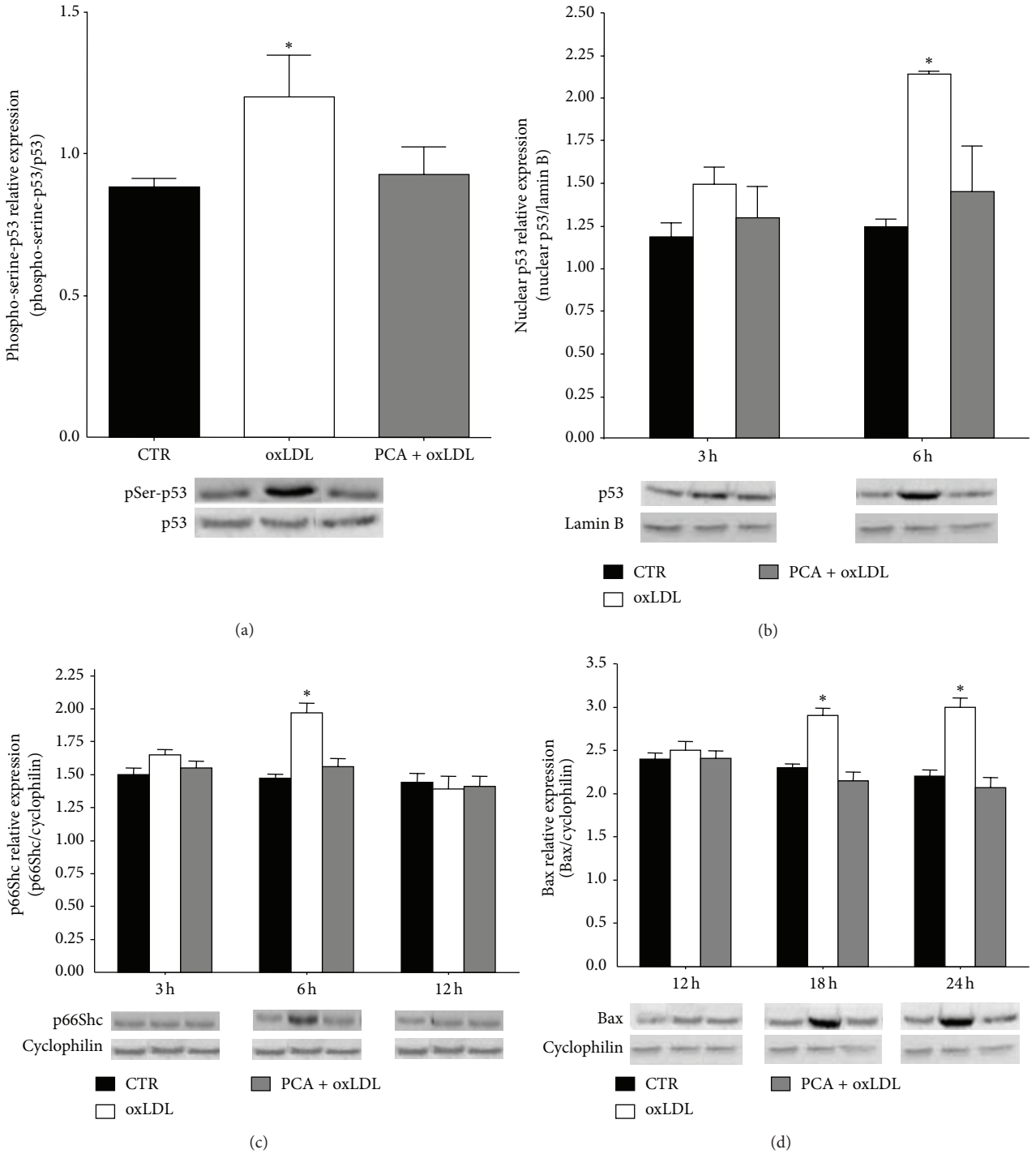

Figure 3: Protocatechuic acid counteracted oxLDL-induced activation of p53 and expression of its target genes p66Shc and Bax. Blotting analyses of (a) serine-phosphorylated p53 (1 h after oxLDL treatment) detected in anti-p53 immunoprecipitate and normalized to the nonphosphorylated protein. (b) p53 content determined in nuclear extract and normalized to lamin B protein, (c) p66Shc protein expression, and (d) Bax protein expression, normalized to cyclophilin protein. Values obtained in control cells (CTR), oxLDL-treated cells (oxLDL), and oxLDL-treated cells preincubated with 25 μM PCA (PCA + oxLDL) were compared. Representative blots are shown. Results are expressed as mean ± S.E.M. ($^*P < 0.05$ versus controls) of at least three independent experiments.

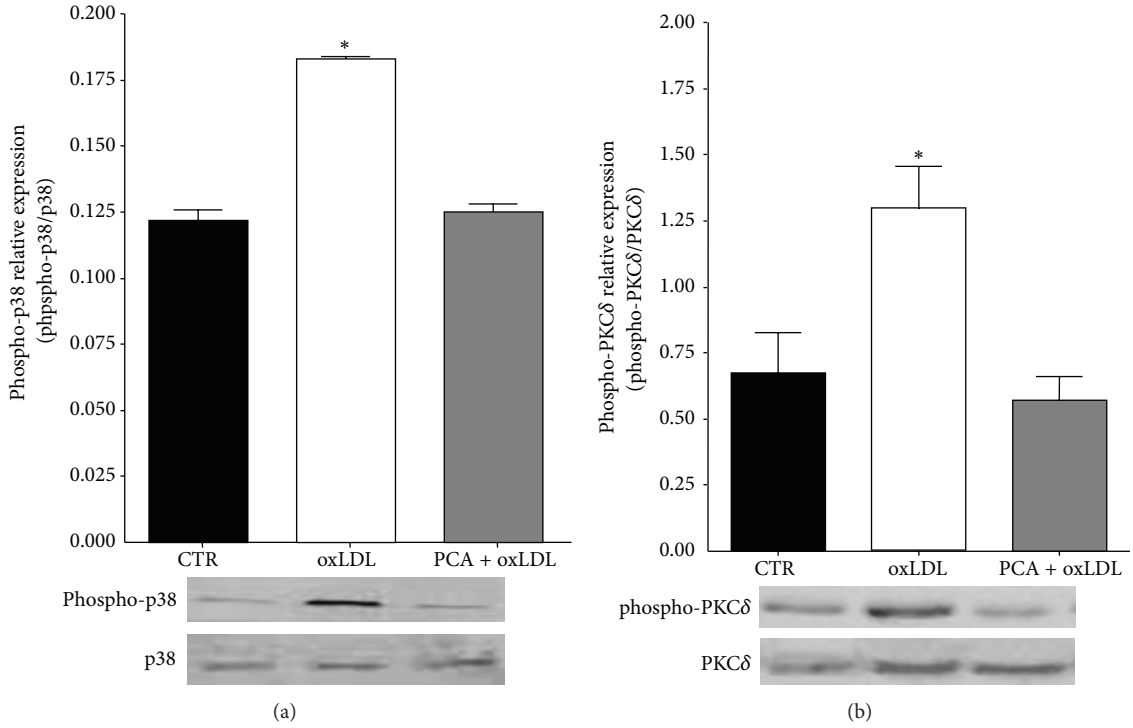

FIGURE 4: Protocatechuic acid prevented oxLDL-induced p38 and PKCδ activation. (a) phospho-p38 expression or (b) phospho-PKCδ expression (30′ after oxLDL treatment) in control cells (CTR), oxLDL-treated cells (oxLDL), and oxLDL-treated cells preincubated with 25 μM PCA (PCA + oxLDL). Results were normalized to the respective nonphosphorylated proteins. Each panel shows representative blots. Values are the mean ± SEM. $^{*}P < 0.05$ versus controls of at least three independent experiments.

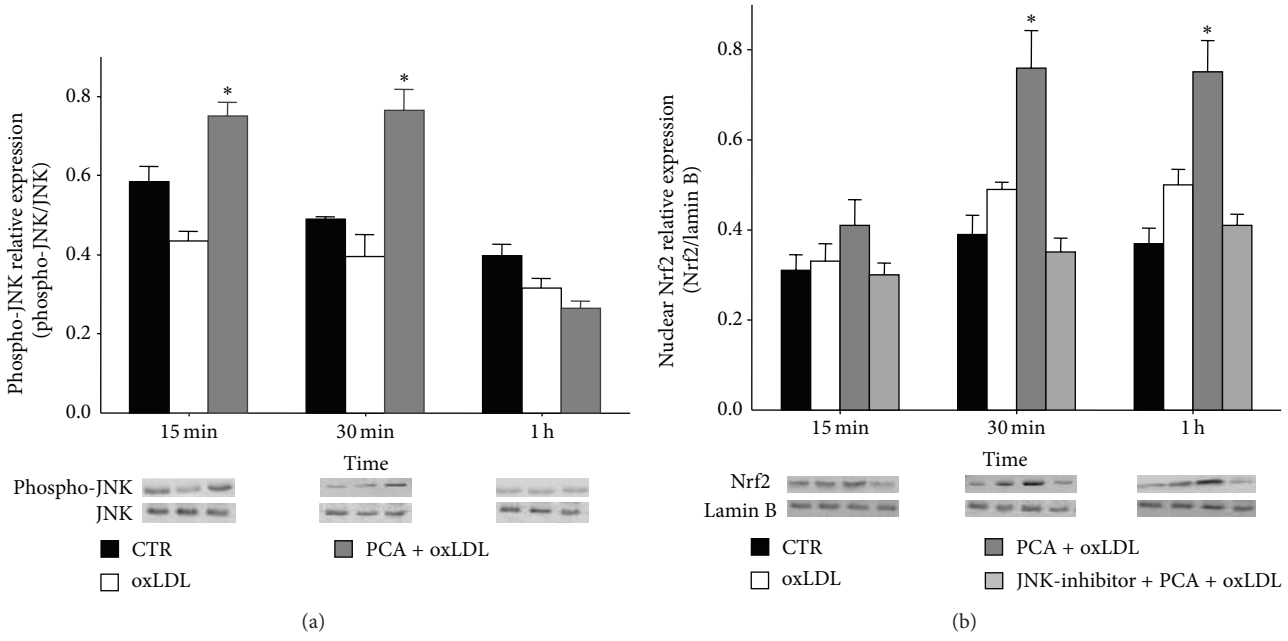

FIGURE 5: Protocatechuic acid activated JNK/Nrf2 pathway in J774A.1 cells treated with oxLDL. (a) Phospho-JNK expression normalized to the nonphosphorylated form in oxLDL-treated cells or in oxLDL-treated cells incubated with PCA, with respect to controls. (b) Nrf2 content determined in nuclear extract and normalized to lamin B protein in oxLDL-treated cells incubated with PCA in the presence or absence of the JNK1/2 inhibitor with respect to oxLDL-treated cells. The inhibitor tested on control cells and oxLDL-treated cells did not show any effect (data not shown). Representative blots from at least three independent experiments are shown. Results are expressed as mean ± S.E.M. $^{*}P < 0.05$ versus controls.

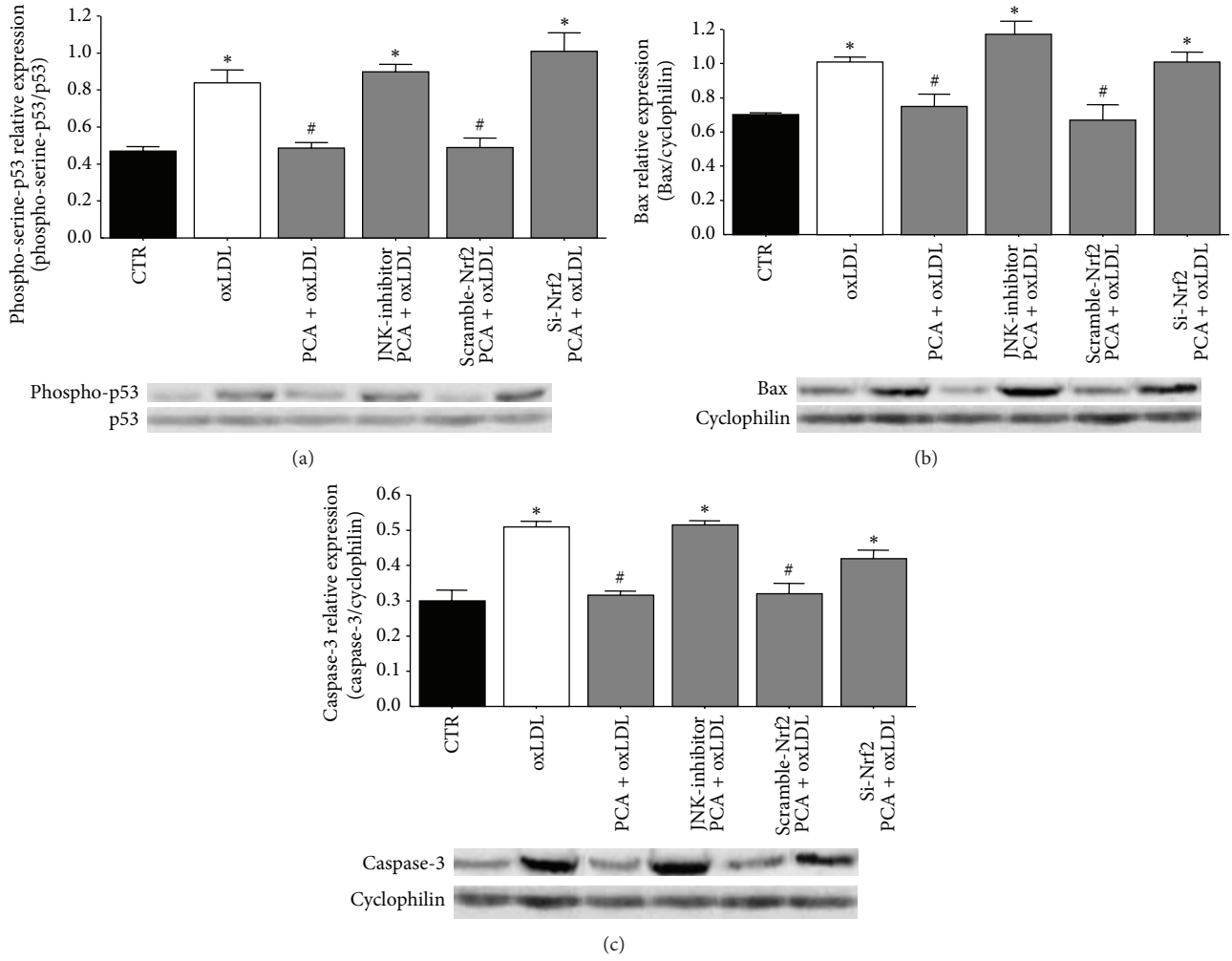

Figure 6: Protocatechuic acid activation of JNK and JNK-dependent activation of Nrf2 lead p53-dependent apoptosis inhibition. Blotting analyses of (a) serine-phosphorylated p53 (1 h after oxLDL treatment) detected in anti-p53 immunoprecipitate and normalized to the nonphosphorylated protein. (b) Bax protein expression (18 h after oxLDL treatment), normalized to cyclophilin protein. (c) caspase-3 active form expression (18 h after oxLDL treatment) normalized to cyclophilin protein. Values obtained in oxLDL-treated cells were compared with values obtained in (i) cells treated with oxLDL and PCA or (ii) cells treated with oxLDL and PCA in the presence of the JNK1/2 inhibitor SP600125 or cells treated with (iii) oxLDL and PCA transfected with anti-Nrf2 siRNA or (iv) the corresponding scrambled RNAs. The inhibitor tested on control cells and oxLDL-treated cells did not show any effect (data not shown). Representative blots from at least three independent experiments are shown. Results are expressed as mean ± S.E.M.; $^{*}P < 0.05$ versus controls; $^{\#}P < 0.05$ versus oxLDL-treated cells.

since the occurrence of apoptosis, which mainly concerns macrophages in advanced vessel lesions, is a crucial event in the progression of the disease.

Modulation of apoptosis is one of the main mechanisms responsible for the protective action of polyphenols against degenerative diseases [8, 38, 39]. PCA exerts antiapoptotic activity by acting as antioxidant and by modulating a number of pro- and antiapoptotic factors [40, 41]. However, PCA is also able to induce apoptosis and its proapoptotic activity appears specifically directed towards transformed cells [42, 43].

In the first place, this study demonstrated that the treatment with PCA of cells exposed to oxLDL prevented the decrease of cellular GSH, even if in the presence of ROS overproduction. In addition, it is worth noting that the biphasic increase of ROS found in oxLDL-exposed cells did not occur when the cells were treated with PCA. The inhibition of early ROS production and GSH saving by PCA treatment seem to be the key events responsible for the protective effect of PCA against oxLDL-induced apoptosis. Thus, PCA might act directly on the main factors that control the different stages of activation, induction, and execution of programmed cell death induced by oxLDL. Apoptosis induced by oxLDL in J774A.1 macrophages occurs mainly by activating p53 that regulates, positively or negatively, the transcription of tens of effector genes coding for proteins such as Bax, a proapoptotic member of the Bcl-2 protein family, and p66Shc. The latter is the oxidative stress-sensor that acts as a cytochrome c oxidoreductase and has been considered the main signal leading to irreversible cell apoptosis, because it triggers a

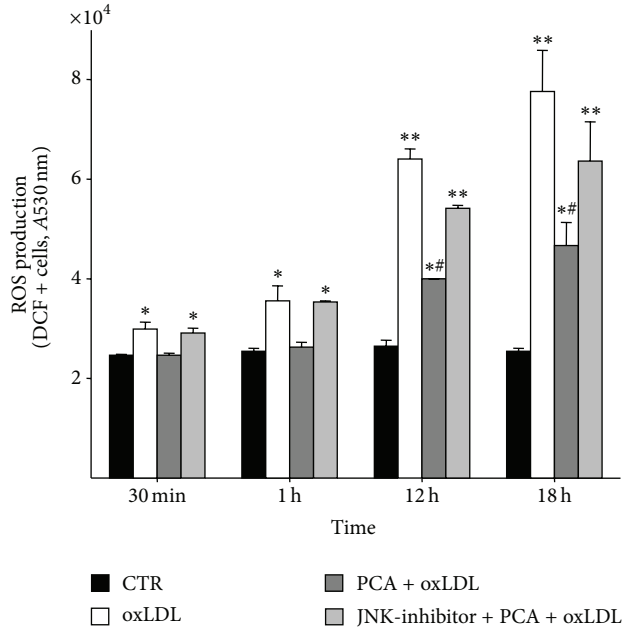

Figure 7: Protocatechuic acid activation of JNK is responsible for the inhibition of ROS hyperproduction. Time course of intracellular ROS production in control cells (CTR), oxLDL exposed cells (oxLDL), oxLDL and PCA-treated cells (PCA + oxLDL), and cells treated with oxLDL and PCA in the presence of the JNK-inhibitor (JNK-inhibitor + PCA + oxLDL). The inhibitor tested on control cells and oxLDL-treated cells did not show any effect (data not shown). Values are the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ versus controls; $^{**}P < 0.001$ versus controls; $^{*\#}P < 0.05$ versus oxLDL-treated cells and versus controls.

further irreparable mitochondrial ROS hyperproduction [44, 45]. The presence of PCA inhibited p53 phosphorylation and, consequently, the expression of two of its target genes BAX and p66SHC.

The activation of p53 in cells treated with oxLDL appears to be controlled by ROS hyperproduction and by the activation/phosphorylation of p38MAPK and PKCδ both necessary for oxLDL-induced proapoptotic signal [30].

In addition to reduce ROS production, we showed that PCA completely decreased the cellular content of phosphorylated p38MAPK and PKCδ to the levels observed in control cells, thus blocking the entire signalling pathway responsible for apoptosis induced by oxLDL.

Furthermore, since GSH concentration in PCA-treated cells exposed to oxLDL remained as high as in the controls, notwithstanding the presence of significantly increased ROS, we hypothesized that PCA might strengthen the endogenous antioxidant defences of the cells.

In this regard, we have demonstrated that PCA, *per se*, like other polyphenols [37], can induce the ARE-dependent antioxidant/detoxifying phase II enzymes such as GR and GPx by activating the transcription factor Nrf2 through JNK-mediated phosphorylation [45]. Herein, in the presence of oxLDL-induced oxidative stress, PCA determined a significant increase of nuclear Nrf2. This most likely depended on JNK activation since it was abolished when the specific JNK1/2 inhibitor was added to the cell culture. This finding is also consistent with our previous data obtained by silencing JNK in. A.1 incubated with PCA alone [37].

It is worth noting that the early (15 min) and transient activation of JNK by PCA seems to play a prominent role in PCA-promoted cell survival. In fact, when JNK was inhibited by SP600125 in oxLDL-treated cells, the ROS overproduction, the activation of p53, the expression of BAX, and the active form of caspase-3 took place even in the presence of PCA. The antiapoptotic effects of PCA were also hindered by transfecting the cells with anti-Nrf2 siRNA. Taken together, these results strongly suggest that JNK/Nrf2 pathway played a major role in counteracting the apoptotic cell death induced by oxLDL in macrophages and add new evidence of the dual role, proapoptotic or antiapoptotic, of JNK signalling pathway.

To the best of our knowledge, this is the first demonstration of PCA-dependent activation of antiapoptotic cell survival signals via the JNK/Nrf2 pathway in macrophages. Furthermore, only few studies have shown that JNK may be responsible for cell survival, proliferation, and differentiation [46]. In fact, the bulk of the studies have provided evidence of a functional role of JNK signalling pathway in promoting proapoptotic responses to stress. The different cellular context as well as the time at which activation takes place, the type of stimulation (transient and prolonged in time), and the ability of JNK to interact with other procell survival signalling pathways has been indicated as responsible for the different activity of JNK [47–49]. In our study, JNK activation might act as a temporary, limited, intracellular signal capable of regulating the cellular antistress and antiapoptotic responses induced by PCA.

In conclusion, our study gives convincing evidence of the essential role of JNK/Nrf2 signalling pathway in the antiapoptotic activity exerted by PCA in oxidatively stressed macrophages by improving the endogenous cellular antioxidant system.

PCA deserves great nutritional interest as the main human metabolite of cyanidin 3-glucoside (C3G), which is in turn the most representative dietary anthocyanins (ACN). Actually, few studies have specifically investigated ACN and phenolic acid bioavailability in humans [16, 50, 51]. PCA has been demonstrated to account for almost 73% of ingested C3G [16]. After ingestion of 1 L of blood orange juice containing 71 mg C3G, the serum maximal concentrations of C3G and PCA were 1.9 nmol/L and 492 nmol/L, respectively [16]. However, it should be considered that the bioavailability can be affected by "chronic" exposure to polyphenols, as the daily consumption of ACN-rich food can provide. Furthermore, the polyphenols might concentrate in the tissue microenvironment [37]. From this point of view, the concentration of PCA tested in our experiments, although higher than that reported after ingestion of food rich in ACN, can be reached plausibly at cellular level. Thus, although the physiological *in vivo* context in which dietary polyphenols exert their influence is undoubtedly much more complex than that available from an *in vitro* system, it could be argued that micromolar concentrations of PCA, which are comparable to those achieved *in vivo* following

a Mediterranean type diet, can help fight cell damage, associated with the onset and progression of atherosclerosis.

The new knowledge achieved on the molecular mechanism that allows PCA to exert protective effects against oxidative injury might represent a useful basis for further *in vivo* studies necessary to design novel diet-based preventive and therapeutic strategies aimed at counteracting oxidative stress-induced pathologies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] F. Saura-Calixto and I. Goni, "Definition of the mediterranean diet based on bioactive compounds," *Critical Reviews in Food Science and Nutrition*, vol. 49, no. 2, pp. 145–152, 2009.

[2] M.-I. Covas, "Olive oil and the cardiovascular system," *Pharmacological Research*, vol. 55, no. 3, pp. 175–186, 2007.

[3] V. M. Victor, N. Apostolova, R. Herance, A. Hernandez-Mijares, and M. Rocha, "Oxidative stress and mitochondrial dysfunction in atherosclerosis: mitochondria-targeted antioxidants as potential therapy," *Current Medicinal Chemistry*, vol. 16, no. 35, pp. 4654–4667, 2009.

[4] C. A. Rice-Evans, N. J. Miller, P. G. Bolwell, P. M. Bramley, and J. B. Pridham, "The relative antioxidant activities of plant-derived polyphenolic flavonoids," *Free Radical Research*, vol. 22, no. 4, pp. 375–383, 1995.

[5] H. Kaneto, N. Katakami, M. Matsuhisa, and T.-A. Matsuoka, "Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis," *Mediators of Inflammation*, vol. 2010, Article ID 453892, 11 pages, 2010.

[6] M. B. de Moura, L. S. dos Santos, and B. van Houten, "Mitochondrial dysfunction in neurodegenerative diseases and cancer," *Environmental and Molecular Mutagenesis*, vol. 51, no. 5, pp. 391–405, 2010.

[7] Y. Curin and R. Andriantsitohaina, "Polyphenols as potential therapeutical agents against cardiovascular diseases," *Pharmacological Reports*, vol. 57, pp. 97–107, 2005.

[8] M. D'Archivio, C. Santangelo, B. Scazzocchio et al., "Modulatory effects of polyphenols on apoptosis induction: relevance for cancer prevention," *International Journal of Molecular Sciences*, vol. 9, no. 3, pp. 213–228, 2008.

[9] C. Giovannini, B. Scazzocchio, R. Varì, C. Santangelo, M. D'Archivio, and R. Masella, "Apoptosis in cancer and atherosclerosis: polyphenol activities," *Annali dell'Istituto Superiore di Sanita*, vol. 43, no. 4, pp. 406–416, 2007.

[10] M. Sanz, E. Cadahía, E. Esteruelas et al., "Phenolic compounds in cherry (*Prunus avium*) heartwood with a view to their use in cooperage," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 8, pp. 4907–4914, 2010.

[11] S. S. Beevi, M. L. Narasu, and B. B. Gowda, "Polyphenolics profile, antioxidant and radical scavenging activity of leaves and stem of Raphanus sativus L," *Plant Foods for Human Nutrition*, vol. 65, no. 1, pp. 8–17, 2010.

[12] J. H. Kwak, H. J. Kim, K. H. Lee, S. C. Kang, and O. P. Zee, "Antioxidative iridoid glycosides and phenolic compounds from Veronica peregrina," *Archives of Pharmacal Research*, vol. 32, no. 2, pp. 207–213, 2009.

[13] M. Rossetto, A. Lante, P. Vanzani, P. Spettoli, M. Scarpa, and A. Rigo, "Red chicories as potent scavengers of highly reactive radicals: A study on their phenolic composition and peroxyl radical trapping capacity and efficiency," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 21, pp. 8169–8175, 2005.

[14] A. Carrasco-Pancorbo, L. Cerretani, A. Bendini, A. Segura-Carretero, T. Gallina-Toschi, and A. Fernández-Gutiérrez, "Analytical determination of polyphenols in olive oils," *Journal of Separation Science*, vol. 28, no. 9-10, pp. 837–858, 2005.

[15] F. Galvano, P. Vitaglione, G. li Volti et al., "Protocatechuic acid: the missing human cyanidins'metabolite," *Molecular Nutrition and Food Research*, vol. 52, no. 3, pp. 386–388, 2008.

[16] P. Vitaglione, G. Donnarumma, A. Napolitano et al., "Protocatechuic acid is the major human metabolite of cyanidin-glucosides," *The Journal of Nutrition*, vol. 137, no. 9, pp. 2043–2048, 2007.

[17] X. Wu, G. R. Beecher, J. M. Holden, D. B. Haytowitz, S. E. Gebhardt, and R. L. Prior, "Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 11, pp. 4069–4075, 2006.

[18] C. D. Kay, G. Mazza, B. J. Holub, and J. Wang, "Anthocyanin metabolites in human urine and serum," *British Journal of Nutrition*, vol. 91, no. 6, pp. 933–942, 2004.

[19] C. D. Kay, P. A. Kroon, and A. Cassidy, "The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products," *Molecular Nutrition and Food Research*, vol. 53, no. 1, pp. S92–S101, 2009.

[20] M.-P. Gonthier, J. L. Donovan, O. Texier, C. Felgines, C. Remesy, and A. Scalbert, "Metabolism of dietary procyanidins in rats," *Free Radical Biology and Medicine*, vol. 35, no. 8, pp. 837–844, 2003.

[21] R. Masella, R. Varì, M. D'Archivio et al., "Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes," *The Journal of Nutrition*, vol. 134, no. 4, pp. 785–791, 2004.

[22] D. Wang, X. Wei, X. Yan, T. Jin, and W. Ling, "Protocatechuic acid, a metabolite of anthocyanins, inhibits monocyte adhesion and reduces atherosclerosis in apolipoprotein E-deficient mice," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 24, pp. 12722–12728, 2010.

[23] D. Wang, T. Zou, Y. Yang, X. Yan, and W. Ling, "Cyanidin-3-O-β-glucoside with the aid of its metabolite protocatechuic acid, reduces monocyte infiltration in apolipoprotein E-deficient mice," *Biochemical Pharmacology*, vol. 82, no. 7, pp. 713–719, 2011.

[24] M. Hidalgo, S. Martin-Santamaria, I. Recio et al., "Potential anti-inflammatory, anti-adhesive, anti/estrogenic, and angiotensin-converting enzyme inhibitory activities of anthocyanins and their gut metabolites," *Genes & Nutrition*, vol. 7, no. 2, pp. 295–306, 2012.

[25] R. Salvayre, N. Auge, H. Benoist, and A. Negre-Salvayre, "Oxidized low-density lipoprotein-induced apoptosis," *Biochimica et Biophysica Acta*, vol. 1585, no. 2-3, pp. 213–221, 2002.

[26] V. N. Bochkov, O. V. Oskolkova, K. G. Birukov, A.-L. Levonen, C. J. Binder, and J. Stöckl, "Generation and biological activities of oxidized phospholipids," *Antioxidants and Redox Signaling*, vol. 12, no. 8, pp. 1009–1059, 2010.

[27] J. M. Isner, M. Kearney, S. Bortman, and J. Passeri, "Apoptosis in human atherosclerosis and restenosis," *Circulation*, vol. 91, no. 11, pp. 2703–2711, 1995.

[28] R. Y. Ball, E. C. Stowers, J. H. Burton, N. R. B. Cary, J. N. Skepper, and M. J. Mitchinson, "Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma," *Atherosclerosis*, vol. 114, no. 1, pp. 45–54, 1995.

[29] D. M. Schrijvers, G. R. Y. de Meyer, M. M. Kockx, A. G. Herman, and W. Martinet, "Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 6, pp. 1256–1261, 2005.

[30] C. Giovannini, R. Var, B. Scazzocchio et al., "OxLDL induced p53-dependent apoptosis by activating p38MAPK and PKCδ signaling pathways in J774A.1 macrophage cells," *Journal of Molecular Cell Biology*, vol. 3, no. 5, pp. 316–318, 2011.

[31] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," *The Journal of Biological Chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[32] K. Yagi, "Lipid peroxides and human diseases," *Chemistry and Physics of Lipids*, vol. 45, no. 2–4, pp. 337–351, 1987.

[33] W. Li, D. Wang, Y. Chi et al., "7-Ketocholesteryl-9-carboxynonanoate enhances the expression of ATP-binding cassette transporter A1 via CD36," *Atherosclerosis*, vol. 226, no. 1, pp. 102–109, 2013.

[34] M. Rosenblat, N. Volkova, and M. Aviram, "Pomegranate phytosterol (β-sitosterol) and polyphenolic antioxidant (punicalagin) addition to statin, significantly protected against macrophage foam cells formation," *Atherosclerosis*, vol. 226, no. 1, pp. 110–117, 2013.

[35] C. Giovannini, E. Straface, D. Modesti et al., "Tyrosol, the major olive oil biophenol, protects against oxidized-LDL- induced injury in Caco-2 cells," *The Journal of Nutrition*, vol. 129, no. 7, pp. 1269–1277, 1999.

[36] R. Masella, R. Varì, M. D'Archivio et al., "Oxidised LDL modulate adipogenesis in 3T3-L1 preadipocytes by affecting the balance between cell proliferation and differentiation," *FEBS Letters*, vol. 580, no. 10, pp. 2421–2429, 2006.

[37] R. Varì, M. D'Archivio, C. Filesi et al., "Protocatechuic acid induces antioxidant/detoxifying enzyme expression through JNK-mediated Nrf2 activation in murine macrophages," *The Journal of Nutritional Biochemistry*, vol. 22, no. 5, pp. 409–417, 2011.

[38] P. M. Kris-Etherton and C. L. Keen, "Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health," *Current Opinion in Lipidology*, vol. 13, no. 1, pp. 41–49, 2002.

[39] D. F. Birt, S. Hendrich, and W. Wang, "Dietary agents in cancer prevention: flavonoids and isoflavonoids," *Pharmacology and Therapeutics*, vol. 90, no. 2-3, pp. 157–177, 2001.

[40] C. Giovannini, B. Scazzocchio, P. Matarrese et al., "Apoptosis induced by oxidized lipids is associated with up-regulation of p66Shc in intestinal Caco-2 cells: protective effects of phenolic compounds," *Journal of Nutritional Biochemistry*, vol. 19, no. 2, pp. 118–128, 2008.

[41] S. Guan, B. Jiang, Y. M. Bao, and L. J. An, "Protocatechuic acid suppresses MPP^+-induced mitochondrial dysfunction and apoptotic cell death in PC12 cells," *Food and Chemical Toxicology*, vol. 44, no. 10, pp. 1659–1666, 2006.

[42] E. C. H. Yip, A. S. L. Chan, H. Pang, Y. K. Tam, and Y. H. Wong, "Protocatechuic acid induces cell death in HepG2 hepatocellular carcinoma cells through a c-Jun N-terminal kinase-dependent mechanism," *Cell Biology and Toxicology*, vol. 22, no. 4, pp. 293–302, 2006.

[43] T.-H. Tseng, C.-J. Wang, E.-S. Kao, and H.-Y. Chu, "Hibiscus protocatechuic acid protects against oxidative damage induced by tert-butylhydroperoxide in rat primary hepatocytes," *Chemico-Biological Interactions*, vol. 101, no. 2, pp. 137–148, 1996.

[44] M. Trinei, E. Migliaccio, P. Bernardi, F. Paolucci, P. Pelicci, and M. Giorgio, "P66Shc, mitochondria, and the generation of reactive oxygen species," *Methods in Enzymology*, vol. 528, pp. 99–110, 2013.

[45] R. Masella, R. di Benedetto, R. Varì, C. Filesi, and C. Giovannini, "Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes," *Journal of Nutritional Biochemistry*, vol. 16, no. 10, pp. 577–586, 2005.

[46] R. J. Davis, "Signal transduction by the JNK group of MAP kinases," *Cell*, vol. 103, no. 2, pp. 239–252, 2000.

[47] P. Hess, G. Pihan, C. L. Sawyers, R. A. Flavell, and R. J. Davis, "Survival signaling mediated by c-Jun NH_2-terminal kinase in transformed B lymphoblasts," *Nature Genetics*, vol. 32, no. 1, pp. 201–205, 2002.

[48] J. A. Lamb, J.-J. Ventura, P. Hess, R. A. Flavell, and R. J. Davis, "JunD mediates survival signaling by the JNK signal transduction pathway," *Molecular Cell*, vol. 11, no. 6, pp. 1479–1489, 2003.

[49] J.-J. Ventura, A. Hübner, C. Zhang, R. A. Flavell, K. M. Shokat, and R. J. Davis, "Chemical genetic analysis of the time course of signal transduction by JNK," *Molecular Cell*, vol. 21, no. 5, pp. 701–710, 2006.

[50] A. Prawan, Y.-S. Keum, T. O. Khor et al., "Structural influence of isothiocyanates on the antioxidant response element (ARE)-mediated heme oxygenase-1 (HO-1) expression," *Pharmaceutical Research*, vol. 25, no. 4, pp. 836–844, 2008.

[51] H. K. Na, E. H. Kim, J. H. Jung, H. H. Lee, J. W. Hyun, and Y. J. Surh, "(-)-Epigallocatechin gallate induces Nrf2-mediated antioxidant enzyme expression via activation of PI3K and ERK in human mammary epithelial cells," *Archives of Biochemistry and Biophysics*, vol. 476, no. 2, pp. 171–177, 2008.

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Review Article

Potential Effects of Pomegranate Polyphenols in Cancer Prevention and Therapy

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Cancer is the second leading cause of death and is becoming the leading one in old age. Vegetable and fruit consumption is inversely associated with cancer incidence and mortality. Currently, interest in a number of fruits high in polyphenols has been raised due to their reported chemopreventive and/or chemotherapeutic potential. Pomegranate has been shown to exert anticancer activity, which is generally attributed to its high content of polyphenols. This review provides a comprehensive analysis of known targets and mechanisms along with a critical evaluation of pomegranate polyphenols as future anticancer agents. Pomegranate evokes antiproliferative, anti-invasive, and antimetastatic effects, induces apoptosis through the modulation of Bcl-2 proteins, upregulates p21 and p27, and downregulates cyclin-cdk network. Furthermore, pomegranate blocks the activation of inflammatory pathways including, but not limited to, the NF-κB pathway. The strongest evidence for its anticancer activity comes from studies on prostate cancer. Accordingly, some exploratory clinical studies investigating pomegranate found a trend of efficacy in increasing prostate-specific antigen doubling time in patients with prostate cancer. However, the genotoxicity reported for pomegranate raised certain concerns over its safety and an accurate assessment of the risk/benefit should be performed before suggesting the use of pomegranate or its polyphenols for cancer-related therapeutic purposes.

1. Introduction

Cancer is the second leading cause of death and is becoming the leading one in old age. It has been estimated that by 2030 the number of new cancer cases will increase by 70% worldwide due to demographic changes alone [1].

The process of cancer development is a consequence of genetic and epigenetic alterations that lead to disruption of basic biological functions, such as cell division, differentiation, angiogenesis, and migration, and transform normal epithelium to preneoplastic lesions and then to invasive carcinoma. The presence of precursor lesions that represent intermediate stages between normal and malignant cells, the slow growth, likely for decades, before symptoms arise and diagnosis is established, a long latency period, and the age-dependent incidence of most cancers indicate that the carcinogenic process occurs during a protracted interval, thus providing the opportunity to block or delay the process, thereby preventing the development of invasive cancer.

Vegetable and fruit consumption is inversely associated with cancer incidence and mortality [2]. Currently, interest in a number of fruits high in polyphenolic compounds has been raised due to their reported chemopreventive and/or chemotherapeutic potential. Pomegranate (*Punica granatum* L.) has been shown to exert anticancer activity, which is generally attributed to its high content of polyphenols including ellagitannins, ellagic acid, and other flavonoids (quercetin, kaempferol, and luteolin glycosides) [3].

The aim of the present review is to critically discuss the cumulative evidence suggesting that pomegranate consumption possesses multiple biological actions and may be helpful in the prevention and therapy of cancer and to provide insight into its anticancer mechanisms.

2. Pomegranate Polyphenols

Pomegranate is a fruit-bearing deciduous shrub and the predominant member of two species comprising the Punicaceae family [4]. Fruits are widely consumed fresh and as juice, jam, and wine [5]. Among seed, peel, and juice, the peel is the constituent which possesses higher content of polyphenols [6]. This part of the fruit contains ellagitannins.

Punicalagin, a large polyphenol with a molecular weight greater than 1000, is unique to pomegranate and is part of a family of ellagitannins that includes the minor tannins punicalin and gallagic acid. Punicalagin represents the bioactive constituent responsible for >50% of the antioxidant activity of pomegranate juice [7]. Pomegranate also contains other polyphenols, such as anthocyanins (3-glucosides and 3,5-glucosides of delphinidin, cyanidin, and pelargonidin) and flavonols [8]. During the juice processing, the whole fruit is pressed and ellagitannins are released into pomegranate juice in significant levels (over 2 g/L juice) [9].

Ellagitannins are hydrolyzed to ellagic acid in the gut, thus resulting in a prolonged release of this acid into the blood [9]. In humans and different animal models, it has been found that ellagic acid is metabolized by the colon microflora to form urolithins A and B. Urolithins can be absorbed into the enterohepatic circulation, which implies that urolithins are in the systemic bloodstream for a short time and then can be excreted in the urine over 12–56 h, as reported after a single administration of 250 mL of pomegranate juice. The metabolite profile is different among subjects, probably due to differences in colonic microflora, where the ellagitannins are metabolized [10–12]. Ellagic acid and urolithins can circulate in the blood and reach and accumulate in many target organs, including intestine and prostate, where the effects of pomegranate ellagitannins are observed [13, 14].

A study analyzed the bioavailability of ellagic acid hydrolyzed from ellagitannins in 18 volunteers following ingestion of pomegranate juice [15]. The plasma appearance and disappearance rates of ellagic acid have been measured. It was found that punicalagin-derived ellagic acid is transformed into dimethylellagic acid glucuronide in plasma and urine on the day of administration of pomegranate juice. Ellagic acid-derived urolithins appeared in urine after the disappearance of dimethylellagic acid glucuronide about 12 h after the administration of pomegranate juice and persisted for 48 h after pomegranate ingestion.

In another study, 11 volunteers consumed 800 mg capsuled pomegranate extract daily containing 330.4 mg punicalagin and 21.6 mg ellagic acid. Cmax and Tmax for plasma ellagic acid were 33.8 ± 12.7 ng/mL at 1 h after ingestion [16].

3. Anticancer Activity of Pomegranate Polyphenols

Accumulating evidence suggests that pomegranate targets a broad spectrum of genes and proteins to suppress cancer growth and progression. The anticancer activity of pomegranate can be seen in a chemopreventive and/or chemotherapeutic approach. Extensive mechanistic studies were performed to evaluate the anticancer activity of pomegranate and its therapeutic potential in various preclinical models. Two primary mechanisms that have been reported are cell-cycle arrest and induction of apoptosis. Some authors have also found significant inhibition of other important mechanisms involved in cancer development such as angiogenesis and metastasis. Figure 1 illustrates the targets and major mechanisms of pomegranate that have been demonstrated in different cancer models.

3.1. Cancer Chemoprevention by Pomegranate Polyphenols. Most chemopreventive agents are antioxidant in nature. The antioxidant activity of commercial pomegranate juices obtained from whole pomegranate was evaluated and compared to that of red wine and a green tea infusion. It showed an antioxidant activity three times higher than those of red wine and green tea (18–20 Trolox equivalent antioxidant capacity versus 6–8 Trolox equivalent antioxidant capacity, resp.). The study also compared the antioxidant activity of commercials juices to that of experimental juices obtained from the arils only. The activity was higher in commercial juices than in experimental juices (18–20 Trolox equivalent antioxidant capacity versus 12–14 Trolox equivalent antioxidant capacity, resp.). The higher antioxidant activity of commercial juices can be imputable to their high content of punicalagin (1500–1900 mg/L). Only traces of this compound were detected in the experimental juices [9].

Punicalagin (10–40 μM) has been shown to significantly inhibit oxidative DNA products by about 70% [17]. A pulse radiolysis technique confirmed the antioxidant activity of punicalagin and evidenced that its ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as to bind to metal ions such as iron and copper are both involved in its antioxidant activity [18].

Using an electron spin resonance with spin trapping, antioxidant activities have also been reported for the three major anthocyanidins (delphinidin, cyanidin, and pelargonidin) of freeze-dried preparations of a 70% acetone extract of pomegranate [19]. Anthocyanidins exhibited scavenging activity against the OH radical generated by a Fenton reaction through the chelation of ferrous ion. The ID_{50} values (the concentration necessary to scavenge O_2 by 50%) of delphinidin, cyanidin, and pelargonidin were 2.4, 22, and 456 μM, respectively. In contrast, no effect was observed for anthocyanidins on NO scavenge. Anthocyanidins inhibited H_2O_2-induced lipid peroxidation in the rat brain homogenates with ID_{50} values of 0.7, 3.5, and 85 μM for delphinidin, cyanidin, and pelargonidin, respectively. These findings suggest that anthocyanidins contribute to the antioxidant activity of pomegranate.

A study compared the antioxidant activity of pomegranate juice, total pomegranate tannin extract, punicalagin, and ellagic acid (all at 10 μg/mL concentrations) using inhibition of lipid peroxidation and Trolox equivalent antioxidant capacity assays. The trend in antioxidant activity was pomegranate juice > total pomegranate tannin extract > punicalagin > ellagic acid. The highest antioxidant activity of pomegranate juice evidences the multifactorial effects and chemical synergy of the action of multiple compounds of pomegranate compared to single purified active ingredients [3].

Pomegranate and its components have been found to inhibit DNA damage, which represents an event involved in the initiation phase of cancer development. Their antigenotoxic effect is only partly dependent on their antioxidant activity. Pomegranate extract (400, 600, and 800 mg/kg b.w.) significantly reduced cyclophosphamide-induced DNA damage in mouse with concomitant increase in antioxidant

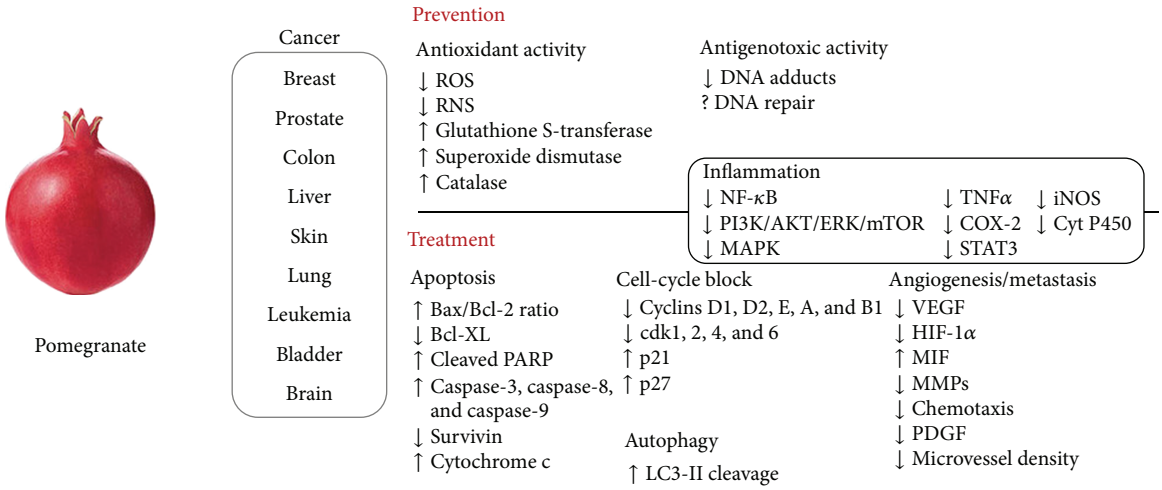

Figure 1: Molecular targets of pomegranate. The arrows reflect changes in protein levels/activities as well as gene expression.

enzymes including glutathione S-transferase, superoxide dismutase, and catalase [20]. Furthermore, punicalagin and ellagic acid (50–500 μM) showed significant inhibition of benzo[a]pyrene-induced DNA adducts, with essentially complete inhibition (97%) at 40 μM by punicalagin and 77% inhibition at 40 μM by ellagic acid [21]. The inhibition of benzo[a]pyrene-induced DNA adducts can be due to the inhibition of the cytochrome P450 activity and/or enhancement of phase II enzymes and due to the direct conjugation with benzo[a]pyrene metabolites. Punicalagin has been shown to inhibit cytochrome P450 1A1 [22], which is directly involved in benzo[a]pyrene bioactivation. Thus, this evidence rules out its ability to scavenge benzo[a]pyrene metabolites [21]. However, a different mechanism can be involved in the antigenotoxic activity of ellagic acid. Indeed, the catechol group of ellagic acid has been found to covalently interact with benzo[a]pyrene metabolites [23]. Apparently, the catechol moieties are protected in the punicalagin molecule but, upon hydrolysis, punicalagin is converted in its active metabolite ellagic acid [9]. Therefore, it is likely that *in vivo* punicalagin is indirectly involved in scavenging benzo[a]pyrene metabolites through its catechol containing moieties.

Punicalagin and ellagic acid markedly antagonized the effect of different mutagens (i.e., sodium azide, methyl methanesulfonate, benzo[a]pyrene, and 2-aminoflourine), with maximum inhibition of mutagenicity up to 90% [21].

Inflammation or proinflammatory conditions can activate cellular signaling and lead to the initiation of cancer by inducing DNA damage and epigenetic changes, making inflammatory signaling pathways a target for cancer prevention. Specifically, chronic inflammation of the colon increases the risk for colon cancer [24]. A study explored the effects of pomegranate juice, total pomegranate tannin extract, and punicalagin on inflammatory cell signaling proteins in a human colon cancer cell line [25]. Cyclooxygenase- (COX-) 1 and cyclooxygenase- (COX-) 2 are implicated in the conversion of free fatty acids to prostanoids. In particular, while COX-1 produces prostanoids that regulate normal tissue homeostasis, COX-2 produces prostanoids inducing inflammation. For this reason, COX-2 overexpression is involved in cancer development. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/nuclear factor kappa B (NFκB) pathway positively affect COX-2 expression. Pomegranate juice (6–50 mg/L), total pomegranate tannin extract (30–200 mg/L), and punicalagin (25–200 mg/L) significantly reduced COX-2 protein expression. Consistent with this observation, the juice inhibited AKT activity and reduced NFκB activation. Pomegranate juice was more potent than total pomegranate tannin and punicalagin, as demonstrated by the recorded COX-2 protein reduction (79% for pomegranate juice, 55% for total pomegranate tannin extract, and 48% for punicalagin), thus indicating that other bioactive polyphenols of pomegranate, such as anthocyanins and flavonols, may contribute to its anti-inflammatory activity.

In vivo studies support the anti-inflammatory activity of pomegranate and its potential in colon cancer prevention. Pomegranate juice (2504.74 mg gallic acid equivalents/L) suppressed the number of aberrant crypt foci and dysplastic aberrant crypt foci in rats injected with azoxymethane. The effect has been found to be associated with a significant downregulation of proinflammatory enzymes such as nitric oxide synthase and COX-2 at both mRNA and protein levels and targeting of anti-inflammatory miR-126-regulated pathways [26].

Recently, ellagic acid was reported to prevent *in vivo* intestinal inflammation and related cancer development. In a model of dextran sulfate sodium-induced acute and chronic colitis, 2% ellagic acid-supplemented diet slightly improved acute colitis. In the chronic model, ellagic acid significantly inhibited the intestinal inflammation, downregulated inflammatory mediators such as COX-2 and iNOS, and blocked the signaling pathways p38 MAPK (mitogen-activated protein kinase), NF-κB, and IL6/STAT3 (signal transducer and activator of transcription 3) in colon tissue [27].

Promising results and targeting of specific mechanisms have been reported for the prevention of certain cancers

including breast, colon, liver and skin by pomegranate and its polyphenols.

3.1.1. Breast. A study performed on mouse mammary organ culture reported a chemopreventive role for pomegranate fractions in breast cancer. Pomegranate fermented juice polyphenols (10 μg/mL) actually caused a 38% inhibition of the incidence of 7,12-dimethylbenz[a]anthracene-induced precancerous mammary lesions. A phenolic fraction of fermented pomegranate juice (10 μg/mL) demonstrated a significantly greater chemopreventive potential, with a 75–90% suppression of lesion formation [28].

Cancer stem cells are thought to be the origin of both primary and secondary breast tumors and thus represent a critical target in breast cancer prevention. Pomegranate extract (5–200 μg/mL) significantly reduced cell viability and blocked cell-cycle progression of a mouse mammary cancer cell line [29], previously characterized as containing a majority of cells possessing stem-cell like characteristics [30], with an IC_{50} (concentration able to decrease cell viability by 50% versus control cultures) of 50 μg/mL [29].

3.1.2. Colon. The Wnt pathway involves a large number of secreted glycoproteins that play a pivotal role in regulating cell fate, differentiation, cell cycle, proliferation, and apoptosis [31]. Aberrant activation of the Wnt signaling has been found to be involved in colon cancer processes [32]. Thus, compounds able to inhibit Wnt signaling may have a role in cancer prevention and treatment. In a 1,2-dimethylhydrazine dihydrochloride-induced rat colon carcinogenesis model, a diet supplemented with 3% (w/w) standardized pomegranate extract inhibited the 1,2-dimethylhydrazine dihydrochloride-induced overexpression of many Wnt-target genes and inhibited tumor incidence. As an example, tumor incidence (66.77%) observed in the 1,2-dimethylhydrazine dihydrochloride group was totally inhibited in the 1,2-dimethylhydrazine dihydrochloride group fed with pomegranate [33]. Also, both ellagic acid and urolithin A inhibited Wnt signaling with IC_{50} (the concentration necessary to inhibit Wnt signaling by 50%) of 19 μg/mL (63 μM) and 9 μg/mL (39 μM), respectively. However, the ellagic acid levels necessary to inhibit Wnt signaling may be near impossible to achieve through regular dietary intake of pomegranate [12, 34]. On the contrary, the inhibition of Wnt signaling achieved by urolithin A is physiologically relevant [35].

Interestingly, significant levels of ellagic acid derivatives and urolithins have been found in human colon tissues from colorectal cancer patients after consumption of pomegranate (900 mg/day for 15 days) [36], thus indicating the induction by pomegranate of a potential molecular preventive environment against colorectal cancer.

The chemopreventive activity of pomegranate on colon carcinogenesis has been found to be also related to its antioxidant activity. Pomegranate peel extract (47 mg (in terms of gallic acid equivalents)/kg of body weight) reduced the incidence of azoxymethane-induced genotoxicity and azoxymethane-induced premalignant lesions by blocking azoxymethane-induced impairment of biochemical indicators of oxidative stress in colonic tissue homogenates [37, 38].

3.1.3. Liver. Pomegranate emulsion has been found to exert chemoprevention of hepatic cancer through antioxidant, antiproliferative, and proapoptotic mechanisms. In particular, Bishayee et al. have found that the emulsion (1 or 10 g/kg) reduces the number and area of γ-glutamyl transpeptidase-positive hepatic foci induced in rat by diethylnitrosamine treatment. The effect was associated with the upregulation of several housekeeping genes under the control of Nrf2, such as glutathione S-transferase, NAD(P)H:quinone oxidoreductase 1, and uridine diphosphate-glucuronosyltransferase [39]. Nrf2 acts as a key mediator of NF-κB-regulated inflammatory pathway. Consistent with this observation, the pomegranate emulsion suppressed several inflammatory markers including NO synthase, 3-nitrotyrosine, heat shock protein 70 and 90, COX-2, and NF-κB induced in rats following exposure to diethylnitrosamine [40]. Since pomegranate juice consumption (377 mL/kg b.w.) has been reported to decrease total hepatic cytochrome P450 content as well as cytochrome P4501A2 expression in rodents [41], Bishayee et al. postulated that the chemopreventive effect of pomegranate emulsion may be also ascribable to the attenuation of diethylnitrosamine activation through pomegranate-induced cytochrome P450 inhibition [39]. However, pomegranate juice as a complex food would never reach the liver. Indeed, *in vivo* studies have not demonstrated unequivocally that the consumption of pomegranate juice may interfere with drug metabolism and clearance [42].

Pomegranate emulsion (1 or 10 g/kg) also reversed hepatic proliferation induced in rat by diethylnitrosamine treatment and induced apoptosis through the upregulation of the proapoptotic protein Bax and the downregulation of the antiapoptotic protein Bcl-2. Canonical NF-κB and Wnt/β-catenin pathways, two interconnected molecular circuits implicated in liver physiology and pathology through regulation of proliferation, differentiation, survival, inflammation, and regeneration [43], have been shown to be involved in the above reported effects and thus in the hepatocarcinogenesis prevention by pomegranate [44].

3.1.4. Skin. Excessive exposure to solar UVB and, to a lesser extent, UVA radiations is the major cause of a variety of cutaneous disorders including skin cancers. In normal human epidermal keratinocytes exposed to UVB, pretreatment with pomegranate fruit extract (10–40 μg/mL) inhibited UVB-dependent activation of NF-κB and UVB-mediated phosphorylation of ERK1/2, JNK1/2 and p38 protein [45], an important group of MAPK pathway signaling proteins that regulate cell proliferation, differentiation, and survival. Furthermore, pomegranate fruit extract (5–60 mg/L) protected human skin fibroblasts from cell death following UV exposure through a reduced activation of the proinflammatory transcription factor NF-κB, downregulation of proapoptotic caspase-3, and an increased G0/G1 phase associated with DNA repair. However, only higher polyphenolic concentrations (500–10,000 mg/L) were able to significantly decrease UV-induced reactive

oxygen species levels and increase intracellular antioxidant capacity [46]. Pomegranate exhibited protective effects also against UVA. Pretreatment of normal human epidermal keratinocytes with pomegranate fruit extract (60–100 μg/mL) actually reduced different cellular pathways including phosphorylation of STAT3, AKT, ERK1/2, mTOR, and p70S6K [47]. The ability of pomegranate fruit extract (0.2%, w/v) to enhance the repair of UVB-mediated DNA damage (i.e., cyclobutane pyrimidine dimers and 8-oxo-7,8-dihydro-2′-deoxyguanosine) recorded in hairless mice [48] contributes to its skin photoprotective activity.

Some studies were designed to extend the skin chemopreventive potential of pomegranate to an *in vivo* situation. Oral feeding of pomegranate fruit extract (0.2% w/v) inhibited skin edema, hyperplasia, infiltration of leukocytes, lipid peroxidation, and hydrogen peroxide generation in the murine skin following UVB exposure. Moreover, it protected against UVB-induced DNA damage and increased p53 and cyclin kinase inhibitor p21 protein levels [48].

Further studies support the ability of pomegranate to prevent skin cancer. In particular, topical application of pomegranate fruit extract (2 mg/mouse) resulted in a significant inhibition of several markers of skin tumor promotion including skin edema and hyperplasia, epidermal ornithine decarboxylase activity, and protein expression of ornithine decarboxylase and COX-2 [49].

The cancer skin chemopreventive effect of pomegranate has been found to be potentiated by diallyl sulfide, a component of garlic. Using a two-stage mouse skin tumorigenesis, pomegranate fruit extract and diallyl sulfide synergistically delayed tumor onset and incidence associated with apoptosis induction and decreased expression of proteins involved in MAPK pathway [50].

3.2. Cancer Chemotherapy by Pomegranate Polyphenols

3.2.1. Breast Cancer. The inhibition of estrogen activity represents a consolidated strategy for the treatment of hormone dependent breast cancer. This strategy includes the antagonism to the estrogen receptor (ER) or the inhibition of estrogen synthesis. The biosynthesis of estrogen is mediated by aromatase enzyme, which converts testosterone to estradiol, a hormonal biomarker that directly correlates with the occurrence of breast cancer.

Pomegranate ellagitannin-derived compounds exhibit antiaromatase activity and affect the growth of breast cancer cells *in vitro* [51]. The antiaromatase activity was tested for ellagic acid, gallic acid, urolithins A and B, and their methylated, acetylated, and sulfate derivatives (all tested at the concentration of 47 μmol/L) and then their antiproliferative potential was tested in human breast cancer cells overexpressing aromatase (MCF-7aro) at concentrations of 2.35 and 4.7 μmol/L. Urolithin B showed the highest antiaromatase activity (about 60% of inhibition) and, as expected, had the highest antiproliferative effect on MCF-7aro [50]. The marked efficacy of urolithin B might be due to its better absorption in cells compared to the other components [52]. Of note, when pomegranate polyphenols were tested together, a higher antiaromatase inhibition has been found, thus suggesting a synergistic effect of the combination. For example, Kim and colleagues demonstrated that polyphenols from pomegranate fermented juice and aqueous pericarp extract (0.02 μg/mL) induced a 60–80% aromatase inhibition [53]. Taken into account that 47 μmol/L (i.e., about 10 μg/mL) of urolithin B are necessary to inhibit the aromatase activity of 40%, it is evident that different compounds should act together for inducting the 60–80% aromatase inhibition reported for 0.02 μg/mL of pomegranate fermented juice and aqueous pericarp extract.

The inhibition of proliferation induced by ellagitannin-derived compounds is also due to a direct antagonism to ER, an antiaromatase-independent mechanism. As breast tumors require estrogen to grow, the 55% inhibition of the estrogenic activity of 17-β-estradiol by lyophilized fresh pomegranate juice (10 mg/mL) in a yeast estrogen screen is relevant [53]. Many pomegranate components, such as luteolin, kaempferol, quercetin, and naringenin, possess the ability to inhibit the estrogenic action of 17-β-estradiol, through competitive binding to ER [54].

Further studies were devoted to test the antiproliferative effect exerted by polyphenols from fermented pomegranate juice and pericarp (starting from 50 μg/mL). A major antiproliferative effect was observed on ER+ cell line (MCF-7), compared to ER− cells (MDA-MB-231) and normal human breast epithelial cells (MCF-10A) [53]. The antiproliferative effect of fermented juice polyphenols was approximately 2-fold higher than that of fresh juice polyphenols [53]. This different activity can be attributed to the fermentation process, which induces breakage of polyphenol-sugar complexes and thus release of free polyphenols [55, 56].

Several studies evidenced the proapoptotic activity of pomegranate. The methanolic extract of pomegranate, whose total phenolic content was 331.28 mg of gallic acid equivalents/g, induced apoptosis on human breast cancer cells at treatment concentration of 100 μg/mL. Additionally, starting from 200 μg/mL, it upregulated the expression of Bax and downregulated Bcl-2 expression [57].

The proapoptotic effect of pomegranate extracts (40 μg/mL) was also investigated on human breast cancer cells in combination with genistein [58], a phytoestrogen isoflavon able to induce apoptosis in ER+ breast cancer cells [59]. Apoptosis induction and cell-growth inhibition of the combination was significantly higher than that of single compounds [58]. These results suggest that the association of genistein and pomegranate could be useful in association with anticancer drugs used for breast tumor.

Tamoxifen is often used against ER+ breast cancer and acts as an ER modulator in breast tissues. Pomegranate fruit extracts (300 μg/mL) additively enhanced tamoxifen-induced inhibition of mitogenic action of estrogen, tamoxifen-induced inhibition of cell cycle, and tamoxifen-induced apoptosis in human breast cancer cells [60]. Furthermore, pomegranate fruit extract restored sensitivity to tamoxifen in tamoxifen-resistant breast tumor cells [60].

Cell-cycle progression is a complex mechanism that leads cells to duplicate their DNA content between phase G1, through phases S and G2, before reaching the cellular division

in M phase. The passage among cell-cycle phases is strictly regulated by cyclins and cyclin-dependent kinase (cdk) complexes. A standardized pomegranate extract inhibited cell growth by inducing cell-cycle arrest in G2/M phase followed by the induction of apoptosis on human breast cancer cells. Treatment of cells with 50 μg/mL of extract for 72 and 96 h resulted in 50% and 80% inhibition of cell growth, respectively [61]. As emerged by comparing the antiproliferative effect of pomegranate extract with that of Trolox and N-acetylcysteine at doses containing equivalent antioxidant activity as the extract, cell-growth inhibition cannot be totally attributed to the consolidated antioxidant properties of pomegranate [61]. The proapoptotic and antiproliferative activity of pomegranate extract was confirmed by data obtained through DNA microarray analysis, which suggests that the cellular effects induced by the extract are related to its ability to affect DNA repair pathway. Pomegranate extract actually downregulated many genes involved in homologous recombination. This effect sensitizes cells to DNA double-strand breaks, a genotoxic event playing a critical role in cancer cell survival [61]. Other anticancer drugs, such as bortezomib, imatinib, and histone deacetylase inhibitors, target homologous recombination [62–64]. This evidence might encourage further studies to test the effect of pomegranate extract in association with the anticancer drugs above reported.

In a mouse mammary cancer cell line derived from MMTV-Wnt-1 mammary mouse tumors (WA4), an arrest in G0/G1 phase was observed after treatment with a commercially available and HPLC-standardized pomegranate extract (Pomella) [29]. The extract exerted a cytotoxic effect also in quiescent WA4 cells, with a dose- and time-dependent activation of caspase-3 that suggests apoptotic cell death (IC_{50} = 200 μg/mL). The cytotoxic effect of the extract has been attributed to its components, such as ellagic acid, ursolic acid, and luteolin, for which an IC_{50} between 5 and 10 μM was recorded. Among these phenolic compounds, ursolic acid exerted the most potent inhibitory effect on cell viability and proliferation. The WA4 cell line is characterized by a majority of stem cells [29]. Therefore, the cytotoxic effects of pomegranate extract on WA4 cells are particularly relevant due to the role of stem cells in primary and secondary breast cancer onset.

Angiogenesis represents the physiological process of new vessels formation and has a crucial role in the development and spread of tumors [65]. Tumor microenvironment requires angiogenesis for supplying oxygen and nutrition, shedding waste metabolites, and allowing tumor growth and progression. Therefore, antiangiogenesis represents a useful anticancer strategy. Accordingly, there are much *in vitro* and *in vivo* evidence on the antiangiogenic properties of pomegranate extract.

Toi and colleagues demonstrated that pomegranate fermented juice polyphenols possess antiangiogenic properties mediated by VEGF downregulation in ER+ breast cancer cells (MCF-7) and immortalized normal human breast epithelial cells (MCF-10A) and upregulation of the angiogenic suppressor migration inhibitory factor (MIF) in ER− breast cancer cells (MDA-MB-231). Additionally, on human umbilical vein endothelial cells (HUVECs), pomegranate extracts (10 μg/mL) showed antiproliferative effects and inhibited tube formation. The same inhibitory activity was confirmed in an *in vivo* angiogenesis model of chicken chorioallantoic membrane, where the inhibition of vessel formation by fermented juice was observed [66].

The matrix metalloproteinases (MMPs), an enzymatic family characterized by a zinc ion in the active site, are critical for maintaining tissue allostasis [67]. An alteration in the regulation of MMPs is common in several tumors and leads to the proteolysis of the extracellular matrix (ECM), a well-established prometastatic mechanism [68, 69]. Multiple MMPs have been associated with tumor progression. As an example, MMP-2, -7, -9, and -11 were identified as responsible for tumor progression in MMP-deficient mice [70]. In the antimetastatic and anti-invasive effect attributed to pomegranate, the inhibition of MMPs represents a fundamental mechanism of action. A polyphenolic extract of pomegranate (0–50 mg/L) inhibited MMP-1, -3, and -13 at posttranscriptional and posttranslational level in human chondrocytes even when administered after treatment with IL-1β [71], an interleukin able to induce upregulation of MMPs, especially MMP-1 and -13 [72]. Furthermore, pomegranate juice (1-2 μL/0.1 mL/well) showed the ability to preserve human reconstituted skin from the formation of MPPs (MMP-1, -2, -3, -7, and -9) when exposed to UVB radiations [73]. Through a semiquantitative RT-PCR, Sreeja and colleagues reported the ability of the pomegranate pericarp extract (160 μg/mL) to downregulate the transcription of MMP-9 in MCF7 [74].

3.2.2. Prostate Cancer. Prostate cancer represents the most common cancer in man. It is estimated for 2014 that in US 233 000 men will develop prostate cancer and 29480 will die from prostate cancer [75]. Nowadays there is lack in the treatment of this disease except for the surgery and radiation approach applicable for prostate cancer in early stage. Among all natural compounds studied for the prevention and/or treatment of prostate cancer, pomegranate has been proven to possess relevant *in vitro* and *in vivo* beneficial effects.

Tissue androgens play a pivotal role in facilitating signaling pathways mediated by androgen receptor leading to prostate cancer progression. During the initial phase, prostate cancer is an androgen-regulated disease that subsequently evolves in an androgen-independent one [76]. Therefore, androgens and their receptors are essential for prostate cancer development, growth, and progression. Treatment of androgen-dependent LNCaP, androgen-independent LNCaP-AP, an engineered cell line overexpressing androgen receptor, and androgen-independent DU145 with pomegranate extract (50 μg/mL) and pomegranate juice (powder form, 100 μg/mL) resulted in the reduction of the expression levels of genes involved in androgen biosynthesis, such as 5α-reductase type I and 3β-hydroxysteroid dehydrogenase type II [77]. Furthermore, a recent study investigated the effects of an ethanolic pomegranate extract on androgen biosynthesis pathways on two human prostate cancer cell lines as well as a murine model of prostate cancer (conditional

PTEN knockout model, representing a comprehensive model for tumor initiation and progression through all stages of prostate cancer to metastatic disease). Pomegranate extract reduced the concentration of testosterone and dihydrotestosterone generated through steroid biosynthesis pathways and decreased the expression of prostate-specific antigen (PSA). *In vivo* data evidenced that pomegranate administered orally in drinking water at a concentration of 0.17 g/L significantly decreased dehydroepiandrosterone, testosterone, and pregnenolone. The decreased ratios indicating the reduced percentages between samples and controls were 42.1%, 80.3%, and 36.5%, respectively [78].

Several groups reported the ability of pomegranate juice or extract to inhibit prostate cancer cell growth *in vitro*. In particular, fermented juice polyphenols and pericarp polyphenols showed cell death induction in three prostate cancer cell lines (PC3, DU145, and LNCaP) [79]. PC3 is an androgen-independent cell line characterized by a high invasive and metastatic potential; DU145 is also an androgen-independent cell line, highly proliferative but with a moderate metastatic potential; LNCaP is an androgen-dependent cell line characterized by functional androgen receptors and the ability to secrete PSA. Fermented juice and pericarp polyphenols in concentration between 20 and 100 μg/mL have been found to inhibit proliferation and induce apoptosis in all three prostate cancer cell lines [79].

In cell-cycle progression, the transition between G1 and S phase is regulated by cyclin D and E. Furthermore, a critical role is played by cdk-cyclin complexes inhibitor such as p21 and p27 [80]. Malik and colleagues identified in the modulation of cdk the main mechanism involved in the proapoptotic and antiproliferative potential of pomegranate [81]. In particular, the pomegranate extract (10–100 μg/mL), obtained from the squeeze of the peeled edible portion of the fruit in 70% acetone/30% distilled water, inhibited PC3 growth through a block in the G1 phase, which was evoked by the modulation of regulatory molecules involved in cell-cycle progression and, in particular, in the G1-S transition. Pomegranate fruit extract actually downregulated cyclins D1, D2, E, cdk2, cdk4, and cdk6 and upregulated p21 and p27 [81, 82]. Furthermore, the induction of apoptosis by pomegranate in PC3 cells was associated with an increased expression of cleaved PARP and Bax and the inhibition of Bcl-2 [81].

Wang and colleagues reported the ability of pomegranate extract (POM Wonderful, Los Angeles, CA, USA), a standardized pomegranate extract containing 37–40% punicalagin and 3.4% free ellagic acid, to induce a potent *in vitro* cytotoxic effect on metastatic castration-resistant prostate cancer cell lines such as C4-2 (IC_{50} = 42 μg/mL), PC3 (IC_{50} = 78 μg/mL), and $ARCaP_M$ (IC_{50} = 161 μg/mL) [83].

The inhibitor-of-apoptosis family member surviving is highly expressed in many cancers and plays a pivotal role in the regulation of cell death, tumor progression, and chemotherapy resistance. In prostate cancer, survivin is frequently overexpressed and associated with poor clinical outcome and resistance to hormone therapy, chemotherapy, and radiation therapy. Based on these pieces of evidence, survivin represents an innovative and promising target for the treatment of prostate cancer [84]. Pomegranate extract (35–150 μg/mL) has been shown to reduce survivin protein and gene expression and modulate its survivin pathway in prostate cancer cells (C4-2, PC3, $ARCaP_M$). STAT3 is an inducer of survivin gene expression. Pomegranate extract actually inhibited STAT3 phosphorylation at Ser727, thus leading to the inactivation of STAT3-dependent transcription of survivin. Furthermore, pomegranate extract (i.p. 60 mg/kg; 3 times/week for 12 weeks) induced apoptosis, retarded cell growth, inhibited survivin, and increased the efficacy of docetaxel (5 mg/kg once a week) in prostate cancer cell-transplanted BALB/c nu/nu mice [83].

Inhibition of prostate cancer cell growth by pomegranate was also reported in immunodepressed mice subcutaneously or orthotopically transplanted with human androgen-dependent CWR22Rv1 prostate cancer cells. Oral administration of pomegranate fruit extract (0.1% and 0.2%, w/v) in drinking fluid to athymic nude mice implanted with androgen-dependent prostate cancer cells (CWR22Rv1) resulted in a significant inhibition in tumor growth concomitant with a significant reduction in secretion of PSA in the serum. As an example, 8 days after cell inoculation, solid tumors were observed in animals receiving water as a drinking fluid. This latency period was prolonged to 11–14 days in animals receiving pomegranate fruit extract. The highest inhibitory effects were observed in animals receiving 0.2% pomegranate fruit extract [81]. Likewise, pomegranate extract (0.8 mg/mouse; ca. 10 times the dose administered to a 70 kg man) induced the same inhibitory effect on androgen-dependent LAPC4 cells implanted in severe combined immunodeficient mice (SCID) [14]. When implanted subcutaneously in murine models, LAPC4 cells produce androgen-dependent tumors; after mouse castration, these cells regrow losing their dependence from androgens [76]. To better predict the effect of pomegranate extract in the clinical response to androgen deprivation caused by castration, Rettig and colleagues used a LAPC4 xenograft model. The authors demonstrated that pomegranate extract is able to delay the growth of LAPC4 androgen-independent tumor through the induction of apoptosis and the inhibition of cell proliferation [85]. NF-κB pathway is one of the main inflammatory signaling pathways involved in cancer development. The constitutive activation of NF-κB pathway is commonly observed in primary prostate cancer and constitutes a risk factor for the development of relapse after radical prostatectomy [86, 87]. NF-κB modulates the transcription of several genes involved in the apoptotic and proliferation process. Rettig and colleagues identified in the inhibition of NF-κB a critical event involved in the induction of apoptosis and inhibition of cell proliferation by pomegranate extract in LCAP4 cells [85].

The inhibition of prostate cancer growth was confirmed in a very recent study by using the murine transgenic adenocarcinoma of the mouse prostate (TRAMP) model. The TRAMP model is widely used in classical chemoprevention protocols since closely mirrors the pathogenesis of human prostate cancer. In a very recent study [88], TRAMP mice received 0.1 and 0.2% pomegranate fruit extract, equivalent to 250 and 500 mL of pomegranate juice, in drinking water, starting at 6 weeks and examined at 12, 20, and 34 weeks of

age. Pomegranate fruit extract supplementation significantly inhibited the development of advanced prostate cancer and its metastasis and doubling the overall survival time. As an example, in water-fed group, 100% mice developed palpable tumors by 20 weeks compared with only 70 and 50% recorded at 34 weeks in the 0.1% and 0.2% pomegranate fruit extract-supplemented mice, respectively. 0.1 and 0.2% pomegranate fruit extract supplementation increased median life expectancy of 30 and 49 weeks, respectively, compared with median survival of 43 weeks recorded in water-fed mice. Of note, in tumors and prostate tissues, supplementation with pomegranate fruit extract resulted in a significant inhibition of mTOR pathway, a master switch of cellular catabolism and anabolism, and thereby a critical regulator of cell growth and proliferation [89].

IGF-I is upregulated in prostate cancer, where it represents a potent mitogen and prosurvival factor and an epidemiologically risk factor for the development of prostate cancer. IGF-I is regulated by 6 different binding proteins (IGFBP). IGFBP-3 is the most abundant in serum and possesses the ability to inhibit IGF-I and stimulate the induction of apoptosis and the inhibition of cell growth [90]. Pomegranate extract (10 μg/mL) in association with IGFBP-3 (1 μg/mL) synergistically induced apoptosis and additively reduced cell proliferation in LNAP4 through the suppression of AKT/mTOR signaling pathway and the increased phosphorylation of JNK [91].

Proof of the antitumor effect of pomegranate on prostate cancer cells have been reported also for some single components of pomegranate. Ellagic acid and urolithin A induced cell-growth inhibition and apoptosis in DU145 and PC3 cells [92].

Hypoxia exerts a key role in the induction of angiogenesis in cancer mainly through the regulation of HIF-1α (hypoxia inducible factor 1α). Pomegranate extract (2.5 μg/mL) exhibited antiangiogenic activity in hypoxic conditions. Both in human prostate cancer cell (LNCaP) and in HUVEC, an inhibition of cell proliferation was observed. VEGF and HIF-1α protein levels became downregulated in hypoxic conditions and this observation supports a direct effect against angiogenesis of pomegranate extract [93]. In SCID mice injected subcutaneously with human prostate cancer cells (LAPC4), pomegranate extract was orally administered (0.8 mg pomegranate extract dissolved in 0.05 mL PBS for 5 days per week). After 4 weeks of treatment, a decrease in tumor growth, microvessel density, HIF-1α and VEGF expression have been found. Pomegranate extract decreased HIF-1α expression, which induced VEGF peptide level downregulation, as already shown in the *in vitro* model. A decreased tumor vessel density and a decreased prostate cancer xenograft size compared to vehicle treatment have been observed [93]. Thus, the antiangiogenic effect can contribute to the inhibition of tumor growth induced by pomegranate extract treatment.

Furthermore, based on the well-known role of inflammation in various types of cancer and the codependence between angiogenesis and inflammation [94], the anti-inflammatory effects of ellagitannins, such as the inhibition of NF-κB and COX-2 [95], can be involved in the inhibition of angiogenesis. A proteomic study exploring the effect of pomegranate fruit juice (7.5 mg/mL) on prostate cancer cells (DU145) identified other targets potentially involved in the antiangiogenic activity of pomegranate. Lee and coworkers observed a significant downregulation of prolidase gene expression [96]. Prolidase can induce the expression of HIF-1α and VEGF and is, therefore, involved in the angiogenic process. Taken together, these observations suggest that the inhibition of prolidase might contribute to the inhibition of angiogenesis and invasion mediated by pomegranate extracts.

Evidence on the antiangiogenic effect of pomegranate is strengthened by the activity of single compounds present in pomegranate, such as ellagic acid. Indeed, ellagic acid (10 μM) has been shown to inhibit the phosphorylation of VEGF receptor and platelet-derived growth factor (PDGF) receptor in muscle cells with consequent inhibition of the signaling of these receptors, including angiogenesis [97].

Cell invasion and migration represent two key steps for tumor metastasization process. Several studies have found the antimetastatic and anti-invasive potential of pomegranate and its polyphenols. Inhibition of proinflammatory chemokines, chemotaxis, and arachidonic acid and hyaluronan metabolism represent some of the main mechanisms modulated by pomegranate treatment in breast and prostate cancer cells.

A marked inhibition of cell invasion induced by pomegranate was reported by several groups in breast cancer and prostate cancer cell lines, through the evaluation of cell passage across a Matrigel membrane. An anti-invasive effect was evoked in PC3 cells by pomegranate fermented juice polyphenols and pomegranate pericarp polyphenols [79], and an increased effect was reported when these pomegranate derivatives were used in association (equally combined with a total concentration of 3 μg/mL) [98]. Notably, ellagic acid (20 and 50 μM) showed the ability to induce a reduction of PC3 invasion and migration.

Arachidonic acid turnover plays a pivotal role in the process of cancer cell survival and invasiveness [99]. Phospholipase A2 (PLA2) induces the release of arachidonic acid from membrane phospholipids and COX metabolizes arachidonic acid in prostaglandins and thromboxanes. PEG2 represents one of the most important metabolites of arachidonic acid. Several studies attributed to PEG2 the ability to promote cancer cell survival and invasion *via* PI3K/AKT pathway activation [100]. In prostate cancer, arachidonic acid turnover is highly increased (10 times compared to healthy cells), and the concentration of cytosolic PLA2 is increased too [101]. Lansky and colleagues associated the anti-invasive effect expressed by fermented juice and pericarp polyphenols (alone and in association) to their ability to modulate the arachidonic acid pathway. In particular, they reduced the PLA2 mRNA expression in PC3 cells (50% by fermented juice and pericarp polyphenols and 80% by their combination, resp., compared to the control) [98]. Moreover, Lansky et al. reported an inhibition of PC3 invasion induced by isolated pomegranate compounds, alone and in combination (at same gross dosage 4 μg/mL), including ellagic acid, caffeic acid, luteolin, and punicic acid [102].

Another important factor involved in the antimetastatic effect of pomegranate is its ability to modulate the hyaluronan metabolism. Hyaluronan, an anionic nonsulfated glycosaminoglycan overexpressed in many tumors, plays a crucial role in tumor progression, supporting cell migration, invasion, and metastasis [103]. Hyaluronan exerts its tumor promoting activity by binding to cell-surface receptor, in particular the hyaluronan-mediated motility receptor (HMMR). This interaction promotes the transduction of many intracellular signals leading to a series of cellular responses, such as protein kinase C, focal adhesion kinase, MAPK, PI3K, tyrosine kinases, RAS, and NF-κB production [104]. A gene expression analysis performed on PC3 cells treated with pomegranate juice and some of its components including luteolin, ellagic acid, and punicic acid (alone and in combination) revealed their ability to downregulate the expression of HMMR [82, 105]. Since the same pomegranate products are responsible for the inhibition of invasion in the same *in vitro* model, this poses the modulation of HMMR and in general of hyaluronan signaling pathway as a crucial mechanism in the inhibition of cancer progression evoked by pomegranate.

Chemokines are small proinflammatory chemoattractant cytokines and represent the main regulators of cell trafficking and adhesion [106]. In particular, the chemokine CXCL12, known as stromal cell-derived factor-1 (SDF1α), binds primarily to the chemokine receptor 4 (CXCR4). The CXCL12/CXCR4 axis is responsible for the regulation of many intracellular signals involved in several pathways such as chemotaxis, cell survival and/or proliferation, increase in intracellular calcium, and gene transcription. The CXCL12/CXCR4 axis plays a critical role in tumor progression, in particular in the angiogenesis, metastasis, and survival processes [107]. Wang and colleagues reported that pomegranate juice and its constituents were able to inhibit chemotaxis acting on CXCL12 in different hormone-dependent and -independent prostate cancer cells (DU145, PC3, and LNCaP) [82, 105]. Furthermore, the inhibition of CXCL12/CXCR4 axis was confirmed by the evaluation of the effect of luteolin, punicic acid, and ellagic acid combination (i.p., 64 μg/component/day) on the formation of metastasis and the expression of CXCR4 on luciferase expressing human prostate cancer cells (PC-3M-luc) implanted in SCID mice. The combination completely inhibited the formation of metastasis and significantly decreased CXCR4 protein levels. Moreover, the combination induced a downregulation of proteins involved in the CXCR4 downstream signaling ($G\alpha_{13}$, PI3K, and p-AKT) [108]. A modulation of chemotaxis toward SDF1α, a chemokine attracting breast cancer cells to the bone, by pomegranate juice (1%) and the combination of luteolin, punicic acid, and ellagic acid (2 and 4 μg/mL), was reported also in ER+ and ER− breast cancer cells by Rocha and colleagues, associated with inhibition of cell growth and migration and induction of cell adhesion [109].

Other effects induced by pomegranate that strengthen the correlation between this fruit and the inhibition of tumor progression were identified in its ability to inhibit cancer cell migration and enhance adhesion, two crucial cellular processes for cancer metastasis. In particular, pomegranate juice and a combination of luteolin, punicic acid, and ellagic acid reduced cell migration through the downregulation of several genes such as type I collagen, tenascin C, and chimerin 1 in prostatic cancer cells (PC3) [82], HMMR, collagen type I alpha1 (COL1A1), anillin (ANLN), and nexilin (NEXN) in breast cancer cells (MCF7) [109]. On the other hand, pomegranate juice and the combination upregulated genes involved in cell adhesion, in particular *E*-cadherin in PC3 cells, claudin 1 (CLDN1) in MCF7 cells, and intercellular adhesion molecule 1 (ICAM1) and myristoylated alanine-rich protein kinase C (MARCKS) in both tumor models [82, 109].

Furthermore, pomegranate juice induced upregulation of anti-invasive miRNAs including miR-335 (regulating *COL1A1*), miR-205, miR-200, and miR-126 and downregulated proinvasive miRNAs such as miR-21 (regulating *MARCKS*) and miR-373 in DU145 cells [82].

On the whole, the above reports unequivocally suggest the potential role of pomegranate in prostate cancer treatment. In this light, the ability of ellagitannins, representing the most abundant polyphenols present in pomegranate juice, and their bioactive metabolites (i.e., urolithin A) to concentrate in mouse prostate tissue after intraperitoneal administration [14] and in the human prostate gland upon consumption of pomegranate juice or extract [110, 111] would represent *per se* a relevant phenomenon in a therapeutic setting and warrants future human tissue bioavailability studies and clinical studies in men with prostate cancer.

3.2.3. Lung Cancer. Pomegranate fruit extract, obtained from acetone extraction of edible seeds, showed anticancer effects on lung tumor both *in vitro* and *in vivo*. The antiproliferative activity of pomegranate fruit extract (50–150 μg/mL) was tested both in adenocarcinomic human alveolar basal epithelial cells (A549) and in normal human bronchial epithelial cells (NHBE) showing a minimal effect in normal cells and a decrease in cell viability up to 47% at the highest tested pomegranate concentration on A549 cells [112]. Furthermore, pomegranate fruit extract treatment of A549 induced a strong cell-cycle arrest in G1 phase, with a 72% cell in G1 at the highest tested concentration (150 μg/mL). The cell-cycle block induced by pomegranate fruit extract was associated with a marked and dose-dependent induction of protein responsible for the transition from G1 to S phase, such as WAF1/p21 and KIP1/p27. Accordingly, pomegranate fruit extract treatment has been found to downregulate cyclins D1, D2, and E and also cdk2, cdk4, and cdk6, all involved in cell-cycle regulation of G1 phase. Moreover, pomegranate fruit extract downregulated (Ki-67 and PCNA) and inhibited (MAPK, PI3K/AKT, and NF-κB/p65) different proliferation markers [112]. The anticancer effect of pomegranate fruit extract reported on lung cell cultures was confirmed by *in vivo* data. In athymic mice implanted with adenocarcinomic human alveolar basal epithelial cells, pomegranate fruit extract (0.1 and 0.2%, w/v) administered by drinking fluid prolonged the latency period for tumor appearance from 15 to 19 days after cell inoculation [112]. Similar results have been found in two different bioassays of lung tumorigenesis, where benzo[a]pyrene and N-nitroso-tris-chloroethylurea were used to induce lung tumors. Oral consumption of 0.2%

pomegranate fruit extract (w/v) in drinking water induced a significant reduction of lung tumor multiplicities. The highest tumor reduction was 61.6% in the benzo[a]pyrene + pomegranate fruit extract group at 140 days and 65.9% in the N-nitroso-tris-chloroethylurea + pomegranate fruit extract group at 240 days. Those effects were associated with the inhibition of several markers of cell proliferation and angiogenesis including phosphorylation of MAPKs, activation of NF-κB, Ki-67, and proliferating cell nuclear antigen, VEGF (vascular endothelial growth factor) [113].

Taken together, these findings indicate pomegranate fruit extract as a promising chemotherapeutic agent in non-small cell lung cancer.

In vivo evidence of the antiangiogenic effect of pomegranate fruit extract 0.2% (w/v) was reported in two A/J mice lung tumors models. In the first one, lung tumor was inducted by benzo[a]pyrene and the second one by N-nitroso-tris-chloroethylurea. NO is a genotoxic reactive nitrogen species synthesized *in vivo* by NO synthases. The inducible form of NO (iNOS) is a common marker of angiogenesis, which resulted in decrease in cells from both lung cancer mice models treated with pomegranate fruit extract. Likewise, a decrease in the number of CD31+ cells was observed, the platelet-derived endothelial cell adhesion protein that represents an index of inhibition of tumor angiogenesis. VEGF expression was also downregulated in mice fed with pomegranate fruit extract and the microvessel density reduced (77.8% in pomegranate treated mice compared to benzo[a]pyrene only-treated mice; 65% in pomegranate treated mice compared to N-nitroso-tris-chloroethylurea only-treated mice) [113].

3.2.4. Colon Cancer. Many studies indicate that pomegranate and its pholyphenols exert a remarkable effect not only in colon cancer chemoprevention but also in chemotherapy, in particular modulating cancer cell death and proliferation.

Pomegranate juice and its constituents such as total pomegranate tannin extract, ellagic acid, and punicalagin were investigated for their *in vitro* antiproliferative and proapoptotic ability on different colon cancer cell lines (SW460, SW620, HT29, and HCT116). Pomegranate juice concentration was normalized to deliver equivalent amounts of punicalagin (w/w). The greatest antiproliferative activity was observed for the juice, for which a 30% to 100% inhibition of cell proliferation was observed at concentrations between 12.5 and 100 μg/mL [3]. Of note, ellagic acid, punicalagin, and total pomegranate tannin extract induced apoptosis in HT29 and HCT116 colon cancer cells at the concentration of 100 μg/mL but failed if cells were treated at doses held equivalent to that detected in pomegranate juice [3]. Again, this underlies the fact that the biological effects of pomegranate come from the synergistic action of pomegranate polyphenols, including flavonols and anthocyanins. Furthermore, on HT29 colon cancer cells, pomegranate juice, ellagic acid, total pomegranate tannin extract and punicalagin induced apoptosis, whereas in the HCT116 colon cancer cells ellagic acid, punicalagin and total pomegranate tannin extract (all at 100 μg/mL) induced apoptosis, but pomegranate juice did not [3]. This evidence might be attributed to the different extent of differentiation of these two cell lines: HCT116 is a highly aggressive cell line unable to differentiate and HT29 retains the ability to differentiate [114].

Pomegranate juice derived ellagitannins (gallic acid, a dimer of gallic acid, ellagic acid, punicalins, and punicalagins) and the intestinal metabolites urolithins exhibited dose- and time-dependent decrease of cell proliferation on HT29 mediated by cell-cycle arrest and followed by the induction of cell death. Among the analyzed ellagitannins, gallic acid dimer was the most effective in the inhibition of proliferation (IC_{50} = 123 μM), also after short time of treatment (12–24 h), whereas the other compounds exhibited antiproliferative effects between 24 and 48 h. Ellagic acid became less effective with an IC_{50} of 462 μM [115]. Cell-cycle arrest induced by ellagitannins was mainly represented by a block in S phase, confirmed by the downregulation of cdk A and B1, necessary for cell progression to G2/M phase. Also urolithins induced a cell-cycle block, but in G2/M phase [115], probably related to its modulation of MAPK, as observed in previous experiments in Caco2 cells [115]. Furthermore, the block of cell cycle in the S phase by ellagic acid and punicalagin, with activation of cyclin E and downregulation of cyclins A and B1, is in accordance with the effects observed in Caco2 cells [13].

The nature of pomegranate-induced cell death in human colon cancer cells was defined. Both ellagitannins and urolithins induced apoptosis by the dose-dependent activation of caspase-3, with the highest induction recorded for punicalagin 100 μM (197% compared to control) [115]. The proapoptotic activity of punicalagin and ellagic acid was Fas- and caspase-8-independent, while the activation of caspase-3 and caspase-9, the release of cytochrome c in the cytosol, and the downregulation of Bcl-XL confirmed the involvement of the apoptotic intrinsic pathway. The effects were recorded at the highest tested concentrations (30 μM for ellagic acid and 100 μM for punicalagin) [13]. Of note, the proapoptotic effect of punicalagin and ellagic acid observed in colon cancer cells was not recorded in normal colon cells [13], thus suggesting a selective action towards cancer cells.

3.2.5. Leukemia. The potential effect of pomegranate juice extract on the inhibition of cell proliferation and induction of apoptosis has been investigated also in leukemia cells. On different leukemia cell lines (four lymphoblastic (Jurkat, SUP-B15, MOLT-3, and CCRF-CEM) and four myeloblastic (HL-60, THP-1, K562, and KG1a) cell lines), pomegranate juice extract (6.25% and 12.5% (v/v)) significantly induced apoptosis. In particular, lymphoblastic cells became the most sensitive to pomegranate, with 2.1% viable cells recorded in CCRF-CEM after treatment with pomegranate 6.25% for 24 h and 0.02% after treatment with pomegranate 12.5%. KG-1a was the most sensitive among the myeloblastic cell lines (31% viable cells after 6.25% pomegranate juice extract treatment and 6.25% viable cells after 12.5%), whereas THP-1 was the less affected by pomegranate (63% viable cells at the maximum tested concentration). The viability of nontumor hematopoietic stem cells (HSCs) was also affected by pomegranate juice extract but at a less extent compared to the majority of the leukemic cells analyzed [116]. The different sensitivity to pomegranate juice extract of lymphoblastic and myeloblastic

cells is not surprising and may be justified by the different molecular pathways altered in those two types of leukemia. These differences are often exploited to obtain antileukemic drugs specific for lymphoblastic rather than myeloblastic leukemia [117, 118]. Other natural compounds showed similar effects. For instance, sulforaphane, a dietary isothiocyanate found in cruciferous, induced apoptosis in different types of leukemia blasts but at a different extent [119].

Pomegranate juice extract exhibited antiproliferative effects on all tested cell lines, including nontumor cells. Treatment with pomegranate at the highest tested dose (12.5% (v/v)) caused a significant S phase arrest in all leukemia cell lines, with the exception of HL-60 and KG-1a, where a small % of cells were blocked in the G0/G1 phase [116]. The different modulation of cell-cycle arrest in HL-60 and KG-1 cells confirms a different effect of pomegranate juice extract in myeloblastic leukemia cells as compared to lymphoblastic leukemia cells, possibly imputable in this case to a different modulation of c-myc expression, overexpressed in HL-60 and whose inhibition has been found to be frequently involved in the G0/G1 block [120]. At the lowest pomegranate juice extract tested concentration (6.25% (v/v)), all cells showed a cell-cycle arrest in G0/G1 phase and the induction of senescence, despite being nonsignificant for all cell lines. Nontumor HSCs showed a behavior similar to leukemic cells [116].

Some different fractions of pomegranate extract have been also tested on leukemia cell lines. Five different fractions of pomegranate juice obtained by solid phase extraction were tested on different leukemia cells (CCRF-CEM, MOLT-3, HL-60, and THP-1) to elucidate which constituents are responsible for the pomegranate antileukemic activity [121]. The acetonitrile fraction has been identified as the responsible for apoptosis induction, cell-cycle arrest, and inhibition of cell proliferation. Acetonitrile fraction was the only one able to decrease ATP levels in leukemia cells. The induction of apoptosis by the acetonitrile fraction, tested at 6.25, 12.5, and 25% (v/v), was in line with the results previously obtained with pomegranate juice extract in terms of cell line sensitivity, with CCRF-CEM becoming the most sensitive and THP-1a the least sensitive. Dose-dependent induction of caspase-3 and nuclear morphology characteristics confirmed the above reported evidence. Acetonitrile fraction also induced a block in S phase with a concomitant decrease of cells in G0/G1 phase in all four cell lines after 48 h of treatment. The acetonitrile fraction became the richest in polyphenols and the HPLC analysis indicated the presence of ellagitannins and ellagic acid and the lack of anthocyanins. Punicalagin was the most active among ellagitannins [121]. Moreover, ellagic acid (25 μM) was found to induce caspase-3-dependent apoptosis and S phase cell-cycle block on the promyelocytic cell line (HL60) [122]. Taken together, these findings confirm that the phenolic components are responsible for the main effects induced by pomegranate in leukemia cells including apoptosis, cell-cycle arrest, and inhibition of cell proliferation.

Another mechanism by which pomegranate constituents impart an antileukemic effect has been reported by Kawaii and Lansky [123]. One of the main characteristics of leukemia is the block of cell differentiation at an early stage. The induction of differentiation is an antileukemic strategy with favorable outcomes for substances such as all-trans retinoic acid. Because of the similar structure between plant flavonoids and differentiation-promoting drugs such as retinoids, it was hypothesized that flavonoid-rich pomegranate extracts might have a similar effect on differentiation. Thus, flavonoid-rich fresh and fermented pomegranate juice and aqueous extracts of pomegranate pericarps were evaluated as potential cytodifferentiating agents. Fermented pomegranate juice and pericarp strongly promoted the differentiation of human HL-60 promyelocytic leukemia cells, while fresh juice showed a milder effect [123]. The milder effect of fresh juice could be attributed to the fact that flavonoids are presumably bound to sugar moieties, whereas after fermentation they are free.

Interestingly, ellagic acid (25 μM) enhanced retinoic acid-induced differentiation of promyelocytic leukemic cells towards granulocytic phenotype [122]. Thus, the association of retinoic acid with ellagic acid might be a promising strategy to reduce the therapeutic dosage of retinoic acid and its cardiorespiratory toxicity [124].

Taking into account the promising antileukemic activity of pomegranate, nanoparticles constituted by partially purified pomegranate ellagitannins and gelatin were produced to potentially increase bioavailability and bioactivities. Comparing the proapoptotic ability of pomegranate purified ellagitannins with that of pomegranate purified ellagitannin gelatin nanoparticle suspension in promyelocytic leukemia cells, ellagitannins encapsuled in nanoparticles were less effective than pomegranate purified ellagitannins in the induction of the early stage of apoptosis, while having similar effects in the induction of late stage of apoptosis [125]. Once embedded in nanoparticles, the effect of an active component might be altered [126], but the differences in activity are principally imputable to nanoparticles' cell uptake that depends on particle size, zeta-potential, and morphology. Even if the functionalized punicalagin-nanoparticles seem to respect the criteria for ideal absorption, the apoptotic activity was lower [125]. Further studies are necessary to optimize the formulation of ellagitannin nanoparticles.

3.2.6. Bladder Cancer. Urinary bladder urothelial carcinoma represents the most frequent cancer affecting the urinary system [75]. Taiwanese pomegranate ethanol extract (50 and 100 μg/mL) inhibited the proliferation of T24 and J82 bladder carcinoma cell lines. The inhibition of cell proliferation induced by pomegranate ethanol extract was related to the induction of S phase block, supported by the inhibition of cyclin A protein level and cdk1 expression. The authors investigated the mechanisms underlying the proapoptotic effect induced by the extract in T24 cells. Pomegranate ethanol extract induced the activation of procaspase-3, procaspase-8, and procaspase-9 and the increase in Bax/Bcl-2 ratio, thus proving that apoptosis in T24 cells was induced by pomegranate through the modulation of both intrinsic and extrinsic pathways. Moreover, pomegranate ethanol extract stimulated procaspase-12 and increased the expression of endoplasmic reticulum stress markers, including CHOP and Bip. This evidence suggests that the endoplasmic reticulum

stress may play a crucial role in the proapoptotic effect of pomegranate ethanol extract [127].

3.2.7. Brain Tumors. Gliomas are the most frequent brain tumors, with still poor prognosis because of their resistance to surgical and medical treatments. Interestingly, punicalagin (1–30 μg/mL) has been found to induce cell death in U87MG human glioma cells [128]. The decrease in cell viability was associated with an increased expression of cyclin E and decreased expression of cyclins A and B. Punicalagin induced apoptosis in U87MG, as shown by the increase in caspase-9 and caspase-3 activity and PARP cleavage. Apoptosis is not the only mechanisms of cell death induced by punicalagin. Indeed, pretreatment with the caspase inhibitor z-DEVD-fmk did not completely prevent cell death. Accordingly, punicalagin caused autophagic cell death, as confirmed by the increased LC3-II cleavage. Although the role of AMPK in determining autophagy remains to be verified, ectopic expression of p27(Kip1) or phosphorylation on Thr 198 increase autophagy. Accordingly, punicalagin raised the level of phosphorylated AMPK and phosphorylated p27 at Thr198. The dose-dependent decrease in punicalagin-induced cell death after chloroquine treatment, a suppressor of autophagy, further confirmed the ability of punicalagin to induce autophagic cell death [128]. These data, albeit preliminary, are encouraging and justify further investigations on the antitumor activity of punicalagin in gliomas.

4. Human Clinical Studies

Copious evidence from preclinical studies indicates the efficacy of pomegranate and its polyphenols against cancer and supports their further development for clinical applications. Interestingly, different clinical studies for human testing of pomegranate have been conducted.

A randomized, placebo-controlled study explored the ability of orally administered 2 g pomegranate extract (delivering pomegranate polyphenols in an amount equivalent to about 250 mL of pomegranate juice) daily for up to 4 weeks to reduce the level of an oxidative stress biomarker (i.e., 8-hydroxy-2′-deoxyguanosine) in 70 patients with a histologic diagnosis of prostate adenocarcinoma [110]. The study evidenced the accumulation of pomegranate extracts in benign and malignant prostate tissues. Furthermore, in benign and cancer tissues, 8-hydroxy-2′-deoxyguanosine levels were 16% and 23% lower in the pomegranate-treated arm, respectively. In both cases, the differences did not reach statistical significance. Taking into account the presumed importance of oxidative stress in prostate cancer development and progression, further and larger studies with longer duration and higher doses are needed to formally test whether pomegranates can alter oxidative stress biomarker levels and its clinical relevance. This is particularly true in that the study found a strong trend between higher urolithin A levels in prostate tissues and lower 8-hydroxy-2′-deoxyguanosine levels.

Several phase II clinical trials have linked oral consumption of pomegranate juice with significant prolongation of PSA doubling time for men with prostate carcinoma with no accompanying serious adverse effects.

In a phase II clinical trial, Pantuck et al. conducted a single-arm phase II trial in 46 patients with recurrent prostate cancer and rising PSA, no prior hormonal therapy, and no evidence of metastases. Patients received 250 mL of pure pomegranate juice (POM Wonderful, containing 570 mg total polyphenol gallic acid equivalents) daily until disease progression. Data on the quantity of punicalagin and ellagic acid were not stated. Although a proper placebo control was not included in the trial, a statistically significant prolongation of PSA doubling time from 15 months at baseline to 54 months after treatment was observed. In addition, a 40% reduction in serum oxidative state was observed in patients. No serious adverse effects were reported [129]. An *in vitro* arm of the trial using patient serum, collected at 9 months after study initiation and incubated with a human prostate cancer cell line, demonstrated that pomegranate juice induced a 12% decrease in cell growth and 17% increase in apoptosis [129].

The above reported finding was further supported by a nonblinded phase II trial in patients with rising PSA and without metastases randomized to receive 1 or 3 g daily of pomegranate extract (containing about 370 mg punicalagin and 30 mg ellagic acid *per* day by HPLC measurement [77]) for up to 18 months [130]. Although a proper placebo control was not included in the trial, the study found that 76% to 82% of patients receiving pomegranate in both arms had longer PSA doubling times values than pretreatment PSA doubling times (from 11.9 to 18.8 months in the low-dose group and 12.2 to 17.5 months in the high-dose group), with no differences in PSA doubling times between arms.

Stenner-Liewen et al. recently investigated the therapeutic impact of pomegranate juice as an adjunct intervention compared to placebo in 98 patients with more advanced or metastatic prostate cancer, the majority (68%) of which had castration-resistant prostate cancer. During the study, patients had to continue their baseline treatment (e.g., androgen deprivation, and zoledronic acid). They consumed 500 mL of pomegranate juice (containing 1147 mg/day polyphenol gallic acid) or 500 mL of placebo beverage daily for 4 weeks. Thereafter, all patients received 250 mL of the pomegranate juice daily (containing 573 mg/day polyphenol gallic acid) for another 4 weeks. Consumption of pomegranate juice did not result in significant PSA declines compared to placebo [131]. There are several differences between the study by Stenner-Liewen et al. [131] and the previous studies reported [129, 130]: (1) the different prostate cancer stage: early prostate cancer stage have been enrolled in the Paller's study and Pantuck's study, while patients with advanced prostate cancer have been enrolled in the Stenner-Liewen's study; (2) PSA levels of patients recruited in the Pantuck's study were 0.5–5 ng/mL versus >5 ng/mL in the study by Stenner-Liewen et al.; (3) the duration of treatment was 12 months in the Pantuck's study, 18 months in the Paller's study, and 2 months in the Stenner-Liewen's study; (4) the observation period was 18 months/progression in the Pantuck's study and Paller' study and 2 months in the Stenner-Liewen's study; (5) taking as a benchmark the daily dosage of 400 mg (370 mg punicalagin and 30 mg ellagic acid) of

the Paller's study, the daily pomegranate polyphenol content used by Stenner-Liewen et al. was 20 times (study period) to 40 times (follow-up period) below the required amount for successful anticancer treatment and far too low to produce a clinical effect [132]. Thus, a careful characterization of the active principles should be mandatory prior to performing more well-controlled human studies aimed at demonstrating the efficacy of pomegranate and its polyphenols and provide a deeper understanding of their therapeutic potential in metastatic prostate cancer treatment.

On the whole, the hitherto published clinical trials on prostate cancer seem to suggest that pomegranate might be useful in early and well differentiated prostate cancer and provide important information for the design and patient selection for further trials with pomegranate.

5. Final Considerations

A number of studies, both *in vitro* and *in vivo*, demonstrated the ability of pomegranate and its polyphenols to contrast various biological events involved in cancer pathogenesis and progression. This scenario paves the way to a double exploitation of pomegranate polyphenols in cancer: as a chemopreventive strategy to reduce the onset of tumors through diet and direct anticancer agents to treat different human cancers at higher dosage regimens more reliably achievable through pharmaceutical delivery of purified compounds.

Pomegranate contains a mixture of phenols, flavonoids, anthocyanins, and tannins able to modulate cellular biochemistry. It is, therefore, difficult to carefully assess the underlying mechanisms that are responsible for its effects and associate them with a single constituent. Each of these constituents could be targeting a different pathway. Most probably, different components of pomegranate modulate different pathways, thus inhibiting several pathways at the same time. This can be highly effective in treating complex diseases such as cancer, characterized by the deregulation of multiple aberrant signalling pathways.

To better understand the mechanism of pomegranate, it is helpful to assess what levels can be reliably attainable in patients. The bioavailability of flavonoids has yet to be unanimously agreed upon. Some studies report that they are poorly absorbed in the upper gastrointestinal tract. Small intestinal absorption can range from 0% to 60% of the administered dose, dependent upon the food source [133]. It has been found that 240 mL *per* day of pomegranate juice (POM Wonderful, standardized to 570 mg of total polyphenol gallic acid equivalents *per* day) used in the Pantuck's study achieved serum ellagic acid concentrations of $0.14 \pm 0.05\,\mu$mol/L [129, 134]. However, another study reported concentrations of ellagic acid of only $0.06\,\mu$mol/L 1 hour after consuming 180 mL of pomegranate juice [135]. Those differences could be attributed to polymorphisms in metabolic enzymes such as uridine 5′-diphospho-glucuronosyltransferases. Another possibility is whether or not ellagic acid represents the optimal metabolite to measure [136]. Thus, whether the high concentrations used for some pomegranate's polyphenols in the *in vitro* study reflect that which would be found *in vivo* is yet to be elucidated.

Because chemopreventive interventions are aimed at healthy populations at high risk for cancer development, it is important to define the toxicological profile of pomegranate. In rats and mice, oral LD_{50} (median lethal dose) for pomegranate peel extract was greater than 5 g/kg b.w. and the intraperitoneal LD_{50} in rats and mice was 217 and 187 mg/kg b.w., respectively. NOAEL (no observed effect level) for pomegranate peel extract following 90-day administration to rats was 600 mg/kg/day [137]. No changes were observed for blood parameters and serum enzymes in 86 overweight human volunteers after oral ingestion of 1420 mg/day (870 mg gallic acid equivalents) of pomegranate fruit extract for a 28-day period [138]. Another study in 10 patients with carotid artery stenosis did not record any toxic effect for kidney, liver, and heart function after pomegranate juice consumption (121 mg/L ellagic acid equivalents) for up to 3 years [139].

Some studies investigated the toxic profile of pomegranate polyphenols. High doses of punicalagin or punicalin (12.5 and 25 mg/kg b.w.) exerted detrimental effects in rat characterized by serum alanine aminotransferase and aspartate aminotransferase increase, hepatic injury, and increase in hepatic lipid peroxidation [140]. However, a recent study [141] did not report tissue alterations or changes in serum biochemical and hematological parameters in rats upon consumption of a 6% punicalagin-containing diet (approximately equivalent to 350 g/day of punicalagin for a 70 kg person [142]) for 37 days.

An *in vitro* and *in vivo* study dealing with the possible genotoxic effect of a pomegranate whole fruit extract was recently performed [143]. The extract induced point mutations (statistically significant at concentrations ≥2 mg/plate in the Ames test and ≥1.5 mg/mL in the *Saccharomyces cerevisiae* assay), sister chromatid exchanges (statistically significant at concentrations $\geq 110\,\mu$g/mL) and chromosome aberrations (statistically significant at concentrations $\geq 45\,\mu$g/mL) *in vitro*. A significant genotoxic effect (statistically significant at doses ≥70 mg/kg b.w.) was recorded also *in vivo* in both sexes of mouse. Furthermore, a very recent study reported an elevated frequency of γ-H2AX foci (i.e., a sensitive measure of DNA double-strand breaks) in mammary cancer cells after treatment with $50\,\mu$g/mL of pomegranate extract [61]. Four potentially mutagenic pyridine alkaloids (i.e., pelletierine, pseudopelletierine, iso-pelletierine, and methylisopelletierine) could account for the genotoxic activity of pomegranate [143, 144].

Genotoxicity is implicated in cancer initiation and in the pathogenesis of different chronic degenerative diseases such as atherosclerosis [145], glaucoma [146], and neurodegenerative diseases [147]. Moreover, the dose-response relationship for genotoxic compounds suggests the lack of a threshold. This means that human exposure to genotoxic agents poses a risk at any level. The genotoxic effects reported for pomegranate raised certain concerns over its safety. Further investigations should be undertaken to evaluate the extent to which pomegranate or its components can be consumed without risk to human health and accurately assess the risk/benefit. Thus, caution should be exercised when

suggesting the use of pomegranate or its polyphenols for cancer-related therapeutic purposes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] R. Siegel, E. Ward, O. Brawley, and A. Jemal, "Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths," *CA Cancer Journal for Clinicians*, vol. 61, no. 4, pp. 212–236, 2011.

[2] H. Boeing, A. Bechthold, A. Bub et al., "Critical review: vegetables and fruit in the prevention of chronic diseases," *European Journal of Nutrition*, vol. 51, no. 6, pp. 637–663, 2012.

[3] N. P. Seeram, L. S. Adams, S. M. Henning et al., "In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice," *Journal of Nutritional Biochemistry*, vol. 16, no. 6, pp. 360–367, 2005.

[4] E. P. Lansky and R. A. Newman, "*Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer," *Journal of Ethnopharmacology*, vol. 109, no. 2, pp. 177–206, 2007.

[5] L. Gu, M. A. Kelm, J. F. Hammerstone et al., "Concentrations of proanthocyanidins in common foods and estimations of normal consumption," *Journal of Nutrition*, vol. 134, no. 3, pp. 613–617, 2004.

[6] A. Faria and C. Calhau, "The bioactivity of pomegranate: impact on health and disease," *Critical Reviews in Food Science and Nutrition*, vol. 51, no. 7, pp. 626–634, 2011.

[7] N. Seeram, R. Lee, M. Hardy, and D. Heber, "Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry," *Separation and Purification Technology*, vol. 41, no. 1, pp. 49–55, 2005.

[8] I. F. F. Benzie and S. Wachtel-Galor, *Herbal Medicine: Biomolecular and Clinical Aspects*, CRC Press, Boca Raton, Fla, USA, 2nd edition, 2011.

[9] M. I. Gil, F. A. Tomas-Barberan, B. Hess-Pierce, D. M. Holcroft, and A. A. Kader, "Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing," *Journal of Agricultural and Food Chemistry*, vol. 48, no. 10, pp. 4581–4589, 2000.

[10] B. Cerdá, R. Llorach, J. J. Cerón, J. C. Espín, and F. A. Tomás-Barberán, "Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice," *European Journal of Nutrition*, vol. 42, no. 1, pp. 18–28, 2003.

[11] B. Cerdá, J. C. Espín, S. Parra, P. Martínez, and F. A. Tomás-Barberán, "The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans," *European Journal of Nutrition*, vol. 43, no. 4, pp. 205–220, 2004.

[12] J. C. Espín, R. González-Barrio, B. Cerdá, C. López-Bote, A. I. Rey, and F. A. Tomás-Barberán, "Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 25, pp. 10476–10485, 2007.

[13] M. Larrosa, F. A. Tomás-Barberán, and J. C. Espín, "The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway," *Journal of Nutritional Biochemistry*, vol. 17, no. 9, pp. 611–625, 2006.

[14] N. P. Seeram, W. J. Aronson, Y. Zhang et al., "Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localize to the mouse prostate gland," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 19, pp. 7732–7737, 2007.

[15] N. P. Seeram, R. Lee, and D. Heber, "Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice," *Clinica Chimica Acta*, vol. 348, no. 1-2, pp. 63–68, 2004.

[16] S. U. Mertens-Talcott, P. Jilma-Stohlawetz, J. Rios, L. Hingorani, and H. Derendorf, "Absorption, metabolism, and antioxidant effects of pomegranate (*Punica granatum* L.) polyphenols after ingestion of a standardized extract in healthy human volunteers," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 23, pp. 8956–8961, 2006.

[17] F. Aqil, R. Munagala, M. V. Vadhanam et al., "Anti-proliferative activity and protection against oxidative DNA damage by punicalagin isolated from pomegranate husk," *Food Research International*, vol. 49, no. 1, pp. 345–353, 2012.

[18] A. P. Kulkarni, H. S. Mahal, S. Kapoor, and S. M. Aradhya, "In vitro studies on the binding, antioxidant, and cytotoxic actions of punicalagin," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 4, pp. 1491–1500, 2007.

[19] Y. Noda, T. Kaneyuki, A. Mori, and L. Packer, "Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 1, pp. 166–171, 2002.

[20] M. V. Dassprakash, R. Arun, S. K. Abraham, and K. Premkumar, "*In vitro* and *in vivo* evaluation of antioxidant and antigenotoxic potential of *Punica granatum* leaf extract," *Pharmaceutical Biology*, vol. 50, no. 12, pp. 1523–1530, 2012.

[21] M. Zahin, I. Ahmad, R. C. Gupta, and F. Aqil, "Punicalagin and ellagic acid demonstrate antimutagenic activity and inhibition of benzo[a]pyrene induced DNA adducts," *BioMed Research International*, vol. 2014, Article ID 467465, 10 pages, 2014.

[22] F. Aqil, M. V. Vadhanam, and R. C. Gupta, "Enhanced activity of punicalagin delivered via polymeric implants against benzo[a]pyrene-induced DNA adducts," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 743, no. 1-2, pp. 59–66, 2012.

[23] J. M. Sayer, H. Yagi, A. W. Wood, A. H. Conney, and D. M. Jerina, "Extremely facile reaction between the ultimate carcinogen benzo[a]pyrene-7,8-diol 9,10-epoxide and ellagic acid," *Journal of the American Chemical Society*, vol. 104, no. 20, pp. 5562–5564, 1982.

[24] C. Pohl, A. Hombach, and W. Kruis, "Chronic inflammatory bowel disease and cancer," *Hepato-Gastroenterology*, vol. 47, no. 31, pp. 57–70, 2000.

[25] L. S. Adams, N. P. Seeram, B. B. Aggarwal, Y. Takada, D. Sand, and D. Heber, "Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 3, pp. 980–985, 2006.

[26] N. Banerjee, H. Kim, S. Talcott, and S. Mertens-Talcott, "Pomegranate polyphenolics suppressed azoxymethane-induced colorectal aberrant crypt foci and inflammation: possible role of

miR-126/VCAM-1 and miR-126/PI3K/ AKT/mTOR," *Carcinogenesis*, vol. 34, no. 12, pp. 2814–2822, 2013.

[27] M. Marín, R. M. Giner, J.-L. Ríos, and M. C. Recio, "Intestinal anti-inflammatory activity of ellagic acid in the acute and chronic dextrane sulfate sodium models of mice colitis," *Journal of Ethnopharmacology*, vol. 150, no. 3, pp. 925–934, 2013.

[28] R. Mehta and E. P. Lansky, "Breast cancer chemopreventive properties of pomegranate (*Punica granatum*) fruit extracts in a mouse mammary organ culture," *European Journal of Cancer Prevention*, vol. 13, no. 4, pp. 345–348, 2004.

[29] Z. Dai, V. Nair, M. Khan, and H. P. Ciolino, "Pomegranate extract inhibits the proliferation and viability of MMTV-Wnt-1 mouse mammary cancer stem cells in vitro," *Oncology Reports*, vol. 24, no. 4, pp. 1087–1091, 2010.

[30] S. Smith, C. Salcido, M. Carlson, S. Hursting, and L. Varticovski, "Wnt-1 mammary tumors are enriched in CD44+/CD24- cells with cancer stem cell characteristics," *AACR Meeting Abstracts*, vol. 2009, article 3087, 2009.

[31] K. M. Cadigan and R. Nusse, "Wnt signaling: a common theme in animal development," *Genes and Development*, vol. 11, no. 24, pp. 3286–3305, 1997.

[32] N. Barker and H. Clevers, "Mining the Wnt pathway for cancer therapeutics," *Nature Reviews Drug Discovery*, vol. 5, no. 12, pp. 997–1014, 2006.

[33] N. A. H. Sadik and O. G. Shaker, "Inhibitory effect of a standardized pomegranate fruit extract on wnt signalling in 1, 2-dimethylhydrazine induced rat colon carcinogenesis," *Digestive Diseases and Sciences*, vol. 58, no. 9, pp. 2507–2517, 2013.

[34] M. V. Selma, J. C. Espín, and F. A. Tomás-Barberán, "Interaction between phenolics and gut microbiota: role in human health," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 15, pp. 6485–6501, 2009.

[35] M. Sharma, L. Li, J. Celver, C. Killian, A. Kovoor, and N. P. Seeram, "Effects of fruit ellagitannin extracts, ellagic acid, and their colonic metabolite, urolithin A, on Wnt signaling," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 7, pp. 3965–3969, 2010.

[36] M. A. Nunez-Sanchez, R. Garcia-Villalba, T. Monedero-Saiz et al., "Targeted metabolic profiling of pomegranate polyphenols and urolithins in plasma, urine and colon tissues from colorectal cancer patients," *Molecular Nutrition & Food Research*, vol. 58, no. 6, pp. 1199–1211, 2014.

[37] M. I. Waly, A. Ali, N. Guizani, A. S. Al-Rawahi, S. A. Farooq, and M. S. Rahman, "Pomegranate (*Punica granatum*) peel extract efficacy as a dietary antioxidant against azoxymethane-induced colon cancer in rat," *Asian Pacific Journal of Cancer Prevention*, vol. 13, no. 8, pp. 4051–4055, 2012.

[38] M. I. Waly, A. S. Al-Rawahi, M. Al Riyami et al., "Amelioration of azoxymethane induced-carcinogenesis by reducing oxidative stress in rat colon by natural extracts," *BMC Complementary and Alternative Medicine*, vol. 14, article 60, 2014.

[39] A. Bishayee, D. Bhatia, R. J. Thoppil, A. S. Darvesh, E. Nevo, and E. P. Lansky, "Pomegranate-mediated chemoprevention of experimental hepatocarcinogenesis involves Nrf2-regulated antioxidant mechanisms," *Carcinogenesis*, vol. 32, no. 6, pp. 888–896, 2011.

[40] A. Bishayee, R. J. Thoppil, A. S. Darvesh, V. Ohanyan, J. G. Meszaros, and D. Bhatia, "Pomegranate phytoconstituents blunt the inflammatory cascade in a chemically induced rodent model of hepatocellular carcinogenesis," *Journal of Nutritional Biochemistry*, vol. 24, no. 1, pp. 178–187, 2013.

[41] A. Faria, R. Monteiro, I. Azevedo, and C. Calhau, "Pomegranate juice effects on cytochrome p450s expression: *in vivo* studies," *Journal of Medicinal Food*, vol. 10, no. 4, pp. 643–649, 2007.

[42] D. Farkas, L. E. Oleson, Y. Zhao et al., "Pomegranate juice does not impair clearance of oral or intravenous midazolam, a probe for cytochrome P450-3A activity: comparison with grapefruit juice," *Journal of Clinical Pharmacology*, vol. 47, no. 3, pp. 286–294, 2007.

[43] Y. Takigawa and A. M. C. Brown, "Wnt signaling in liver cancer," *Current Drug Targets*, vol. 9, no. 11, pp. 1013–1024, 2008.

[44] D. Bhatia, R. J. Thoppil, A. Mandal, K. A. Samtani, A. S. Darvesh, and A. Bishayee, "Pomegranate bioactive constituents suppress cell proliferation and induce apoptosis in an experimental model of hepatocellular carcinoma: role of Wnt/β-catenin signaling pathway," *Evidence-based Complementary and Alternative Medicine*, vol. 2013, Article ID 371813, 15 pages, 2013.

[45] F. Afaq, A. Malik, D. Syed, D. Maes, M. S. Matsui, and H. Mukhtar, "Pomegranate fruit extract modulates UV-B-mediated phosphorylation of mitogen-activated protein kinases and activation of nuclear factor kappa B in normal human epidermal keratinocytes," *Photochemistry and Photobiology*, vol. 81, no. 1, pp. 38–45, 2005.

[46] A. Lisbeth, G. Noratto, L. Hingorani, S. T. Talcott, and S. U. Mertens-Talcott, "Protective effects of standardized pomegranate (*Punica granatum* L.) polyphenolic extract in ultraviolet-irradiated human skin fibroblasts," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 18, pp. 8434–8441, 2008.

[47] D. N. Syed, A. Malik, N. Hadi, S. Sarfaraz, F. Afaq, and H. Mukhtar, "Photochemopreventive effect of pomegranate fruit extract on UVA-mediated activation of cellular pathways in normal human epidermal keratinocytes," *Photochemistry and Photobiology*, vol. 82, no. 2, pp. 398–405, 2006.

[48] F. Afaq, N. Khan, D. N. Syed, and H. Mukhtar, "Oral feeding of pomegranate fruit extract inhibits early biomarkers of UVB radiation-induced carcinogenesis in SKH-1 hairless mouse epidermis," *Photochemistry and Photobiology*, vol. 86, no. 6, pp. 1318–1326, 2010.

[49] F. Afaq, M. Saleem, C. G. Krueger, J. D. Reed, and H. Mukhtar, "Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-κB pathways and inhibits skin tumorigenesis in CD-1 mice," *International Journal of Cancer*, vol. 113, no. 3, pp. 423–433, 2005.

[50] J. George, M. Singh, A. K. Srivastava, K. Bhui, and Y. Shukla, "Synergistic growth inhibition of mouse skin tumors by pomegranate fruit extract and diallyl sulfide: evidence for inhibition of activated MAPKs/NF-κB and reduced cell proliferation," *Food and Chemical Toxicology*, vol. 49, no. 7, pp. 1511–1520, 2011.

[51] L. S. Adams, Y. Zhang, N. P. Seeram, D. Heber, and S. Chen, "Pomegranate ellagitannin-derived compounds exhibit antiproferative and antiaromatase activity in breast cancer cells *In vitro*," *Cancer Prevention Research*, vol. 3, no. 1, pp. 108–113, 2010.

[52] M. Larrosa, A. González-Sarrías, M. T. García-Conesa, F. A. Tomás-Barberán, and J. C. Espín, "Urolithins, ellagic acid-derived metabolites produced by human colonic microflora, exhibit estrogenic and antiestrogenic activities," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 5, pp. 1611–1620, 2006.

[53] N. D. Kim, R. Mehta, W. Yu et al., "Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer," *Breast Cancer Research and Treatment*, vol. 71, no. 3, pp. 203–217, 2002.

[54] D. A. Van Elswijk, U. P. Schobel, E. P. Lansky, H. Irth, and J. Van Der Greef, "Rapid dereplication of estrogenic compounds in pomegranate (*Punica granatum*) using on-line biochemical detection coupled to mass spectrometry," *Phytochemistry*, vol. 65, no. 2, pp. 233–241, 2004.

[55] K.-C. Jeng, C.-S. Chen, Y.-P. Fang, R. C.-W. Hou, and Y.-S. Chen, "Effect of microbial fermentation on content of statin, GABA, and polyphenols in Pu-erh tea," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 21, pp. 8787–8792, 2007.

[56] E. Sato, M. Kohno, H. Hamano, and Y. Niwano, "Increased anti-oxidative potency of garlic by spontaneous short-term fermentation," *Plant Foods for Human Nutrition*, vol. 61, no. 4, pp. 157–160, 2006.

[57] M. Dikmen, N. Ozturk, and Y. Ozturk, "The antioxidant potency of *Punica granatum* L. fruit peel reduces cell proliferation and induces apoptosis on breast cancer," *Journal of Medicinal Food*, vol. 14, no. 12, pp. 1638–1646, 2011.

[58] M. A. L. Jeune, J. Kumi-Diaka, and J. Brown, "Anticancer activities of pomegranate extracts and genistein in human breast cancer cells," *Journal of Medicinal Food*, vol. 8, no. 4, pp. 469–475, 2005.

[59] T. Sakamoto, H. Horiguchi, E. Oguma, and F. Kayama, "Effects of diverse dietary phytoestrogens on cell growth, cell cycle and apoptosis in estrogen-receptor-positive breast cancer cells," *Journal of Nutritional Biochemistry*, vol. 21, no. 9, pp. 856–864, 2010.

[60] S. Banerjee, S. Kambhampati, S. K. Banerjee, and I. Haque, "Pomegranate sensitizes Tamoxifen action in ER-α positive breast cancer cells," *Journal of Cell Communication and Signaling*, vol. 5, no. 4, pp. 317–324, 2011.

[61] A. B. Shirode, P. Kovvuru, S. V. Chittur, S. M. Henning, D. Heber, and R. Reliene, "Antiproliferative effects of pomegranate extract in MCF-7 breast cancer cells are associated with reduced DNA repair gene expression and induction of double strand breaks," *Molecular Carcinogenesis*, vol. 53, no. 6, pp. 458–470, 2014.

[62] Y. Murakawa, E. Sonoda, L. J. Barber et al., "Inhibitors of the proteasome suppress homologous DNA recombination in mammalian cells," *Cancer Research*, vol. 67, no. 18, pp. 8536–8543, 2007.

[63] B. Qiao, M. Kerr, B. Groselj et al., "Imatinib radiosensitizes bladder cancer by targeting homologous recombination," *Cancer Research*, vol. 73, no. 5, pp. 1611–1620, 2013.

[64] B. Ladd, J. J. Ackroyd, J. K. Hicks, C. E. Canman, S. A. Flanagan, and D. S. Shewach, "Inhibition of homologous recombination with vorinostat synergistically enhances ganciclovir cytotoxicity," *DNA Repair*, vol. 12, no. 12, pp. 1114–1121, 2013.

[65] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nature Medicine*, vol. 1, no. 1, pp. 27–31, 1995.

[66] M. Toi, H. Bando, C. Ramachandran et al., "Preliminary studies on the anti-angiogenic potential of pomegranate fractions *in vitro* and *in vivo*," *Angiogenesis*, vol. 6, no. 2, pp. 121–128, 2003.

[67] C. J. Malemud, "Matrix metalloproteinases (MMPs) in health and disease: an overview," *Frontiers in Bioscience*, vol. 11, no. 2, pp. 1696–1701, 2006.

[68] C. M. Overall and C. López-Otín, "Strategies for MMP inhibition in cancer: innovations for the post-trial era," *Nature Reviews Cancer*, vol. 2, no. 9, pp. 657–672, 2002.

[69] I. Stamenkovic, "Matrix metalloproteinases in tumor invasion and metastasis," *Seminars in Cancer Biology*, vol. 10, no. 6, pp. 415–433, 2000.

[70] A. R. Nelson, B. Fingleton, M. L. Rothenberg, and L. M. Matrisian, "Matrix metalloproteinases: Biologic activity and clinical implications," *Journal of Clinical Oncology*, vol. 18, no. 5, pp. 1135–1149, 2000.

[71] S. Ahmed, N. Wang, B. B. Hafeez, V. K. Cheruvu, and T. M. Haqqi, "*Punica granatum* L. extracts inhibits IL-1β-induced expression of matrix metalloproteinases by inhibiting the activation of MAP kinases and NF-κB in human chondrocytes *in vitro*," *Journal of Nutrition*, vol. 135, no. 9, pp. 2096–2102, 2005.

[72] K. S. Mix, J. A. Mengshol, U. Benbow, M. P. Vincenti, M. B. Sporn, and C. E. Brinckerhoff, "A synthetic triterpenoid selectively inhibits the induction of matrix metalloproteinases 1 and 13 by inflammatory cytokines," *Arthritis & Rheumatology*, vol. 44, no. 5, pp. 1096–1104, 2001.

[73] F. Afaq, M. A. Zaid, N. Khan, M. Dreher, and H. Mukhtar, "Protective effect of pomegranate-derived products on UVB-mediated damage in human reconstituted skin," *Experimental Dermatology*, vol. 18, no. 6, pp. 553–561, 2009.

[74] S. Sreeja, T. R. Santhosh Kumar, B. S. Lakshmi, and S. Sreeja, "Pomegranate extract demonstrate a selective estrogen receptor modulator profile in human tumor cell lines and in vivo models of estrogen deprivation," *Journal of Nutritional Biochemistry*, vol. 23, no. 7, pp. 725–732, 2012.

[75] R. Siegel, J. Ma, Z. Zou, and A. Jemal, "Cancer statistics, 2014," *CA: A Cancer Journal for Clinicians*, vol. 64, no. 1, pp. 9–29, 2014.

[76] K. A. Klein, R. E. Reiter, J. Redula et al., "Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice," *Nature Medicine*, vol. 3, no. 4, pp. 402–408, 1997.

[77] M. Y. Hong, N. P. Seeram, and D. Heber, "Pomegranate polyphenols down-regulate expression of androgen-synthesizing genes in human prostate cancer cells overexpressing the androgen receptor," *Journal of Nutritional Biochemistry*, vol. 19, no. 12, pp. 848–855, 2008.

[78] D. S. Ming, S. Pham, S. Deb et al., "Pomegranate extracts impact the androgen biosynthesis pathways in prostate cancer models in vitro and in vivo," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 143, pp. 19–28, 2014.

[79] M. Albrecht, W. Jiang, J. Kumi-Diaka et al., "Pomegranate extracts potently suppress proliferation, xenograft growth, and invasion of human prostate cancer cells," *Journal of Medicinal Food*, vol. 7, no. 3, pp. 274–283, 2004.

[80] K. Vermeulen, D. R. Van Bockstaele, and Z. N. Berneman, "The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer," *Cell Proliferation*, vol. 36, no. 3, pp. 131–149, 2003.

[81] A. Malik, F. Afaq, S. Sarfaraz, V. M. Adhami, D. N. Syed, and H. Mukhtar, "Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 41, pp. 14813–14818, 2005.

[82] L. Wang, A. Alcon, H. Yuan, J. Ho, Q.-J. Li, and M. Martins-Green, "Cellular and molecular mechanisms of pomegranate juice-induced anti-metastatic effect on prostate cancer cells," *Integrative Biology*, vol. 3, no. 7, pp. 742–754, 2011.

[83] Y. Wang, S. Zhang, S. Iqbal et al., "Pomegranate extract inhibits the bone metastatic growth of human prostate cancer cells and enhances the in vivo efficacy of docetaxel chemotherapy," *The Prostate*, vol. 74, no. 5, pp. 497–508, 2014.

[84] S. Fukuda and L. M. Pelus, "Survivin, a cancer target with an emerging role in normal adult tissues," *Molecular Cancer Therapeutics*, vol. 5, no. 5, pp. 1087–1098, 2006.

[85] M. B. Rettig, D. Heber, J. An et al., "Pomegranate extract inhibits androgen-independent prostate cancer growth through a nuclear factor-κB-dependent mechanism," *Molecular Cancer Therapeutics*, vol. 7, no. 9, pp. 2662–2671, 2008.

[86] J. Domingo-Domenech, B. Mellado, B. Ferrer et al., "Activation of nuclear factor-κB in human prostate carcinogenesis and association to biochemical relapse," *British Journal of Cancer*, vol. 93, no. 11, pp. 1285–1294, 2005.

[87] V. Fradet, L. Lessard, L. R. Bégin, P. Karakiewicz, A.-M. Mes Masson, and F. Saad, "Nuclear factor-κB nuclear localization is predictive of biochemical recurrence in patients with positive margin prostate cancer," *Clinical Cancer Research*, vol. 10, no. 24, pp. 8460–8464, 2004.

[88] V. M. Adhami, I. A. Siddiqui, D. N. Syed, R. K. Lall, and H. Mukhtar, "Oral infusion of pomegranate fruit extract inhibits prostate carcinogenesis in the TRAMP model," *Carcinogenesis*, vol. 33, no. 3, pp. 644–651, 2012.

[89] N. Hay, "The Akt-mTOR tango and its relevance to cancer," *Cancer Cell*, vol. 8, no. 3, pp. 179–183, 2005.

[90] S. M. Firth and R. C. Baxter, "Cellular actions of the insulin-like growth factor binding proteins," *Endocrine Reviews*, vol. 23, no. 6, pp. 824–854, 2002.

[91] S. Koyama, L. J. Cobb, H. H. Mehta et al., "Pomegranate extract induces apoptosis in human prostate cancer cells by modulation of the IGF-IGFBP axis," *Growth Hormone and IGF Research*, vol. 20, no. 1, pp. 55–62, 2010.

[92] R. Vicinanza, Y. Zhang, S. M. Henning, and D. Heber, "Pomegranate juice metabolites, ellagic acid and urolithin a, synergistically inhibit androgen-independent prostate cancer cell growth via distinct effects on cell cycle control and apoptosis," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 247504, 12 pages, 2013.

[93] M. R. Sartippour, N. P. Seeram, J. Y. Rao et al., "Ellagitannin-rich pomegranate extract inhibits angiogenesis in prostate cancer *in vitro* and *in vivo*," *International Journal of Oncology*, vol. 32, no. 2, pp. 475–480, 2008.

[94] J. R. Jackson, M. P. Seed, C. H. Kircher, D. A. Willoughby, and J. D. Winkler, "The codependence of angiogenesis and chronic inflammation," *The FASEB Journal*, vol. 11, no. 6, pp. 457–465, 1997.

[95] B. B. Aggarwal and S. Shishodia, "Molecular targets of dietary agents for prevention and therapy of cancer," *Biochemical Pharmacology*, vol. 71, no. 10, pp. 1397–1421, 2006.

[96] S.-T. Lee, Y.-L. Wu, L.-H. Chien, S.-T. Chen, Y.-K. Tzeng, and T.-F. Wu, "Proteomic exploration of the impacts of pomegranate fruit juice on the global gene expression of prostate cancer cell," *Proteomics*, vol. 12, no. 21, pp. 3251–3262, 2012.

[97] L. Labrecque, S. Lamy, A. Chapus et al., "Combined inhibition of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound," *Carcinogenesis*, vol. 26, no. 4, pp. 821–826, 2005.

[98] E. P. Lansky, W. Jiang, H. Mo et al., "Possible synergistic prostate cancer suppression by anatomically discrete pomegranate fractions," *Investigational New Drugs*, vol. 23, no. 1, pp. 11–20, 2005.

[99] K. V. Honn, R. S. Bockman, and L. J. Marnett, "Prostaglandins and cancer: a review of tumor initiation through tumor metastasis," *Prostaglandins*, vol. 21, no. 5, pp. 833–864, 1981.

[100] H. Sheng, J. Shao, M. K. Washington, and R. N. DuBois, "Prostaglandin E2 increases growth and motility of colorectal carcinoma cells," *Journal of Biological Chemistry*, vol. 276, no. 21, pp. 18075–18081, 2001.

[101] A. A. Chaudry, K. W. J. Wahle, S. McClinton, and L. E. F. Moffat, "Arachidonic acid metabolism in benign and malignant prostatic tissue in vitro: effects of fatty acids and cyclooxygenase inhibitors," *International Journal of Cancer*, vol. 57, no. 2, pp. 176–180, 1994.

[102] E. P. Lansky, G. Harrison, P. Froom, and W. G. Jiang, "Pomegranate (*Punica granatum*) pure chemicals show possible synergistic inhibition of human PC-3 prostate cancer cell invasion across Matrigel," *Investigational New Drugs*, vol. 23, no. 2, pp. 121–122, 2005.

[103] S. Seton-Rogers, "Metastasis: multitasking hyaluronic acid," *Nature Reviews Cancer*, vol. 12, no. 4, p. 228, 2012.

[104] E. A. Turley, P. W. Noble, and L. Y. W. Bourguignon, "Signaling properties of hyaluronan receptors," *Journal of Biological Chemistry*, vol. 277, no. 7, pp. 4589–4592, 2002.

[105] L. Wang, J. Ho, C. Glackin, and M. Martins-Green, "Specific pomegranate juice components as potential inhibitors of prostate cancer metastasis," *Translational Oncology*, vol. 5, no. 5, pp. 344–355, 2012.

[106] A. F. Chambers, A. C. Groom, and I. C. MacDonald, "Dissemination and growth of cancer cells in metastatic sites," *Nature Reviews Cancer*, vol. 2, no. 8, pp. 563–572, 2002.

[107] B. A. Teicher and S. P. Fricker, "CXCL12 (SDF-1)/CXCR4 pathway in cancer," *Clinical Cancer Research*, vol. 16, no. 11, pp. 2927–2931, 2010.

[108] L. Wang, W. Li, M. Lin et al., "Luteolin, ellagic acid and punicic acid are natural products that inhibit prostate cancer metastasis," *Carcinogenesis*, vol. 35, no. 10, pp. 2321–2330, 2014.

[109] A. Rocha, L. Wang, M. Penichet, and M. Martins-Green, "Pomegranate juice and specific components inhibit cell and molecular processes critical for metastasis of breast cancer," *Breast Cancer Research and Treatment*, vol. 136, no. 3, pp. 647–658, 2012.

[110] S. J. Freedland, M. Carducci, N. Kroeger et al., "A double-blind, randomized, neoadjuvant study of the tissue effects of POMx pills in men with prostate cancer before radical prostatectomy," *Cancer Prevention Research*, vol. 6, no. 10, pp. 1120–1127, 2013.

[111] A. González-Sarrías, J. A. Giménez-Bastida, M. T. García-Conesa et al., "Occurrence of urolithins, gut microbiota ellagic acid metabolites and proliferation markers expression response in the human prostate gland upon consumption of walnuts and pomegranate juice," *Molecular Nutrition and Food Research*, vol. 54, no. 3, pp. 311–322, 2010.

[112] N. Khan, N. Hadi, F. Afaq, D. N. Syed, M.-H. Kweon, and H. Mukhtar, "Pomegranate fruit extract inhibits prosurvival pathways in human A549 lung carcinoma cells and tumor growth in athymic nude mice," *Carcinogenesis*, vol. 28, no. 1, pp. 163–173, 2007.

[113] N. Khan, F. Afaq, M.-H. Kweon, K. Kim, and H. Mukhtar, "Oral consumption of pomegranate fruit extract inhibits growth and progression of primary lung tumors in mice," *Cancer Research*, vol. 67, no. 7, pp. 3475–3482, 2007.

[114] T. M. Yeung, S. C. Gandhi, J. L. Wilding, R. Muschel, and W. F. Bodmer, "Cancer stem cells from colorectal cancer-derived cell lines," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 8, pp. 3722–3727, 2010.

[115] S. G. Kasimsetty, D. Blalonska, M. K. Reddy, G. Ma, S. I. Khan, and D. Ferreira, "Colon cancer chemopreventive activities of

pomegranate ellagitannins and Urolithins," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 4, pp. 2180–2187, 2010.

[116] H. Dahlawi, N. Jordan-Mahy, M. R. Clench, and C. L. Le Maitre, "Bioactive actions of pomegranate fruit extracts on leukemia cell lines in vitro hold promise for new therapeutic agents for leukemia," *Nutrition and Cancer*, vol. 64, no. 1, pp. 100–110, 2012.

[117] M. Bellido and P. A. W. Te Boekhorst, "JAK2 inhibition: reviewing a new therapeutical option in myeloproliferative neoplasms," *Advances in Hematology*, vol. 2012, Article ID 535709, 6 pages, 2012.

[118] A. J. Marks, M. S. Cooper, R. J. Anderson et al., "Selective apoptotic killing of malignant hemopoietic cells by antibody-targeted delivery of an amphipathic peptide," *Cancer Research*, vol. 65, no. 6, pp. 2373–2377, 2005.

[119] C. Fimognari, E. Turrini, P. Sestili et al., "Antileukemic activity of sulforaphane in primary blasts from patients affected by myelo- and lympho-proliferative disorders and in hypoxic conditions," *PLoS ONE*, vol. 9, no. 7, Article ID e101991, 2014.

[120] A. Renneville, C. Roumier, V. Biggio et al., "Cooperating gene mutations in acute myeloid leukemia: a review of the literature," *Leukemia*, vol. 22, no. 5, pp. 915–931, 2008.

[121] H. Dahlawi, N. Jordan-Mahy, M. Clench, G. J. McDougall, and C. L. Maitre, "Polyphenols are responsible for the proapoptotic properties of pomegranate juice on leukemia cell lines," *Food Science & Nutrition*, vol. 1, no. 2, pp. 196–208, 2013.

[122] Y. Hagiwara, T. Kasukabe, Y. Kaneko, N. Niitsu, and J. Okabe-Kado, "Ellagic acid, a natural polyphenolic compound, induces apoptosis and potentiates retinoic acid-induced differentiation of human leukemia HL-60 cells," *International Journal of Hematology*, vol. 92, no. 1, pp. 136–143, 2010.

[123] S. Kawaii and E. P. Lansky, "Differentiation-promoting activity of pomegranate (*Punica granatum*) fruit extracts in HL-60 human promyelocytic leukemia cells," *Journal of Medicinal Food*, vol. 7, no. 1, pp. 13–18, 2004.

[124] P. Fenaux, C. Chomienne, and L. Degos, "All-trans retinoic acid and chemotherapy in the treatment of acute promyelocytic leukemia," *Seminars in Hematology*, vol. 38, no. 1, pp. 13–25, 2001.

[125] Z. Li, S. S. Percival, S. Bonard, and L. Gu, "Fabrication of nanoparticles using partially purified pomegranate ellagitannins and gelatin and their apoptotic effects," *Molecular Nutrition and Food Research*, vol. 55, no. 7, pp. 1096–1103, 2011.

[126] K. W. Powers, S. C. Brown, V. B. Krishna, S. C. Wasdo, B. M. Moudgil, and S. M. Roberts, "Research strategies for safety evaluation of nanomaterials. Part VI. characterization of nanoscale particles for toxicological evaluation," *Toxicological Sciences*, vol. 90, no. 2, pp. 296–303, 2006.

[127] S.-T. Lee, M.-H. Lu, L.-H. Chien, T.-F. Wu, L.-C. Huang, and G.-I. Liao, "Suppression of urinary bladder urothelial carcinoma cell by the ethanol extract of pomegranate fruit through cell cycle arrest and apoptosis," *BMC Complementary and Alternative Medicine*, vol. 13, article 364, 2013.

[128] S. G. Wang, M. H. Huang, J. H. Li, F. I. Lai, H. M. Lee, and Y. N. Hsu, "Punicalagin induces apoptotic and autophagic cell death in human U87MG glioma cells," *Acta Pharmacologica Sinica*, vol. 34, no. 11, pp. 1411–1419, 2013.

[129] A. J. Pantuck, J. T. Leppert, N. Zomorodian et al., "Phase II study of pomegranate juice for men with rising prostate-specific antigen following surgery or radiation for prostate cancer," *Clinical Cancer Research*, vol. 12, no. 13, pp. 4018–4026, 2006.

[130] C. J. Paller, X. Ye, P. J. Wozniak et al., "A randomized phase II study of pomegranate extract for men with rising PSA following initial therapy for localized prostate cancer," *Prostate Cancer and Prostatic Diseases*, vol. 16, no. 1, pp. 50–55, 2013.

[131] F. Stenner-Liewen, H. Liewen, R. Cathomas, C. Renner, U. Petrausch, and T. Sulser, "Daily pomegranate intake has no impact on PSA levels in patients with advanced prostate cancer—results of a phase IIb randomized controlled trial," *Journal of Cancer*, vol. 4, no. 7, pp. 597–605, 2013.

[132] S. Chrubasik-Hausmann, C. Vlachojannis, and B. Zimmermann, "Pomegranate juice and prostate cancer: importance of the characterisation of the active principle," *Phytotherapy Research*, vol. 28, no. 11, pp. 1676–1678, 2014.

[133] C. Manach and J. L. Donovan, "Pharmacokinetics and metabolism of dietary flavonoids in humans," *Free Radical Research*, vol. 38, no. 8, pp. 771–785, 2004.

[134] N. P. Seeram, Y. Zhang, R. McKeever et al., "Pomegranate juice and extracts provide similar levels of plasma and urinary ellagitannin metabolites in human subjects," *Journal of Medicinal Food*, vol. 11, no. 2, pp. 390–394, 2008.

[135] N. P. Seeram, S. M. Henning, Y. Zhang, M. Suchard, Z. Li, and D. Heber, "Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 hours," *Journal of Nutrition*, vol. 136, no. 10, pp. 2481–2485, 2006.

[136] S. J. Klempner and G. Bubley, "Complementary and alternative medicines in prostate cancer: from bench to bedside?" *Oncologist*, vol. 17, no. 6, pp. 830–837, 2012.

[137] C. Patel, P. Dadhaniya, L. Hingorani, and M. G. Soni, "Safety assessment of pomegranate fruit extract: acute and subchronic toxicity studies," *Food and Chemical Toxicology*, vol. 46, no. 8, pp. 2728–2735, 2008.

[138] D. Heber, N. P. Seeram, H. Wyatt et al., "Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 24, pp. 10050–10054, 2007.

[139] M. Aviram, M. Rosenblat, D. Gaitini et al., "Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation," *Clinical Nutrition*, vol. 23, no. 3, pp. 423–433, 2004.

[140] C. C. Lin, Y. F. Hsu, T. C. Lin, and H. Y. Hsu, "Antioxidant and hepatoprotective effects of punicalagin and punicalin on acetaminophen-induced liver damage in rats," *Phytotherapy Research*, vol. 15, no. 3, pp. 206–212, 2001.

[141] B. Cerdá, J. J. Cerón, F. A. Tomás-Barberán, and J. C. Espín, "Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 11, pp. 3493–3501, 2003.

[142] M. Azorín-Ortuño, C. Urbán, J. J. Cerón et al., "Safety evaluation of an oak-flavored milk powder containing ellagitannins upon oral administration in the rat," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 8, pp. 2857–2865, 2008.

[143] A. Sánchez-Lamar, G. Fonseca, J. L. Fuentes et al., "Assessment of the genotoxic risk of *Punica granatum* L. (Punicaceae) whole fruit extracts," *Journal of Ethnopharmacology*, vol. 115, no. 3, pp. 416–422, 2007.

[144] European Food Safety Authority, "Compendium of botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern," *EFSA Journal*, 2009.

[145] P. C. Shukla, K. K. Singh, B. Yanagawa, H. Teoh, and S. Verma, "DNA damage repair and cardiovascular diseases," *Canadian Journal of Cardiology*, vol. 26, pp. 13A–16A, 2010.

[146] A. O. Khan, "Genetics of primary glaucoma," *Current Opinion in Ophthalmology*, vol. 22, no. 5, pp. 347–355, 2011.

[147] E. Storkebaum, A. Quaegebeur, M. Vikkula, and P. Carmeliet, "Cerebrovascular disorders: molecular insights and therapeutic opportunities," *Nature Neuroscience*, vol. 14, no. 11, pp. 1390–1397, 2011.

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Review Article

Age-Related Cognitive Impairment as a Sign of Geriatric Neurocardiovascular Interactions: May Polyphenols Play a Protective Role?

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It is known that endothelial dysfunction plays an important role in the development and progression of cardiovascular diseases implicated also in cognitive decline. Experimental studies pointed to the fact that the modification of NO levels via NOS activity may affect the blood pressure level as well as several higher nervous functions—for example, learning and memory. There are emerging evidences from *in vitro* and animal studies suggesting that polyphenols may potentially have a protective effect on the development of neurodegenerative diseases and may improve cognitive function as well as positively affecting the blood pressure regulatory mechanisms. This review accentuates the need for precisely defined clinically controlled studies as well as for use of adequate experimental procedures discriminating between the human higher brain functions and the only overall activation of the brain cortex. The physiological neurocardiovascular interactions are implicated in the increased healthy life span as well.

1. Introduction

The European health report of WHO from 2012 points to the fact that the group of people aged 65 or more years constituted 15% of the total by the end of 2010 and is projected to comprise more than 25% by 2050. Consequently, related health care costs will rise significantly. Therefore, it is important to search for possibilities to maintain health and cognitive health with age as well. It is referred that 25–30% of people aged 85 or older have some degree of age-associated cognitive decline which may significantly influence their instrumental activities of daily living [1]. Age-associated cognitive decline differs in extent among the individuals. The cognitive decline is one of the most feared aspects of becoming old. Cognitive functions are under the influence of many factors and their mechanisms are poorly understood. The aging and cardiovascular diseases are either directly or indirectly responsible for cognitive decline. The age related cognitive decline which is in relation to cerebrovascular dysfunction in senescence becomes a very serious problem of geriatric medicine and care. The pathological studies have pointed to the fact that up to the 34% of dementing illnesses show postmortem confirmed significant vascular pathology [2]. The identification of factors helping people maintain their cognitive health becomes the very important current public health challenge.

2. NO and Basic Neurocardiovascular Interactions

It is now known that a lot from all biochemical factors modulating the blood flow regulate also the neuronal functions including the freely diffusing nitric oxide (NO) molecule [3]. Patterning and branching of blood vessels and nerves are strongly molecularly linked [4]. Diffusible messengers can directly impact neural activity which increases with increased blood volume. That is, microvascular endothelial cells participate in signal processing in the brain by generating tonic and phasic NO signals; the endothelial NO production leads to a net depolarization in proximal nerve fibers [5]. It is a very important finding when assuming the fact that the brain functions are made possible by interconnected structure of neurons, glia, and microvessels. To do it the brain utilizes over

100 billion cells and 600 km of micro vessels [6]. The neurons, glia, and vascular cells meet in the so-called neurovascular unit which is the basic structural and functional brain unit for information processing, integration as well as energy supply. All brain networks consist of three compartments, neuronal, glial, and capillary, forming the brain complex cellular networks. This complex cellular network is a base for the brain functional hyperaemia which is known as the increased blood flow and volume within the activated brain region [7]. Moreover, the endogenous NO production in the thalamus, which is very important in the gating mechanisms, varies with the behavioural state [8]. It points to the important role of NO within the regulatory mechanisms for interacting components of neuronal networks coherent firing of which creates a basis for the human higher brain functions, that is, for cognitive, strategic, and affective neuronal networks mainly. Their proper regulatory mechanisms are very important concerning the healthy aging.

Healthy aging and physical well-being depend in a great extent on properly functioning vascular and nervous system. There is a bulk of literature describing the beneficiary effects of the polyphenols upon the cardiovascular system. Their ability to improve endothelial function by increasing production of the signalling nitric oxide molecule leading to relaxation of the endothelial smooth muscle resulting in a greater control of blood pressure was documented repeatedly. Increased NO level inhibits the enzymes like cyclo-oxygenase (COX-2), reactive C protein, and the atheromatous plaque adhesion molecules known to be involved in inflammation [9]. Endothelial dysfunction plays a role in the development and progression of cardiovascular diseases implicated in cognitive decline and is also an important mechanism to be considered in development of neurodegenerative disorders [10]. In the review article, Vauzour et al. [11] pointed to the suggested mechanism for the action of polyphenols on vascular function which involves their ability to modulate the levels of and activity of nitric oxide synthase (eNOS) and therefore nitric oxide (NO) bioavailability to the endothelium. This regulation of vascular nitric oxide is thought to involve the ability of polyphenols to interact with kinase signaling pathways such as the PI3-kinase/Akt pathway and intracellular Ca^{2+} on eNOS phosphorylation and subsequent NO production. They also pointed out that polyphenols were shown to act to prevent age-related vascular injury. Recently, Valls-Pedret et al. [12] have shown that increased consumption of polyphenols in the group of 447 elderly subjects at high cardiovascular risk was associated also with better cognitive performance. The effect of polyphenols on cognitive functions is as a hot topic now the subject of the randomized, placebo controlled, double-blinded clinical trials [13].

On the other hand, the essential hypotension has been shown to cause prolonged execution times in the attention tasks; moderately decreased accuracy was found in the tests assessing sustained attention and working memory and hypotensive subjects showed smaller elevations in BP during the execution of the cognitive tasks [14]. Moreover, relentless brain hypoperfusion may be responsible for protein synthesis abnormalities that later result in neurodegenerative lesions and thus induce progressive cognitive impairment [15]. Unfortunately, the cognitive decline due to the hypotension is mostly ignored. The age-related cognitive impairment as seen at subtly diminished some neuropsychological functions [16, 17] may be taken as an indicator of the level of the geriatric neurocardiovascular interactions.

3. Polyphenols and Brain Regulatory Mechanisms

The role of diet and other lifestyle factors in successful aging belong to the current topics of scientific interest [18]. Several findings suggest that both the physiological and psychological diet components might help to delay the onset and/or slow the progression of age-associated cognitive decline [19]. Oxidative damage, among others, is implicated in aging and age-associated cognitive decline. Dietary antioxidants found in fruits and vegetables may help protect against oxidative damage and several studies support a link between the antioxidant status and cognitive functions in senescence [20]. The polyphenols are proposed as the mostly rich natural substances with antioxidant potential in our diet [21].

The neurological benefits of polyphenols, especially of resveratrol, a potential antioxidant from red wine polyphenols, were documented experimentally in cerebral ischemia, brain oedema, Parkinson disease, amyotrophic lateral sclerosis, brain tumours, pain, cognitive impairments, aging, and several other CNS disorders [22]. According to Hollman [23], there is no doubt that polyphenols are excellent antioxidants *in vitro*, but systemic antioxidant effects *in vivo* are hard to prove. The preclinical studies have shown that polyphenols display neuroprotective effects, enhance neuronal functions, stimulate brain flow, induce neurogenesis and prevent age-related damage through their antioxidant and anti-inflammatory activities. Kesse-Guyot et al. [24] have pointed out that high intake of specific polyphenols may help to preserve verbal memory which is a salient vulnerable domain in pathological brain aging. As Vauzour et al. have stressed [11] the polyphenols can cross the blood brain barrier but the question of their dose remains to be answered. The effects of polyphenols upon the brain functions are associated with nitric oxide signalling. The effects of polyphenols on cognition and against neurodegenerative processes appear to be mediated via their interactions with neuronal and glial signaling pathways that affect gene expression and interfere with the cell death mechanisms. One NO signalling pathway affects the brain derived neurotrophic factor (BDNF), secretory protein, neurophilin, which regulates neuronal surviving and differentiation. It means that BDNF affects the axonal and dendritic growth and via such a way the synapses and components of release of the neurotransmitters [25, 26] NO act in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain. BDNF stimulates nNOS and NO, produced by the proliferating cells, and signals to begin differentiating into neural cell phenotypes [27]. The regulation of neuronal growth and synaptic metabolism is essential for cognitive functioning.

There exist a small number of clinical trials about the therapeutic benefits of the natural substances. It is also known that the diet and the other important factors of lifestyle are not so exactly controlled as in animal studies. But Letenneur and coworkers [28] published the results of PAQUID study (Personnes Agées Quid) on prospectively examined flavonoid intake in relation to cognitive functions and decline. At the start of study, 1640 subjects aged 65 or older free from dementia were adjusted for age, sex, and educational level. During the following 10 years' period, they were four times reexamined at home by psychologist by means of Mini-Mental State Examination, Benton's Visual Retention Test and "Isaacs" set test and a comprehensive dietary survey was performed. Subjects within the two highest quartiles of flavonoid intake had significantly better cognitive functions as well as evolution of performance over time.

It is now known that dietary polyphenols are extensively metabolized *in vivo* and the chemical, biophysical, and biological properties of their metabolites are quite different from those of parent molecule. The systemic effects of polyphenols are associated with nitric oxide signalling. Actually, NO plays a dual role in tissues and cells. It belongs to essential physiological signalling molecule mediating various cell functions but when present in excess it induces cytotoxic and mutagenic effects [29], as for neurons also [30]. While the normal production of NO is associated with normal function of eNOS its overproduction (toxic) is associated with increase in inducible NOS (iNOS). Results of many studies demonstrated clearly that inhibition of endothelial nitric oxide synthase (eNOS) reduces blood flow and may contribute to cognitive dysfunction [31]. Cai et al. [32] stated that the downregulation of iNOS and upregulation of eNOS may protect from cognitive impairment. Figure 1 shows how the changes of eNOS and iNOS activity can affect human cognitive functions. Animal model studies pointed to the fact that the modulation of NO levels via NOS activity, for example, by administration of polyphenols, may affect aging and dementia [33], memory deficits and long-term memory [34], spatial working memory [35], and performance in cognitive tasks relating to learning and memory as well [36].

Moreover, the role of NO and NOS was described in psychiatric disorders also. Several studies have shown a prominent role of NO in the pathogenesis of major depression. Nitric oxide modulates norepinephrine, serotonin, dopamine, and glutamate, the major neurotransmitters involved in the neurobiology of major depression [37]. The role of NO and NOS was described in schizophrenia as well [38, 39] and an increased level of NO was characterized as a link between cognitive impairment in Alzheimer disease patients [40]. In the human studies, the question of genetic polymorphisms of relation between NO and psychiatric disorders was neglected. Quite recently, it was found that the variability in nNOS gene is associated with schizophrenia [41]. A functional promoter polymorphism in neuronal NOS (nNOS) was proposed to be associated with personality traits related to impulsivity [42]. It may open new field for study disorders with difficulty to control impulses as primary feature (e.g., kleptomania, pyromania, and others) and disorders with difficulty to control impulses that is not their primary feature as for example, attention-deficit/hyperactivity disorder, or manic state of bipolar disorder [43].

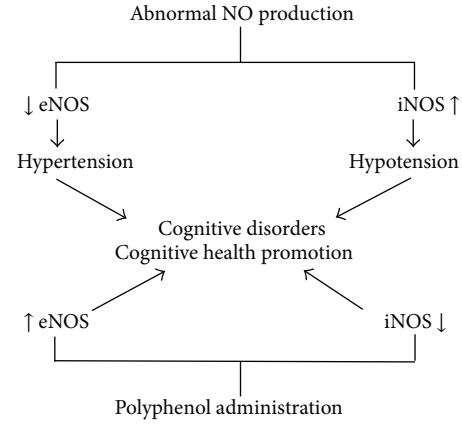

Figure 1: Changes of eNOS and iNOS activity and their effects upon human cognitive function.

Several of our own studies have shown [44–47] that administration of polyphenols may evoke following effects in the animal brain: (1) antioxidant activity, mainly inhibition of the NADPH oxidase and subsequent reactive oxygen species generation; (2) an activator effect on endothelial and inhibitory action upon both neuronal (nNOS) and inducible nitric oxide synthase activity; (3) downregulation of the proinflammatory transcription factors such as NF-κB; and (4) modulation of signalling pathways such as mitogen-activated protein kinase cascade and cAMP response element-binding protein leading to the improvement of memory and cognitive performance. Nevertheless, the question still remains whether the polyphenols may have also an influential general beneficial effect in relation to the behavioural and brain functions of senior persons.

4. Polyphenols and Cognitive Functions

Weichselbaum and Buttriss [48] pointed to the fact that there is emerging evidence from *in vitro* and a few animal studies suggesting that polyphenols may potentially have a protective effect on the development of neurodegenerative diseases and may improve cognitive function in patients when such diseases are established but the controlled human studies are needed. At the same time Macready et al. [49] reviewed 15 human randomized controlled trial studies on the effect of flavonoids upon the cognitive functions. As the authors pointed out, the findings from this selection of human cognition studies using flavonoid treatments are suggestive of a positive association between flavonoid consumption and cognitive function. The reviewed studies employed a total of 55 different cognitive tests covering a broad range of cognitive domains and most studies incorporated at least one measure of executive function/working memory, with nine reporting significant improvements in performance as a function of flavonoid supplementation compared to a control group. This publication clearly draws attention to the fact that to characterize the influence of biologically active substance

upon the cognitive functions requires exact definition of the measured executive function, type of memory, motor function with or without a cognitive component, and also type of measured IQ. Consequently, the authors stressed the fact that a great deal of work still needs to be carried out to identify tasks that are sensitive to flavonoid-related cognitive changes in healthy human populations. Moreover, it seems that there may exist differences in cognitive decline related to the consumption of nutrients available in fruits and vegetables [50]. Our own results suggested also the need to use adequate experimental procedures for discriminating among such higher brain functions as perception, memory, and attention as opposed to the only overall activation of the brain cortex [51, 52].

5. Polyphenols and Visual-Oculomotor Integration

In our preliminary study concerning the effect of single polyphenol substance administration in normotensive young healthy persons, we hypothesized that it may positively influence some human higher brain functions which are usually mostly affected in age-cognitive decline and/or disorders of the central nervous system [52]. Because the interaction between subjects, between subject and animal, and also between subject and environment is subserved by sensory-motor integration and the most tight and subtle one is the visual-oculomotor integration we decided to analyse the accuracy of saccadic eye movements. The saccades are the rapid jumps of eyes by means of which the subject is scanning the visual environment. In our experimental examinations, we recorded the saccadic eye movements by means of the electrooculography. At the beginning of examinations, the saccades were elicited by switching on the 0.3° circular visual targets in the visual field (the visual-guided saccade task—VGS). The subject has to fixate as rapid and accurate as possible the visual targets which appeared suddenly in the visual field. The subjects are not aware of the control mechanisms for execution of these reflexive movements. But one can split the visual-oculomotor integration by using the memory-guided saccades paradigm. Immediately after the end of the VGS task the memory-guided saccade task (MGS) was introduced [53]. Subjects have to fixate a central visual target and to continue its fixation while another visual stimulus was briefly flashed into the periphery of the visual field. He/she has to remember the location of the peripheral visual stimulus. After the central fixation target was switched off subject had to make a saccade to the remembered peripheral target location. Following the registration of VGS and MGS tasks in one group of volunteers the polyphenolic substance extracted from red wine (Provinols, 4 mg/kg of body weight) was administered; in the second group the placebo was used and in the third group nothing was administered. The whole procedure was repeated two and three hours later (the composition of Provinols was as follows: proanthocyanidins 480, total anthocyanidins 61, free anthocyanidins 19, catechin 38, hydroxycinnamic acid 18, flavonols 14, and polymeric tanins 37 mg/g of Provinols).

In average, the 95% of VGS are accurate contrarily to 50–60% of MGS. Two hours after the administration of Provinols, the accuracy of MGS was substantially and significantly increased but only slightly of VGS. The results confirmed the significantly increased number of more accurate memory guided saccades after the Provinols administration, which suggested a better performance in the MGS test. Nevertheless, the question remains what actually the administration of the polyphenolic substance affected: spatial memory, working memory, encoding of visual stimuli, execution of MGS, visual perception, or attention.

Another interesting finding concerns the relation of horizontal saccadic eye movements to the functional brain asymmetry. The VGS directed to the motor dominant hemisphere are more accurate—they are followed by less number of corrective saccades. Moreover, when the reflexive saccade toward the visual target is inaccurate and is followed by a corrective saccade the corrective ones are of significantly shorter latency when directed towards the motor dominant hemisphere. The shortest latency is recorded when the correction is directed from the periphery of the visual field towards its centre [54]. In this case, the functional brain asymmetry helps the person to catch and process the visual stimulus as early and as accurate as possible. This relation is not present after the administration of Provinols. Similar finding we had seen in patients suffering from depression and panic disorder [55, 56]. Administration of Provinols increased the overall activation level of the brain cortex [57]. The question is open about what is done with NO signalling when the level of activation and/or excitation is enhanced by intensive focusing of the attention on the subjective problems or by administration of some biologically active substance as, for example, Provinols?

In a subsequent study, the whole experimental design was repeatedly used and, in addition to the electrooculography, the electroencephalography and the blood pressure changes were recorded [50]. In general, the accuracy of VGS and MGS saccades was the same as in previous study. No differences in EEG evoked potentials time-locked to saccadic eye movement onsets among the three groups of subjects were found. There were no differences in preparation and execution of saccades as well as in the time of first encoding the new visual information within the primary visual cortex. As expected, the single administration of polyphenolic substance did not affect the blood pressure. As for the EEG power spectral densities, during the preparatory and execution periods for saccades guided by memory information the significant decrease within the slow EEG bands was registered, alpha power mainly. It means the after the Provinols administration the heightened cortical activation appeared in areas playing the role in attention spatial orienting and memorizing as well.

Up to now, it is not possible to separate attention and memory components in our results but they clearly demonstrate that the polyphenolic extract from red wine Provinols positively affects some cognitive/integrative higher brain functions and probably the attention and activation status of the human brain as well. Taking into the consideration also the above mentioned results of PAQUID study by Letenneur et al. [28], the preventive as well as activation

effects of polyphenols upon human cognitive functions are quite promisingly suggested at least in young healthy persons. In order to differentiate the effects of polyphenols upon the cognitive, affective and integrative human higher brain functions the more precise experimental set up should be introduced for the future studies. Because of the changes in metabolism rate in elderly subjects the question of the above mentioned effects of administration of polyphenolic substances upon the MGS have to be experimentally proved also.

6. NO, Human Aging Problems, and Life Span

Because the NO plays a significant role in vascularization and neurogenesis, it is quite obvious that some authors point to its possible effect on life span [58]. Cheng et al. [59] discovered that NO acts in a positive feedback loop with brain derived neurotrophic factor (BDNF) to regulate neural progenitor cell proliferation and differentiation in the mammalian brain. BDNF stimulates nNOS and NO produced by the proliferating cells signals to start differentiation into neural cell phenotypes. Ungvari et al. [60] summarized their review article pointing that increased bioavailability of NO, decreased vascular reactive oxygen species generation, activation the antioxidant response element pathway, induction of reactive oxygen species detoxification systems exert anti-inflammatory effects and, thereby, suppressed initiation and/or progression of vascular disease that accompany aging. The study of Montesanto et al. [61] has shown that genetic variability of NOS genes has an effect on common age related phenotypes and longevity in humans as well as previously reported for model organisms.

Physiological aging is accompanied by several mental changes which are more or less "normal" symptoms of aging as well as of old age. The dynamics of psychic activities decreases overall and central fatigue increases. The increased extracellular glutamate concentration was described in neurodegenerative and inflammatory brain diseases [62–64] and the potential role of glutamate transport in mental fatigue was hypothesized [65]. Hu et al. [66] found a dose-dependent inhibition of astrocyte glutamate uptake by a mechanism in which NO plays a role.

The human motor manifestations during aging are decreased also. It was reported that NO plays an important role in the control of behaviour [67] and locomotion as well [68–70]. The more recent human studies indicate that exercise training for a period ranging from days to several weeks enhances basal release of nitric oxide from the aorta, active and inactive muscle, and coronary arteries. This may contribute to the reduction in resting blood pressure that can be observed after as little as 4 weeks of training [71, 72]. Increased NO production with a wide range of intensities and duration of physical trainings that progresses to structural and other sustained adaptations were documented, [73, 74]. The positive effect of physical exercises upon the aging brain in clinical as well as nonpathological populations was also repeatedly documented [75] In spite of the fact that physical exercise promotes brain and cognitive vitality into older adulthood the more intervention research is needed as Kramer et al. [76] pointed out taking into the consideration also their previous meta-analysis of the relevant literature from 1966 to 2001.

Disorders of memory functions during aging steadily and gradually increase. General principles on how NO and memory are related were recently described by Susswein et al. [77]. However, the authors stressed the fact that the role of NO in memory formation is extremely variable. It seems to be strongly dependent not only on the particular animal investigated but also on the specific behaviour and training paradigm examined.

In the course of aging, the expansive and confrontational symptoms are losing and the depressive and anxiety reactions are coming to the fore. Of course, there are more "normal" symptoms of aging but in the majority of them the NO signalling may play a role. The NO and aging are closely related and it seems that NO system in each brain region may influence chemical and structural changes in the CNS during aging [78]. Moreover, it was pointed out that the mechanisms by which NOS enzymes promote vascular dysfunction in aging are specific for each enzyme isoform [79]. The question of the specific/general mechanisms of the effects of polyphenols in relation to healthy aging is still open to be solved.

Normal aging is a very complex process under the influence of many factors that vary from individual to individual. The main problem is how to change lifestyle and habits of older population. Further clinically controlled studies and adequate experimental procedures are needed. It is now clear that the multi-/transdisciplinary approach only may reveal the causal mechanisms underlying the neurocardiovascular interactions serving as a prerequisite for physiological aging, cognitive health promotion and a longer healthy life span. Such a demand is very important because of the need for controlled clinical trials to determine the effect of reducing vascular risk factors upon decreasing a risk for cognitive decline, improving cognitive function and lengthening the healthy life span [80].

7. Conclusion

For many people, aging is associated with little cognitive decline, for some memory declines significantly with age and for others aging is associated with severe cognitive deficits. Moreover, not all cognitive functions are affected equally by age. Several health factors and behaviours may be protective in maintaining cognitive function, nutrition being one of them. Our own data have shown that even the single administration of polyphenols may significantly increase the level of the overall activation of the brain cortex and performance in tests of cognitive functions; the effect is time limited. Literary data support the opinion that the chronic consumption of polyphenols rich diet can promote the "healthy" aging, that is, to delay the cognitive decline and to prolong the healthy life span and protect the good execution in daily activities of seniors. We have stressed the importance of further controlled clinical trials with very carefully defined cognitive tasks to be administered as also the use of the registration of appropriate sensorimotor function

as a valid indicator of the effect of polyphenol administration and of the level of neurocardiovascular interaction of the aged person.

Conflict of Interests

The authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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References

[1] M. P. Lawton and E. M. Brody, "Assessment of older people: self-maintaining and instrumental activities of daily living.," *Gerontologist*, vol. 9, no. 3, pp. 179–186, 1969.

[2] G. G. Leblanc, J. F. Meschia, D. T. Stuss, and V. Hachinski, "Genetics of vascular cognitive impairment: the opportunity and the challenges," *Stroke*, vol. 37, no. 1, pp. 248–255, 2006.

[3] T. Kitagami, K. Yamada, H. Miura, R. Hashimoto, T. Nabeshima, and T. Ohta, "Mechanism of systemically injected interferon-alpha impeding monoamine biosynthesis in rats: role of nitric oxide as a signal crossing the blood-brain barrier," *Brain Research*, vol. 978, no. 1-2, pp. 104–114, 2003.

[4] Y. Honma, T. Araki, S. Gianino et al., "Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons," *Neuron*, vol. 35, no. 2, pp. 267–282, 2002.

[5] G. Garthwaite, K. Bartus, D. Malcolm et al., "Signaling from blood vessels to CNS axons through nitric oxide," *The Journal of Neuroscience*, vol. 26, no. 29, pp. 7730–7740, 2006.

[6] J. LeDoux, *Synaptic Self*, Penguin Books, London, UK, 2002.

[7] D. R. Harder, N. J. Alkayed, A. R. Lange, D. Gebremedhin, and R. J. Roman, "Functional hyperemia in the brain: hypothesis for astrocyte-derived vasodilator metabolites," *Stroke*, vol. 29, no. 1, pp. 229–234, 1998.

[8] J. A. Williams, S. R. Vincent, and P. B. Reiner, "Nitric oxide production in rat thalamus changes with behavioural state, local depolarization, and brain stem stimulation," *The Journal of Neuroscience*, vol. 17, no. 1, pp. 420–427, 1997.

[9] R. N. Kalaria, "Cerebrovascular disease and mechanisms of cognitive impairment. Evidence from clinicopathological studies in humans," *Stroke*, vol. 43, no. 9, pp. 2526–2534, 2012.

[10] N. Sattar and G. Ferns, "Endothelial dysfunction," in *Cardiovascular Disease: Diet, Nutrition and Emerging Risk Factors*, S. Stanner, Ed., pp. 63–77, Blackwell, Oxford, UK, 2005.

[11] D. Vauzour, A. Rodriguez-Mateos, G. Corona, M. J. Oruna-Concha, and J. P. E. Spencer, "Polyphenols and human health: prevention of disease and mechanisms of action," *Nutrients*, vol. 2, no. 11, pp. 1106–1131, 2010.

[12] C. Valls-Pedret, R. M. Lamuela-Raventós, A. Medina-Remón et al., "Polyphenol-rich foods in the mediterranean diet are associated with better cognitive function in elderly subjects at high cardiovascular risk," *Journal of Alzheimer's Disease*, vol. 29, no. 4, pp. 773–782, 2012.

[13] Clinical Trials gov, Identifier NCT020636446, 2014-2015.

[14] S. Duschek, E. Matthias, and R. Schandry, "Essential hypotension is accompanied by deficits in attention and working memory," *Behavioral Medicine*, vol. 30, no. 4, pp. 149–158, 2005.

[15] J. C. De La Torre, "Cardiovascular risk factors promote brain hypoperfusion leading to cognitive decline and dementia," *Cardiovascular Psychiatry and Neurology*, vol. 2012, Article ID 367516, 15 pages, 2012.

[16] S. R. Waldstein, S. B. Manuck, C. M. Ryan, and M. F. Muldoon, "Neuropsychological correlates of hypertension: review and methodologic considerations," *Psychological Bulletin*, vol. 110, no. 3, pp. 451–468, 1991.

[17] S. R. Waldstein, C. M. Ryan, S. B. Manuck, D. K. Parkinson, and E. J. Bromet, "Learning and memory function in men with untreated blood pressure elevation," *Journal of Consulting and Clinical Psychology*, vol. 59, no. 4, pp. 513–517, 1991.

[18] F. Jagla, "Over-mineralization: an attempt for the alternative and unifying theory of aging?" in *Lifestyle and Risk Factors of Cardiovascular Diseases: Proceedings of ESH Satellite Symposium, June 14–16, 2010, Bratislava, Slovak Republic*, pp. 9–10, Institute of Normal and Pathological Physiology, 2010.

[19] L. M. King, "Nutrition and cognitive functioning: multifaceted analysis of physiological and psychological components," *Pure Insight*, vol. 1, pp. 26–30, 2012.

[20] K. van Dyk and M. Sano, "The impact of nutrition on cognition in the elderly," *Neurochemical Research*, vol. 32, no. 4-5, pp. 893–904, 2007.

[21] F. Saura-Calixto, J. Serrano, and I. Goñi, "Intake and bioaccessibility of total polyphenols in a whole diet," *Food Chemistry*, vol. 101, no. 2, pp. 492–501, 2007.

[22] S. Doré, "Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity," *NeuroSignals*, vol. 14, no. 1-2, pp. 61–70, 2005.

[23] P. C. H. Hollman, "The 4th International Conference on Polyphenols and Health," *Nutrition Bulletin*, vol. 35, no. 2, pp. 183–185, 2010.

[24] E. Kesse-Guyot, L. Fezeu, V. A. Andreeva et al., "Total and specific polyphenol intakes in midlife are associated with cognitive function measured 13 years later," *The Journal of Nutrition*, vol. 142, no. 1, pp. 76–83, 2012.

[25] M. V. Chao, "Neurotrophins and their receptors: a convergence point for many signalling pathways," *Nature Reviews Neuroscience*, vol. 4, no. 4, pp. 299–309, 2003.

[26] J. Garthwaite, "Concepts of neural nitric oxide-mediated transmission," *European Journal of Neuroscience*, vol. 27, no. 11, pp. 2783–2802, 2008.

[27] A. Cheng, S. Wang, J. Cai, M. S. Rao, and M. P. Mattson, "Nitric oxide acts in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain," *Developmental Biology*, vol. 258, no. 2, pp. 319–333, 2003.

[28] L. Letenneur, C. Proust-Lima, A. Le Gouge, J. F. Dartigues, and P. Barberger-Gateau, "Flavonoid intake and cognitive decline over a 10-year period," *The American Journal of Epidemiology*, vol. 165, no. 12, pp. 1364–1371, 2007.

[29] E. Bakan, S. Taysi, M. F. Polat et al., "Nitric oxide levels and lipid peroxidation in plasma of patients with gastric cancer," *Japanese Journal of Clinical Oncology*, vol. 32, no. 5, pp. 162–166, 2002.

[30] K. M. Boje and P. K. Arora, "Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death," *Brain Research*, vol. 587, no. 2, pp. 250–256, 1992.

[31] J. C. De La Torre and G. Aliev, "Inhibition of vascular nitric oxide after rat chronic brain hypoperfusion: spatial memory and immunocytochemical changes," *Journal of Cerebral Blood Flow and Metabolism*, vol. 25, no. 6, pp. 663–672, 2005.

[32] Z.-Y. Cai, Y. Yan, S.-Q. Sun et al., "Minocycline attenuates cognitive impairment and restrains oxidative stress in the hippocampus of rats with chronic cerebral hypoperfusion," *Neuroscience Bulletin*, vol. 24, no. 5, pp. 305–313, 2008.

[33] R. L. Galli, B. Shukitt-Hale, K. A. Youdim, and J. A. Joseph, "Fruit polyphenolics and brain aging: nutritional interventions targeting age-related neuronal and behavioral deficits," *Annals of the New York Academy of Sciences*, vol. 959, pp. 128–132, 2002.

[34] J. Cho, S. K. Jong, H. L. Pham, J. Jing, Y. Back, and K.-S. Chung, "Antioxidant and memory enhancing effects of purple sweet potato anthocyanin and cordyceps mushroom extract," *Archives of Pharmacal Research*, vol. 26, no. 10, pp. 821–825, 2003.

[35] C. M. Williams, M. A. El Mohsen, D. Vauzour et al., "Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels," *Free Radical Biology and Medicine*, vol. 45, no. 3, pp. 295–305, 2008.

[36] C. Andres-Lacueva, B. Shukitt-Hale, R. L. Galli, O. Jauregui, R. M. Lamuela-Raventos, and J. A. Joseph, "Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory," *Nutritional Neuroscience*, vol. 8, no. 2, pp. 111–120, 2005.

[37] A. Dhir and S. K. Kulkarni, "Nitric oxide and major depression," *Nitric Oxide*, vol. 24, no. 3, pp. 125–131, 2011.

[38] C. N. Karson, W. S. Griffin, R. E. Mrak et al., "Nitric oxide synthase (NOS) in schizophrenia: increases in cerebellar vermis," *Molecular and Chemical Neuropathology*, vol. 27, no. 3, pp. 275–284, 1996.

[39] K. V. Chowdari, M. N. Bamne, and V. L. Nimgaonkar, "Genetic association studies of antioxidant pathway genes and schizophrenia," *Antioxidants and Redox Signaling*, vol. 15, no. 7, pp. 2037–2045, 2011.

[40] A. R. West, M. P. Galloway, and A. A. Grace, "Regulation of striatal dopamine neurotransmission by nitric oxide: effector pathways and signaling mechanisms," *Synapse*, vol. 44, no. 4, pp. 227–245, 2002.

[41] T. Shinkai, O. Ohmori, H. Hori, and J. Nakamura, "Allelic association of the neuronal nitric oxide synthase (NOS1) gene with schizophrenia," *Molecular Psychiatry*, vol. 7, no. 6, pp. 560–563, 2002.

[42] G. Donohoe, J. Walters, D. W. Morris et al., "Influence of NOS1 on verbal intelligence and working memory in both patients with schizophrenia and healthy control subjects," *Archives of General Psychiatry*, vol. 66, no. 10, pp. 1045–1054, 2009.

[43] A. Reif, C. P. Jacob, D. Rujescu et al., "Influence of functional variant of neuronal nitric oxide synthase on impulsive behaviors in humans," *Archives of General Psychiatry*, vol. 66, no. 1, pp. 41–50, 2009.

[44] M. Kovacsova, A. Barta, J. Parohova, S. Vrankova, and O. Pechanova, "Neuroprotective mechanisms of natural polyphenolic compounds," *Activitas Nervosa Superior Rediviva*, vol. 52, no. 3, pp. 181–186, 2010.

[45] J. Parohova, S. Vrankova, A. Barta, M. Kovacsova, D. Bartko, and O. Pechanova, "The cross-talk of nuclear factor kappa B and nitric oxide in the brain," *Activitas Nervosa Superior Rediviva*, vol. 51, pp. 123–126, 2009.

[46] O. Pechanova, "Contribution of central nervous system to hypertension: role of angiotensin II and nitric oxide," *Activitas Nervosa Superior Rediviva*, vol. 52, no. 4, pp. 223–227, 2010.

[47] O. Pecháňová, R. Rezzani, P. Babál, I. Bernátová, and R. Andriantsitohaina, "Beneficial effects of Provinols: cardiovascular system and kidney," *Physiological Research*, vol. 55, no. 1, pp. S17–S30, 2006.

[48] E. Weichselbaum and J. L. Buttriss, "Polyphenols in the diet," *Nutrition Bulletin*, vol. 35, no. 2, pp. 157–164, 2010.

[49] A. L. Macready, O. B. Kennedy, J. A. Ellis, C. M. Williams, J. P. E. Spencer, and L. T. Butler, "Flavonoids and cognitive function: a review of human randomized controlled trial studies and recommendations for future studies," *Genes and Nutrition*, vol. 4, no. 4, pp. 227–242, 2009.

[50] J. H. Kang, A. Ascherio, and F. Grodstein, "Fruit and vegetable consumption and cognitive decline in aging women," *Annals of Neurology*, vol. 57, no. 5, pp. 713–720, 2005.

[51] F. Jagla, O. Pecháňová, R. Andriantsitohaina, L. Pilšáková, and I. Riečanský, "The influence of nitric oxide on memory guided saccades," in *Neuro-Psycho-Physiological and Social Research and Interventions from an Interdisciplinary Point of View*, S. Colombo, M. G. Cassitto, G. F. Goldwurm, and S. Masaraki, Eds., pp. 39–41, Mimesis—la fabbrica dei libri, Milano, Italy, 2006.

[52] F. Jagla, B. Cimrová, S. Budac, M. Jergelová, S. Bendzala, and O. Pechanova, "Red wine polyphenols may influence human space memory," in *Proceedings of the World Congress: Oxidants and Antioxidants in Biology (OCC '10)*, Translational Redox Science. Book of Abstracts, p. 114, Fess Parker A Doubletree Resort, Santa Barbara, Calif, USA, 2010.

[53] C. Pierrot-Deseilligny, S. Rivaud, B. Gaymard, and Y. Agid, "Cortical control of memory-guided saccades in man," *Experimental Brain Research*, vol. 83, no. 3, pp. 607–617, 1991.

[54] F. Jagla, V. Zikmund, T. R. Mashonkina, and N. A. Yakimoff, "The accuracy of saccadic eye movements is associated with their horizontal and vertical direction," *Bratislavske Lekarske Listy*, vol. 93, no. 6, pp. 287–290, 1992.

[55] F. Jagla, P. Kukumberg, and M. Jergelová, "Central and peripheral oculomotor manifestations in panic disorder," in *Proceeding of the International CIANS Conference in Neurophysiology and Behavioural Intervention in Psychosomatics, Stress Disorders and Health Promotion*, G. F. Goldwurm, F. Colombo, and S. Masaraki, Eds., pp. 171–176, CIANS, Milan, Italy, 2001.

[56] M. Jergelová and F. Jagla, "Central and peripheral correlates of eye movements in selected mood disorders," *Neuroendocrinology Letters*, vol. 31, no. 6, pp. 731–737, 2010.

[57] B. Cimrová, S. Budáč, U. Melicherová, M. Jergelová, and F. Jagla, "Electrophysiological evidence of the effect of natural polyphenols upon the human higher brain functions," *Neuroendocrinology Letters*, vol. 32, no. 4, pp. 464–468, 2011.

[58] T. Hayashi, H. Nomura, M. Osawa, J. Funami, A. Miyazaki, and A. Iguchi, "Nitric oxide metabolites are associated with survival in older patients," *Journal of the American Geriatrics Society*, vol. 55, no. 9, pp. 1398–1403, 2007.

[59] A. Cheng, S. Wang, J. Cai, M. S. Rao, and M. P. Mattson, "Nitric oxide acts in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain," *Developmental Biology*, vol. 258, no. 2, pp. 319–333, 2003.

[60] Z. Ungvari, C. Parrado-Fernandez, A. Csiszar, and R. de Cabo, "Mechanisms underlying caloric restriction and lifespan regulation: implications for vascular aging," *Circulation Research*, vol. 102, no. 5, pp. 519–528, 2008.

[61] A. Montesanto, P. Crocco, F. Tallaro et al., "Common polymorphisms in nitric oxide synthase (*NOS*) genes influence quality of aging and longevity in humans," *Biogerontology*, vol. 14, no. 2, pp. 177–186, 2013.

[62] C. Bossuet, F. Vaufrey, F. Condé et al., "Up-regulation of glutamate concentration in the putamen and in the prefrontal cortex of asymptomatic SIVmac251-infected macaques without major brain involvement," *Journal of Neurochemistry*, vol. 88, no. 4, pp. 928–938, 2004.

[63] Z. Liang, J. Valla, S. Sefidvash-Hockley, J. Rogers, and R. Li, "Effects of estrogen treatment on glutamate uptake in cultured human astrocytes derived from cortex of Alzheimer's disease patients," *Journal of Neurochemistry*, vol. 80, no. 5, pp. 807–814, 2002.

[64] M. Arundine and M. Tymianski, "Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury," *Cellular and Molecular Life Sciences*, vol. 61, no. 6, pp. 657–668, 2004.

[65] L. Rönnbäck and E. Hansson, "On the potential role of glutamate transport in metal fatigue," *Journal of Neuroinflammation*, vol. 1, article 22, 2004.

[66] S. Hu, W. S. Sheng, L. C. Ehrlich, P. K. Peterson, and C. C. Chao, "Cytokine effects on glutamate uptake by human astrocytes," *NeuroImmunoModulation*, vol. 7, no. 3, pp. 153–159, 2000.

[67] D. S. Reddy and S. K. Kulkarni, "Inhibition of neuronal nitric oxide synthase (n-cNOS) reverses the corticotrophin-induced behavioral effects in rats," *Molecular and Cellular Biochemistry*, vol. 183, no. 1-2, pp. 25–38, 1998.

[68] O. Pecháňová, L. Horniaková, and Z. Csizmadiová, "The effect of red wine and non- alcoholic red wine polyphenolic extract on the behavior of normotensive and spontaneous hypertensive rats: a comparative study," *Psychopharmacology & Biological Narcology*, vol. 78, pp. 2–3, 2004.

[69] O. Pecháňová, L. Horniaková, W. J. Zenebe, and R. Andriantsitohaina, "Effects of red wine polyphenolic compound on the motor activity of normotensive and spontaneous hypertensive rats," *Homeostasis in Health and Disease*, vol. 42, no. 5, pp. 222–224, 2003.

[70] O. Pechánová, L. Jendeková, S. Kojšová, and F. Jagla, "Possible role of nitric oxide in the locomotor activity of hypertensive rats," *Behavioural Brain Research*, vol. 174, no. 1, pp. 160–166, 2006.

[71] G. Jennings, L. Nelson, P. Nestel et al., "The effects of changes in physical activity on major cardiovascular risk factors, hemodynamics, sympathetic function, and glucose utilization in man: a controlled study of four levels of activity," *Circulation*, vol. 73, no. 1, pp. 30–40, 1986.

[72] B. A. Kingwell and G. L. Jennings, "Effects of walking and other exercise programs upon blood pressure in normal subjects," *Medical Journal of Australia*, vol. 158, no. 4, pp. 234–238, 1993.

[73] S. M. Bode-Böger, R. H. Böger, E. P. Schröder, and J. C. Frölich, "Exercise increases systemic nitric oxide production in men," *Journal of Cardiovascular Risk*, vol. 1, no. 2, pp. 173–178, 1994.

[74] D. J. Green, A. Maiorana, G. O'Driscoll, and R. Taylor, "Effect of exercise training on endothelium-derived nitric oxide function in humans," *Journal of Physiology*, vol. 561, no. 1, pp. 1–25, 2004.

[75] U. Laufs, A. Urhausen, N. Werner et al., "Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects," *European Journal of Preventive Cardiology*, vol. 12, no. 4, pp. 407–414, 2005.

[76] A. F. Kramer, K. I. Erickson, and S. J. Colcombe, "Exercise, cognition, and the aging brain," *Journal of Applied Physiology*, vol. 101, no. 4, pp. 1237–1242, 2006.

[77] A. J. Susswein, A. Katzoff, N. Miller, and I. Hurwitz, "Nitric oxide and memory," *Neuroscientist*, vol. 10, no. 2, pp. 153–162, 2004.

[78] J. Jung, C. Na, and Y. Huh, "Alterations in nitric oxide synthase in the aged CNS," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 718976, 7 pages, 2012.

[79] S. B. A. Cau, F. S. Carneiro, and R. C. Tostes, "Differential modulation of nitric oxide synthases in aging: therapeutic opportunities," *Frontiers in Physiology*, vol. 3, article 208, Article ID Article 218, 2012.

[80] F. Jagla and O. Pechanova, "Cognitive decline as an indicator of neuro-cardiovascular interactions: may polyphenols play a role?" in *Proceedings of the Joint Meeting of the 8th International Symposium Nitric Oxide: From Basic Regulations to Lifestyle-Related Diseases and the 2nd Genetic and Environmental Factors in Hypertension*, I. Bernátová, Ed., pp. 143–149, Institute of Normal and Pathological Physiology SAS, Vrsar, Croatia, September 2014.

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Review Article

Role of Polyphenols and Other Phytochemicals on Molecular Signaling

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Optimized nutrition through supplementation of diet with plant derived phytochemicals has attracted significant attention to prevent the onset of many chronic diseases including cardiovascular impairments, cancer, and metabolic disorder. These phytonutrients alone or in combination with others are believed to impart beneficial effects and play pivotal role in metabolic abnormalities such as dyslipidemia, insulin resistance, hypertension, glucose intolerance, systemic inflammation, and oxidative stress. Epidemiological and preclinical studies demonstrated that fruits, vegetables, and beverages rich in carotenoids, isoflavones, phytoestrogens, and phytosterols delay the onset of atherosclerosis or act as a chemoprotective agent by interacting with the underlying pathomechanisms. Phytochemicals exert their beneficial effects either by reducing the circulating levels of cholesterol or by inhibiting lipid oxidation, while others exhibit anti-inflammatory and antiplatelet activities. Additionally, they reduce neointimal thickening by inhibiting proliferation of smooth muscle cells and also improve endothelium dependent vasorelaxation by modulating bioavailability of nitric-oxide and voltage-gated ion channels. However, detailed and profound knowledge on specific molecular targets of each phytochemical is very important to ensure safe use of these active compounds as a therapeutic agent. Thus, this paper reviews the active antioxidative, antiproliferative, anti-inflammatory, or antiangiogenesis role of various phytochemicals for prevention of chronic diseases.

1. Introduction

Overwhelming evidence from epidemiological, *in vivo, in vitro*, and clinical trial data indicates that the plant-based diet can reduce the risk of chronic diseases (e.g., cardiovascular disease, hypertension, diabetes, and cancer) due to presence of biologically active plant compounds or phytochemicals. Steinmetz and Potter identified more than a dozen classes of phytochemicals from plant based diets (fruits, vegetable, nuts, etc.), such as carotenoids, phenolic compounds (flavonoids, isoflavonoids, and lignin), phenolic acid, phytosterols and phytostanols, tocotrienols, organosulfur compounds, and nondigestible carbohydrates (dietary fiber, Table 1) [1]. According to many researchers regular consumption of diet rich in vegetables, fruits, whole grains, herbs, nuts, seeds, which contain plenty of phenolic compounds, terpenoids, phytosterols, organosulfur exert their beneficial effect in disease prevention, by regulating several cellular molecular pathways like regulation of inflammation, redox potentials, metabolic disorder, apoptosis, and so forth [2]. Polyphenols (flavonoids, lignans, stilbenes, etc., see Table 1) are the most diverse group of phytochemicals distributed in vegetables, fruits, olive oil, and wine and exhibit wide range of protective roles such as hypolipidemic, antioxidative, antiproliferative, and anti-inflammatory effects to reduce the onset of disease progression [3–5]. Similarly terpenoids (carotene, lycopene, etc.), another important chemically active natural hydrocarbon, contain oxygen rich moieties like alcohol, aldehydes, and ketones. Epidemiological studies have shown that the oral supplementation of tomato extract (rich in carotenoid, lycopene) significantly control the risk of hyperlipidemia, CVD, metabolic syndrome by regulating several physiological phenonmenon like reduction of blood pressure of low density lipoprotein oxidation, hypertension [6]. Organosulfur, phytosterols are the other widely distributed active phytochemicals which have been found to reduce the generation

Table 1: Classification of phytochemicals (adapted from Gonzalez-Castejon and Rodriguez-Casado [36, 92, 107]).

Dietary phytochemicals	Functional derivatives		Sources	Therapeutics effect
Polyphenols	Phenolic acid	Caffeic acid Ferulic acid Chlorogenic acid	Coffee beans, soybeans	Antioxidative effect: (1) Curcumin, resveratrol, tea polyphenol, and isothiocyanates ↑ the phase II detoxifying and antioxidant enzymes like HO-1, GST by modulation transcription factor Nrf2 [20, 24]. (2) Curcumin protects against neuronal degeneration by ↓ ROS neutralizing NO induced free radicals. Curcumin ↑ intracellular GSH pool by triggering specific transcription factors (e.g., TPA, EpRE) [44, 45]. (3) Isoflavones target eNOS and redox sensitive gene expression and regulate vascular reactivity [37, 40]. (4) Aged rats fed soy protein diet rich in genistein and daidzein, which interact with estrogen receptors, ↑ eNOS and antioxidant enzymes expression [39]. (5) Resveratrol, anthocyanin, blocking activation of NF-kB, MAPK, and PGE2, protect neuronal disorder [51, 52]. Anti-inflammatory effect: (1) Procyanidins ↓ expression of iNOS and COX-2 inhibit inflammatory phenomenon. Procyanidins also significantly ↓ expression of TNF-α and IL-1β by blocking NF-kB activity via ↓ of MAPK and P38 pathways [52]. (2) Tea and/or red wine polyphenol inhibit expression of COX-2, LOX and exert anti-inflammatory effect. Similarly kaempferol, a flavonoid rich in fruits, vegetables (broccoli) ↓ reduce inflammation by inhibiting the generation of PGE2 [64–66]. (3) Oral supplementation of curcumin (45 mg/kg) in male mice ↓ stemic inflammation by blocking the release of TNF-α and CRP [68]. (4) Cinnamon bark extract (content TAPP) protects from AHR or asthma by reducing inflammatory mediators like IL-4 and IL-13 [69]. (5) Cinnamon bark extract protects against systemic inflammation in rheumatoid arthritis by reducing CRP and stimulating autoimmune system by ↓ expression of many inflammatory mediators (prostaglandin E2, NO) in RA rats [69]. Metabolism regulation: (1) Anthocyanin and myrtillin of blueberry extract show hypoglycemic effect in humans. Supplementation of 3% blueberry enriched diet for 8 weeks and/or 0.5% GSE-supplemented diet significantly reduced the arterial blood pressure in SHRs via endothelium mediated stimulation of NO metabolism and activation of COX-induced product [74, 75]. (2) TAPP, the main active component of Cinnamon extract, significantly ↓ the blood HbA1c level and improves the insulin signaling in diabetic animal study [73]. (3) Phenolic compounds, namely, chlorogenic acid and ferulic acid, and a plant alkaloid, berberine, are considered as potent antidiabetic agent, as these phytochemicals enhance the uptake of 2DG in time- and dose-dependent manner and significantly upregulate the expression of GLUT4 and PPAR-γ and PKI3K expression [90, 91]. Antiproliferative effect: (1) Tea polyphenols exert antiproliferative effects by interacting with MMP system. Tea polyphenol ↓ SMC proliferation by blocking cyclin D1 and Cyclin E and/by inhibiting the cell growth markers PCNA [94, 95]. (2) Resveratrol ↑ apoptosis by upregulation of tumor suppressor genes p21Cip1/WAF1, p53, the proapoptotic protein Bax expression, ↑ caspase apoptotic signals, and ↓ antiapoptotic proteins Bcl-2, Bcl-XL, and survivin expression [103, 104]. (3) Polyphenols (resveratrol, genistein, curcumin, C-phycocyanin, and quercetin) inhibit proliferation and motility of cells by suppression of cell adhesion molecule CD44 expression [102]. (4) Ellagic acid, isovitexin ↓ SMC proliferation might be by ↓ ROS generation and ↓ of ERK1/2 and iNOS expression [101]. Antiangiogenic effect: (1) Ellagic acid significantly ↓ angiogenesis in hamster buccal punch by ↓ PI3K/Akt and MAPK and VEGF signaling pathways, suppressing HDAC6 and hypoxia-inducible HIF-1α responses [105]. (2) *Tephrocia purpurea* rich in flavonoids was found to exert wound healing effect by significant ↑ of angiogenesis or blood vessels formation, fibroblast cells, and generation of collagen fibres [106]. (3) Astaxanthin (non-provitamin A carotenoid) predominantly distributed in microalgae, fungi, plants, and sea foods, inhibits tumor progression by regulating STAT3/JAK-2 [108].
	Curcuminoids	Curcumin	Turmeric	
	Flavonoids	Flavonols, Flavanones Anthocyanins Flavanonoles	Tea, fruits (citrus, apple, grapes) Vegetables	
	Chalcones	Phlorizin	Tomatoes, tea	
	Stilbenes	Resveratol	Grapes, red wine	
	Lignans	Sesamin Pinoresinol	Whole grains, legumes	
	Isoflavonoids	Genistein Daidzein	Soya	

Table 1: Continued.

Dietary phytochemicals	Functional derivatives		Sources	Therapeutics effect
Terpenoids	Carotenoids	Lycopene Lutein Carotene	Tomatoes, spinach, carrot	Antioxidative effect: consumption of lycopene (rich in tomato, spinach, etc.) significantly ↑ antioxidant enzymes SOD, GSH-Px, GR, and GSH and ↓ levels of MDA in hypertensive patients. Lycopene ↓ MDA levels and ↑ GSH levels in postmenopausal women and protects from cardiovascular disorder. Anti-inflammatory effect: lycopene ↓ the release of proinflammatory cytokine TNF-α by ↓ NF-kB activation and induces anti-inflammatory effect. Moreover significant inhibition of lipid peroxidation by the combination of ascorbic acid and α-tocopherol is the complementary to the anti-inflammatory effect of lycopene.
	Sesquiterpenes	Acyclic compound (Farnesol, Nerolidol) Cyclic compound (Abscisic acid)	Fruits, vegetables	
Organosulfur		Allicin Allyl sulfide	Garlic, onion	Metabolism regulation: (1) *In vivo* antidiabetic effect of garlic is well documented in diabetic rats and mice. Allicin (main active component) induces pancreatic secretion of insulin or its release from bound insulin [80, 81]. (2) Garlic and garlic protein diet significantly ↓ serum cholesterol, triglyceride, and LDL cholesterol by allicin mediated inhibition of cholesterol synthesis [83–85]. (3) Gamma-glutamylcysteines component of garlic ↓ blood pressure by inhibiting angiotensin-converting enzyme [14, 80, 85].
Phytosterols	Sterol, Stanols, Campestanols	Diosgenin	Fenugreek, wild yam	Metabolic regulation: phytosterol, inhibiting cholesterol absorption [83, 85, 89]

of inflammatory signaling molecules and also modulate antioxidative effect by inhibition of NF-κB pathways [6, 7]. These phytochemicals (organosulfur, phytosterols) also show protective effect against atherosclerosis by regulating of serum total and LDL-cholesterol levels. Phytochemicals such as resveratrol, epigallocatechin gallate, gingerol, phytosterol, and myricetin directly influence various molecular signal transduction pathways like inflammation cascade, cell proliferation/migration, oxidative stress, and metabolic disorders, which are involved in the development of several noncommunicable diseases. Although beneficial effects of plant based diets or phytochemicals in reducing the risk of chronic diseases have been shown through various epidemiological and *in vitro*/*in vivo* studies, much more mechanistic and clinical evidence is required to define a particular phytochemical as an inhibitor of specific cellular pathways or identify the plant derived active compound with specific therapeutics properties. Therefore, this review is focused on the cellular targets of naturally occurring active compounds from various sources in order to explore the protective/therapeutics role as an antioxidative, anti-inflammatory regulation of metabolic disorder as well as antiproliferative properties by targeting specific molecular signal transduction, which might play a crucial role in the pathogenesis of chronic disease.

2. Bioavailability of Phytochemicals

Bioavailability of individual compounds of interest at the target site is one of the important challenges/parameters to determine the therapeutic efficiency of the target drug [8]. Although accurate bioavailability of particular compound is not possible to predict according to Lipinski et al. a compound might have better bioavailability when it contains maximum 5 hydrogen-bond donors, and 10 hydrogen-bond acceptors, in association with having a molecular mass not more than 500 daltons; a partition coefficient log P value should not be more than 5 and contains less than 10 rotatable bonds [9]. Most of the phytochemicals, including polyphenols such as curcumin and green tea polyphenols, do not satisfy all these chemical specifications and exhibit low bioavailability [10]. Although compounds such as genistein and biochanin A have all those good absorptive chemical properties, high rate excretion in the gut by efflux mechanism limits their bioavailability [10]. Therefore, other factors like solubility of the compounds, stability due to gastric and colonic pH, metabolism by gut microflora, absorption across the intestinal wall, active efflux mechanism, and first-pass metabolic effects may also play crucial role in limiting the bioavailability of phytochemicals [11]. As an example, limited bioavailability of epigallocatechin gallate is mainly due to poor absorption and rapid first-pass metabolism. Similarly low stability, increased oxidation, and high hepatic uptake are also responsible for the limited the bioavailability of some widely distributed polyphenols such as flavonoids (flavonols, anthocyanines) resveratol 8 (grapes) [12].

Moreover it has been also reported that rapid conjugation of a phytochemicals, especially by glucuronidation in the intestine and liver, in association with reaction of cytochrome P450 (C-P450) enzymes, which are recognized as important clearance mechanisms, is primarily responsible for their poor bioavailability [12]. Many researchers have shown that co-delivery of lead target molecules (phytochemicals) with an agent that can modulate the activity of glucuronidation or inhibit C-P450 mediated clearance mechanism is possible to increase the bioavailability of active compound of interest at the target site [12, 13]. Piperine, a component of black pepper, inhibits glucuronidation and is able to enhance the bioavailability of several bioactive compounds by altering the C-p450 mediated enzymatic biotransformation [8, 14]. Rapid mobilization in the intestine is largely responsible for low bioavailability of curcumin at target site; however consumption of curcumin along with piperin, a known inhibitor of intestinal and hepatic β-glucuronidation of curcumin, may induce the bioavailability of curcumin about 20-fold [8, 14]. Many phytochemicals were identified as promising therapeutics agent in the preliminary *in vitro* studies. However, when those compounds were tested into *in vivo* studies, many of them failed to translate the preclinical/clinical findings, because compounds either were unstable in the gut or exhibited poor bioavailability [8, 14]. Therefore, detailed preclinical and clinical studies on bioavailability of phytochemicals/active compounds are urgently needed to understand their therapeutic limitation as well as to find out the better compound delivery system to achieve the best efficiency level of the drug at the target organ.

3. Molecular Mechanism of Photochemical in Redox Modulation

Cells use enzymes and oxygen to perform its normal physiological functions are continuously exposed to free radicals. Free radicals are oxygen containing highly reactive molecules with one or more unpaired electrons. This highly reactive oxygen species (ROS) includes numerous partially reduced oxygen metabolites such as hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and superoxide anions ($O\bullet^-_2$) radicals. ROS are generated intracellularly as a byproduct of normal metabolism and as second messengers in various signal transduction pathways [15]. ROS are also generated exogenously due to direct uptake by cells from the extracellular sources or produced during exposure of cells to some environmental triggers. It has been well established that ROS are heterogeneous and can exert their beneficial or detrimental effect depending on the concentrations at which they are present in the cellular level. At low levels, ROS can be driven by NADPH/NADPH oxidase and is required to maintain homeostatic signaling events as well as inducing cell proliferation and survival through the posttranslational modification of kinases and phosphatase [16]. However, overproduction of ROS or exposure of cells to ROS for extended period may cause irreversible damage to DNA, protein, and lipids. Therefore, numerous innate defense systems have developed to detoxify or prevent the detrimental effect of ROS. These include nonenzymatic molecules (e.g., glutathione, vitamins A, C, and E, and flavonoids) as well as induction of phase II detoxifying/antioxidative enzymes (e.g., superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px)

and hemeoxygenase-1 (HO-1), which are involved to eliminate or inactivate the ROS from cellular level) [17]. An imbalance between ROS generation and defense mechanism or inadequate presence of antioxidant molecules results in the state known as oxidative stress. Growing evidence indicates that chronic and acute excess generation of ROS under pathophysiologic conditions is pivotal in the development of cardiovascular diseases (CVD) or premature atherosclerosis progression, cancer, pulmonary fibrosis, and neurodegenerative disorder [18–20]. Oxidative modification of low density lipoprotein (Ox-LDL) regulates many signaling pathways, which causes inhibition of endothelial nitric oxide synthase (eNOS) and promotes vasoconstriction, expression of adhesion molecules, and progression of platelet aggregation. Additionally proliferation of smooth muscle cell (SMC) by Ox-LDL stimulates hypertension due to reduction of blood vessel lumen. Similarly excessive generation of ROS, particularly H_2O_2, has been detected in cancer cells. Though the exact sources of H_2O_2 generation in cancer cells are not known, the higher amount of H_2O_2 increases level of hydroxyl radical (OH), which in turn mediates oxidative damage of DNA and ultimately results in genomic instability. Transcriptional activation of some genes like cyclooxygenase-2 (COX-2), matrix metalloproteinases (MMPs), and cyclin B1 has been reported to be due to oxidative stress or induced generation of H_2O_2 [21–23]. Overexpression of these genes and H_2O_2 generations are also observed in breast cancer tissue, colon cancer, and cervical cancer tissue. Compelling evidence from epidemiological, clinical, and experimental studies demonstrated that phenolic and other naturally occurring compounds present in cereals, legumes, nuts, olive oil, vegetables, fruits, tea, and red wine exert antioxidative/anti-inflammatory effect through their free radical scavenging properties, as well as enhancement of antioxidative enzymes [18, 20].

Phytochemicals like curcumin, resveratrol, terpenoids, epigallocatechin-3-gallate (EGCG), and isothiocyanates share common properties and play an important role to activate the phase II detoxifying and antioxidant enzymes like HO-1, GSH-Px, and glutathione-S-transferase (GST) by targeting the common transcription factor Nrf2 [nuclear factor (erythroid derived 2) related factor] [20, 24]. Epidemiological studies reported regular consumption of lycopene (rich in tomato, spinach, etc.) significantly induced the antioxidant enzymes like SOD, GSH-Px, and glutathione reductase (GR) and reduced form of glutathione (GSH); moreover this bioactive compound also reduced the levels of lipid peroxide malondialdehyde (MDA), LDL oxidation, and ROS mediated DNA damage. Additionally, lycopene was found to induce cardioprotective effect by reducing MDA levels and increasing GSH levels in postmenopausal women [25, 26]. The redox-sensitive transcription factor Nrf2 plays an important role in regulating induction of phase II detoxifying or antioxidant enzymes (HO-1, SOD, etc.) which result in cellular defense against oxidative stress and exert cytoprotective mechanism [27]. Many researchers have elucidated the molecular mechanisms responsible for activation of Nrf2. A cytoskeleton binding protein called Kelch-like erythroid CNC homologue- (ECH-) associated protein 1 (Keap1) binds to Nrf2 regulating its translocation to the nucleus or its activation [28]. Following nuclear translocation, Nrf2 binds not only to the specific consensus *cis*-element called antioxidative responsive element (ARE) or electrophile response element (EpRE) present in the promoter region of genes encoding many antioxidant enzymes but also to other *trans*-acting factors such as small Maf-F/G/K as well as the coactivators of ARE including cAMP response element binding protein (CREB-binding protein or CBP), p300 that can coordinately regulate the ARE-driven antioxidant gene transcription [28–30]. Tea extract contain large amount of polyphenols (theaflavins, catechins, epicatechins, epicatechins-3-gallate, EGCG), which are characterized by 2 or more aromatic rings with at least one hydroxyl group linked by a carbon bridge. Hydroxyl group of tea polyphenols (TPs) are actually the main source for electron donor and efficiently scavenging singlet oxygen (e.g., NO, peroxynitrite). *In vitro* study by Leung et al. found significant reduction of oxidized LDL following application of theaflavins and catechins [31]. Similar response was also observed by Lee et al. showing decrease in oxidized LDL in plasma of individuals after 4 weeks of green tea consumption [32, 33]. Many researchers have reported that antioxidative properties of tea polyphenols (theaflavins and catechins) might be due to inhibition of ROS-generating enzymes (e.g., iNOS), which contribute for the production of NO mediated free radicals. The molecular mechanism of antioxidant enzyme induction by EGCG or other TPs and polyphones still remain unexplored in large extent [28]. A widely accepted model for induction of ARE-mediated antioxidant gene expression involves phosphorylation of serine/threonine residues of Nrf2 by protein kinases, leading to enhanced nuclear accumulation of Nrf2 and subsequent ARE binding. Furthermore, Wu et al. observed induction of HO-1 enzyme in endothelial cells due to activation of Akt and Nrf2 by EGCG, which impart protective measure of endothelial cell against H_2O_2 mediated oxidative stress [34]. Supplementation of curcumin as well as EGCG has been reported to enhance Nrf2 nuclear translocation and upregulation of HO-1 by Akt, EKR1/2, and p38 MAPK signaling in human breast epithelial cells as wells as B lymphoblasts [27, 28, 35]. Other plausible mechanisms of EGCG-induced Nrf2 activation are oxidation or modification of cysteine thiols present in Keap1 by ROS and/or via active form of EGCG during its redox-cycling [28]. Although EGCH mediated ARE-mediated upregulation of antioxidative gene may also be plausible through activation of MAPKs, which finally activate Nrf2. However, in contrast many studies have reported that inhibition of MAPKs by phytochemicals finally induced Nrf2 activity [27, 28]. Procyanidin B2 was found to increase nuclear translocation of Nrf2 via regulation of ERKs and p38 signaling [36]. Similarly soy isoflavones and polyphenols rich in red wine, tea, and dark chocolate regulate vascular reactivity by targeting endothelial nitric oxide synthase (eNOS) and inducing nuclear accumulation of Nrf2. Induced endothelium-dependent NO generation in response to phytochemicals (polyphenol) may further activate cellular sensor(s) for oxidative stress and thereby enhance NO bioavailability [37]. NO can react further with superoxide anions ($O\bullet^-_2$) to form peroxynitrite, which further upregulates nuclear accumulation of Nrf2 and activates

ARE dependent transcription of phase II and antioxidant defense enzymes [37, 38]. Epidemiological studies have demonstrated that supplementation of soy isoflavones for longer period improves arterial compliance in men and postmenopausal women, induces plasma nitrite/nitrate levels, and decreases plasma endothelin-1 levels. However, in contrast, an isoflavone deficient diet fed from conception throughout adult life might result in decreased GSH concentrations and mRNA levels for eNOS. *In vivo* studies on aged male rats by Mahn et al. have shown that supplementation of soy protein diet rich in genistein and daidzein, found to interact with estrogen receptors, increases mRNA levels of eNOS and antioxidant enzymes [39]. Hence, vascular protection and antioxidative effect of soy isoflavone diets mostly related to an upregulation of eNOS expression and activity, NO bioavailability in association with accumulation of Nrf2, and ARE dependent activation of antioxidant defense enzymes [37, 40]. Impaired metabolism and overproduction of ROS also contribute for neuronal degeneration and onset of neuronal diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Therefore, neutralization of ROS and other types of free radicals by endogenous antioxidative enzymes (HO, GST) or regulation of oxidative stress by inducing supplementary intake of natural antioxidant is considered as a primary preventive therapeutic measure for the clinicians to protect from chronic neuronal disorder. Numerous pieces of evidence reported curcumin (rhizome of *Curcuma longa*), EGCG (tea), and resveratrol (grapes, berry) as potential natural occurring bioactive neuroprotective compounds because of their antioxidative properties [41, 42]. Curcumin has been shown to exert protective effect against neuronal degeneration by scavenging ROS and neutralizing NO induced free radicals. *In vivo* study on Alzheimer's disease (AD) transgenic mouse model by Lim et al. demonstrated that curcumin reduces neuronal oxidative stress by inducing expression of cytoprotective proteins or antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), heme oxygenase 1 (HO-1), and glutathione-S-transferase (GST) [43]. Additionally curcumin is also able to increase the intracellular glutathione (GSH) pool by affecting the nuclear content or by triggering specific transcription factors such as 12-tetradecanoate 13-acetate (TPA), electrophilic response element (EpRE) [44, 45]. Reversal of intracellular GSH pool in AD patient by curcumin makes it unique therapeutics for AD treatment as depletion of cellular GSH level plays a pivotal role in AD pathogenesis. However because of curcumin's low water solubility and poor bioavailability the major challenge to use curcumin as therapeutic agent for the treatment of AD is to cross the blood brain barrier (BBB) [42]. However EGCG, main phenolic component of green tea, has been reported to have slow rate of BBB penetration and about 5% bioavailability following oral consumption [46]. Series of rodent studies have shown the pleiotropic effect of EGCG, which exert the neuroprotective measure by modulation of antioxidative enzyme activity (SOD, GST) in association with suppression of ROS generation and protection of neuronal cells from glycation induced neurotoxicity.

Resveratrol is one of the most widely distributed phenolic compounds in fruits (apple, berry, and grapes), nuts, and so forth. Extensive study over resveratrol has shown its antioxidant properties and protective role in many chronic health issues like neural disorder (AD, PD), inflammation, CVD, and so forth. Central nervous system is one of the target organs for resveratrol as it is able to cross the BBB, although bioavailability of resveratrol is very low as it is metabolized quickly to glucuronide and sulfate conjugate. Similar to curcumin and EGCG, administration of resveratrol also mediates its neuroprotective effect via stimulation/upregulation of various antioxidant enzymatic activities such as HO, GST, and SOD. Additionally administration of resveratrol on transgenic mouse model of AD has shown protective role of this particular phytochemical against neuronal impairments mainly through inhibition of NF-κB modulated expression of several pathways like iNOS, prostaglandin E2(PGE2) [47–50]. Recent study by D'evoli et al. has shown the antioxidative and cytoprotective effects of red chicory leaf extract to improve intestinal complications are mainly due to its high content of both anthocyanins and phenolic compounds. Molecular targets of chemopreventive dietary phytochemicals are nuclear transcription factors NF-κB and hypoxia inducible transcription factor (HIF) [51]. This finding has been further supported recently by Bak et al., who demonstrated that wild grape seed procyanidins (WGP) effectively suppressed the generation of oxidative stress mediators like ROS and nitric oxide (NO) mainly by preventing the activation of NF-κB and p38 mitogen-activated protein kinases (MAPKs) pathway in LPS-RAW264.7 cells [52]. Bak et al. have also shown that antioxidative and chemopreventive effects of WGP are associated with induction of nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway in the human hepatoma HepG2 cell line [52].

Induced oxidative stress and oxidative modification of LDL by ROS are one of the key risk factors for atherosclerotic plaque formation, which restricts blood flow and results in high blood pressure. Numerous phenolic/flavonoid compounds are potent inhibitors of LDL oxidation and exert their cardioprotective role by inducing antiplatelet and anti-inflammatory effects at localized (microvascular) and/or systemic level. Additionally polyphenols may also increase HDL levels and improve endothelial function. The mechanism of antioxidant activity of phenolics/flavonoid compounds can be characterized by direct scavenging or quenching of oxygen free radicals, which are mostly attributed to the o-dihydroxyl group in the A and/or B ring (catechol group) of their diphenylpropane structure [53]. Catechol type flavonoids (e.g., quercetin, heliosin) therefore possess powerful antioxidant activity.

Human study by Aviram et al. reported small but significant (6%) decrease of lipid peroxidation in plasma following 2 wk consumption of pomegranate juice (PJ, 50 mL/day) by 13 healthy, nonsmoking men aged 20–35 years compared with plasma obtained before study entry [54]. Additionally, a significant (9%) increase in plasma total antioxidant status was also observed after 2 wk of PJ consumption. Supplementation with 50 mL PJ/d for additional one week resulted in a further 21% decrease in plasma lipid peroxidation, whereas

an additional increase in PJ supplementation to 80 mL PJ/d for another week did not inhibit plasma susceptibility to lipid peroxidation further. The inhibitory effect of PJ consumption on plasma lipid peroxidation was maintained for 2 wk after PJ supplementation ended. This study therefore showed that daily consumption of PJ may reduce the progression of atherosclerotic lesions by reducing the plasma lipid peroxidation and by virtue of its ability to attenuate platelet activations [54].

4. Role of Phytochemical in Inflammatory Process

Inflammation is defined as a series of immunological, biochemical, and/or cellular alterations in response to exogenous or endogenous stimuli. Both chronic and acute phase inflammatory processes act locally and systematically to activate the cells associated with inflammatory process (macrophages, endothelial cells, and fibroblast) to induce the inflammatory mediators like ROS, NO, prostaglandin E2, and proinflammatory mediators such as cytokines, TNF-alpha, and COX-2. Multiple studies have shown that overexpression of proinflammatory genes including TNF-alpha and interleukin is associated with activation of transcription factor NF-κB [55]. Activated transcription factor translocates to the nucleus and either regulates the release of inflammatory mediators or induces the upregulation of inflammatory gene expression by binding with the DNA. Furthermore, phosphorylation of MAPK plays important role in chronic inflammation by regulating production of NO and proinflammatory genes from macrophages as well as in the activation of NF-κB [52, 56]. Hence, suppression or inhibition of those inflammatory/proinflammatory mediators is one of the major targets for treatment of many chronic diseases (cancer, CVD, and diabetes) using anti-inflammatory compounds.

Wild grapes procyanidins (WGP) induced anti-inflammatory effect and the molecular mechanism of procyanidins was studied in detail by Bak et al. using lipopolysaccharide (LPS) stimulated RAW 264.7 [52]. In this study Bak et al. have shown that incubation of RAW 264.7 cells by WGP significantly blocked inflammatory phenomenon by reducing protein expression of iNOS and COX-2, two important inducible enzymes which play critical role in NO, PGE2 generation [52]. Moreover, authors have shown that WGP treatment significantly reduced LPS stimulated expression of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) in RAW 264.7 cells. These effects of WGP might be due to suppression of nuclear factor-κB (NF-κB) activity via downregulation of MAPK and p38 pathways. Hence, suppression of NF-κB by WGP would be an important protective measure to reduce the chances of inflammation mediated chronic health issues, as transcription factor NF-κB activity plays a central role in inflammatory process [52]. Similarly brassica derived phytochemicals like sulforaphane (SFN), phenethyl-isothiocyanate (PEITC), and indole-3-carbinol (I3C) are also found to exert anti-inflammatory effect by downregulation of LPS induced expression of COX-2, iNOS in mouse macrophages mainly by inhibiting NF-κB pathways [57–59]. *In vivo* study on C57BL/6 showed that pretreatment of mice with SFN resulted in significant reduction of dextran-sodium-sulfate induce colitis compared to PBS treated control mice [60]. Similar to brassica phytochemicals, tea polyphenols, primarily EGCG, epicatechin-3-gallate (ECG), and epigallocatechin (EGC) work as chemopreventive compounds by inducing anti-inflammatory effect in cancer cells. Both EGCG and theaflavins reduced LPS-induced TNF-alpha generation and also iNOS expression by preventing activation of NF-κB [19, 61]. *In vivo* study by Chen et al. demonstrated that tea flower extract (TEE), rich with many polyphenols (EGCG, EGC, and ETC), possesses anti-inflammatory effect against chronic inflammation. In this study authors have shown that oral administration of TEE in mice is associated with significant reduction of tissue specific (liver) acute inflammation by blocking cytokines (TNF-alpha, IL-1β) expression and NO production [62]. Recently increasing interest on beneficial effect of extra-virgin olive oil (EVOO) is mainly focused on the anti-inflammatory effect of phenolic compounds present in the EVOO. Glycoside oleuropein, hydroxytyrosol, and tyrosol are the major phenolic component of EVOO and have been able to inhibit inflammation by blocking eicosanoids (prostaglandin I2, Leukotriene B_4) production enzymes such as COX-2, lipoxygenase (LOX), and phospholipase A2 (PLA2) in animal and human cells [63]. Additionally, hydroxytyrosol one of the main component of EVOV reported to impart anti-coagulatory/anti-atherosclerotic effect in association with their anti-inflammatory activity in individuals with type 1 diabetic by reducing the production and accumulation of thromboxan B2 (TXB2) and hydroxyeicosatetraenoic 27 acids (HETE) in serum, which results in reduced platelet aggregation. Moreover, this particular phenolic component of EVOV can also be considered as potent anti-inflammatory agent as this is able to prevent the expression of COX-2 and iNOS in LPS-stimulated macrophages [64, 65].

Polyphenol present in red wine and black tea, for instance, quercetin, EGCG, ECG, and theflavins, are able to inhibit COX-2 and LOX in dose dependent manner following application to LPS activated murine macrophage RAW 264 cells [66]. Likewise, kaempferol, a flavonoid widely distributed in many natural sources including apples, grapes, cabbage, and tomato, significantly reduces inflammation by inhibiting the generation of PGE2. Researchers have shown that cocoa polyphenols (flavonols, anthocyanidins, catechins, etc.) decrease the inflammation by numerous mechanisms such as inhibition of mitogen induced activation of T cells and reduced expression of IL-2 and other cytokines (IL-6, TNF-alpha). Curcumin has been identified as naturally occurring active component with wide range of medicinal effects against many chronic diseases like CVD, cancer, and metabolic disorder due to its strong antioxidative and anti-inflammatory response *in vitro* and *in vivo*. Aggarwal and Harikumar reported oral application of 70–100 mg/kg curcumin reduces systemic (plasma) and tissue specific (aortic tissue) inflammatory response as well as LDL oxidation and hypocholesteromic effects in rodents [67]. Similarly, Nemmar et al. have shown that supplementation of curcumin as oral gavage (45 mg/kg) in male mice significantly reduces systemic inflammation by preventing the release of TNF-α and C-reactive protein (CRP) [68]. CRP is an acute

phase protein and has been identified to play an important pathogenic role in the progression of many chronic degenerative diseases like CVD, arthritis. Increased level of CRP has been observed in almost every inflammation mediated diseases onset. Similar to curcumin Pauwels et al. have shown antiarthritic/inflammatory potentials of type-A procyanidine polyphenol (TAPP) extracted from cinnamon bark. Rheumatoid arthritis (RA) is an acute inflammatory condition in joint with functional impairments mainly involved with localized (skeletal join) excessive prostaglandin synthesis as well as systemic inflammation characterized by increased level of serum CRP. Oral consumption of cinnamon bark extract and its polyphenol (procyanidine) has been found to regulate systemic inflammation by reducing CRP and was also able to stimulate autoimmune system by blocking expression of many inflammatory mediators (prostaglandin E2, NO) in RA rats [69]. Results showed the therapeutic value of this particular polyphenol and its potential to reduce or reversal of RA progression. Due to its strong anti-inflammatory potentials TAPP is found to be successfully used to regulate airway hyperresponsiveness (AHR) or asthma by reducing inflammatory mediators like IL-4 and IL-13, which played critical role in mucus hyper-secretion and the onset of AHR [69]. Recently Hazewindus et al. have shown synergistic effect of bioactive compounds such as lycopene, ascorbic acid, and α-tocopherol, rich in tomato. In this study authors have shown that lycopene alone significantly exerts anti-inflammatory effect by reducing the release of proinflammatory cytokine TNF-α via regulation of NF-κB activation. Moreover significant inhibition of lipid peroxidation by the combination of ascorbic acid and α-tocopherol is the complementary to the anti-inflammatory effect of lycopene.

5. Role of Phytochemicals in Metabolism Regulation

Metabolism is the cascade of cellular chemical transformation regulated by a pool of enzymes which break down the organic matter, harvest the energy (digestion), and allow the cells to grow, reproduce, and respond to molecular signal by maintaining inter- and intracellular homeostasis. Any alteration of this chain of chemical event may lead to metabolic disorder or unregulated metabolism which ultimately results in high plasma glucose level (diabetic), obesity, high blood pressure (hypertension), CVD, organ failure, and so forth. Diseases or cluster of physiological changes induced mostly by metabolic disorder like diabetes, CVD are the major health concern for morbidity with high socioeconomic burden worldwide. Modification of dietary pattern in association with regulation in life style (regular exercise, weight loss) plays an effective role to slow the deleterious effect metabolic syndrome [70]. Epidemiological, randomized, and controlled dietary studies on human or rodents provide a lot of evidence showing that consumption of dietary fibers improves the indices of diabetes risk by regulating glycemic and plasma glucose level. The important pathogenic factors responsible for the development of metabolic disorder, insulin resistance, β-cells dysfunction, and finally diabetes are oxidative stress and tissue specific (localized) and/or systemic inflammation. Phytochemicals present in the whole grains including phytosterol, flavanols, anthocyanidins, and cinnamic acids modulate the diabetic risk by exerting their antioxidative and anti-inflammatory effect [70, 71]. Increasing number of controlled epidemiological studies has reported significant reduction of plasma cytokines (IL-6) or CRP in healthy individuals following regular consumption of rye bran and whole wheat bread. Furthermore, short-chain fatty acid of cereal fibre prevents inflammatory response in colonic mucosa by binding to the G-protein coupled receptor and also by blocking transcription factor NF-κB. Similarly consumption of brown and black rice for 4 months was found to exert cardioprotective effect by regulating oxidative stress in individuals with preexisting complications, coronary heart disease (CHD) [72]. Although the detailed molecular mechanism is still unexplored, controlled epidemiological studies have reported the significant reduction of oxidized plasma malondialdehyde and urine prostaglandin in subjects with CHD. Similar to wholegrain, fenugreek seed and cinnamon extract has been widely used for its medicinal value in the treatment of diabetes. The hypoglycemic and hypocholesterolemic effect of fenugreek seed is mainly attributed to its high concentration of soluble fiber content, which helps to decrease the postprandial blood glucose [70]. TAPP, the main active component of cinnamon extract significantly lowered the blood HbA1c level and also improved the insulin signaling in murine models of diabetes [73]. Hypoglycemic effect of blueberry extract in humans is mainly due to presence of anthocyanin and myrtillin. Significant reduction of blood pressure in salt-sensitive spontaneously hypertensive rats (SHRs) supplemented with 3% blueberry enriched diet for 8 weeks might be due to vasodilator effect of blueberry polyphenol through an endothelium mediated stimulation of NO metabolism and activation of COX-induced product [74, 75]. Similarly, grape seed extract (GSE), which is a rich source of polyphenols (approximately 90% of which are procyanidins and 7% other polyphenol compounds), has become popular for the treatment and prevention of chronic cardiac disease and other disorders. *In vivo studies on* spontaneously hypertensive rats have shown that regular consumption of 0.5% GSE-supplemented diet significantly reduced the arterial blood pressure possible due to induction of endothelium dependent vascular dialtion [76]. Aldolase reductase (AR), a rate limiting enzyme in polyol pathways plays an important role in the progression of diabetic complications and other chronic metabolic disorder. Detailed phytochemicals analysis by Termentzi et al. has shown *Sorbus domestica* fruits extract content high concentration of flavonoids and hydroxycinnamoyl esters. Further evaluation on *in vitro* study of *Sorbus domestica* fruits extract indicates high content hydroxycinnamoyl esters possess AR blocking activity. Thus regular consumption of this particular fruit extract can be the promising natural therapeutic measure for regulation of chronic or long-term diabetic complications [77, 78]. Furthermore, Russian tarragon (*Artemisia dracunculus* L.) extract was demonstrated to attribute antidiabetic/antihyperglycemic effect in both streptozotocin induced genetically diabetic KKAy murine models. *In vitro* study on hepatic cells showed

that bioactive components like 6-demethoxycapillarisin and 2′,4′-dihydroxy-4-methoxydihydrochalcone are involved in hepatic glucose output by inhibiting the transcription factor for a primary enzyme, phosphoenol pyruvate carboxykinase (PEPCK) [79]. Regular consumption of fresh bitter melon juice (popular vegetables of Asian origin) or dried whole vegetables have been found to significantly regulate the blood glucose level and diabetes-related complications including nephropathy, insulin resistance, and early cataract formation. The observed hypoglycemic effect of bitter melon might be due to presence of cucurbitane-type triterpenoids steroidal saponins called charantins, insulin-like peptides and alkaloids, which are associated with hypoglycemic activity [70]. Similar as bitter melon, antidiabetic effect of garlic was not well documented in human studies, but garlic was found to be an effective in regulating blood glucose level in streptozotocin-induced as well as alloxan-induced diabetes mellitus in rats and mice. Although the exact mechanism of garlic as an antidiabetic agent is not clearly understood, but several *in vitro* studies proposed that allicin (main component of garlic) may enhance serum insulin following effective combination with cystein, or garlic can simply exert its antidiabetic effect by inducing secretion of insulin from the pancreatic beta cells or its release from bound insulin [80, 81]. However, cardioprotective role of garlic has been well documented both *in vivo* and controlled human studies mainly by regulation of metabolism and due to its anti-atherosclerotic effect. Many *in vivo* studies reported supplementation of 1–4% garlic and garlic protein diet in hypercholesterolemic rats significantly reduced serum cholesterol, triglyceride, and LDL cholesterol. Similarly, controlled human studies have shown that a dose of 4 grams of garlic cloves per day, 6 days a week for 6 months, significantly affects the LDL-C or other plasma lipid concentrations in adults (30–65 years) with moderate hypercholesterolemia [82]. Garlic extract contains high levels of water soluble phytochemicals such as S-allyl-cystein (SAC) as well as small amount of oil soluble compounds [14]. *In vitro* studies further demonstrated that this cholesterol-lowering effect of garlic might be because garlic triggers the formation of allicin through action of alliinase enzymes and allicin inhibits cholesterol synthesis *in vitro* [83, 84]. Even *in vitro* studies reveled that antiatherosclerotic and antiatherogenic activities of garlic are due to reduction of lipid content and/or LDL oxidations by allicin and SAC present in the garlic extract [80]. Moreover, antihypertensive effect of garlic extract was also evaluated both in hypertensive rats and in controlled human (hypertensive) studies. The gamma-glutamylcysteine component of garlic might be responsible for lowering blood pressure by inhibiting angiotensin-converting enzyme *in vitro* [14, 80, 85]. Similar to organosulfur compound in garlic, phytosterols (including sitosterol, campesterol, and stanols) are also efficiently inducing cholesterol lowering effect both in animal and in human studies. The primary sources of phytosterols are vegetables, nuts, fruits seeds, and vegetable oil (olive oil, canola oil). Sitosterol and campesterol are the most frequent plant sterols and constitute about 60% and 35%, respectively, of plant sterols in food [86]. Phytosterols and cholesterol are structurally similar but are metabolized differently. Due to their structural similarity to cholesterol, plant sterols are well studied for their ability to inhibit cholesterol absorption. In addition to their cholesterol lowering effect, plant sterols may possess antiatherosclerosis, anti-inflammation, and antioxidative activities [87]. Pelletier et al. demonstrated that consumption of 0.7 g of soy sterols fed to 12 normocholesterolemic individuals reduced LDL cholesterol by 15.2% relative to the control [88]. Many *in vitro* studies on caco-2 cells have shown that the hypocholesterolemic effect of plant stanols might be by reduced intestinal cholesterol due to structural similarity between plant stanols and cholesterol [89].

Phenolic compounds, namely, chlorogenic acid and ferulic acid, and a plant alkaloid, berberine, are also considered as potent antidiabetic agent with high therapeutic efficiency as well as less side effect. All these three phytochemicals are reported to enhance the uptake of 2 deoxyglucose (2DG) in time- and dose-dependent manner. Reduced glucose transporter (GLUT4) translocation along with impaired glucose transport is the major pathogenesis of diabetes progression. Treatment of L6 myoblasts with these above mentioned phytochemicals shows that chlorogenic acid and berberine significantly upregulate the expression of GLUT4 and peroxisomal proliferator-activated receptors-gamma (PPAR-γ) expression whereas treatment of L6 myoblasts with ferulic acid results in significant induction of GLUT4 and phosphoinositide-3-kinase (PI3K) gene expression. Based on this study researchers concluded that chlorogenic acid and berberine exert their antidiabetic effect or increased glucose uptake in PI3K independent manner; however increased glucose uptake with ferulic acid is highly dependent on PI3K pathway [90, 91]. Several *in vivo* and *in vitro* studies have demonstrated the synergistic effect of resveratrol, quercetin, and genistein in preventing obesity/weight gain and adipogenesis and also contribute to control multiple metabolic disorders like dyslipidemia, insulin resistance, and so forth. Hypolipidemic effect of resveratrol has been found to exert significant reduction of serum total cholesterol, triglycerides, and lipid content in hepatic tissue in high-fat diet hamster [92]. Lagouge et al. revealed that the protecting activity of resveratrol on regulation of metabolism is mainly achieved due to enhancement of sirtuin 1 (Sirt1), a NAD+-dependent deacetylase activity, which improving the cellular insulin sensitivity. Therefore, consumption of plant based diet imparts beneficial effect by preventing the development and progression of age chronic diseases that are highly associated with metabolic disorder [93].

6. Role of Phytochemicals in Cell Migration and Proliferation

Abnormal proliferation and migration of vascular smooth muscle cells (VSMC) are the primary event contributing to the pathogenesis of atherosclerosis or restenosis and is also linked with other cellular process such as localized inflammation, arterial hypertension due to narrowing of blood vessel. Similarly excessive cell proliferation and impaired programmed cell death or apoptosis represent major causative factors for the development and progression

of cancer [19]. Therefore, this particular event is considered as a main target for the reversal of the neovascularization mediated health effect (atherosclerosis, cancer, etc.). Series of *in vitro* and *in vivo* studies has demonstrated tea as healthy beverage as it is rich source of many polyphenols (catechins, EGC, and EGCG) and is capable of reducing or slowing the progression of atherosclerosis by inhibiting the SMC proliferation and arrested the cells in G1 phase [94, 95]. EGCG is found to be effectively inducing SMC arrest by blocking cyclin D1 and cyclin E; beside this EGCG is also inhibiting the cell growth markers PCNA [94, 95]. Tea polyphenols exert antiproliferative effects by interacting also with matrix metalloproteinase (MMP) system. Expression of various MMPs has been found to be upregulated mostly in any type of cancer cells. Many studies have already shown that MMP transcription is induced and regulated by many regulators like cytokines, growth factor, ROS, and so forth. Several MMPs have been found to be as key agonists in tumor invasion, metastasis, and angiogenesis, including MMPs 1, 2, 3, 9, and 14 [96–98]. Two particular subtypes of this family, MMP 2 and MMP 9, are found to be actively involved in the turnover of basement membrane collagen and alteration of matrix proteins during angiogenesis and also plays important role for tissue remodeling. Both catechins and EGCG reduce MMP2/MMP-9 secretion in VSMC by preventing NfKB and AP-1 activities [95]. Khan et al. have reported administration of green tea polyphenol (e.g., EGCG) in transgenic adenocarcinoma mice results in significant inhibition of cancer progression or metastasis of prostrate tissue by regulating MMP 2 and MMP 9 expression [99]. Additionally it has been shown that both catechins and EGCG suppress PDGF-BB induced activation of PDGF-receptor mediated signal transduction pathways in VSMC by blocking tyrosine phosphorylation and their downstream molecular targets [99]. These findings are consistent with our recent study on antiproliferative effect of *Gentiana lutea* and its main constituent isovitexin where Kesavan et al. have reported aqueous root extract of *G. lutea* and isovitexin significantly inhibit PDGF-BB induced proliferation of SMC by downregulation of ERK1/2 and iNOS expression [100]. Similarly in another study we have shown significant antiproliferative effect of ellagic acid (EA), phenolic compound present mainly in barriers, which might be due to prevention of PDGF-BB receptor tyrosine phosphorylation/activation, reduced ROS generation, and downstream stimulation of ERK1/2 [101]. In this study authors have also demonstrated that treatment of streptozotocin-induced diabetic rats with EA reduced the onset of atheroma formation by blocking SMC proliferation and downregulation of cyclin D1 expression [101]. Hence all these findings suggest that regular consumption of phytochemicals is associated with prevention of cardiovascular complications. Antiproliferative effect of phytochemicals are not only inhabit the onset of CVD progressionbut also plays a significant role in cancer prevention. Curcumin and its derivatives have been found to inhibit the proliferation of breast cancer (BC) cell lines and by downregulation of matrix metalloproteinase-1 (MMP-1) expression [102]. Resveratrol is also effectively preventing cell proliferation by stimulating cell cycle arrest via regulation of cell cycle proteins such as cyclins E and D1. Furthermore, resveratrol induces apoptosis by controlling series of events including upregulation of tumor suppressor genes p21Cip1/WAF1, p53, the proapoptotic protein Bax expression, activating caspase apoptotic signals, and downregulation of antiapoptotic proteins Bcl-2, Bcl-XL, and survivin expression. Likewise, quercetin may also act as a plant derived anticancer drug by upregulating expression of Bax, which finally leads to cell apoptosis and is able to impart antiproliferative effect by suppression of Bcl-2 protein activity and stimulation of DNA fragmentation procedure [103, 104]. Many studies reported that antimetastatic effects of phytochemicals are mainly due to alteration of activity or expression of cell adhesion molecules (CD44), which are significantly upregulated during metastasis and primarily contribute to the cancer cell growth. Ouhtit et al. have shown that treatment of BC cell line with cocktail of six phytochemicals (indole-3-carbinol, resveratrol, genistein and curcumin, C-phycocyanin, quercetin) caused a marked inhibition of proliferation and motility of cells in combination with suppression of cell adhesion molecule CD44 expression, which plays an important role as a metastasis initiating factor [102].

7. Role of Phytochemicals in Angiogenesis

Angiogenesis is a complex controlled phenomenon for growth and development with proangiogenic and antiangiogenenic factors. Angiogenesis is well orchestrated physiological balance between the stimulatory and inhibitory signals for new blood vessels development or preexisting vasculature. Any alteration in normal angiogenesis results in either poor vascularisation or abnormal vasculature. Chronic ischemic wound is the result of insufficient blood vessel formation or reduced angiogenesis. In contrast cancer cells or tumors induced uncontrolled angiogenesis or abnormal blood vessel growth to spread metastasis by secreting proangiogenic factors like vascular endothelial growth factors (VEGF). In a very recent work Kowshik et al. have demonstrated antiangiogenic effects of ellagic acid in a hamster model of oral oncogenesis by examining the transcript and protein expression of hypoxia-induced VEGF signaling cascade. In this study authors have found ellagic acid significantly inhibiting angiogenesis in hamster buccal punch maybe by abolishing phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K/Akt) and MAPK and VEGF signaling pathways, which involves suppression of histone deacetylase 6 (HDAC6) and hypoxia-inducible factor 1-alpha (HIF-1α) responses [105]. Wound healing capacity of *Tephrosia purpurea* was studied by Lodhi et al. in rats with three different types of wound such as incision wound, excision wound, and dead space wound. Histopathological study following treatment of wound area with *T. purpurea* ethanolic extract has shown significant increase in angiogenesis or blood vessels formation, fibroblast cells, and collagen fibres generation due to presence of large amount of flavonoids in the extract [106]. Several *in vivo* and *in vitro* studies have shown combination of EGCG and curcumin exhibited synergistic growth inhibition of premalignant and malignant cells through the suppression of angiogenesis, cell proliferation, and upregulation of

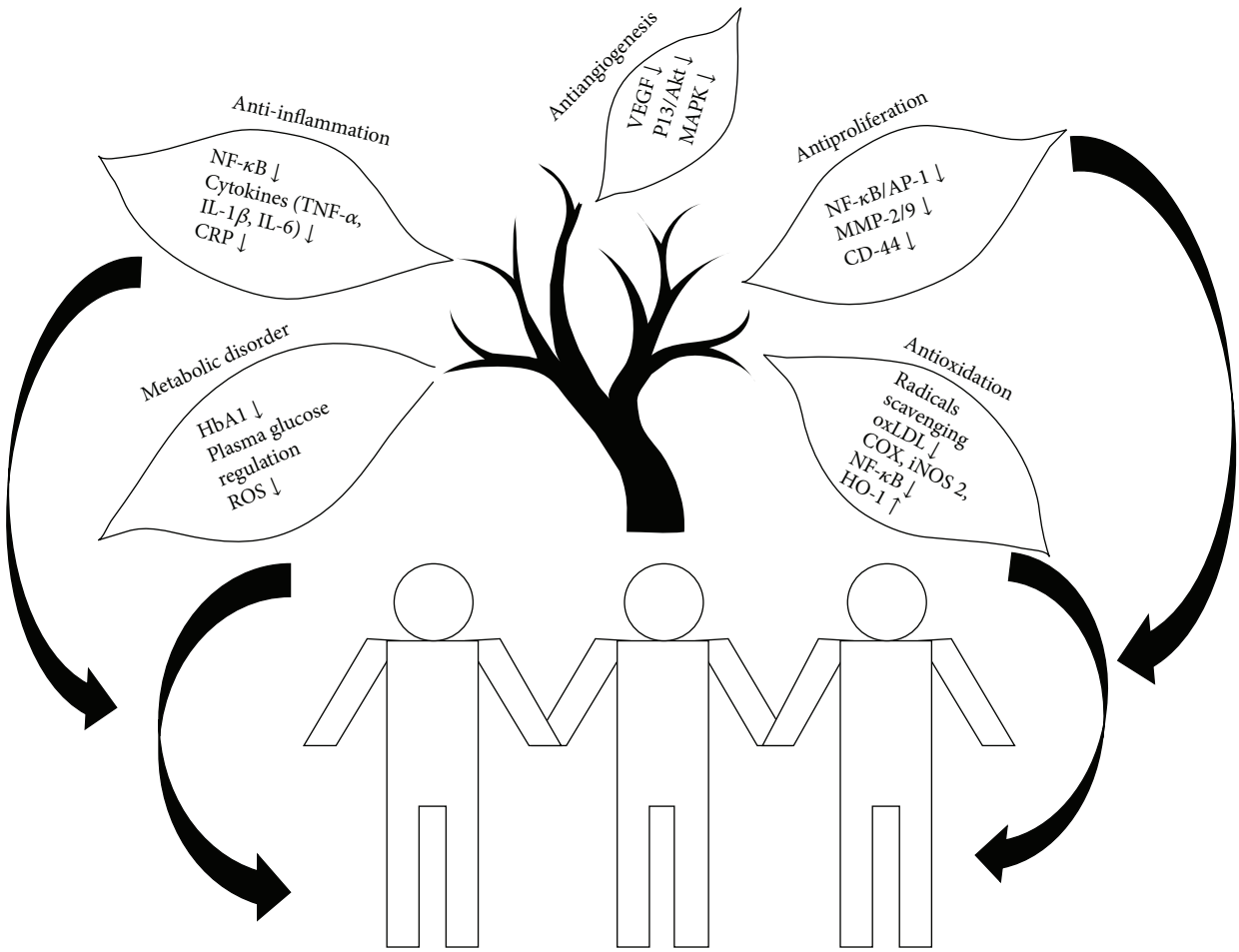

Figure 1: The molecular mechanism/signaling targeted by phytochemicals to exert the protective effect: antioxidation, anti-inflammation, antiproliferation, metabolic disorder, and antiangiogenesis.

apoptosis [107]. Similarly Kowshik et al. have reported role of astaxanthin as a cancer preventive agent by suppression of angiogenesis. Astaxanthin, which is a non-provitamin A carotenoid predominantly distributed in microalgae, fungi, plants, and sea foods, is found to inhibit the onset of tumor progression by targeting signal transducer and activator of transcription 3 (STAT3)/Janus kinase 2 (JAK-2) [108].

8. Summary: Future of Phytochemicals and Therapeutic Mechanism

Relationship between phytochemicals and disease prevention has been a major focus of health research for almost half a century. Epidemiological and clinical studies indicate that the risk of chronic or noncommunicable diseases is reduced by a diet rich in fruits, vegetables, and unrefined grains. Other foods such as mono- and polyunsaturated fats, brans, nuts, plant sterols, and soy proteins have all been shown to have a favorable effect on pathogenesis of CVD (e.g., lipid profile and blood pressure lowering effect), cancer, and/or neurodegeneration. The progression in the knowledge of both the disease pathomechanisms and the targeted pathways by dietary components to exert their medicinal effect may provide new avenues to develop dietary strategies to prevent and/or to treat the numerous disorders. Based on the epidemiological and/or clinical evidence, it has been found that phytochemicals and/or naturally occurring active compounds are having broad range of physiological effects, which include reduction of inflammatory cascades, oxidative stress, improved metabolic disorder, vascular homeostasis, or antiproliferation (Figure 1). However, it is still not clear whether an individual component of the diet or a combination of nutrients and dietary habits is responsible for the observed protective effects. Therefore, screening of large scale of potential beneficial molecules present in the regular diet may provide lead molecules that may be used in the future as inexpensive dietary supplements specific to disease prevention. The products being naturally occurring in the markets would be easily available for all strata of the society. This would also open up a huge possibility of herbal product based markets and scopes of employment. The area of phytochemicals and its protective effect will only grow successfully if preclinical and/or clinical research is able to integrate credible science with thorough consumer understanding, uncompromised taste, and convenience, along with awareness about the preventive role of dietary product on the development of chronic diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] K. A. Steinmetz and J. D. Potter, "Vegetables, fruit, and cancer. I. Epidemiology," *Cancer Causes and Control*, vol. 2, no. 5, pp. 325–357, 1991.

[2] T. Maraldi, D. Vauzour, and C. Angeloni, "Dietary polyphenols and their effects on cell biochemistry and pathophysiology 2013," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 576363, 3 pages, 2014.

[3] E. M. Alissa and G. A. Ferns, "Functional foods and nutraceuticals in the primary prevention of cardiovascular diseases," *Journal of Nutrition and Metabolism*, vol. 2012, Article ID 569486, 16 pages, 2012.

[4] T. W. George, C. Niwat, S. Waroonphan, M. H. Gordon, and J. A. Lovegrove, "Effects of chronic and acute consumption of fruit- and vegetable-puree- based drinks on vasodilation, risk factors for CVD and the response as a result of the eNOS G298T polymorphism," *Proceedings of the Nutrition Society*, vol. 68, no. 2, pp. 148–161, 2009.

[5] R. H. Liu, "Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals," *The American Journal of Clinical Nutrition*, vol. 78, no. 3, supplement, pp. 517S–520S, 2003.

[6] S. Hirai, T. Uemura, N. Mizoguchi et al., "Diosgenin attenuates inflammatory changes in the interaction between adipocytes and macrophages," *Molecular Nutrition and Food Research*, vol. 54, no. 6, pp. 797–804, 2010.

[7] S. C. Sahu, "Dual role of organosulfur compounds in foods: a review," *Journal of Environmental Science and Health C: Environmental Carcinogenesis & Ecotoxicology Reviews*, vol. 20, no. 1, pp. 61–76, 2002.

[8] F. Aqil, R. Munagala, J. Jeyabalan, and M. V. Vadhanam, "Bioavailability of phytochemicals and its enhancement by drug delivery systems," *Cancer Letters*, vol. 334, no. 1, pp. 133–141, 2013.

[9] C. A. Lipinski, F. Lombardo, B. W. Dominy, and P. J. Feeney, "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings," *Advanced Drug Delivery Reviews*, vol. 46, no. 1–3, pp. 3–26, 2001.

[10] S. Gao and M. Hu, "Bioavailability challenges associated with development of anti-cancer phenolics," *Mini-Reviews in Medicinal Chemistry*, vol. 10, no. 6, pp. 550–567, 2010.

[11] C. S. Yang, S. Sang, J. D. Lambert, and M.-J. Lee, "Bioavailability issues in studying the health effects of plant polyphenolic compounds," *Molecular Nutrition and Food Research*, vol. 52, supplement 1, pp. S139–S151, 2008.

[12] C. Manach, A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez, "Polyphenols: Food sources and bioavailability," *The American Journal of Clinical Nutrition*, vol. 79, no. 5, pp. 727–747, 2004.

[13] B. Wu, K. Kulkarni, S. Basu, S. Zhang, and M. Hu, "First-pass metabolism via UDP-glucuronosyltransferase: a barrier to oral bioavailability of phenolics," *Journal of Pharmaceutical Sciences*, vol. 100, no. 9, pp. 3655–3681, 2011.

[14] I. Rahman, S. K. Biswas, and P. A. Kirkham, "Regulation of inflammation and redox signaling by dietary polyphenols," *Biochemical Pharmacology*, vol. 72, no. 11, pp. 1439–1452, 2006.

[15] V. J. Thannickal and B. L. Fanburg, "Reactive oxygen species in cell signaling," *American Journal of Physiology: Lung Cellular and Molecular Physiology*, vol. 279, no. 6, pp. L1005–L1028, 2000.

[16] R. A. Cairns, I. S. Harris, and T. W. Mak, "Regulation of cancer cell metabolism," *Nature Reviews Cancer*, vol. 11, no. 2, pp. 85–95, 2011.

[17] J. L. Martindale and N. J. Holbrook, "Cellular response to oxidative stress: signaling for suicide and survival," *Journal of Cellular Physiology*, vol. 192, no. 1, pp. 1–15, 2002.

[18] G. Loo, "Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation," *The Journal of Nutritional Biochemistry*, vol. 14, no. 2, pp. 64–73, 2003.

[19] V. Stangl, H. Dreger, K. Stangl, and M. Lorenz, "Molecular targets of tea polyphenols in the cardiovascular system," *Cardiovascular Research*, vol. 73, no. 2, pp. 348–358, 2007.

[20] F. Tosetti, D. M. Noonan, and A. Albini, "Metabolic regulation and redox activity as mechanisms for angioprevention by dietary phytochemicals," *International Journal of Cancer*, vol. 125, no. 9, pp. 1997–2003, 2009.

[21] M. L. Parkett, R. E. Harris, F. S. Joarder, M. S. Ross, K. P. Clausen, and F. M. Robertson, "Cyclooxygenase-2 gene expression in human breast cancer," *International Journal of Oncology*, vol. 10, no. 3, pp. 503–507, 1997.

[22] H. Sano, Y. Kawahito, R. L. Wilder et al., "Expression of cyclooxygenase-1 and -2 in human colorectal cancer," *Cancer Research*, vol. 55, no. 17, pp. 3785–3789, 1995.

[23] J. Westermarck and V.-M. Kähäri, "Regulation of matrix metalloproteinase expression in tumor invasion," *The FASEB Journal*, vol. 13, no. 8, pp. 781–792, 1999.

[24] K. T. Liby, M. M. Yore, and M. B. Sporn, "Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer," *Nature Reviews Cancer*, vol. 7, no. 5, pp. 357–369, 2007.

[25] K. S. C. Bose and B. K. Agrawal, "Effect of lycopene from cooked tomatoes on serum antioxidant enzymes, lipid peroxidation rate and lipid profile in coronary heart disease," *Singapore Medical Journal*, vol. 48, no. 5, pp. 415–420, 2007.

[26] D. V. Ratnam, D. D. Ankola, V. Bhardwaj, D. K. Sahana, and M. N. V. R. Kumar, "Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective," *Journal of Controlled Release*, vol. 113, no. 3, pp. 189–207, 2006.

[27] J. H. Lee, T. O. Khor, L. Shu, Z.-Y. Su, F. Fuentes, and A.-N. T. Kong, "Dietary phytochemicals and cancer prevention: Nrf2 signaling, epigenetics, and cell death mechanisms in blocking cancer initiation and progression," *Pharmacology and Therapeutics*, vol. 137, no. 2, pp. 153–171, 2013.

[28] H.-K. Na and Y.-J. Surh, "Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG," *Food and Chemical Toxicology*, vol. 46, no. 4, pp. 1271–1278, 2008.

[29] S. Dhakshinamoorthy and A. K. Jaiswal, "Small Maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:quinone oxidoreductase1 gene," *Journal of Biological Chemistry*, vol. 275, no. 51, pp. 40134–40141, 2000.

[30] G. Shen, V. Hebbar, S. Nair et al., "Regulation of Nrf2 transactivation domain activity: the differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein," *Journal of Biological Chemistry*, vol. 279, no. 22, pp. 23052–23060, 2004.

[31] L. K. Leung, Y. Su, R. Chen, Z. Zhang, Y. Huang, and Z.-Y. Chen, "Theaflavins in black tea and catechins in green tea are equally effective antioxidants," *Journal of Nutrition*, vol. 131, no. 9, pp. 2248–2251, 2001.

[32] P. V. A. Babu and D. Liu, "Green tea catechins and cardiovascular health: an update," *Current Medicinal Chemistry*, vol. 15, no. 18, pp. 1840–1850, 2008.

[33] M.-J. Lee, Z.-Y. Wang, H. Li et al., "Analysis of plasma and urinary tea polyphenols in human subjects," *Cancer Epidemiology Biomarkers and Prevention*, vol. 4, no. 4, pp. 393–399, 1995.

[34] C. C. Wu, M. C. Hsu, C. W. Hsieh, J. B. Lin, P. H. Lai, and B. S. Wung, "Upregulation of heme oxygenase-1 by Epigallocatechin-3-gallate via the phosphatidylinositol 3-kinase/Akt and ERK pathways," *Life Sciences*, vol. 78, no. 25, pp. 2889–2897, 2006.

[35] C. K. Andreadi, L. M. Howells, P. A. Atherfold, and M. M. Manson, "Involvement of Nrf2, p38, B-Raf, and nuclear factor-κB, but not phosphatidylinositol 3-kinase, in induction of hemeoxygenase-1 by dietary polyphenols," *Molecular Pharmacology*, vol. 69, no. 3, pp. 1033–1040, 2006.

[36] I. Rodríguez-Ramiro, S. Ramos, L. Bravo, L. Goya, and M. Á. Martín, "Procyanidin B2 induces Nrf2 translocation and glutathione S-transferase P1 expression via ERKs and p38-MAPK pathways and protect human colonic cells against oxidative stress," *European Journal of Nutrition*, vol. 51, no. 7, pp. 881–892, 2012.

[37] G. E. Mann, D. J. Rowlands, F. Y. L. Li, P. de Winter, and R. C. M. Siow, "Activation of endothelial nitric oxide synthase by dietary isoflavones: role of NO in Nrf2-mediated antioxidant gene expression," *Cardiovascular Research*, vol. 75, no. 2, pp. 261–274, 2007.

[38] T. W. Kensler, N. Wakabayashi, and S. Biswal, "Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway," *Annual Review of Pharmacology and Toxicology*, vol. 47, pp. 89–116, 2007.

[39] K. Mahn, C. Borrás, G. A. Knock et al., "Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo," *The FASEB Journal*, vol. 19, no. 12, pp. 1755–1757, 2005.

[40] P. Nestel, A. Fujii, and L. Zhang, "An isoflavone metabolite reduces arterial stiffness and blood pressure in overweight men and postmenopausal women," *Atherosclerosis*, vol. 192, no. 1, pp. 184–189, 2007.

[41] K. C. Das and C. W. White, "Redox systems of the cell: possible links and implications," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, pp. 9617–9618, 2002.

[42] S. Davinelli, N. Sapere, D. Zella, R. Bracale, M. Intrieri, and G. Scapagnini, "Pleiotropic protective effects of phytochemicals in Alzheimer's disease," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 386527, 11 pages, 2012.

[43] G. P. Lim, T. Chu, F. Yang, W. Beech, S. A. Frautschy, and G. M. Cole, "The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse," *Journal of Neuroscience*, vol. 21, no. 21, pp. 8370–8377, 2001.

[44] D. A. Dickinson, K. E. Iles, H. Zhang, V. Blank, and H. J. Forman, "Curcumin alters EpRE and AP-1 binding complexes and elevates glutamate-cysteine ligase gene expression," *The FASEB Journal*, vol. 17, no. 3, pp. 473–475, 2003.

[45] H. Liu, L. E. Harrell, S. Shenvi, T. Hagen, and R.-M. Liu, "Gender differences in glutathione metabolism in Alzheimer's disease," *Journal of Neuroscience Research*, vol. 79, no. 6, pp. 861–867, 2005.

[46] L.-C. Lin, M.-N. Wang, T.-Y. Tseng, J.-S. Sung, and T.-H. Tsai, "Pharmacokinetics of (-)-epigallocatechin-3-gallate in conscious and freely moving rats and its brain regional distribution," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 4, pp. 1517–1524, 2007.

[47] J. A. Baur, K. J. Pearson, N. L. Price et al., "Resveratrol improves health and survival of mice on a high-calorie diet," *Nature*, vol. 444, no. 7117, pp. 337–342, 2006.

[48] J. A. Baur and D. A. Sinclair, "Therapeutic potential of resveratrol: the in vivo evidence," *Nature Reviews Drug Discovery*, vol. 5, no. 6, pp. 493–506, 2006.

[49] D. Kim, M. D. Nguyen, M. M. Dobbin et al., "SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis," *The EMBO Journal*, vol. 26, no. 13, pp. 3169–3179, 2007.

[50] Y. A. Kim, S.-Y. Lim, S.-H. Rhee et al., "Resveratrol inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in β-amyloid-treated C6 glioma cells," *International Journal of Molecular Medicine*, vol. 17, no. 6, pp. 1069–1075, 2006.

[51] L. D'evoli, F. Morroni, G. Lombardi-Boccia et al., "Red chicory (*Cichorium intybus* L. cultivar) as a potential source of antioxidant anthocyanins for intestinal health," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 704310, 8 pages, 2013.

[52] M.-J. Bak, V. L. Truong, H.-S. Kang, M. Jun, and W.-S. Jeong, "Anti-inflammatory effect of procyanidins from wild grape (*Vitis amurensis*) seeds in LPS-induced RAW 264.7 cells," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 409321, 11 pages, 2013.

[53] J. Terao, "Dietary flavonoids as antioxidants," *Forum of Nutrition*, vol. 61, pp. 87–94, 2009.

[54] M. Aviram, M. Rosenblat, D. Gaitini et al., "Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation," *Clinical Nutrition*, vol. 23, no. 3, pp. 423–433, 2004.

[55] R. Korhonen, A. Lahti, H. Kankaanranta, and E. Moilanen, "Nitric oxide production and signaling in inflammation," *Current Drug Targets: Inflammation and Allergy*, vol. 4, no. 4, pp. 471–479, 2005.

[56] Y. Shan, R. Zhao, W. Geng et al., "Protective effect of sulforaphane on human vascular endothelial cells against lipopolysaccharide-induced inflammatory damage," *Cardiovascular Toxicology*, vol. 10, no. 2, pp. 139–145, 2010.

[57] E. Heiss, C. Herhaus, K. Klimo, H. Bartsch, and C. Gerhäuser, "Nuclear factor κB is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms," *Journal of Biological Chemistry*, vol. 276, no. 34, pp. 32008–32015, 2001.

[58] A. Prawan, C. L. L. Saw, T. O. Khor et al., "Anti-NF-κB and anti-inflammatory activities of synthetic isothiocyanates: effect of

chemical structures and cellular signaling," *Chemico-Biological Interactions*, vol. 179, no. 2-3, pp. 202–211, 2009.

[59] A. E. Wagner, A. M. Terschluesen, and G. Rimbach, "Health promoting effects of brassica-derived phytochemicals: from chemopreventive and anti-inflammatory activities to epigenetic regulation," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 964539, 12 pages, 2013.

[60] A. E. Wagner, O. Will, C. Sturm, S. Lipinski, P. Rosenstiel, and G. Rimbach, "DSS-induced acute colitis in C57BL/6 mice is mitigated by sulforaphane pre-treatment," *Journal of Nutritional Biochemistry*, vol. 24, no. 12, pp. 2085–2091, 2013.

[61] Y.-L. Lin, S.-H. Tsai, S.-Y. Lin-Shiau, and C.-T. Ho, "Theaflavin-3,3'-digallate from black tea blocks the nitric oxide synthase by down-regulating the activation of NF-κB in macrophages," *European Journal of Pharmacology*, vol. 367, no. 2-3, pp. 379–388, 1999.

[62] B.-T. Chen, W.-X. Li, R.-R. He et al., "Anti-inflammatory effects of a polyphenols-rich extract from tea (*Camellia sinensis*) flowers in acute and chronic mice models," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 537923, 7 pages, 2012.

[63] C. Santangelo, R. Varì, B. Scazzocchio, R. di Benedetto, C. Filesi, and R. Masella, "Polyphenols, intracellular signalling and inflammation," *Annali dell'Istituto Superiore di Sanita*, vol. 43, no. 4, pp. 394–405, 2007.

[64] C. L. Léger, M. A. Carbonneau, F. Michel et al., "A thromboxane effect of a hydroxytyrosol-rich olive oil wastewater extract in patients with uncomplicated type 1 diabetes," *European Journal of Clinical Nutrition*, vol. 59, no. 5, pp. 727–730, 2005.

[65] M. C. Maiuri, D. De Stefano, P. Di Meglio et al., "Hydroxytyrosol, a phenolic compound from virgin olive oil, prevents macrophage activation," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 371, no. 6, pp. 457–465, 2005.

[66] D. Ichikawa, A. Matsui, M. Imai, Y. Sonoda, and T. Kasahara, "Effect of various catechins on the IL-12p40 production by murine peritoneal macrophages and a macrophage cell line, J774.1," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 9, pp. 1353–1358, 2004.

[67] B. B. Aggarwal and K. B. Harikumar, "Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 1, pp. 40–59, 2009.

[68] A. Nemmar, D. Subramaniyan, and B. H. Ali, "Protective effect of curcumin on pulmonary and cardiovascular effects induced by repeated exposure to diesel exhaust particles in mice," *PLoS ONE*, vol. 7, no. 6, Article ID e39554, 2012.

[69] R. A. Pauwels, J. C. Kips, R. A. Peleman, and M. E. van der Straeten, "The effect of endotoxin inhalation on airway responsiveness and cellular influx in rats," *American Review of Respiratory Disease*, vol. 141, no. 3, pp. 540–545, 1990.

[70] B. L. Graf, I. Raskin, W. T. Cefalu, and D. M. Ribnicky, "Plant-derived therapeutics for the treatment of metabolic syndrome," *Current Opinion in Investigational Drugs*, vol. 11, no. 10, pp. 1107–1115, 2010.

[71] A. García-Lafuente, E. Guillamón, A. Villares, M. A. Rostagno, and J. A. Martínez, "Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease," *Inflammation Research*, vol. 58, no. 9, pp. 537–552, 2009.

[72] Y. V. Bobryshev, S. M. Cherian, S. J. Inder, and R. S. A. Lord, "Neovascular expression of VE-cadherin in human atherosclerotic arteries and its relation to intimal inflammation," *Cardiovascular Research*, vol. 43, no. 4, pp. 1003–1017, 1999.

[73] J.-J. Dugoua, D. Seely, D. Perri et al., "From type 2 diabetes to antioxidant activity: a systematic review of the safety and efficacy of common and cassia cinnamon bark," *Canadian Journal of Physiology and Pharmacology*, vol. 85, no. 9, pp. 837–847, 2007.

[74] A. Z. Kalea, K. Clark, D. A. Schuschke, and D. J. Klimis-Zacas, "Vascular reactivity is affected by dietary consumption of wild blueberries in the sprague-dawley rat," *Journal of Medicinal Food*, vol. 12, no. 1, pp. 21–28, 2009.

[75] A. Z. Kalea, K. Clark, D. A. Schuschke, A. S. Kristo, and D. J. Klimis-Zacas, "Dietary enrichment with wild blueberries (*Vaccinium angustifolium*) affects the vascular reactivity in the aorta of young spontaneously hypertensive rats," *Journal of Nutritional Biochemistry*, vol. 21, no. 1, pp. 14–22, 2010.

[76] N. Peng, J. T. Clark, J. Prasain, H. Kim, C. R. White, and J. M. Wyss, "Antihypertensive and cognitive effects of grape polyphenols in estrogen-depleted, female, spontaneously hypertensive rats," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 289, no. 3, pp. R771–R775, 2005.

[77] A. Termentzi, P. Alexiou, V. J. Demopoulos, and E. Kokkalou, "The aldose reductase inhibitory capacity of *Sorbus domestica* fruit extracts depends on their phenolic content and may be useful for the control of diabetic complications," *Pharmazie*, vol. 63, no. 9, pp. 693–696, 2008.

[78] C. Veeresham, A. R. Rao, and K. Asres, "Aldose reductase inhibitors of plant origin," *Phytotherapy Research*, vol. 28, no. 3, pp. 317–333, 2014.

[79] D. Govorko, S. Logendra, Y. Wang et al., "Polyphenolic compounds from Artemisia dracunculus L. inhibit PEPCK gene expression and gluconeogenesis in an H4IIE hepatoma cell line," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 293, no. 6, pp. E1503–E1510, 2007.

[80] S. K. Banerjee and S. K. Maulik, "Effect of garlic on cardiovascular disorders: a review," *Nutrition Journal*, vol. 1, article 4, 2002.

[81] Y.-Y. Yeh and L. Liu, "Cholesterol-lowering effect of garlic extracts and organosulfur compounds: human and animal studies," *Journal of Nutrition*, vol. 131, no. 3, pp. 989S–993S, 2001.

[82] C. D. Gardner, L. M. Chatterjee, and J. J. Carlson, "The effect of a garlic preparation on plasma lipid levels in moderately hypercholesterolemic adults," *Atherosclerosis*, vol. 154, no. 1, pp. 213–220, 2001.

[83] R. Gebhardt and H. Beck, "Differential inhibitory effects of garlic-derived organosulfur compounds on cholesterol biosynthesis in primary rat hepatocyte cultures," *Lipids*, vol. 31, no. 12, pp. 1269–1276, 1996.

[84] A. Sendl, G. Elbl, B. Steinke, K. Redl, W. Breu, and H. Wagner, "Comparative pharmacological investigations of *Allium ursinum* and *Allium sativum*," *Planta Medica*, vol. 58, no. 1, pp. 1–7, 1992.

[85] K. Rahman and G. M. Lowe, "Garlic and cardiovascular disease: a critical review," *Journal of Nutrition*, vol. 136, no. 3, pp. 736S–740S, 2006.

[86] O. Weingärtner, M. Böhm, and U. Laufs, "Controversial role of plant sterol esters in the management of hypercholesterolaemia," *European Heart Journal*, vol. 30, no. 4, pp. 404–409, 2009.

[87] A. Berger, P. J. H. Jones, and S. S. Abumweis, "Plant sterols: factors affecting their efficacy and safety as functional food ingredients," *Lipids in Health and Disease*, vol. 3, article 5, 2004.

[88] S. Pelletier, S. Kundrat, and C. M. Hasler, "Effects of a functional foods nutrition education program with cardiac rehabilitation patients," *Journal of Cardiopulmonary Rehabilitation*, vol. 23, no. 5, pp. 334–340, 2003.

[89] J. Plat and R. P. Mensink, "Increased intestinal ABCA1 expression contributes to the decrease in cholesterol absorption after plant stanol consumption," *The FASEB Journal*, vol. 16, no. 10, pp. 1248–1253, 2002.

[90] P. K. Prabhakar and M. Doble, "Synergistic effect of phytochemicals in combination with hypoglycemic drugs on glucose uptake in myotubes," *Phytomedicine*, vol. 16, no. 12, pp. 1119–1126, 2009.

[91] P. K. Prabhakar and M. Doble, "Interaction of phytochemicals with hypoglycemic drugs on glucose uptake in L6 myotubes," *Phytomedicine*, vol. 18, no. 4, pp. 285–291, 2011.

[92] M. González-Castejón and A. Rodriguez-Casado, "Dietary phytochemicals and their potential effects on obesity: a review," *Pharmacological Research*, vol. 64, no. 5, pp. 438–455, 2011.

[93] M. Lagouge, C. Argmann, Z. Gerhart-Hines et al., "Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α," *Cell*, vol. 127, no. 6, pp. 1109–1122, 2006.

[94] K.-Y. Chyu, S. M. Babbidge, X. Zhao et al., "Differential effects of green tea-derived catechin on developing versus established atherosclerosis in apolipoprotein E-null mice," *Circulation*, vol. 109, no. 20, pp. 2448–2453, 2004.

[95] C.-H. Kim and S.-K. Moon, "Epigallocatechin-3-gallate causes the p21/WAF1-mediated G_1-phase arrest of cell cycle and inhibits matrix metalloproteinase-9 expression in TNF-α-induced vascular smooth muscle cells," *Archives of Biochemistry and Biophysics*, vol. 435, no. 2, pp. 264–272, 2005.

[96] X. W. Cheng, M. Kuzuya, S. Kanda et al., "Epigallocatechin-3-gallate binding to MMP-2 inhibits gelatinolytic activity without influencing the attachment to extracellular matrix proteins but enhances MMP-2 binding to TIMP-2," *Archives of Biochemistry and Biophysics*, vol. 415, no. 1, pp. 126–132, 2003.

[97] X. W. Cheng, M. Kuzuya, T. Sasaki et al., "Green tea catechins inhibit neointimal hyperplasia in a rat carotid arterial injury model by TIMP-2 overexpression," *Cardiovascular Research*, vol. 62, no. 3, pp. 594–602, 2004.

[98] J. El Bedoui, M.-H. Oak, P. Anglard, and V. B. Schini-Kerth, "Catechins prevent vascular smooth muscle cell invasion by inhibiting MT1-MMP activity and MMP-2 expression," *Cardiovascular Research*, vol. 67, no. 2, pp. 317–325, 2005.

[99] N. Khan, F. Afaq, M. Saleem, N. Ahmad, and H. Mukhtar, "Targeting multiple signaling pathways by green tea polyphenol (−)-epigallocatechin-3-gallate," *Cancer Research*, vol. 66, no. 5, pp. 2500–2505, 2006.

[100] R. Kesavan, U. R. Potunuru, B. Nastasijević, T. Avaneesh, G. Joksić, and M. Dixit, "Inhibition of vascular smooth muscle cell proliferation by Gentiana lutea root extracts," *PLoS ONE*, vol. 8, no. 4, Article ID e61393, 2013.

[101] U. P. Rani, R. Kesavan, R. Ganugula et al., "Ellagic acid inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and prevents atheroma formation in streptozotocin-induced diabetic rats," *Journal of Nutritional Biochemistry*, vol. 24, no. 11, pp. 1830–1839, 2013.

[102] A. Ouhtit, R. L. Gaur, M. Abdraboh et al., "Simultaneous inhibition of cell-cycle, proliferation, survival, metastatic pathways and induction of apoptosis in breast cancer cells by a phytochemical super-cocktail: genes that underpin its mode of action," *Journal of Cancer*, vol. 4, no. 9, pp. 703–715, 2013.

[103] J. Duo, G.-G. Ying, G.-W. Wang, and L. Zhang, "Quercetin inhibits human breast cancer cell proliferation and induces apoptosis via Bcl-2 and Bax regulation," *Molecular Medicine Reports*, vol. 5, no. 6, pp. 1453–1456, 2012.

[104] A. Murakami, H. Ashida, and J. Terao, "Multitargeted cancer prevention by quercetin," *Cancer Letters*, vol. 269, no. 2, pp. 315–325, 2008.

[105] J. Kowshik, H. Giri, T. K. Kishore et al., "Ellagic acid inhibits VEGF/VEGFR2, PI3K/Akt and MAPK signaling cascades in the hamster cheek pouch carcinogenesis model," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 14, no. 9, pp. 1249–1260, 2014.

[106] S. Lodhi, R. S. Pawar, A. P. Jain, and A. K. Singhai, "Wound healing potential of *Tephrosia purpurea* (Linn.) Pers. in rats," *Journal of Ethnopharmacology*, vol. 108, no. 2, pp. 204–210, 2006.

[107] J. W. Kim, A. R. M. R. Amin, and D. M. Shin, "Chemoprevention of head and neck cancer with green tea polyphenols," *Cancer Prevention Research*, vol. 3, no. 8, pp. 900–909, 2010.

[108] J. Kowshik, A. B. Baba, H. Giri et al., "Astaxanthin inhibits JAK/STAT-3 signaling to abrogate cell proliferation, invasion and angiogenesis in a hamster model of oral cancer," *PLoS ONE*, vol. 9, no. 10, Article ID e109114, 2014.

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Research Article

Polymethoxyflavone Apigenin-Trimethylether Suppresses LPS-Induced Inflammatory Response in Nontransformed Porcine Intestinal Cell Line IPEC-J2

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The *in vitro* anti-inflammatory effect of apigenin and its trimethylated analogue (apigenin-trimethylether) has been investigated in order to evaluate whether these flavonoids could attenuate LPS-induced inflammation in IPEC-J2 non-transformed intestinal epithelial cells. Levels of IL-6, IL-8, TNF-α, and COX-2 mRNA were measured as a marker of inflammatory response. The extracellular H_2O_2 level in IPEC-J2 cells was also monitored by Amplex Red assay. Our data revealed that both compounds had significant lowering effect on the inflammatory response. Apigenin (at 25 μM) significantly decreased gene expression of IL-6 in LPS-treated cells, while apigenin-trimethylether in the same concentration did not influence IL-6 mRNA level. Both apigenin and apigenin-trimethylether reduced IL-8 gene expression significantly. TNF-α mRNA level was decreased by apigenin-trimethylether, which was not influenced by apigenin. Treatment with both flavonoids caused significant reduction in the mRNA level of COX-2, but the anti-inflammatory effect of the methylated analogue was more effective than the unmethylated one. Furthermore, both flavonoids reduced significantly the level of extracellular H_2O_2 compared to the control cells. In conclusion, the methylated apigenin analogue could avoid LPS-induced intestinal inflammation and it could be applied in the future as an effective anti-inflammatory compound.

1. Introduction

Flavonoids are naturally occurring polyphenolic compounds which are part of the regular human diet, because of their presence in vegetables, fruits, and beverages such as wine, coffee, and tea. A broad spectrum of beneficial effects (e.g., anticancer, antibacterial, and anti-inflammatory) is attributed to these molecules [1–3]. Many of the positive biological actions of flavonoids have been assigned to their antioxidant properties. However, there is an emerging view that flavonoids and their *in vivo* metabolites do not act only as conventional hydrogen-donating antioxidants but could modulate protein kinase signalling pathways in cells, influencing transcription factor nuclear factor kappa B (NF-κB) [2].

Polymethoxylated flavones (PMFs) or polymethoxyflavones are flavones substituted with two or more methoxy groups. They are coming into center of interest more and more due to their documented wide spectrum of biological activity including anti-inflammatory, anticarcinogenic, and antiatherogenic properties [4–7]. Some of the polymethoxylated citrus flavonoids have also demonstrated antiproliferative properties in preliminary studies [8, 9]. A novel tangeretin derivative, 5-acetyl-6,7,8,4′-tetramethylnortangeretin, inhibited MCF-7 breast cancer cell growth through both caspase-dependent intrinsic apoptosis and caspase-independent apoptosis pathways [10].

The antiproliferative effects of methoxylated versus hydroxylated flavones were directly compared in SCC-9 human oral squamous carcinoma cells [6]. Apigenin-trimethylether was about eight times more potent than apigenin, one of the most studied hydroxyflavones. More recent research data demonstrated that citrus PMFs are directly associated with the inhibition of enzymes involved in the inflammation [11–13]. From previous reports, it seemed that flavonoids with

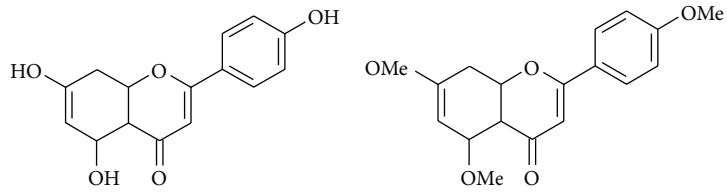

Figure 1: Structure of apigenin (3′,4′,5-trihydroxyflavone) (a) and apigenin-trimethylether (3′,4′,5-trimethoxyflavone) (b).

free hydroxyl groups are more physiologically active than their methylated derivatives because of their stronger free radical scavenging activity. Hence, hydroxylated polyphenols scavenge free radical species better, whereas fully methoxylated flavonoids can also effectively inhibit the enzymes like inducible nitric oxide synthase (iNOS) and NADPH oxidase that generate free radicals like NO and superoxide anion [11, 14]. Nobiletin, the most studied element of the PMF family, has been shown to inhibit the production of prostaglandin E2 (PGE2) in human synovial fibroblasts by selectively downregulating COX-2 [13, 15]. Gene expression of proinflammatory cytokines, such as IL-1α, IL-1β, TNF-α, and IL-6, was found downregulated by nobiletin, tangeretin, and 3,5,6,7,8,3′,4′-heptamethoxyflavone [15, 16]. It has been shown in an LPS-induced mouse macrophage model that nobiletin, tangeretin, and their derivatives moderately attenuated iNOS and COX-2 gene expression [16, 17]. Nevertheless, the available information about the biological effect of methoxylated flavonoids is not fully explored.

Methoxyflavones originate from plant-derived foods, especially from citrus peel, and could be released from hydroxyflavones by the catechol-O-methyltransferase [18] in the enterocytes. Due to their increased bioavailability [6], methoxylated flavones are more able to induce beneficial effects *in vivo* as compared to their hydroxylated analogs.

Intestinal epithelial cells play an important role in the innate immune response against pathogenic bacteria. Besides acting as a physical barrier, previous studies suggest that epithelial cells also have significant role in generating signals by the production of several cytokines, chemokines, and other signalling molecules [19–21]. The phosphoglycolipid LPS, component of the outer membrane in Gram-negative bacteria, is recognized by epithelial toll-like receptor-4 (TLR-4) [22]. Ligation of TLR initiates a signalling cascade that results in the activation of the transcription factor NF-κB and subsequent upregulation of costimulatory molecules as well as inflammatory cytokines and chemokines [23]. NF-κB also regulates the expression of COX-2, affecting the production of prostaglandins.

In vitro gut models offer a suitable alternative to *in vivo* animal experiments. Cancerous cell lines such as Caco-2 and HT-29 are widely used for this tool. However, the major disadvantage of cell lines originated from cancer tissues is that their glycosylation pattern, proliferation rate, and colonisation ability significantly differ from healthy tissues. The nontransformed porcine intestinal epithelial cell line IPEC-J2, originally isolated from jejunal epithelia of a neonatal unsuckled piglet, models *in vivo* structure and function of the small intestine more closely than colon tumorigenic cell lines. This cell line forms polarized monolayers with high transepithelial electrical resistance when cultured on 0.4 μm pore-size filters, developing apical and basolateral part [24]. Because of the abovementioned facts, IPEC-J2 cell line is a realistic and representative tool for mimicking the human as well as the pig small intestine. It can be good tool for pharmacology research, toxicity, microbiology, bioavailability, and metabolism studies in the field of human as well as veterinary medicine and food science [25].

The main aim of this study is to investigate the possible protective effects of methoxyflavones in intestinal epithelial cells under the condition of inflammation. Apigenin (3′,4′,5-trihydroxyflavone, Figure 1(a)), a well-studied and abundant flavonoid, and their methylated analogue (3′,4′,5-trimethoxyflavone, Figure 1(b)) were chosen as test compounds in order to study the abovementioned subject. This is the first report, which describes the effect of apigenin in intestinal inflammation using nontransformed intestinal epithelial cell line. Moreover, comparison of the anti-inflammatory effect of an unmethylated flavonoid and its methylated analogue in a nontransformed intestinal cell model was also performed at first time.

2. Materials and Methods

2.1. Chemicals. Apigenin (≥97%), dimethyl sulfoxide (≥99.7%, sterile-filtered, BioReagent), LPS (derived from *Salmonella enterica* ser. Typhimurium), and H_2O_2 (30%) were purchased from Sigma-Aldrich (Steinheim, Germany). Apigenin-trimethylether (≥99%) was from INDOFINE Chemical Company (Hillsborough, NJ, USA).

2.2. Cell Line and Culture Conditions. The nontransformed porcine intestinal epithelial cell line IPEC-J2, originally isolated from jejunal epithelia of a neonatal unsuckled piglet [24], was a kind gift from Dr. Jody Gookin, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA. IPEC-J2 cells were grown and maintained in complete medium, which consisted of a 1 : 1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) (plain medium) supplemented with 5% foetal bovine serum (FBS), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 5 ng/mL epidermal growth factor, and 1% penicillin-streptomycin (all from Fisher Scientific, Loughborough, UK).

Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Cell cultures were tested by PCR and they were found to be free of *Mycoplasma* contamination.

For the experiments, IPEC-J2 cells between passages 42 and 48 were seeded onto six-well Transwell polyester membrane inserts (Corning Inc., Corning, NY, USA), the latter coated with 8 μg/cm^2 rat tail collagen type I (Sigma-Aldrich, Steinheim, Germany), at a density of 1.5×10^5 cells/mL (the volume of complete medium was 1.5 mL on the apical side and 2.5 mL on the basolateral side per well according to the manufacturer's instructions). Cells were allowed to adhere for 24 h before being washed and refed every other day until confluence was reached. Transepithelial electrical resistance (TEER) measurement of monolayers was performed on alternate days after seeding, from days 5 to 21 of culture, using an EVOM Epithelial Tissue Volt/Ohmmeter (World Precision Instruments, Berlin, Germany).

2.3. Neutral Red Uptake Assay for Cell Viability. Influence of apigenin and apigenin-trimethylether on the viability of enterocytes in different concentrations (25, 50, and 100 μM) was tested. Flavonoids were dissolved in DMSO and diluted in cell culture medium. The final concentration of DMSO at the cells was 0.1%. In control experiments, this concentration did not show any effects on the measured parameters. IPEC-J2 cells were seeded in a 96-well plate and incubated with polyphenols for 1, 2, 4, and 24 h, respectively. Viability of IPEC-J2 cells was measured 24 hours after treatment by Neutral Red uptake assay as described by Repetto et al. [26].

2.4. Treatment of Cells with LPS. Before treatment, confluent monolayers of the IPEC-J2 cells were washed with plain medium. LPS solutions were prepared freshly prior to each experiment. LPS was added in plain medium at 10 μg/mL on the apical side of the IPEC-J2 layer. After 1 h incubation with LPS and flavones, cells were washed with plain medium and cultured for additional 1 h for PCR studies. TEER measurements were performed both before and after the LPS treatment.

2.5. Measurement of Extracellular H_2O_2. Fluorescent ROS measurement was based on the detection of H_2O_2 using the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen, Molecular Probes) keeping the IPEC-J2 cells on the 96-well plate. In the presence of horseradish peroxidase (HRP), Amplex Red reacts with H_2O_2 in a 1 : 1 stoichiometry producing a highly fluorescent resorufin [27].

IPEC-J2 cells were treated with LPS in phenol-red free DMEM and the H_2O_2 concentrations in the medium were determined using the working solution of 100 μM Amplex Red reagent and 0.2 U/mL HRP. H_2O_2 determination was also performed after 1 h LPS treatment immediately and 24 h incubation with phenol-red free DMEM. After 30 min incubation with the dye at room temperature the quantitative analyses of H_2O_2 contents were accomplished, the excitation wavelength was set at 560 nm, and emission was measured at 590 nm (Victor X2 2030 fluorometer, Perkin Elmer, Waltham, MA, USA).

2.6. Quantitative Real-Time PCR. One hour after the 1 h LPS treatment, culture medium was removed and 1 mL of ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to the IPEC-J2 samples. Samples were collected and kept at −80°C until further processing. Total RNA was isolated from the cells according to the manufacturer's instructions. To prevent DNA contamination, the isolated RNA (2 μg) was treated with AMP-D1 DNase I (Sigma). Quantity A_{260}/A_{280} and A_{260}/A_{230} ratios of the extracted RNA were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Quality and quantity control of the isolated RNA were carried out both before and after the DNase treatment.

Synthesis of the first strand of cDNA from 1000 ng of total RNA was achieved using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's recommendations, using the random hexamer as a priming method. Quantitative real-time PCR (qRT-PCR) was performed using the iQ SYBR Green Supermix kit (BioRad, Hercules, CA, USA) on the MiniOpticon System (BioRad). The cDNA was diluted 5-fold, before equal amounts were added to duplicate qRT-PCR reactions. Tested genes of interest were IL-6, IL-8, TNF-α, COX-2, and Hsp70. Hypoxanthine phosphoribosyl transferase (HPRT) and Cyclophilin-A (CycA) were used as reference genes. Primer sequences are listed in Table 1. For each PCR reaction, 2.5 μL cDNA was added directly to a PCR reaction mixture and set to a final volume of 25 μL, containing 1x concentrated iQ SYBR Green Supermix and 0.2 μM of the appropriate primers. The thermal profile for all reactions was 3 min at 95°C, then 40 cycles of 20 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. At the end of each cycle, the fluorescence monitoring was set for 10 seconds. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. In order to determine the efficiencies of the PCR reactions, standard curves were obtained for each target and reference gene, using serial dilutions of a reference cDNA. Real-time PCR efficiencies (E) were calculated according to the equation: $E = 10(-1/\text{slope})$. To determine the stability of the reference genes, the geNorm (version 3.5) was used.

2.7. Statistics. Relative gene expression levels of the genes of interest were calculated by the Relative Expression Software Tool (REST) 2009 Software (Qiagen GmbH, Hilden, Germany). Statistical analysis of other data was performed with STATISTICA 10 software (StatSoft Inc., Tulsa, USA). Differences between means were evaluated by one-way ANOVA, with data of normal distribution, and homogeneity of variances was confirmed. To compare treated groups to controls we used Dunnett post-hoc test. For the comparison of different treatments we used Fisher LSD test. Level of significance was set at $P < 0.05$. All values were expressed as means ± standard deviations.

3. Results

3.1. Viability of IPEC-J2 Cells after Flavonoid Treatment. Viability of IPEC-J2 cells was monitored after apigenin and

Table 1: Sequence of primer sets used for quantitative real-time.

Gene symbol	Accession number	Primer sequences	Product size (bp)	Efficiency	R^2	Reference
IL-8	NM_213867	F 5′-AGAGGTCTGCCTGGACCCCA-3′ R 5′-GGGAGCCACGGAGAATGGGT-3′	126	1.972	0.999	[28]
IL-6	NM_214399	F 5′-TTCACCTCTCCGGACAAAAC-3′ R 5′-TCTGCCAGTACCTCCTTGCT-3′	122	1.970	0.995	[29]
TNF-α	NM_214022	F 5′-TTCCAGCTGGCCCCTTGAGC-3′ R 5′-GAGGGCATTGGCATACCCAC-3′	146	1.873	0.982	[30]
COX-2	NM_214321	F 5′-AGAAGCGAGGACCAGCTTTC-3′ R 5′-AAAGCGGAGGTGTTCAGGAG-3′	215	1.905	0.981	NCBI/Primer-Blast
Hsp70	NM_001123127	F 5′-GCCCTGAATCCGCAGAATA-3′ R 5′-TCCCCACGGTAGGAAACG-3′	152	2.0	0.993	[31]
CycA	NM_214353	F 5′-GCGTCTCCTTCGAGCTGTT-3′ R 5′-CCATTATGGCGTGTGAAGTC-3′	160	1.907	0.998	[30]
HPRT	NM_001032376	F 5′-GGACTTGAATCATGTTTGTG-3′ R 5′-CAGATGTTTCCAAACTCAAC-3′	91	1.963	0.997	[32]

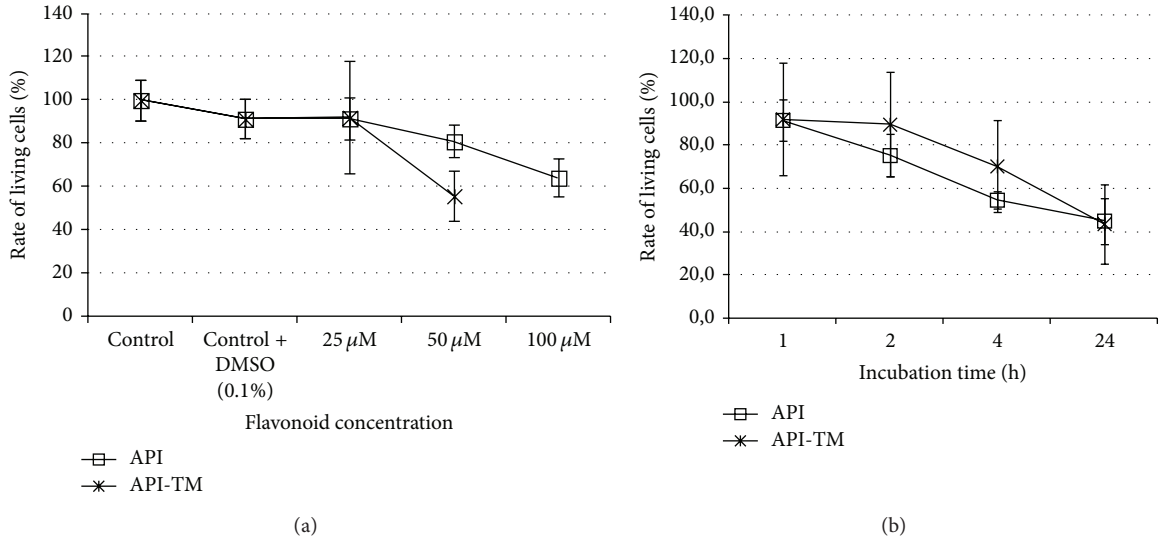

Figure 2: Rate of living IPEC-J2 cells after apigenin and apigenin-trimethylether treatment. Cell viability was tested using Neutral Red uptake method. Effect of flavones on cell viability was studied in a dose- (a) and time-dependent (b) manner. Data are shown as means + standard deviations.

apigenin-trimethyether treatment, respectively (see Figures 2(a) and 2(b)). Neutral Red uptake assay showed that there was no significant difference in number of viable IPEC-J2 cells incubated with plain DMEM containing 0.1% DMSO. At 25 μM treatment dose in case of both flavones for 1 h did not reduce the number of viable enterocytes significantly. More than 44% of the IPEC-J2 cells were killed by 1 h treatment when apigenin-trimethylether was applied at 50 μM concentrations, while incubation with 100 μM destroyed 55.9 ± 8.53% of cells. After 1 h exposure of 50 μM and 100 μM apigenin, reduced viability of IPEC-J2 cells was detected (rate of living cells decreased to 80.9±7.49% and 64.1±8.53%, resp., compared to the control samples). On the basis of the above-mentioned data, it seemed to be safe to use both flavones in 25 μM concentration for further experiments. Viability was tested also on a time-dependent manner, using 25 μM concentrations of polyphenols. Living cell rate decreased to 75.3 ± 9.9% after 2 h apigenin-trimethylether exposure, while 4 h and 24 h treatment greatly reduced number of living IPEC-J2 cells (rate of viable cells was 70.3 ± 20.1 and 43.6 ± 18.3, resp.). Reduced viability was observed when enterocytes were treated with 25 μM apigenin for 4 h and 24 h, respectively (in preliminary studies rate of viable cells was 54.7 ± 3.84 and 45.0 ± 10.5, resp.). Therefore, 1 h treatment period was used in further experiments.

3.2. Inflammatory Response in IPEC-J2 Cells after LPS Treatment. In order to verify the integrity of the IPEC-J2 cell layer, TEER values between apical and basolateral compartment were measured. Experiments were performed with confluent

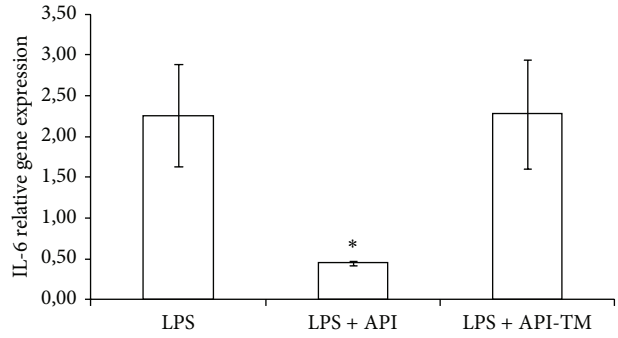

Figure 3: Relative gene expression of IL-6 in IPEC-J2 cells exposed to LPS treatment (at 1 μg/mL; 1 h treatment). Effect of apigenin (25 μM) and apigenin-trimethylether (25 μM) on the IL-6 mRNA levels (n = 3-4/group; $^*P < 0.05$). Data are shown as means + standard deviations. API = apigenin; API-TM = apigenin-trimethylether.

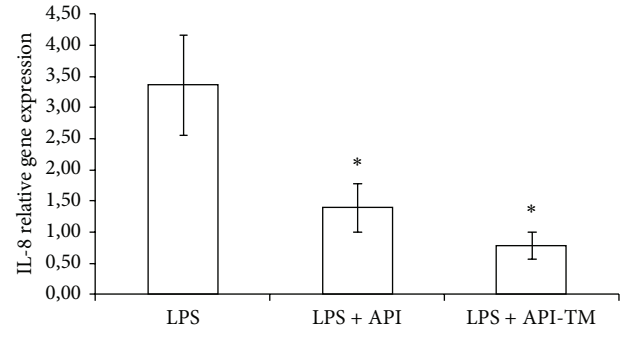

Figure 4: Relative gene expression of IL-8 in IPEC-J2 cells exposed to LPS treatment (at 1 μg/mL; 1 h treatment). Effect of apigenin (25 μM) and apigenin-trimethylether (25 μM) on the IL-8 mRNA levels (n = 3-4/group; $^*P < 0.05$). Data are shown as means + standard deviations. API = apigenin; API-TM = apigenin-trimethylether.

polarized IPEC-J2 cells with high TEER values. The integrity of the cell monolayer was not altered after LPS treatment (data not shown).

Relative gene expressions of IL-6, IL-8, TNF-α, COX-2, and Hsp70 in LPS-triggered IPEC-J2 cells were determined. LPS treatment significantly increased TNF-α ($P = 0.018$), IL-6 ($P = 0.044$), and IL-8 ($P = 0.001$) relative gene expression levels. Significant upregulation of COX-2 gene was also observed after 1 h 10 μg/mL LPS administration ($P = 0.012$). There was no alteration in the mRNA level of heat shock protein 70 ($P = 0.375$) after LPS treatment.

3.3. Effect of Apigenin and Apigenin-Trimethylether on the Relative Gene Expression of Cytokines TNF-α, IL-6, and IL-8 in LPS-Treated Enterocytes. At 25 μM apigenin treatment dose, relative gene expression of IL-6 significantly decreased compared to LPS-treated cells ($P = 0.0348$). Apigenin-trimethylether in the same concentration did not influence the IL-6 mRNA level. Both apigenin ($P = 0.0009$) and apigenin-trimethylether ($P = 0.0014$) caused significant reduction in the IL-8 gene expression. There was no significant difference in the IL-8 gene expression reducing effect of apigenin and its unmethylated analogue. TNF-α mRNA level was decreased by 25 μM apigenin-trimethylether treatment ($P = 0.0081$), while apigenin did not suppress TNF-α mRNA level compared to the LPS-treated enterocytes. Figures 3–5 show the effect of flavonoids on proinflammatory cytokine gene expressions.

3.4. Cox-2 Relative Gene Expression. Apigenin and apigenin-trimethylether treatment caused significant reduction in the mRNA level of COX-2 ($P = 0.0287$, $P = 0.0006$) Effect of apigenin and apigenin-trimethylether was compared using one-way ANOVA (Fisher LSD test). It was shown that there is a significant difference between the effect of hydroxy and methoxy-analogue ($P = 0.0264$). The influence of apigenin and its trimethylated derivative on COX-2 relative gene expression could be seen in Figure 6.

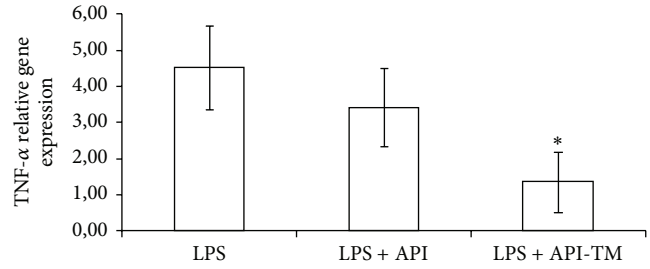

Figure 5: Relative gene expression of TNF-α in IPEC-J2 cells exposed to LPS treatment (at 1 μg/mL; 1 h treatment). Effect of apigenin (25 μM) and apigenin-trimethylether (25 μM) on the TNF-α mRNA levels (n = 3-4/group; $^*P < 0.05$). Data are shown as means + standard deviations. API = apigenin; API-TM = apigenin-trimethylether.

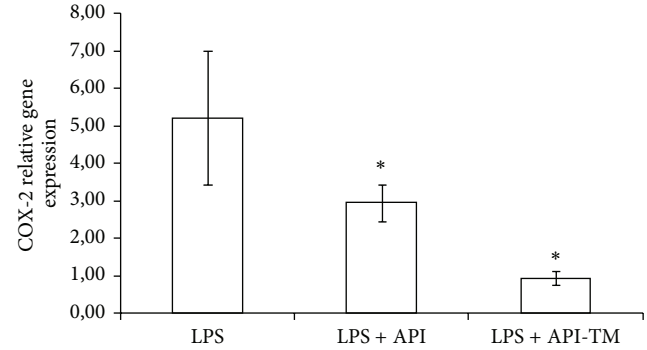

Figure 6: Relative gene expression of COX-2 in IPEC-J2 cells exposed to LPS treatment (at 1 μg/mL; 1 h treatment). Effect of apigenin (25 μM) and apigenin-trimethylether (25 μM) on the COX-2 mRNA levels (n = 3-4/group; $^*P < 0.05$). Data are shown as means + standard deviations. API = apigenin; API-TM = apigenin-trimethylether.

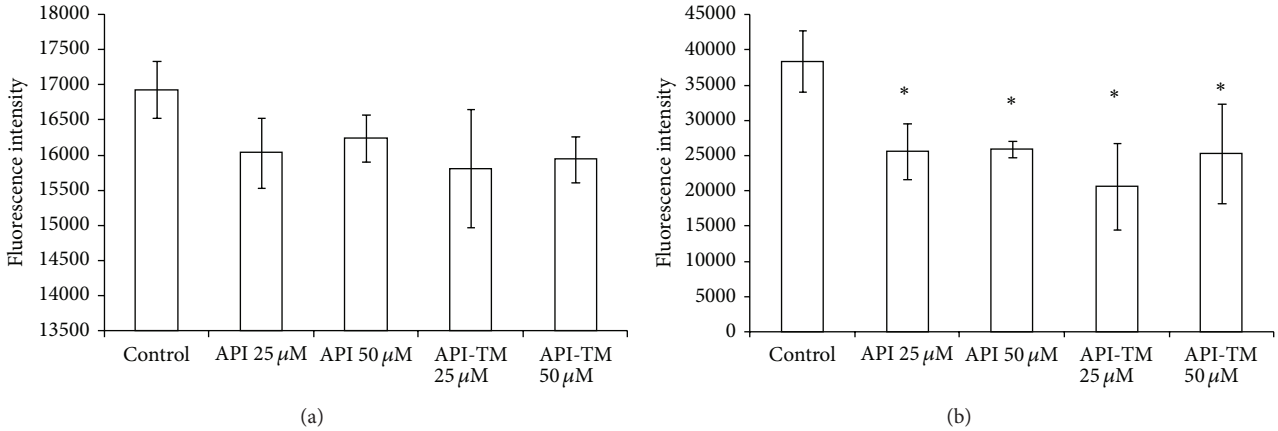

FIGURE 7: Level of extracellular H_2O_2 in IPEC-J2 cells exposed to flavonoid treatment (25 and 50 μM; 1 h). Effect of apigenin and apigenin-trimethylether on the relative extracellular H_2O_2 levels (n = 3-4/group; $^*P < 0.05$). Fluorescence measurement was performed by Amplex Red method. Fluorescence was detected immediately (a) and 24 h after (b) flavonoid treatment. Data are shown as means + standard deviations. API = apigenin; API-TM = apigenin-trimethylether.

3.5. Extracellular H_2O_2 Production in IPEC-J2 Cells after LPS and Flavonoid Treatment. Figure 7 shows the relative extracellular H_2O_2 level in IPEC-J2 cells after flavonoid treatment. LPS only at higher concentration (at 50 μg/mL) increased the level of extracellular H_2O_2; however, IPEC-J2 cells are irreversibly damaged using LPS in this concentration (data not shown). Flavonoids showed different effects on the rate of H_2O_2 production, depending on the duration of incubation in DMEM after flavonoid treatment. In case of short time effect measurements (1 h incubation with flavonoids and detection immediately after the incubation period), neither apigenin nor its trimethylated analogue decreased mitochondrial H_2O_2 production rate effectively. Long time effects of flavonoid treatment (Amplex Red measurement was performed 24 h after the 1 h flavonoid incubation) were also studied. Extracellular H_2O_2 level was significantly decreased in case of both lower (25 μM) and higher (50 μM) concentration apigenin treatments. The same reducing effect was found, when 25 μM and 50 μM apigenin-trimethylether were applied.

4. Discussion

LPS, a major integral component of the outer membrane of Gram-negative bacteria, is widely used to induce inflammation in case of several cell types, including small intestinal cell lines such as IPEC-J2. NF-κB activation resulted in the expression of a wide range of genes, many of which are markedly upregulated in response to microbial infection and inflammation. Some of these are cytokines and chemokines (IL-1, IL-6, IL-8, and TNF-α) and inflammatory enzymes such as COX-2. The inhibition of signal transduction pathways at any point of the inflammatory cascade reduces the production of these proteins, which modulates the inflammation locally [33].

Considering the literature [21, 34, 35] and our previous results, detection of relative gene expression of proinflammatory cytokines in IPEC-J2 intestinal epithelial cells was optimal after 1 and 2 hours [36, 37]. In our experiments, 1 h incubation of IPEC-J2 cells with LPS (and further 1 h incubation with medium) resulted in significantly high relative IL-6, IL-8, and TNF-α as well as COX-2 gene expression level. Relative expression of the abovementioned genes was tested 4 h after LPS treatment as well, and those results did not differ significantly from the expression data from 1 h LPS treatment. Expression of TLR-4, a key element of the activation of NF-κB, in IPEC-J2 cells after LPS treatment was also measured in our previous study [37]. No difference in the TLR-4 expression was detected compared to the control samples, using different concentrations, incubation times, and different types of LPS. Our data agrees with the results of other studies [23, 38, 39], that epithelial cell lines often show a low level expression of TLR-4, explained by the fact that intestinal epithelial cells are relative resistant to the permanent exposure to Gram-negative commensal bacteria.

The *in vitro* anti-inflammatory effect of apigenin was studied in many cases. However, these reports were performed using cancerous cell lines or immune cells. LPS-stimulated human peripheral blood mononuclear cells were cultured in the presence of apigenin, and TNF-α, IL-1β, and IL-6 were measured in the cell culture supernatant [40]. Apigenin inhibited proinflammatory cytokine production dose-dependently. Murine macrophage cells and mice were treated with LPS and herbal constituents by Smolinski and Pestka [41]. IL-6 and TNF-α were measured from serum and supernatant by ELISA. All three constituents including apigenin inhibited LPS-induced IL-6 and TNF-α production in murine macrophage culture. Inhibition of these two cytokines in mice did not display the same patterns of inhibition as cell culture data. *In vitro* cotreatment with apigenin reduced LPS-induced IL-6, but not TNF-α production. Cotreatment with 3.7 and 37 mM of apigenin significantly reduced IL-6, while inhibition of TNF-α was not observed. However, a study of Mastuda et al. [42] showed that apigenin, at IC50 = 5.3 mM, inhibited antigen-IgE-mediated TNF-α secretion in RBL-2H3 mast cells. Apigenin also inhibited

TNF-α production as well as iNOS expression and NO production in LPS-activated macrophages, and this effect has been associated with the inhibition of the NF-κB pathway [43]. Apigenin inhibited TNF-α secretion in a concentration-dependent manner. It shows only a slight effect on TNF-α release that did not reach 25 μM concentration, whereas apigenin decreases TNF-α release by 41.6% at 50 μM. In our model, apigenin (25 μM) treatment reduced both IL-6 and IL-8 mRNA level significantly compared to the LPS-treated group, while it did not affect TNF-α. A potential reason for the differences in results could be the differences in stimuli and cell types used.

In a study of Wang and Huang [44] the anti-inflammatory effects of apigenin in *Helicobacter pylori*-infected human stomach adenocarcinoma cells (MKN45) were investigated and expression of I-κB-a, COX-2, and proinflammatory cytokines was measured. Apigenin treatment (9.3–74 μM) significantly increased I-κB-a expression and thus inhibited NF-κB activation. Expression of IL-8, IL-6, and COX-2 was significantly decreased. Apigenin inhibited the production of NO and PGE2 by suppressing the expression of iNOS and COX-2 protein in murine microglia cell line model [45]. Moreover, it suppressed p38 mitogen-activated protein kinase and c-Jun N-terminal kinase (JNK) phosphorylation without affecting the activity of extracellular signal-regulated kinase (ERK).

In terms of anti-inflammatory effect, only a few studies have reported methoxyflavones. The most studied methoxylated flavones originated from citrus peel. *Citrus aurantium* L. extract containing nobiletin, naringin, and hesperidin inhibited the proinflammatory mediators including cytokines, COX-2, and iNOS by blocking NF-κB and mitogen-activated protein kinase (MAPK) signalling in LPS-stimulated macrophages. The molecular mechanism was associated with inhibition of the phosphorylation of I-κB-a and nuclear translocation of the NF-κB p-65 as well as phosphorylation of MAPK by flavonoids [46]. Contrarily, a formulated product from citrus peel extract (Gold Lotion) inhibited the gene expression of iNOS, but not COX-2 in a mouse skin inflammatory model. These differential effects may be explained due to the degree of dependency of iNOS and COX-2 promoters on various transcription factors [47]. Nobiletin has been shown to significantly suppress the activation of activator protein 1, NF-κB, and cAMP response element-binding protein (CREB) [13].

During and Larondelle [48] studied whether methoxylated flavones versus their unmethylated analogs can modulate the intestinal inflammatory response. After IL-1β stimulus of Caco-2 cells, the anti-inflammatory activity of apigenin, chrysin, luteolin, and quercetin was investigated. Their results indicate that methoxylation of chrysin improves its anti-inflammatory effect significantly, probably through a more explicit inhibition of the NF-κB signaling pathway. Nevertheless, the abovementioned effect was not observed in case of other flavonoids. Furthermore, neither apigenin nor its methylated analogue reduced IL-8 level significantly, but IL-6 and PGE-2 concentration were reduced by both compounds. It seems that hydroxyflavones and their methoxylated analogues share the same signal transduction pathways; however, methoxyflavones needs further investigations.

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, referred to as reactive oxygen species (ROS), and their elimination by protective mechanisms, so-called antioxidants [49]. This loss of equilibrium leads to damage of important biomolecules, with potential impact on the whole organism. Oxidative stress could activate a variety of transcription factors including NF-κB and lead to chronic inflammation. Flavonoid antioxidants could protect cells against the damaging effects of ROS [50].

In this study, some oxidative stress markers were also followed. It was found that neither the mRNA level of Hsp70 nor level of extracellular H_2O_2 was affected by 10 μg/mL LPS treatment. LPS in higher concentration (50 μg/mL) increased extracellular H_2O_2 level significantly; however, IPEC-J2 cells are irreversibly damaged (data not shown).

Nevertheless, effect of flavones on oxidative stress markers is worthy to be investigated, because they could act not only as antioxidants but also as prooxidants; that is, they are potential inducers of oxidative stress. The prooxidant potential of apigenin was studied by Miyoshi et al. [51]. Flow cytometric measurements and immunoblotting showed the intracellular accumulations of ROS and protein carbonyls in the cells treated with apigenin in a dose-dependent manner. In HL-60 cells treated with 50 μM apigenin for 1 h, ROS level was 3.5-fold increased compared to control cells. Structure-activity relationship data suggested that presence of 4-hydroxy group and also the absence of 3-and 3′-hydroxy groups are important for prooxidant effect. Treatment cells with 25 μM apigenin did not affect the extracellular ROS status. On the basis of our measurements in IPEC-2 intestinal epithelial cells, no prooxidant effect was observed. Moreover, extracellular H_2O_2 level in IPEC-J2 cells was significantly decreased 24 h after flavonoid treatment in case of both apigenin and apigenin-trimethylether. Deng et al. [52] compared the cytoprotective effect of methylated polyphenols and their unmethylated analogues. They found that methylation largely reduced chemical antioxidant capacity, but methylated flavons can still effectively protect lymphocytes from hydrogen peroxide-induced cytotoxicity.

The biological fate of the flavonoids is very complex, and it is dependent on a large number of parameters. Different activities could be influenced by the different absorption rate and metabolism. Absorption and metabolism studies of hydroxyflavones and methoxyflavones have been investigated primarily in cancer studies [6, 53]. Caco-2 cell model was established to test the absorption as well as permeability of polymethoxyflavones in the human intestine. Three compounds (3′-hydroxy-5,6,7,4′-tetramethoxyflavone, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and 3-hydroxy-5,6,7,8,3′,4′-hexamethoxyflavone) showed very high permeability [54, 55]. The membrane transporters as well as metabolic enzymes could influence the cellular availability of polyphenols, affecting their anticancer potential. Nevertheless, absorptive and metabolism properties of these compounds could influence their anti-inflammation activities in the same way.

In conclusion, methylated flavonoids including apigenin-trimethylether may be useful tools in the treatment of intestinal inflammations in human as well in veterinary medicine. Future perspectives should include understanding the molecular basis for inhibitory effects of these compounds on proinflammatory cytokine gene expression and the role of altered absorption and metabolism.

Abbreviations

LPS: Lipopolysaccharide
IL-6: Interleukin-6
IL-8: Interleukin-8
TNF-α: Tumor necrosis factor alpha
COX-2: Cyclooxygenase 2.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] K. Bisht, K.-H. Wagner, and A. C. Bulmer, "Curcumin, resveratrol and flavonoids as anti-inflammatory, cyto- and DNA-protective dietary compounds," *Toxicology*, vol. 278, no. 1, pp. 88–100, 2010.

[2] R. J. Williams, J. P. E. Spencer, and C. Rice-Evans, "Flavonoids: antioxidants or signalling molecules?" *Free Radical Biology and Medicine*, vol. 36, no. 7, pp. 838–849, 2004.

[3] B. H. Havsteen, "The biochemistry and medical significance of the flavonoids," *Pharmacology and Therapeutics*, vol. 96, no. 2-3, pp. 67–202, 2002.

[4] A. Murakami, Y. Nakamura, K. Torikai et al., "Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion mice," *Cancer Research*, vol. 60, no. 18, pp. 5059–5066, 2000.

[5] S. Li, C. Y. Lo, and C. T. Ho, "Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 12, pp. 4176–4185, 2006.

[6] T. Walle, N. Ta, T. Kawamori, X. Wen, P. A. Tsuji, and U. K. Walle, "Cancer chemopreventive properties of orally bioavailable flavonoids—methylated versus unmethylated flavones," *Biochemical Pharmacology*, vol. 73, no. 9, pp. 1288–1296, 2007.

[7] U. K. Walle and T. Walle, "Bioavailable flavonoids: cytochrome P450-mediated metabolism of methoxyflavones," *Drug Metabolism and Disposition*, vol. 35, no. 11, pp. 1985–1989, 2007.

[8] J. A. Manthey, K. Grohmann, and N. Guthrie, "Biological properties of citrus flavonoids pertaining to cancer and inflammation," *Current Medicinal Chemistry*, vol. 8, no. 2, pp. 135–153, 2001.

[9] J. A. Manthey and N. Guthrie, "Antiproliferative activities of citrus flavonoids against six human cancer cell lines," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 21, pp. 5837–5843, 2002.

[10] J. Wang, Y. Duan, D. Zhi et al., "Pro-apoptotic effects of the novel tangeretin derivate 5-acetyl-6,7,8,4′-tetramethylnortangeretin on MCF-7 breast cancer cells," *Cell Biochemistry and Biophysics*, vol. 70, no. 2, pp. 1255–1263, 2014.

[11] S.-Y. Choi, H.-C. Ko, S.-Y. Ko et al., "Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits," *Biological and Pharmaceutical Bulletin*, vol. 30, no. 4, pp. 772–778, 2007.

[12] A. Murakami, S. Kuwahara, Y. Takahashi et al., "In vitro absorption and metabolism of nobiletin, a chemopreventive polymethoxyflavonoid in citrus fruits," *Bioscience, Biotechnology and Biochemistry*, vol. 65, no. 1, pp. 194–197, 2001.

[13] A. Murakami, T. Shigemori, and H. Ohigashi, "Zingiberaceous and citrus constituents, 1′-acetoxychavicol acetate, zerumbone, auraptene, and nobiletin, suppress lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 murine macrophages through different modes of action," *Journal of Nutrition*, vol. 135, no. 12, supplement, pp. 2987S–2992S, 2005.

[14] A. Murakami, Y. Nakamura, Y. Ohto et al., "Suppressive effects of citrus fruits on free radical generation and nobiletin, an anti-inflammatory polymethoxyflavonoid," *BioFactors*, vol. 12, no. 1–4, pp. 187–192, 2000.

[15] N. Lin, T. Sato, Y. Takayama et al., "Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages," *Biochemical Pharmacology*, vol. 65, no. 12, pp. 2065–2071, 2003.

[16] J. A. Manthey, K. Grohmann, A. Montanari, K. Ash, and C. L. Manthey, "Polymethoxylated flavones derived from citrus suppress tumor necrosis factor-alpha expression by human monocytes," *Journal of Natural Products*, vol. 62, no. 3, pp. 441–444, 1999.

[17] S. Li, S. Sang, M.-H. Pan et al., "Anti-inflammatory property of the urinary metabolites of nobiletin in mouse," *Bioorganic and Medicinal Chemistry Letters*, vol. 17, no. 18, pp. 5177–5181, 2007.

[18] H. Lu, X. Meng, and C. S. Yang, "Enzymology of methylation of tea catechins and inhibition of catechol-O-methyltransferase by (−)-epigallocatechin gallate," *Drug Metabolism and Disposition*, vol. 31, no. 5, pp. 572–579, 2003.

[19] F. Bäckhed, S. Normark, E. K. H. Schweda, S. Oscarson, and A. Richter-Dahlfors, "Structural requirements for TLR4-mediated LPS signalling: a biological role for LPS modifications," *Microbes and Infection*, vol. 5, no. 12, pp. 1057–1063, 2003.

[20] R. S. Pitman and R. S. Blumberg, "First line of defense: the role of the intestinal epithelium as an active component of the mucosal immune system," *Journal of Gastroenterology*, vol. 35, no. 11, pp. 805–814, 2000.

[21] C. Arce, M. Ramírez-Boo, C. Lucena, and J. J. Garrido, "Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from *Salmonella typhimurium*," *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 33, no. 2, pp. 161–174, 2010.

[22] M. T. Abreu, "Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function," *Nature Reviews Immunology*, vol. 10, no. 2, pp. 131–143, 2010.

[23] T. E. Burkey, K. A. Skjolaas, S. S. Dritz, and J. E. Minton, "Expression of Toll-like receptors, interleukin 8, macrophage migration inhibitory factor, and osteopontin in tissues from pigs challenged with *Salmonella enterica* serovar Typhimurium or serovar Choleraesuis," *Veterinary Immunology and Immunopathology*, vol. 115, no. 3-4, pp. 309–319, 2007.

[24] P. Schierack, M. Nordhoff, M. Pollmann et al., "Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine," *Histochemistry and Cell Biology*, vol. 125, no. 3, pp. 293–305, 2006.

[25] T. Langerholc, P. A. Maragkoudakis, J. Wollgast, L. Gradisnik, and A. Cencic, "Novel and established intestinal cell line models—an indispensable tool in food science and nutrition," *Trends in Food Science and Technology*, vol. 22, no. 1, pp. S11–S20, 2011.

[26] G. Repetto, A. del Peso, and J. L. Zurita, "Neutral red uptake assay for the estimation of cell viability/cytotoxicity," *Nature Protocols*, vol. 3, no. 7, pp. 1125–1131, 2008.

[27] J. G. Mohanty, J. S. Jaffe, E. S. Schulman, and D. G. Raible, "A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative," *Journal of Immunological Methods*, vol. 202, no. 2, pp. 133–141, 1997.

[28] E. Paszti-Gere, E. Csibrik-Nemeth, K. Szeker, R. Csizinszky, C. Jakab, and P. Galfi, "Acute oxidative stress affects IL-8 and TNF-alpha expression in IPEC-J2 porcine epithelial cells," *Inflammation*, vol. 35, no. 3, pp. 994–1004, 2012.

[29] R. Sakumoto, T. Komatsu, E. Kasuya, T. Saito, and K. Okuda, "Expression of mRNAs for interleukin-4, interleukin-6 and their receptors in porcine corpus luteum during the estrous cycle," *Domestic Animal Endocrinology*, vol. 31, no. 3, pp. 246–257, 2006.

[30] K. A. Hyland, D. R. Brown, and M. P. Murtaugh, "*Salmonella enterica* serovar Choleraesuis infection of the porcine jejunal Peyer's patch rapidly induces IL-1β and IL-8 expression," *Veterinary Immunology and Immunopathology*, vol. 109, no. 1-2, pp. 1–11, 2006.

[31] X. Zhong, T. Wang, X. Zhang, and W. Li, "Heat shock protein 70 is upregulated in the intestine of intrauterine growth retardation piglets," *Cell Stress and Chaperones*, vol. 15, no. 3, pp. 335–342, 2010.

[32] A.-B. Nygard, C. B. Jørgensen, S. Cirera, and M. Fredholm, "Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR," *BMC Molecular Biology*, vol. 8, article 67, 2007.

[33] J. J. Malago, J. F. J. G. Koninkx, and J. E. van Dijk, "The heat shock response and cytoprotection of the intestinal epithelium," *Cell Stress & Chaperones*, vol. 7, no. 2, pp. 191–199, 2002.

[34] M. M. Geens and T. A. Niewold, "Preliminary characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enterotoxigenic *Escherichia coli*, *Escherichia coli*, and *E. coli* lipopolysaccharide," *Comparative and Functional Genomics*, vol. 2010, Article ID 469583, 11 pages, 2010.

[35] T. Angrisano, R. Pero, S. Peluso et al., "LPS-induced IL-8 activation in human intestinal epithelial cells is accompanied by specific histone H3 acetylation and methylation changes," *BMC Microbiology*, vol. 10, article 172, 2010.

[36] E. Paszti-Gere, G. Matis, O. Farkas et al., "The effects of intestinal LPS exposure on inflammatory responses in a porcine enterohepatic co-culture system," *Inflammation*, vol. 37, no. 1, pp. 247–260, 2014.

[37] O. Farkas, G. Matis, E. Paszti-Gere et al., "Effects of *Lactobacillus plantarum* 2142 and sodium n-butyrate in lipopolysaccharide-triggered inflammation: comparison of a porcine intestinal epithelial cell line and primary hepatocyte monocultures with a porcine enterohepatic co-culture system," *Journal of Animal Science*, vol. 92, no. 9, pp. 3835–3845, 2014.

[38] M. T. Abreu, P. Vora, E. Faure, L. S. Thomas, E. T. Arnold, and M. Arditi, "Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide," *Journal of Immunology*, vol. 167, no. 3, pp. 1609–1616, 2001.

[39] J. Alvarado, P. Taylor, J. R. del Castillo, and L. E. Thomas, "Interferon gamma bound to extracellular matrix changes the hyporesponsiveness to LPS in crypt but not villous intestinal epithelial cells," *Immunology Letters*, vol. 99, no. 1, pp. 109–112, 2005.

[40] S. Hougee, A. Sanders, J. Faber et al., "Decreased pro-inflammatory cytokine production by LPS-stimulated PBMC upon in vitro incubation with the flavonoids apigenin, luteolin or chrysin, due to selective elimination of monocytes/macrophages," *Biochemical Pharmacology*, vol. 69, no. 2, pp. 241–248, 2005.

[41] A. T. Smolinski and J. J. Pestka, "Modulation of lipopolysaccharide-induced proinflammatory cytokine production in vitro and in vivo by the herbal constituents apigenin (chamomile), ginsenoside Rb1 (ginseng) and parthenolide (feverfew)," *Food and Chemical Toxicology*, vol. 41, no. 10, pp. 1381–1390, 2003.

[42] H. Mastuda, T. Morikawa, K. Ueda, H. Managi, and M. Yoshikawa, "Structural requirements of flavonoids for inhibition of antigen-induced degranulation, TNF-α and IL-4 production from RBL-2H3 cells," *Bioorganic & Medicinal Chemistry*, vol. 10, no. 10, pp. 3123–3128, 2002.

[43] M. Comalada, I. Ballester, E. Bailón et al., "Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship," *Biochemical Pharmacology*, vol. 72, no. 8, pp. 1010–1021, 2006.

[44] Y.-C. Wang and K.-M. Huang, "In vitro anti-inflammatory effect of apigenin in the *Helicobacter pylori*-infected gastric adenocarcinoma cells," *Food and Chemical Toxicology*, vol. 53, pp. 376–383, 2013.

[45] S. K. Ha, P. Lee, J. A. Park et al., "Apigenin inhibits the production of NO and PGE_2 in microglia and inhibits neuronal cell death in a middle cerebral artery occlusion-induced focal ischemia mice model," *Neurochemistry International*, vol. 52, no. 4-5, pp. 878–886, 2008.

[46] S. R. Kang, K. I. Park, H. S. Park et al., "Anti-inflammatory effect of flavonoids isolated from Korea Citrus aurantium L. on lipopolysaccharide-induced mouse macrophage RAW 264.7 cells by blocking of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways," *Food Chemistry*, vol. 129, no. 4, pp. 1721–1728, 2011.

[47] M.-H. Pan, S. Li, C.-S. Lai, Y. Miyauchi, M. Suzawa, and C.-T. Ho, "Inhibition of citrus flavonoids on 12-O-tetradecanoylphorbol 13-acetate-induced skin inflammation and tumorigenesis in mice," *Food Science and Human Wellness*, vol. 1, no. 1, pp. 65–73, 2012.

[48] A. During and Y. Larondelle, "The O-methylation of chrysin markedly improves its intestinal anti-inflammatory properties: structure-activity relationships of flavones," *Biochemical Pharmacology*, vol. 86, no. 12, pp. 1739–1746, 2013.

[49] S. Reuter, S. C. Gupta, M. M. Chaturvedi, and B. B. Aggarwal, "Oxidative stress, inflammation, and cancer: how are they linked?" *Free Radical Biology and Medicine*, vol. 49, no. 11, pp. 1603–1616, 2010.

[50] C. Rice-Evans, "Flavonoid antioxidants," *Current Medicinal Chemistry*, vol. 8, no. 7, pp. 797–807, 2001.

[51] N. Miyoshi, K. Naniwa, T. Yamada, T. Osawa, and Y. Nakamura, "Dietary flavonoid apigenin is a potential inducer of intracellular oxidative stress: the role in the interruptive apoptotic signal," *Archives of Biochemistry and Biophysics*, vol. 466, no. 2, pp. 274–282, 2007.

[52] D. W. Deng, J. L. Zhang, J. M. Cooney et al., "Methylated polyphenols are poor 'chemical' antioxidants but can still effectively protect cells from hydrogen peroxide-induced cytotoxicity," *FEBS Letters*, vol. 580, no. 22, pp. 5247–5250, 2006.

[53] T. Walle, "Absorption and metabolism of flavonoids," *Free Radical Biology and Medicine*, vol. 36, no. 7, pp. 829–837, 2004.

[54] L. Wang, J. Wang, L. Fang et al., "Anticancer activities of citrus peel polymethoxyflavones related to angiogenesis and others," *BioMed Research International*, vol. 2014, Article ID 453972, 10 pages, 2014.

[55] S. Li, M.-H. Pan, C.-Y. Lo et al., "Chemistry and health effects of polymethoxyflavones and hydroxylated polymethoxyflavones," *Journal of Functional Foods*, vol. 1, no. 1, pp. 2–12, 2009.

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Research Article

Effect of the French Oak Wood Extract Robuvit on Markers of Oxidative Stress and Activity of Antioxidant Enzymes in Healthy Volunteers: A Pilot Study

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We examined *in vitro* antioxidant capacity of polyphenolic extract obtained from the wood of oak *Quercus robur* (QR), Robuvit, using TEAC (Trolox equivalent antioxidant capacity) method and the effect of its intake on markers of oxidative stress, activity of antioxidant enzymes, and total antioxidant capacity in plasma of 20 healthy volunteers. Markers of oxidative damage to proteins, DNA, and lipids and activities of Cu/Zn-superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined in the erythrocytes. We have found an *in vitro* antioxidant capacity of Robuvit of 6.37 micromole Trolox equivalent/mg of Robuvit. One month intake of Robuvit in daily dose of 300 mg has significantly decreased the serum level of advanced oxidation protein products (AOPP) and lipid peroxides (LP). Significantly increased activities of SOD and CAT as well as total antioxidant capacity of plasma after one month intake of Robuvit have been shown. In conclusion, we have demonstrated for the first time that the intake of Robuvit is associated with decrease of markers of oxidative stress and increase of activity of antioxidant enzymes and total antioxidant capacity of plasma *in vivo*.

1. Introduction

The French oak wood extract Robuvit (Horpag Research Ltd.) is a registered proprietary water extract obtained from the wood of *Quercus robur* (QR). The plant belongs to the plant family Fagacae, genus *Quercus*.

The oak wood used for Robuvit originates exclusively from oak trees grown in France. Oak wood contains a specific profile of tannins named roburins that are part of the ellagitannins (ETs). Robuvit is standardized and specified to contain at least 20% of roburins (A, B, C, D, and E) including grandinin. The two most abundant ETs in the Robuvit are stereoisomers vescalagin and castalagin, which were originally isolated and described by Mayer et al. [1]. Roburins and grandinin are dimers of these compounds or differ by the presence of a pentose substituent. They were isolated and identified later by du Penhoat et al. [2]. Further to the roburins, Robuvit contains monomeric vescalagin and castalagin as well as ellagic acid (EA) and gallic acid (GA) [3].

Owing to their unique molecular structure roburins are very potent antioxidants. Humans have been exposed to these compounds for centuries from wine and spirits that matured in oak wood barrels. Oak wood is currently the only known food source of roburins, and, according to this specificity, the major source of roburins in human diets results from the consumption of wine and spirits (cognac and whiskey) traditionally matured, aged, and stored in oak barrels [4].

Little is known about roburins bioavailability and biological effects. Natella et al. [3] found out that Robuvit is bioavailable to humans and its consumption is associated with increase of antioxidant capacity at hydrophilic conditions. They identified beside GA and EA also metabolites of ETs named urolithins in the plasma of volunteers after intake of Robuvit. Urolithins are metabolites of EA, which are released from ETs by hydrolysis in the intestine by gut microflora [5]. Effects of EA, one of compounds of Robuvit, were examined in many models *in vitro* and *in vivo*. EA is characterized by antioxidant [6–9], anticarcinogenic [10–13], antiproliferative [9, 14], anti-inflammatory [15, 16], proapoptotic [17], and antiplatelet properties [18].

The aim of our study was to examine *in vitro* antioxidant capacity of Robuvit using TEAC method and the *in vivo* effect of its intake on oxidative stress in healthy volunteers. The level of markers of oxidative stress (markers of oxidative damage to proteins, lipids, and DNA), activities of antioxidant enzymes Cu/Zn-superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and total antioxidant capacity of plasma were investigated.

2. Material and Methods

2.1. Subjects. Twenty healthy volunteers (12 women and 8 men, mean age 54.2 ± 6.56; range: 45–65 years) were included in the study. All participants gave a written informed agreement to participate in the study. The study was approved by the Ethical Committee of University Hospital and Faculty of Medicine, Comenius University in Bratislava, Slovakia. Subjects with acute inflammatory, renal and cardiovascular diseases, and diabetes mellitus and women with hormone substitution therapy were excluded from the study.

The 8-week experiment was divided into three periods. In the first period (run-in period) volunteers were instructed to control their diet for 2 weeks. No additional antioxidants like vitamins C, E or coenzyme Q or excess of chocolate, red wine, or beer should be consumed. Drinking a cup of green tea, 2 dL red wine, or 1 beer daily was allowed. After run-in period, Robuvit administration began (week 0). Volunteers took 1 capsule (100 mg) of extract Robuvit three times a day (300 mg daily) for 4 weeks, followed by two-week wash-out period in which capsules were not administered. The extract was supplied by Horphag Research Ltd., Switzerland.

2.2. Sample Preparation. The venous blood was collected after 12-hour overnight fast into commercial serum tubes and EDTA coated tubes. Within 1 hour of collection, blood was centrifuged (700 ×g, 5 min); serum and plasma were aliquoted and frozen at −80°C until analysis. For isolation of erythrocytes, blood was washed three times with 0.15 mol/L NaCl solution. After final centrifugation (700 ×g, 7 min), erythrocytes were haemolysed by addition of triple volume of distilled water and haemolysate was frozen at −20°C until analysis.

The samples of blood were taken after run-in period (week 0), at the end of intervention period (week 4) and at the end of wash-out period (week 6).

In the serum of the basic biochemical parameters, the concentrations of advanced oxidation protein products (AOPP) and lipid peroxides (LP) were determined; in the plasma the levels of advanced glycation end-products (AGEs), 8-isoprostanes (8-isoP), protein carbonyls, and total antioxidant capacity were examined. In isolated lymphocytes marker of oxidative damage to DNA and 8-oxoguanin (8-oxoG) was assessed. The concentration of haemoglobin by Drabkin method and SOD, CAT, and GPx activities were measured in haemolysate of erythrocytes.

2.3. Chemicals. ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), dextran sulphate, chloramine T, igepal, o-phenylenediamine, guanidine hydrochloride, Tween 20, bovine serum albumin (BSA), histopaque 1083, low melting point (LMP) agarose, normal melting point (NMP) agarose, Triton X-100, hydrogen peroxide, AAPH [2,2′-azobis (2-amidinopropane) dihydrochloride], and formamidopyrimidine-DNA glycosylase (Fpg) were purchased from Sigma-Aldrich (USA); sodium azide and DAPI (4,6-diamidino-2-phenylindole dihydrochloride) were purchased from Merck (Germany); TPTZ (2,4,6-tripyridyl-s-triazine), disodium fluorescein, 2,4-dinitrophenylhydrazine (DNPH), and glycerol were purchased from Fluka (Germany); all other chemicals were purchased from Lachema (Czech republic); Robuvit was obtained from Horphag Research Ltd. (Geneva, Switzerland).

2.4. Measurement of Basic Biochemical Parameters. Basic biochemical parameters (bilirubin, glucose, gamma-glutamyl transferase (GMT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), uric acid (UA), total proteins (TP), total cholesterol (TCh), triacylglycerols (TAG), HDL-cholesterol (HDL-chol), LDL-cholesterol (LDL-chol), and VLDL-cholesterol (VLDL-chol)) were determined at an accredited clinical biochemistry and haematology laboratory using a Hitachi 911 analyzer by Roche diagnostics kits (Switzerland).

2.5. Measurement of In Vitro Antioxidant Capacity of Robuvit—TEAC (Trolox Equivalent Antioxidant Capacity) Assay. 57.8 mg of Robuvit was dissolved in 10 mL of distilled water, vortexed for 5 min, centrifuged (3000 ×g/10 min), and diluted to concentrations of 10^{-4} to 10^{-9} g/mL with distilled water.

In vitro antioxidant capacity of Robuvit was measured by method according to Re et al. [19]. It is a decolourization assay for measurement of both lipophilic and hydrophilic antioxidants. The radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen-donating antioxidants into colourless form. Quantification was performed using the dose-response curve for reference antioxidant Trolox water soluble form of vitamin E. Robuvit antioxidant capacity was calculated as Trolox equivalents per mg of Robuvit.

2.6. Determination of Advanced Oxidation Protein Products (AOPP). Serum concentration of AOPP was measured by modified method according to Witko-Sarsat et al. [20]. 200 μL of blood serum diluted 1 : 5 with phosphate buffer saline (PBS, pH 7.4), 200 μL of chloramine T (0–100 μmol/L) for calibration, and 200 μL of PBS as blank were applied on a microtiter plate. 10 μL of 1.16 mol/L potassium iodide was added to standards and 20 μL of acetic acid was added into all samples. Absorbance at 340 nm was measured and results are presented in μmol/L.

2.7. Determination of Protein Carbonyls. The concentration of protein carbonyls in plasma was measured by ELISA method according to Buss et al. [21]. Briefly, carbonyls of sample were derivatized by 2,4-dinitrophenylhydrazine (DNPH). A biotin-conjugated rabbit IgG raised against DNPH was used as a primary antibody and a monoclonal goat anti-rabbit IgG antibody labelled by horseradish peroxidase (HRP) as a secondary antibody. Product of enzymatic reaction catalyzed by HRP has absorption maximum at 490 nm. Results were calculated from calibration curve using oxidized BSA as a standard and expressed in ng/mL.

2.8. Determination of Advanced Glycation End-Products (AGEs). The concentration of plasma AGEs was determined by modified method according to Kalousová et al. [22]. Determination was based on measurement of total fluorescence at wavelengths (exc./emis.) 345 nm/465 nm in the plasma diluted with 0.01 mol/L PBS. Results are expressed in AU/g of proteins.

2.9. Determination of Oxidative DNA Damage by Comet Assay. Oxidative DNA damage in lymphocytes was evaluated by the enzymatically modified comet assay using formamidopyrimidine DNA glycosylase (Fpg) recognizing oxidized purines [23]. Lymphocytes were isolated using Histopaque 1083 according to the manufacturer's protocol. Oxidative DNA damage was expressed as a number of 8-oxoG per 10^6 guanines according to ESCODD [24].

2.10. Determination of 8-Isoprostanes (8-isoP). 8-isoP were determined by the commercial EIA kit (Cayman Chemical Company, USA). This assay is based on the competition between 8-isoP and an 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-isoprostane Tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. Because the concentration of the 8-isoprostane Tracer is held constant while the concentration of 8-isoP varies, the amount of 8-isoprostanes Tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 8-isoP in the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AchE) is added to the well. The product of enzymatic reaction has a distinct yellow colour and absorbs the light at 405 nm. The concentration of 8-isoP is expressed in pg/mL.

2.11. Determination of Lipid Peroxides (LP). Concentration of LP in serum was measured by method according to El-Saadani et al. [25]. The analysis was based on the ability of LP to convert iodide to iodine, which can be measured spectrophotometrically at 365 nm. Results are presented in nmol/mL of plasma.

2.12. Measurement of Activity of Cu/Zn-Superoxide Dismutase (SOD). Activity of SOD was determined by the commercial kit number 19160 (Fluka, Germany), using bovine Cu/Zn-SOD as a standard (Sigma, Germany). Superoxide radical is generated from xanthine and oxygen by xanthine oxidase. Superoxide radical reduces Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to form a yellow water-soluble formazan dye with maximum absorption at 450 nm. At the presence of SOD the reduction of WST-1 is decreased. 1 U of SOD activity is defined as the enzyme activity causing 50% inhibition of reduction of WST-1. SOD activity is expressed in U SOD/mg Hb.

2.13. Measurement of Activity of Catalase (CAT). Catalase activity was determined by modified method of Bergmeyer [26]. 250 μL of H_2O_2 was added into 50 mL phosphate buffer saline (PBS, 50 mmol/L, pH = 7.0) and formed mixture was diluted to get absorbance 0.7–1.0. 30 μL of sample was added into 2 mL of PBS + H_2O_2 mixture. Change of absorbance at wavelength 240 nm was measured for 1 min. Distilled water was used as blank. Activity of catalase is expressed in μkat/g Hb.

2.14. Measurement of Activity of Glutathione Peroxidase (GPx). Activity of GPx was determined by the commercial kit (Sigma-Aldrich, USA). This kit uses an indirect determination method. It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase and NADPH. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of GPx activity. Activity of GPx is expressed in U/g Hb.

2.15. Measurement of Total Antioxidant Capacity of Plasma—ORAC (Oxygen Radical Absorbance Capacity) Assay. The ORAC assay was measured by modified method according to Huang et al. [27]. The method is based on the oxygen radical absorbance capacity where a peroxyl radical (AAPH) oxidizes fluorescein in competition with an antioxidant (in the plasma). AAPH leads to production of ROO^- that reacts with the fluorescein. This results in a gradual loss of fluorescence intensity. Antioxidants, which are present in the plasma, inhibit the reaction between ROO^- and the fluorescent probe, which slows down or inhibits the fluorescence degradation. The ORAC assay indicates the antioxidant capacity of the plasma by using the area under the curve in combination with inhibition time and inhibition potency. Results are presented as mmol of Trolox/L.

2.16. Measurement of Total Antioxidant Capacity—FRAP (Ferric Reducing Ability of Plasma) Assay. The FRAP was determined by the method according to Benzie and Strain [28].

The method is based on the reduction of Fe^{3+}-TPTZ complex to the ferrous form at low pH (pH 3.6). This reduction is monitored spectrophotometrically at 593 nm. Quantification was performed using the dose-response curve for reference antioxidant Trolox. Results are presented as mmol of Trolox/mL.

2.17. Statistical Analysis. Results are presented as a mean ± standard deviation (SD) or median and interquartile range (IQ, 1st quartile, 3rd quartile). Parametric Student's paired t-test was used for statistical analysis of data with a Gaussian distribution and nonparametric Mann-Whitney U-test or Wilcoxon's signed ranks test was used for data with a non-Gaussian distribution. Value $P < 0.05$ was considered statistically significant. Statistical software StatsDirect 2.3.7. (StatsDirect Sales, Sale, Cheshire M33 3UY, UK) was used.

3. Results

Administration of Robuvit for 4 weeks decreased weight of volunteers, but because of high interindividual variability of body weight this change (−0.7 kg) has no relevance. Values of BMI were not significantly changed after week 4 and week 6.

Basic biochemical parameters were investigated in fasting venous blood in examination periods 0, 4, and 6: bilirubin, glucose, GMT, ALP, AST, ALT, UA, TP, TCh, TAG, HDL-chol, LDL-chol, and VLDL-chol. All values of mentioned biochemical parameters were in the physiological ranges.

We did not observe serious unwanted effects of Robuvit administration.

In vitro antioxidant capacity of Robuvit was calculated as 6.37 micromole Trolox equivalent/mg of Robuvit.

Concentrations of markers of oxidative damage to proteins (AOPP, protein carbonyls, and AGEs), DNA (8-oxoG), and lipids (8-isoP and LP), activities of antioxidant enzymes (SOD, CAT, and GPx), and total antioxidant capacity (ORAC and FRAP) are summarized in Table 1. Four weeks administration of Robuvit significantly decreased the concentration of AOPP in serum by 42.09% and LP by 21.53%. This effect persisted after week 6 where the level of AOPP remained decreased by 42.85% and LP by 19.79%.

The concentrations of protein carbonyls, AGEs, 8-oxoG, and 8-isoP were not significantly changed after week 4. Only after week 6 we found out significantly decreased level of protein carbonyls by 7.77% in comparison to week 0.

We measured activities of SOD, CAT, and GPx in haemolysate of erythrocytes. Administration of Robuvit significantly increased activity of SOD by 11.76% and this effect persisted after week 6 where the activity of SOD was even more increased by 13.22%. We also found a significantly higher activity of CAT after week 4 by 15.54%. Activity of GPx was not influenced by intake of Robuvit.

Total antioxidant capacity of plasma was determined by two methods with different mechanism of action: ORAC and FRAP. We did not find significant changes in ORAC values in weeks 4 and 6 in comparison to week 0. However, we found out significantly increased level of FRAP values by 6.31% after week 4.

4. Discussion

We have studied *in vitro* antioxidant capacity of the French oak wood extract Robuvit and the *in vivo* effect of its administration on markers of oxidative damage to biomolecules, activity of antioxidant enzymes, and total antioxidant capacity in 20 healthy volunteers. Our results represent the first investigation of extract from the wood of *Quercus robur*, Robuvit on oxidative stress in humans.

We have determined *in vitro* antioxidant capacity of Robuvit by TEAC method. Natella et al. [3] determined the antioxidant capacity of Robuvit by ORAC assay as 648 nmol Trolox equivalents per mg extract in Robuvit, 10 times lower than our result (6370 nmol Trolox equivalents per mg), which can be caused by different method used or rather by different way of evaluation.

In our human trial we have found that Robuvit was able to protect significantly proteins and lipids against oxidation as we found significantly decreased levels of AOPP and LP in serum.

In addition, 4-week intake of Robuvit significantly increased the intracellular defence against oxidative stress by enhancing the activities of SOD and CAT in erythrocytes. We also found significantly increased FRAP values of total antioxidant capacity in the plasma (by 6.3%) after week 4 in healthy volunteers, but this small change may not have a biological relevance. Also Natella et al. [3] observed significantly increased total antioxidant capacity in the hydrophilic component of plasma (by 5.55%) measured by ORAC assay.

The mechanism for the antioxidant effect of polyphenols in Robuvit *in vivo* is most probably based on the antioxidant and biomodulating activity of the EA and metabolites of the roburins, the urolithins.

Following consumption of the oak wood extract Robuvit, besides EA and GA, three different urolithins were identified in plasma of volunteers [3]. Antioxidant effects of EA and urolithins were examined in many models *in vivo* and *in vitro*. They were associated with reduction of intracellular reactive oxygen species (ROS) and malondialdehyde (MDA) levels and increase of SOD activity in H_2O_2-treated T24 cells [9]. Similar results were observed also by Kim et al. [8] who found out that EA significantly reduced ROS level and MDA concentration in paraquat-induced A549 cells. Kavitha et al. [7] observed significant increased expression of antioxidant enzymes SOD, CAT, and GPx by EA in 7,12-dimethylbenz[a]anthracene- (DMBA-) induced hamster buccal pouch carcinogenesis model. Celik et al. [6] also found significantly increased activities of CAT and GPx in rat liver microsomes by EA treatment. Urolithins accumulate in different tissues of the body and they have been suggested to be responsible for biological effects observed as a consequence of the ingestion of ellagitannins-rich foods [29]. Our results show rather indirect antioxidant effects of Robuvit than direct ones. Although we observed significantly increased total antioxidant capacity, this change is too small to have important biological relevance. Robuvit increases antioxidant protection against oxidative damage to biomolecules probably through the stimulation of expression or activities of antioxidant enzymes, what is supported by

Table 1: Markers of oxidative stress, activity of antioxidant enzymes, and total antioxidant capacity after weeks 0, 4, and 6.

Parameter	Week 0	Week 4	P (0 versus 4)	Week 6	P (0 versus 6)
AOPP (μmol/L)	58.06 (49.32; 59.27)	33.62 (30.49; 35.56)	<0.0001	33.18 (28.44; 34.03)	<0.0001
PC (ng/mL)	0.296 ± 0.059	0.288 ± 0.052	n.s.	0.273 ± 0.054	0.0037
AGEs (AU/g prot.)	273.17 (249.01; 305.33)	296.17 (237.34; 323.05)	n.s.	286.07 (258.07; 322.95)	n.s.
8-oxoG/10^6 G	0.61 (0.35; 0.91)	0.79 (0.47; 1.43)	n.s.	0.78 (0.38; 1.62)	n.s.
8-isoP (pg/mL)	24.59 (21.16; 33.77)	34.02 (24.16; 39.01)	n.s.	31.63 (23.98; 44.66)	n.s.
LP (nmol/mL)	42.55 (33.6; 61.54)	33.39 (28.17; 44.19)	0.0174	34.13 (28.10; 48.37)	0.0038
SOD (U/mg Hb)	42.42 ± 6.83	47.41 ± 9.93	0.0198	48.03 ± 5.27	0.0240
CAT (μkat/g Hb)	5.60 (4.76; 6.12)	6.47 (6.11; 7.41)	0.0012	4.59 (3.72; 6.46)	n.s.
GPx (U/g Hb)	39.55 ± 9.16	40.74 ± 8.23	n.s.	36.21 ± 8.96	n.s.
ORAC (mmol/L)	8.97 (8.34; 9.61)	8.72 (8.03; 9.67)	n.s.	8.69 (8.08; 9.72)	n.s.
FRAP (mmol/mL)	0.586 ± 0.098	0.623 ± 0.115	0.0091	0.603 ± 0.124	n.s.

Data are presented as mean ± SD or median and IQ range (1st quartile; 3rd quartile); AOPP: advanced oxidation protein products; PC: protein carbonyls; AGEs: advanced glycation end-products; AU: arbitrary units; 8-isoP: 8-isoprostanes; LP: lipid peroxides; SOD: Cu/Zn-superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; ORAC: oxygen radical absorbance capacity; FRAP: ferric reducing ability of plasma; $P < 0.05$: significant; n.s.: not significant ($P > 0.05$).

increased activities of SOD and CAT after supplementation of Robuvit. However, precise mechanism of antioxidant effects is yet unknown and there are another studies needed.

5. Conclusions

In conclusion, we have confirmed for the first time that the intake of the French oak wood extract Robuvit is associated with a decreased damage to proteins and lipids, a stimulation of antioxidant enzymes (SOD and catalase), and a moderate increase of total antioxidant capacity of plasma in humans.

Conflict of Interests

The authors declare that there is no conflict of interests.

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References

[1] W. Mayer, W. Gabler, A. Riester, and H. Korger, "On tannins compounds from the wood of chestnut and oak wood-IIs. Isolation of castalagin, vescalagin, castalin and vescaline," *Liebigs Annual of Chemistry*, vol. 707, pp. 177–181, 1967.

[2] C. L. M. H. du Penhoat, V. M. F. Michon, S. Peng, C. Viriot, A. Scalbert, and D. Gage, "Structural elucidation of new dimeric ellagitannins from *Quercus robur*," *Journal of the Chemical Society*, no. 7, pp. 1653–1660, 1991.

[3] F. Natella, G. Leoni, M. Maldini et al., "Absorption, metabolism, and effects at transcriptome level of a standardized French oak wood extract, Robuvit , in healthy volunteers: pilot study," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 2, pp. 443–453, 2014.

[4] A. Glabasnia and T. Hofmann, "Sensory-directed identification of taste-active ellagitannins in American (Quercus alba L.) and European oak wood (Quercus robur L.) and quantitative analysis in bourbon whiskey and oak-matured red wines," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 9, pp. 3380–3390, 2006.

[5] N. P. Seeram, S. M. Henning, Y. Zhang, M. Suchard, Z. Li, and D. Heber, "Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 hours," *The Journal of Nutrition*, vol. 136, no. 10, pp. 2481–2485, 2006.

[6] G. Celik, A. Semiz, S. Karakurt, S. Arslan, O. Adali, and A. Sen, "A comparative study for the evaluation of two doses of ellagic acid on hepatic drug metabolizing and antioxidant enzymes in the rat," *BioMed Research International*, vol. 2013, Article ID 358945, 9 pages, 2013.

[7] K. Kavitha, P. Thiyagarajan, J. Rathna, R. Mishra, and S. Nagini, "Chemopreventive effects of diverse dietary phytochemicals against DMBA-induced hamster buccal pouch carcinogenesis via the induction of Nrf2-mediated cytoprotective antioxidant, detoxification, and DNA repair enzymes," *Biochimie*, vol. 95, no. 8, pp. 1629–1639, 2013.

[8] Y. S. Kim, T. Zerin, and H. Y. Song, "Antioxidant action of ellagic acid ameliorates paraquat-induced A549 cytotoxicity," *Biological and Pharmaceutical Bulletin*, vol. 36, no. 4, pp. 609–615, 2013.

[9] Z. Qiu, B. Zhou, L. Jin et al., "In vitro antioxidant and antiproliferative effects of ellagic acid and its colonic metabolite, urolithins, on human bladder cancer T24 cells," *Food and Chemical Toxicology*, vol. 59, pp. 428–437, 2013.

[10] Y. Chung, L. Lu, M. Tsai, Y. Chen, S. Yao, and C. Hsu, "The inhibitory effect of ellagic acid on cell growth of ovarian carcinoma cells," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 306705, 12 pages, 2013.

[11] S. Umesalma, P. Nagendraprabhu, and G. Sudhandiran, "Antiproliferative and apoptotic-inducing potential of ellagic acid against 1, 2-dimethyl hydrazine-induced colon tumorigenesis in Wistar rats," *Molecular and Cellular Biochemistry*, vol. 388, no. 1-2, pp. 157–172, 2014.

[12] L. Vanella, C. di Giacomo, R. Acquaviva et al., "Effects of ellagic acid on angiogenic factors in prostate cancer cells," *Cancers*, vol. 5, no. 2, pp. 726–738, 2013.

[13] M. Zhao, S. Tang, J. L. Marsh, S. Shankar, and R. K. Srivastava, "Ellagic acid inhibits human pancreatic cancer growth in Balb c nude mice," *Cancer Letters*, vol. 337, no. 2, pp. 210–217, 2013.

[14] M. R. Loizzo, A. Said, R. Tundis et al., "Antioxidant and antiproliferative activity of *Diospyros lotus* L. extract and isolated compounds," *Plant Foods for Human Nutrition*, vol. 64, no. 4, pp. 264–270, 2009.

[15] M. Marín, R. M. Giner, J. L. Ríos, and M. C. Recio, "Intestinal anti-inflammatory activity of ellagic acid in the acute and chronic dextrane sulphate sodium model of mice colitis," *Journal of Ethnopharmacology*, vol. 150, no. 3, pp. 925–934, 2013.

[16] M. A. Rosillo, M. Sánchez-Hidalgo, A. Cárdeno et al., "Dietary supplementation of an ellagic acid-enriched pomegranate extract attenuates chronic colonic inflammation in rats," *Pharmacological Research*, vol. 66, no. 3, pp. 235–242, 2012.

[17] M. Larrosa, F. A. Tomás-Barberán, and J. C. Espín, "The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway," *Journal of Nutritional Biochemistry*, vol. 17, no. 9, pp. 611–625, 2006.

[18] Y. Chang, W. Chen, K. Lin et al., "Novel bioactivity of ellagic acid in inhibiting human platelet activation," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 595128, 9 pages, 2013.

[19] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radical Biology and Medicine*, vol. 26, no. 9-10, pp. 1231–1237, 1999.

[20] V. Witko-Sarsat, M. Friedlander, C. Capeillère-Blandin et al., "Advanced oxidation protein products as a novel marker of oxidative stress in uremia," *Kidney International*, vol. 49, no. 5, pp. 1304–1313, 1996.

[21] H. Buss, T. P. Chan, K. B. Sluis, N. M. Domigan, and C. C. Winterbourn, "Protein carbonyl measurement by a sensitive ELISA method," *Free Radical Biology and Medicine*, vol. 23, no. 3, pp. 361–366, 1997.

[22] M. Kalousová, J. Škrha, and T. Zíma, "Advanced glycation end-products and advanced oxidation protein products in patients with diabetes mellitus," *Physiological Research*, vol. 51, pp. 597–604, 2002.

[23] A. R. Collins, V. L. Dobson, M. Dušinská, G. Kennedy, and R. Štětina, "The comet assay: what can it really tell us?" *Mutation Research*, vol. 375, no. 2, pp. 183–193, 1997.

[24] C. M. Gedik, A. Collins, and ESCODD, "Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study," *The FASEB Journal*, vol. 19, no. 1, pp. 82–84, 2005.

[25] M. El-Saadani, H. Esterbauer, M. El-Sayed, M. Goher, A. Y. Nassar, and G. Jurgens, "A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent," *Journal of Lipid Research*, vol. 30, no. 4, pp. 627–630, 1989.

[26] H. U. Bergmeyer, *Methods of Enzymatic Analysis, Volume III: Enzymes 1: Oxidoreductases, Transferases*, Verlag Chemie, Weiheim, Germany, 1987.

[27] D. Huang, B. Ou, M. Hampsch-Woodill, J. A. Flanagan, and R. L. Prior, "High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 16, pp. 4437–4444, 2002.

[28] I. F. F. Benzie and J. J. Strain, "The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay," *Analytical Biochemistry*, vol. 239, no. 1, pp. 70–76, 1996.

[29] J. C. Espín, M. Larrosa, M. T. García-Conesa, and F. Tomás-Barberán, "Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far," *Evidence-based Complementary and Alternative Medicine*, vol. 2013, Article ID 270418, 15 pages, 2013.