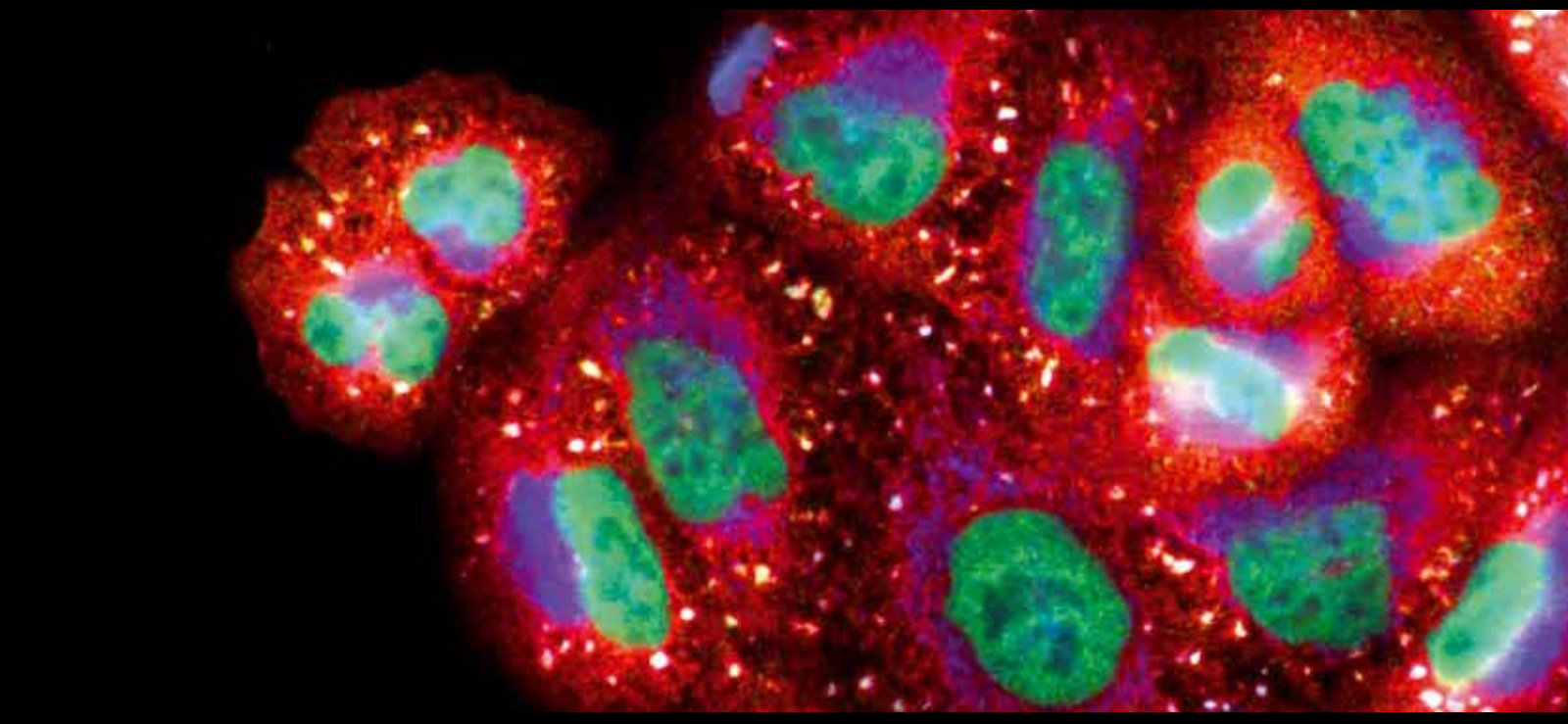
Oxidative Medicine and Cellular Longevity

Dietary Polyphenols and Their Effects on Cell Biochemistry and Pathophysiology

Guest Editors: Cristina Angeloni, Luciano Pirola, David Vauzour, and Tullia Maraldi



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Editorial Board

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Editorial

Dietary Polyphenols and Their Effects on Cell Biochemistry and Pathophysiology

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Polyphenols, occurring in fruit and vegetables, wine, tea, extra virgin olive oil, chocolate, and other cocoa products, have been demonstrated to exert beneficial effects in a large array of disease states, including cancer, cardiovascular disease, and neurodegenerative disorders. Many of the biological effects of polyphenols have been attributed to their antioxidant properties, either through their reducing capacities per se or through their possible influences on intracellular redox status. As such, polyphenols may protect cell constituents against oxidative damage and have been reported to limit the risk of various degenerative diseases associated with oxidative stress, including cardiovascular diseases, type 2 diabetes, and cancer. However, accumulating evidence suggests that the classical hydrogen-donating antioxidant activity is unlikely to be the sole explanation for their cellular effects *in vivo*. Indeed, it has recently become clear that, in complex biological systems, polyphenols are able to exhibit several additional properties which are yet poorly understood. It is evident that polyphenols are potent bioactive molecules and a clear understanding of their precise mechanisms of action as either antioxidants or modulators of cell signaling is crucial to the evaluation of their potential as chemopreventive or anticancer agents and inhibitors of neurodegeneration.

This special issue comprises 14 original research articles that further expand our understanding of the biological functions of polyphenols from different sources and 9 review articles that summarize the current knowledge on the beneficial effects of polyphenols on health.

Potential Effect of Polyphenols in Cancer. Although epidemiological studies have not yet provided conclusive results on the chemopreventive and anticancer effect of tea polyphenols, there is an increasing trend to employ these substances as conservative management for patients diagnosed with less advanced prostate cancer. Two groups (S. Cimino et al. and P. Davalli et al.) review the most recent observations related to tea polyphenols and human prostate cancer risk, in an attempt to better outline their potential employment for preventing prostate cancer.

The original paper by C. Oleaga et al. shows that polyphenolic extracts from coffee, as well as the single constituent caffeic acid, decrease cyclin D1 in HT29 colon cells, thus suggesting chemopreventive properties for both substances. P. Baumeister et al. presented data regarding antimutagenic effects of curcumin and epigallocatechin-3-gallate in human oropharyngeal mucosa cultures exposed to cigarette smoke condensate indicating that dietary polyphenols are capable of preventing tobacco-related genotoxicity in the mucosa of the upper aerodigestive tract.

Regarding other cancer types, two groups (D. Zhang et al. and C. Widén et al.) report the antioxidant and growth inhibiting effects of flavonoid extracts on erythrocyte and an erythroleukemia cell line, respectively.

Caffeic, syringic, and protocatechuic acids are phenolic acids deriving directly from food intake or generated following gut metabolism of polyphenols. L. Zambonin et al. explore the antioxidant activity of these compounds in membrane models and in a leukaemia cell line, HEL.

These compounds showed a chain-breaking antioxidant in membrane models and were able to contrast the intracellular ROS increase due to exogenous oxidative stress in both leukaemia and normal cells. These data further support the antioxidant ROS-depleting approach as a valuable strategy in cancer therapy.

Potential Effect of Polyphenols in Cardiovascular Diseases. Quercetin, a naturally occurring flavonoid, has been shown to down regulate inflammatory responses and to exert cardioprotection. C. Angeloni and S. Hrelia present a study on the protective effect of quercetin on rat cardiac dysfunction during sepsis and demonstrate that this flavonoid is involved in the inhibition of cell growth as well as the induction of apoptosis. These results suggest that quercetin might serve as a valuable protective agent in cardiovascular inflammatory diseases.

Vascular protective effects and antiatherogenic properties of anthocyanins have now been recognized. M. Akhlaghi and B. Bandy compared the protective effect elicited by different flavonoids using rat embryonic ventricular H9c2 cells subjected to simulated ischemia-reperfusion and to tert-butyl hydroperoxide. They also tried to distinguish between indirect (preconditioning) effects versus direct effects. The results supported the possibility that catechins have the capacity to act as preconditioning agents while in acute situations of oxidative stress quercetin and epigallocatechin gallate are the most potent antioxidants amongst the flavonoids tested.

The study by J. Paixão et al. expands our knowledge about the molecular mechanisms underlying the vascular protection afforded by anthocyanins in the context of prevention of endothelial dysfunction and atherosclerosis. Data reported that malvidin-3-glucoside decreases the formation of reactive species after cell aggression and inhibits mitochondrial apoptotic signaling pathways induced by peroxynitrite.

Moreover, positive effects were also determined *in vivo* for the administration of red wine extracts on hypertensive rats, by A. Kondrashov et al.

Potential Effect of Polyphenols in Neurodegenerative Diseases. C. P. Dias et al. discuss in a review article some recent findings addressing the effects of different dietary polyphenols on hippocampal cell proliferation and differentiation, models of anxiety and depression. Dietary polyphenols appear to exert positive effects on anxiety and depression, possibly in part via the regulation of adult hippocampal neurogenesis (AHN). Studies on the effects of dietary polyphenols on behavior and AHN may play an important role in the approach to use diet as part of the therapeutic interventions for mental health related conditions.

Wine polyphenolic compounds are well known for the antioxidant properties such as their ability to scavenge free radicals and also regulate NO activity. Numerous neuroprotective mechanisms of action have been proposed, suggesting that polyphenols may exert their activities by inhibiting the production of reactive oxygen species, therefore reducing inflammation and modulating the activity of intracellular signal transduction molecules, as reported by A. Basli et al.

An extensive review by D. Vauzour reports recent evidence suggesting that polyphenols beneficial effects involve decreases in oxidative/inflammatory stress signaling, increases in protective signaling, and neurohormetic effects leading to the expression of genes that encode antioxidant enzymes, phase-2 enzymes, neurotrophic factors, and cytoprotective proteins. Specific examples of such pathways include the sirtuin-FoxO pathway, the NF-κB pathway, and the Nrf-2/ARE pathway. Together, these processes act to maintain brain homeostasis and play important roles in neuronal stress adaptation and, thus, polyphenols have the potential to prevent the progression of neurodegenerative pathologies.

In another review by S. Davinelli et al., the potential neuroprotective role of some polyphenols has been discussed. In particular, the authors have highlighted the correlations between the neuroprotective functions of the selected polyphenols and their potential therapeutic value in Alzheimer disease.

D. Hu et al. showed that schisandrin (SCH) significantly improved $A\beta_{1--42}$ induced short term and spatial reference memory impairments *in vivo*. Furthermore, in the cerebral cortex and hippocampus of mice, SOD and GSH-peroxidase activities, GSH level, and GSH/GSSG ratio were increased, and levels of malondialdehyde and GSSG were decreased by the treatment of SCH. These results suggest that SCH is a potential cognitive enhancer against Alzheimer's disease through antioxidative action.

In the study by J. Bournival et al., the effects of quercetin and sesamin on neuroinflammation induced by the parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP+) in a glial-neuronal system have been investigated. These data demonstrate that quercetin and the lignan sesamin diminish MPP+-evoked microglial activation and suggest that both these molecules may be regarded as potent, natural, anti-inflammatory compounds.

Role of Polyphenols in Redox Modulation. M. Chohan et al. investigate the effect of cooking and digestion on the anti-inflammatory activity of the culinary herbs. This study shows that the culinary herbs, rosemary, sage, and thyme, in quantities used for cooking, possess significant anti-inflammatory activity that may be due to their polyphenol content.

Mate tea is obtained by the aqueous extraction of the leaves of *Ilex paraguariensis* and it is consumed every day by millions of individuals in South America. B. Scolaro et al. investigate the influence of acute and chronic intake of mate tea on the effects elicited by acute and chronic administration of ethanol. The results showed that acute and chronic mate tea administration prevented oxidative stress in the hippocampus and blood of rats, caused by the *in vivo* administration of ethanol. This suggests that mate tea may have a high antioxidant capacity, probably due to its bioactive components and that mate tea ingestion could prevent oxidative stress-related diseases.

J. Baran et al. address the relationship between apoptosis and delayed luminescence in human leukemia Jurkat T cells under oxidative stress and the protective effects of two flavonoids, quercetin and epigallocatechin gallate applied alone or in combination with menadione or H_2O_2. Menadione, epigallocatechin gallate, and H_2O_2, but not quercetin, interacted with flavine mononucleotide (FMN) and altered its electronic configuration, thus reducing delayed luminescence emission.

Relatively to review articles, I. Andújar et al. discuss the potential health benefits of cocoa polyphenols, P. Oyetakinwhite et al. survey the protective mechanisms of polyphenols from green tea with special emphasis of the effects on skin, and M. Ciz et al. summarized contemporary knowledge on the effects of various flavonoids on the respiratory burst of mammalian neutrophils.

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Review Article

Cocoa Polyphenols and Their Potential Benefits for Human Health

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This paper compiles the beneficial effects of cocoa polyphenols on human health, especially with regard to cardiovascular and inflammatory diseases, metabolic disorders, and cancer prevention. Their antioxidant properties may be responsible for many of their pharmacological effects, including the inhibition of lipid peroxidation and the protection of LDL-cholesterol against oxidation, and increase resistance to oxidative stress. The phenolics from cocoa also modify the glycemic response and the lipid profile, decreasing platelet function and inflammation along with diastolic and systolic arterial pressures, which, taken together, may reduce the risk of cardiovascular mortality. Cocoa polyphenols can also modulate intestinal inflammation through the reduction of neutrophil infiltration and expression of different transcription factors, which leads to decreases in the production of proinflammatory enzymes and cytokines. The phenolics from cocoa may thus protect against diseases in which oxidative stress is implicated as a causal or contributing factor, such as cancer. They also have antiproliferative, antimutagenic, and chemoprotective effects, in addition to their anticariogenic effects.

1. Introduction

Plant phenols from numerous plant species are being actively studied as potential treatments for various metabolic and cardiovascular diseases. For example, resveratrol from red wine [1, 2], epigallocathechin-3-gallate from green tea [3–5], curcumin from turmeric [6], and quercetin [7–12] from different sources have all been studied as potential therapeutic agents, to induce weight loss, lower blood pressure, attenuate glucose levels and insulin resistance (resveratrol), and improve hemoglobin A1c and lipid profile in humans (epigallocathechin-3-gallate). Other studies also carried out in humans have shown the beneficial effects of grape seeds, chokeberries, coffee, carob, and cocoa [13]. This paper reviews the most recent research on this last substance and its potential benefits for human health.

Since the seventeenth century, cocoa and chocolate have been described as potential medicines. In Europe, various historical documents refer to chocolate's medicinal value; thus, although it was drunk as a beverage with a pleasurable taste, it was primarily eaten as a food to treat a number of disorders, including angina and heart pain [14]. Today, *Theobroma cacao* L. (Sterculiaceae) and its products are consumed worldwide and are studied mainly because of the antioxidant and antiradical properties *in vitro* of some of their polyphenolic constituents, specially procyanidins and flavan-3-ols [15]. Many studies have described cocoa phenolics as being bioactive compounds, especially prominent for their metabolic and cardiovascular effects. These effects are due, in part, to the antioxidant [16] and antiradical properties of cocoa phenolics [17], which increase the plasma level of antioxidants to prevent the oxidation of LDL-cholesterol [18]. Along with their known antiplatelet effects [19], these particular properties are related to the protective mechanism of cocoa phenolics in heart disease [18]. Other relevant properties include their ability to modulate the immune response [20–24] and their anti-inflammatory [25–27] and anticarcinogenic properties [26, 28]. In this paper, we have focused on the beneficial effects of cocoa polyphenols on human health, especially with regard to cardiovascular and inflammatory diseases, metabolic disorders, and cancer prevention as well as on their antioxidant properties.

2. Polyphenols Content in Cocoa

Cocoa contains about 380 known chemicals, 10 of which are psychoactive compounds. In their natural state, cocoa beans are virtually inedible because of their high concentration of polyphenols, which gives them an extremely bitter flavor. In a final cocoa product such as chocolate, polyphenol content might decrease from 100% to 10% throughout the different manufacturing processes [29]. For this reason, various authors have focused on the effects of polyphenol-enriched extracts from cocoa [27, 30–32].

Wollgast and Anklam [15] reviewed the polyphenol content of cocoa depending on its origin and the manufacturing process of the final product. Three groups of polyphenols can be identified in cocoa beans: catechins, which constitute about 37% of the polyphenol content in the beans, anthocyanidins (about 4%), and proanthocyanidins (about 58%). Of the catechins, (−)-epicatechin is the most abundant (up to 35%), while (+)-catechin, (+)-gallocatechin, and (−)-epigallocatechin are present in smaller quantities (Figure 1). In the case of anthocyanidins, the main compounds are cyanidin-3-α-L-arabinoside and cyanidin-3-β-D-galactoside, while in the case of procyanidins, the main compounds are dimers, trimers, or oligomers of flavan-3,4-diol linked by 4 → 8 or 4 → 6 bonds. Of these, the main procyanidins are B1, B2, B3, B4, B5, C1, and D. The proportions of these derivatives usually change during the manufacturing process. For example, in the dried, fat-free mass of fresh cocoa beans, the total soluble polyphenol content is 15–20%, which is equivalent to 6% in air-dried, nondefatted cocoa beans. In contrast, it is only about 5% in fermented, nondefatted beans because during the fermentation process epicatechin and soluble polyphenol content is reduced by approximately 10 to 20%, anthocyanidins disappear, and procyanidins decrease 3- to 5-fold. There is also a great amount of variation in the phenolic content of cocoa depending on its origin. For example, (−)-catechin concentration in fermented, defatted cocoa beans varies from 16.52 mg/g in Costa Rican cocoa to 2.66 mg/g in cocoa grown in Jamaica.

3. Bioavailability of Phenolics from Cocoa

There is a great controversy surrounding the bioavailability of phenolics in general and of cocoa derivatives in particular. For example, epicatechin is well absorbed, with a maximum plasma concentration at around 2 h and with approximately 20% of consumed epicatechin being excreted in the urine [33]. In the case of dimers, their presence has been also described in plasma. For example, Holt et al. [34] described the presence of procyanidin dimers, especially B2 (epicatechin-(4β-8)-epicatechin) in peripheral blood of healthy adults who had consumed a cocoa beverage.

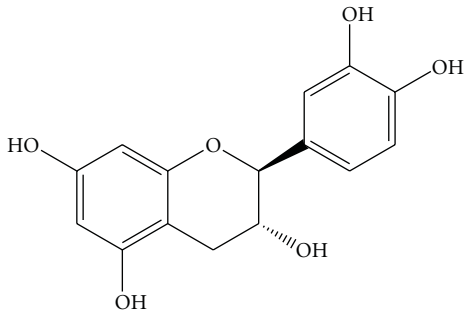
3.1. Gastric Degradation of Cocoa Phenolics. There have been contradictory findings concerning the gastric stability of phenols from cocoa. For example, Spencer et al. [35] reported that, in the acidic environment of the gastric milieu, procyanidin oligomers are hydrolyzed to mixtures of epicatechin monomers and dimers. However, Rios et al. [36] observed that, during gastric transit, procyanidins were remarkably stable in the stomach environment and reached the small intestine intact, available for absorption or metabolism. In addition, their limited absorption in the small intestine could influence the digestive process or the physiology of the gut through direct interaction with the gut mucosa and gut lumen solutes. Consequently, some of the health effects of procyanidins may be associated with the formation by microflora of low-molecular-weight metabolites once the procyanidins reach the colon. This hypothesis has been corroborated by Gonthier et al. [37], who demonstrated that while proanthocyanidins of high molecular weight are not present in plasma or urine, the monomers and aromatic acids produced during their metabolism by intestinal microflora can be found in the latter. These researchers also demonstrated that catechin is well absorbed in the small intestine, with recoveries of 20–40%.

3.2. Intestinal Absorption of Phenols from Cocoa. Ortega et al. [38] coupled an *in vitro* digestion method with a continuous-flow dialyzed duodenal step to construct a model for examining the digestibility and bioaccessibility of polyphenols in cocoa food matrices. Their results show that the fat content of the cocoa samples tested enhances the digestibility of some phenolic compounds, especially procyanidins, during duodenal digestion. The possible mechanism is probably related to the fat fraction's ability to interact with certain phenol compounds following a better micellization of the digested phenols.

A human bioavailability trial with chocolate polyphenols using 40 g of dark chocolate (892 mg of total polyphenols expressed as gallic acid equivalent and 82 mg of epicatechin) showed that epicatechin was indeed absorbed into the blood. The compound was detectable in plasma as glucuronide-conjugated metabolites and sulfate groups after 2 h and was still present after 8 h. The clearance of epicatechin from the plasma compartment was very fast, with an elimination $t_{1/2}$ of 1.9 and 2.3 h for 40 and 80 g of chocolate, respectively [29].

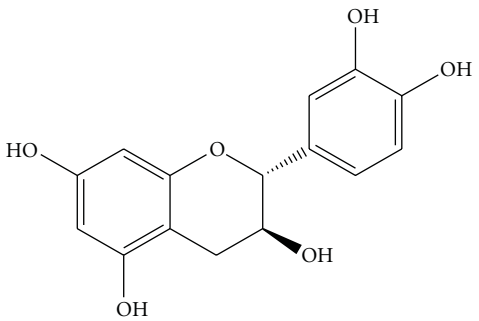
Manach et al. [39] found that the metabolism of simple phenols occurs via a common pathway, being absorbed in the small intestine, and oligomers larger than trimers are unlikely to be absorbed in the small intestine in their native forms. *In vitro* experiments using single layers of Caco-2 cells as a model of absorption in the small intestine have corroborated the *in vivo* studies because they showed that only the dimers and trimers of flavan-3-ols are able to cross the intestinal epithelium; thus, procyanidin B2 is poorly absorbed in rats whereas procyanidin B3 is not absorbed at all [39].

In a study on the bioavailability of cocoa polyphenols in healthy volunteers, Tomás-Barberán et al. [30] demonstrated that the flavonoid composition (mainly flavanol monomers and dimers) of cocoa powder can be enhanced through improved processing of the raw material and that these flavanol-enriched samples lead to increases in the cocoa flavan-3-ols present in plasma and urine, thus increasing their bioavailability.



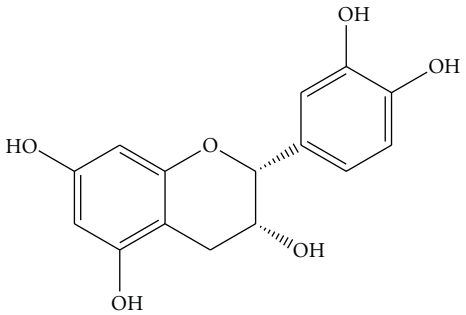
(−)-catechin

(a)



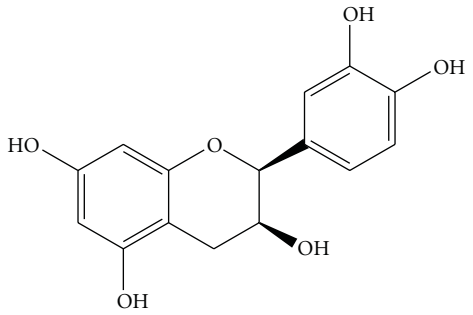
(+)-catechin

(b)



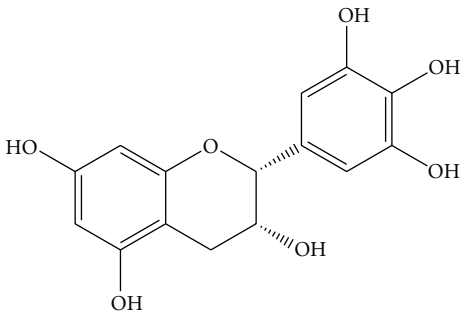
(−)-epicatechin

(c)



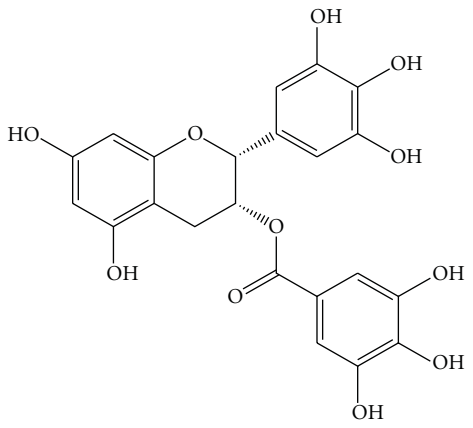
(+)-epicatechin

(d)



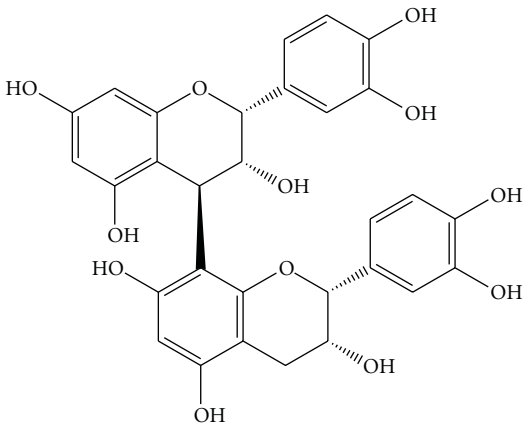
(+)-epigallocatechin

(e)



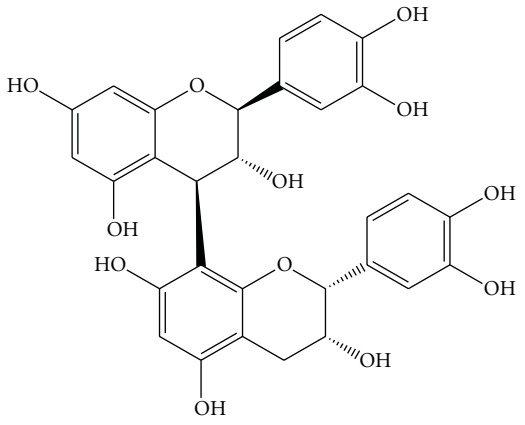
(−)-epigallocatechin gallate

(f)



Procyanidin B2

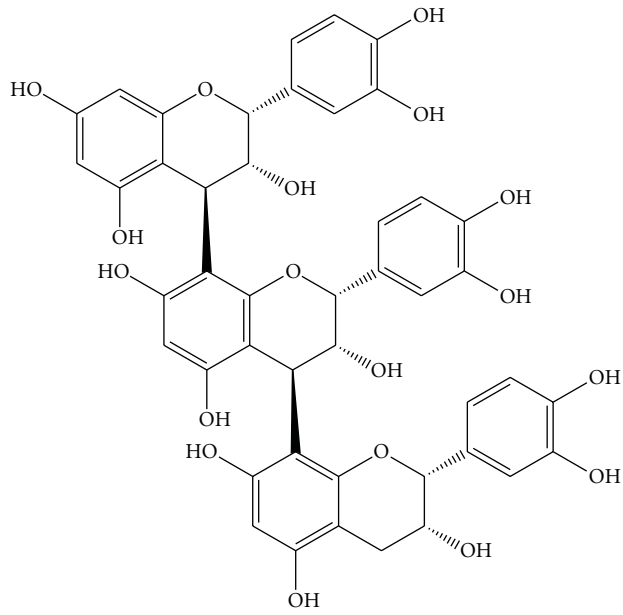
(g)



Procyanidin B3

(h)

Figure 1: Continued.



Procyanidin C1

(i)

Figure 1: Chemical structure of major cocoa phenolics.

4. Antioxidant Properties of Cocoa Phenolics

4.1. Scavenging Activity. Cocoa has more phenolics and higher antioxidant capacity than green tea, black tea, or red wine. In fact, Lee et al. [40] found that cocoa contains 611 mg/serving of gallic acid equivalents and 564 mg/serving of epicatechin equivalents. The values for gallic acid equivalents were 1.8, 3.7, and 4.9 higher than those obtained for red wine, green tea, and black tea, respectively, while the epicatechin equivalents were 3.5, 12.0, and 16.6, respectively, for the same beverages. The values for antioxidant activity, measured with the aid of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^+$) and 2,2-diphenyl-1-pycrylhydrazyl (DPPH) methods, were 1128 and 836 mg/serving, respectively, expressed as vitamin C equivalent antioxidant capacity. When compared against that of red wine, green tea, or black tea, the relative activity of cocoa was 1.8, 2.5, and 4.4 times higher, respectively, in the $ABTS^+$ test, and 1.7, 2.4, and 4.3 times higher, respectively, in the DPPH test. The authors thus concluded that on a perserving basis, cocoa has higher flavonoid content and antioxidant capacity than red wine (2 times), green tea (2-3 times), and black tea (4-5 times), respectively.

Schinella et al. [16] studied the antioxidant activity of different cocoa extracts and demonstrated their effects on different *in vitro* experimental models, proving that the antioxidant effects cannot be fully characterized with one single method. The extracts' scavenging capacity was measured as the ability to bleach the stable radicals DPPH and $ABTS^+$, their reducing ability was assessed with the ferric reducing-antioxidant power (FRAP) assay, and their scavenging properties against H_2O_2, HClO, and peroxynitrite were also evaluated. The extracts acted both as a free radical scavenger (DPPH, $ABTS^+$, superoxide, hypochloride, and peroxinitrite) and a lipid peroxidation inhibitor way that was directly proportional to their polyphenol content. The extracts also inhibited superoxide production by polymorphonuclear leukocytes. These findings indicate that cocoa extracts may modify the biological functions of these cells, which are implicated in the initiation and maintenance of inflammation. The extracts also showed scavenging activity against peroxynitrite, both in the presence and in the absence of a physiological concentration of bicarbonate. Under physiological conditions, bicarbonate levels are usually high and significant amounts of nitrosoperoxycarbonate anion adduct can be produced; however, in inflamed tissues where the pH value is lower, different reactions may occur independent of the presence of CO_2. Because peroxynitrite anion can attack a wide range of biological molecules, its inhibition can provide protection against the damage induced by this free radical.

In a previous study, Schroeder et al. [41] examined the effect of a specific cocoa flavanol, (−)-epicatechin in a cellular model (murine aortic endothelial cells) for examining its protective action against peroxynitrite effects, and found that while it effectively blocked the nitration of protein tyrosine residues by peroxynitrite (IC_{50} value = approximately 0.02 mol epicatechin/mol peroxynitrite), its ability to block the oxidative inactivation of glyceraldehyde-3-phosphate dehydrogenase or soybean lipoxygenase (LOX)-1 was not significant (IC_{50} > 1 mol epicatechin/mol peroxynitrite).

4.2. Lipid Peroxidation. Schinella et al. [16] evaluated the ability of cocoa powder to bring about lipid peroxidation

in brain homogenates and human plasma using nonenzymatic generation systems. Cocoa powder inhibited lipid peroxidation in both the homogenates and the plasma but had no effect on the hydroxyl radical, nor did it exhibit ferrous chelating activity. Metal chelating capacity reduces the concentration of the catalyzing transition metal in lipid peroxidation. It has previously been reported that the chelation efficiency of some compounds with a phenyl group on Fe^{2+} depends on the number of hydroxyl groups on the benzene ring and that hydroxyl substitution in the ortho position results in a higher chelating effect. However, because the polyphenol-rich cocoa extract showed neither ferrous nor copper chelating activity, it most likely acts as a chain-breaking antioxidant.

Verstraeten et al. [42] studied the antioxidant and membrane effects of procyanidin B2 (dimer) and procyanidin C1 (trimer) from cocoa in phosphatidyl choline liposomes. Both procyanidins inhibited lipid oxidation of liposomes in a concentration (0.33–5 μM)-dependent manner; moreover, both compounds also increased the membrane surface potential. They did this by decreasing membrane fluidity, an effect that extended into the hydrophobic region of the bilayer, protecting the lipid bilayer from disruption by Triton X-100. Procyanidins can thus interact with membrane phospholipids, probably through their polar head group, to provide protection against the attack of oxidants and other molecules that challenge the integrity of the bilayer.

4.3. Resistance to Oxidative Stress. Martorell et al. [43] used model organisms to study the effect of cocoa polyphenols on resistance to oxidative stress. A protocol of acute oxidative stress was optimized in order to test the best conditions to induce oxidative damage in *Saccharomyces cerevisiae*: doses of 4 mg/mL of a polyphenol-enriched cocoa powder led to an increased resistance to hydrogen peroxide (2 and 3 mM) tolerance, similar to those observed in cells treated with vitamin C. To discard the possibility that this was a strain-related effect, the authors repeated the test with other strains belonging to *S. cerevisiae* species. To discover the metabolic target with which the polyphenol-enriched cocoa powder might interact, authors evaluated the transcriptional profile concluding that the protection upon oxidative stress is mediated at least by sirtuins in *S. cerevisiae*. In order to clarify the function of sirtuin proteins as metabolic targets of cocoa flavonoids, different trials were performed with a more complex model, the nematode *Caenorhabditis elegans.* Worms fed with the cocoa powder were more resistant toward the oxidative stressor hydrogen peroxide, increasing their survival rate from 36% to 53% and their lifespan in a 17% after being fed with the cocoa powder. However, mutant strains of the worm for sirtuin and for the transcription factor DAF-1 did not show resistance to oxidative stress nor prolonged lifespan, suggesting that the effects of cocoa powder are mediated at least through these genes. This work highlights the potential of polyphenol-enriched cocoa powder as a functional ingredient with resistance to oxidative stress.

In a clinical study, Spadafranca et al. [44] investigated the effects of both dark chocolate (860 mg of polyphenols, of which 58 mg were epicatechin) and white chocolate (5 mg of polyphenols, undetectable epicatechin) on plasma epicatechin levels, DNA damage to mononuclear blood cells, and total plasma antioxidant activity in 20 healthy subjects who followed a balanced diet for 4 weeks. Detectable epicatechin levels were only observed 2 h after dark chocolate intake, at which timeless DNA damage to mononuclear blood cells was likewise observed, but both effects were no longer evident after 22 h. No effect on total plasma antioxidant activity was noted. These results demonstrate that dark chocolate improves DNA resistance to oxidative stress in healthy subjects but that this effect is transient, probably due to flavonoid kinetics. Because oxidative stress not only affects both the inflammatory process and atherogenesis, but also has mutagenic power over the human genome, the protective effect of cocoa polyphenols may have important consequences for oncogene expression and cancer pathogenesis.

5. Phenolics from Cocoa in Metabolic and Endocrine Disorders

Cocoa extracts and cocoa procyanidins are potent inhibitors of key enzymes in the digestion of carbohydrates and lipids; these inhibitory activities are related not only to their polyphenol content, but also to the degree of polymerization of cocoa procyanidins, all of which are dramatically affected by the cocoa processing. For example, Gu et al. [45] assessed their inhibitory effects of different cocoa extracts against pancreatic α-amylase, pancreatic lipase, and secreted phospholipase A_2 (PLA_2) *in vitro* and characterized the kinetics of this inhibition and demonstrated that cocoa extracts and the procyanidins found in them dose-dependently inhibited all three enzymes. They also observed that the inhibitory potency of cocoa procyanidins increases as a function of their degree of polymerization, but they did not examine whether cocoa extracts and/or cocoa procyanidins can inhibit digestive enzymes *in vivo* and affect related downstream pathways such as aberrant eicosanoid metabolism at dose levels achievable in the diet.

The principal aspects to be treated in this section are the effects of cocoa polyphenols on carbohydrate and lipoprotein metabolism.

5.1. Effects on Carbohydrate Metabolism. Hanhineva et al. [46] published an interesting review compiling the effect of different polyphenols, including flavonoids and proanthocyanidins, on carbohydrate metabolism at many levels. In animal models and in a limited number of human studies, polyphenols have been found to attenuate postprandial glycemic responses and fasting hyperglycemia while improving acute insulin secretion and insulin sensitivity. The possible mechanisms of action include inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose release from the liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of intracellular signaling

pathways and gene expression. These positive effects on glucose homeostasis are also supported by epidemiological evidence on polyphenol-rich diets.

Grassi et al. [47] compared the effects of flavanol-rich dark chocolate and flavanol-free white chocolate with regard to endothelial function, insulin sensitivity, β-cell function, and blood pressure in a randomized crossover trial with 19 hypertensive patients with impaired glucose tolerance. The group of patients treated with flavanol-rich dark chocolate (100 g/day for 15 days) showed decreased insulin resistance and increased insulin sensitivity and β-cell function. Moreover, subjects taking flavanol-rich dark chocolate experienced decreases in systolic and diastolic blood pressure, increased flow-mediated dilation, and decreased total cholesterol and LDL-cholesterol with respect to the control group [47].

Similar effects were described by other authors when reviewing systematically the effect of cocoa polyphenols on cardiovascular risk factors. Shrime et al. [48] did a systematic review and meta-analysis of randomized, controlled trials to evaluate the effect of a flavonoid-rich cocoa fraction on cardiovascular risk factors and showed that the cocoa phenolics decreased insulin resistance and increased flow-mediated vascular dilation, with a maximum effect observed at a flavonoid dose of 500 mg/d.

Hooper et al. [49] in a systematic review of 42 acute or short-term chronic randomized controlled trials including 1297 patients demonstrated a significant reduction of different negative biomarkers of cardiovascular disease, such as insulin resistance. Some parameters were improved in patients after chronic and acute intake of chocolate or cocoa, with significant reductions in serum insulin, and in flow-mediated dilatation. In addition, the reductions in diastolic blood pressure and mean arterial pressure were observed, as well as a slight but significant reduction of LDL-cholesterol and an increase of HDL-cholesterol levels. These effects will be reviewed in the following sections.

5.2. Effects on Lipoprotein Metabolism. Cocoa phenolics increase apolipoprotein (Apo) A1 and decrease ApoB production in both the human hepatoma cell line HepG2 and the human intestinal cell line Caco2. Cells incubated for 24 h in the presence of cocoa polyphenols such as (−)-epicatechin, (+)-catechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 at 10 μM increased both ApoA1 protein levels and mRNA expression, even though ApoB protein and mRNA expression decreased slightly. These effects were the result of the upregulation of the mature form of sterol regulatory element binding proteins (SREBPs) and increased LDL receptor activity induced by the cocoa polyphenols. As cholesterol metabolism is known to be regulated by several different mechanisms, it was hypothesized that cocoa polyphenols, like other plant polyphenols, may act via multiple pathways as a regulatory receptor agonist or ligand. The mechanism responsible for the increase in high-density lipoprotein (HDL)-cholesterol associated with daily cocoa intake was also identified [50]. However, other authors have obtained somewhat contradictory results in clinical trials. Khan et al. [51] had previously evaluated the effects of chronic cocoa consumption (42 g/day for 4 weeks) on lipid profile, oxidized LDL particles, and plasma antioxidant vitamin concentrations in 42 high-risk volunteers in a randomized, crossover feeding trial. The analysis of 24 h urine revealed significant increases of phase II metabolites, including glucuronide and sulfate conjugates. The concentration of HDL-cholesterol was significantly higher after cocoa intake in comparison with the control group, which received only milk, while the oxidized LDL levels decreased. No significant changes were registered for total plasma cholesterol, triglycerides, LDL-cholesterol, ApoA1 and ApoB, lipoprotein concentrations, or the LDL/HDL-cholesterol ratio. Other biochemical parameters such as fibrinogen and homocysteine did not vary, and no significant changes were found in the plasma concentrations of vitamins B_1, B_6, B_{12}, C, and E, or in plasma and intraerythrocyte folic acid concentrations. The authors studied the correlation between changes in polyphenols, lipids, and oxidized LDL measurements and observed higher increments in the urinary excretion of cocoa (−)-epicatechin metabolites. Regarding cocoa metabolites derived from intestinal microbial metabolism, an increase in the urinary excretion of 3-hydroxyphenylacetic and vanillic acids was observed in parallel with a significant increase in plasma HDL-cholesterol concentration and a decrease in plasma-oxidized LDL levels.

Jia et al. [52] studied the effect of cocoa on total cholesterol, LDL-cholesterol, and HDL-cholesterol in 8 trials involving 215 participants. Whereas LDL-cholesterol was significantly lowered and total cholesterol marginally lowered, no significant changes in HDL-cholesterol were observed after cocoa consumption. There was no evidence of a dose-effect relationship, of any effect in healthy subjects, or of any change in HDL-cholesterol. The authors concluded that short-term cocoa consumption significantly reduced blood cholesterol, but the changes depended on the amount of cocoa consumed and the health status of participants; indeed, there was no dose response and no effect in healthy participants. To assess the dose-response effect, Almoosawi et al. [53] carried out a randomized, single-blind, crossover study in which 14 overweight/obese subjects were randomized to take either 20 g of dark chocolate with 500 mg of polyphenols and then 20 g of dark chocolate with 1000 mg of polyphenols or vice versa. Participants followed each diet for 2 weeks separated by a 1-week washout period.

Mellor et al. [31] conducted a randomized, placebo-controlled double-blind crossover trial comparing the effects of chocolate with high polyphenol content (16.6 mg of epicatechins in 45 g of chocolate) with those of chocolate with low-polyphenol content (<2 mg of epicatechins in 45 g of chocolate) on lipid profiles, weight, and glycemic control in 12 individuals with type 2 diabetes on stable medication. HDL-cholesterol increased significantly with high polyphenol chocolate, with a decrease in the total cholesterol/HDL ratio and no changes in any of the other parameters under study. The authors concluded that high-polyphenol chocolate is effective in improving the atherosclerotic cholesterol profile in patients with diabetes by increasing HDL-cholesterol and improving the cholesterol/HDL ratio without

affecting weight, inflammatory markers, insulin resistance, or glycemic control. However, as stated above, marginally significant effects on LDL- and HDL-cholesterol were found by Hooper et al. [49], although there were no significant effects on total cholesterol, after acute or chronic intake. As a secondary analysis, they assessed the effects on body weight, body mass index, and waist circumference, but few trials reported these outcomes.

Some of these indices were recently evaluated by Shrime et al. [48] who studied the cardioprotective effects of a flavonoid-rich cocoa fraction. The most relevant risk factors evaluated were blood pressure, pulse, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, body mass index, C-reactive protein, flow-mediated vascular dilation (FMD), fasting glucose, fasting insulin, serum isoprostane, and insulin sensitivity/resistance indices. Results showed that LDL-cholesterol decreased by 0.077 mmol/L, and HDL-cholesterol increased by 0.046 mmol/L, whereas total cholesterol, triglycerides, and C-reactive protein remained the same.

6. Phenolics from Cocoa in Cardiovascular Diseases

Epidemiological studies demonstrate that regular dietary intake of cocoa polyphenols reduces the risk of coronary heart disease and stroke and is inversely associated with the risk of cardiovascular disease. This effect was described for first time in the Kuna Indians (Panama), who belong to one of the few cultures protected against the age-dependent increase in blood pressure and the development of arterial hypertension. These healthy effects have been correlated with the consumption of high amounts of cocoa. Clinical studies revealed that, compared with other Pan-American citizens, the Kunas have lower blood pressure rates and lower mortality resulting from cardiovascular events [54].

6.1. Effects on Arachidonic Acid Metabolism. The consumption of cocoa/chocolate increases plasma antioxidant capacity, diminishes platelet function and inflammation, and decreases diastolic and systolic arterial pressures. Currently available data indicate that daily consumption of cocoa-rich chocolate or polyphenol-enriched cocoa extracts could partially lower cardiovascular disease risk [55, 56]. The mechanism by which the cardiovascular protection of cocoa polyphenols develops is complex, with different mediators and agents being implicated. For example, the effects of cocoa polyphenols on inflammatory mediators such as leukotrienes (LT), nitric oxide (NO) metabolism, and myeloperoxidase- (MPO-) induced modification of LDL indicate that the antioxidant properties of polyphenol-enriched cocoa extracts could be of great interest for developing therapies to prevent cardiovascular disease since oxidative modification of LDL appears to be crucial for atherogenesis. In fact, cocoa polyphenols decrease the plasma concentration of proinflammatory cysteinyl leukotrienes through inhibition of 5-LOX, as demonstrated by Sies et al. [57] in isolated recombinant human 5-LOX (IC_{50} of (–)-epicatechin = 22 μM). Recombinant human platelet 12-LOX, a representative of another subfamily of mammalian lipoxygenases, also was inhibited by (–)-epicatechin (IC_{50} = 15 μM), therefore concluding that this compound and related flavan-3-ols are general inhibitors of mammalian LOX. The inhibition of human 5-LOX by cocoa flavonoids suggests that these compounds exert antileukotriene actions, which may confer a degree of anti-inflammatory, vasoprotective, and antibronchoconstrictory capacity because the principal metabolite of 5-LOX, LTB_4, causes neutrophils to adhere to endothelial cells, thus acting as a potent chemotactic agent for these and other inflammatory cells. It also stimulates the release of lysosomal enzymes and the generation of superoxide anion in neutrophils, while the cysteinyl-LTs increase vascular permeability and contract airway smooth muscle.

Another oxidant enzyme with pronounced effects on inflammation and atherogenesis is MPO, which has also been reported to act as an NO oxidase. *In vitro*, MPO is capable of binding to LDL, catalyzing oxidative modification and rendering it atherogenic. (–)-epicatechin and procyanidins have been shown to block the MPO/nitrite-mediated and peroxynitrite-mediated lipid peroxidation of LDL; for example, (–)-epicatechin is able to suppress it at concentrations as low as 0.1 μmol/L [58].

Unlike 5-LOX and platelet-type 12-LOX, the reticulocyte-type lipoxygenases (12-LOX and 15-LOX) are catalysts of enzymatic lipid peroxidation. Oxidative modification of LDL by 15-LOX-1, MPO, and peroxynitrite renders its atherogenicity and plays a relevant role in the early developmental stages of atherosclerotic lesions. Agents such as cocoa polyphenols that interfere with the oxidative modification of LDL should therefore be protective for vascular endothelium, including that of coronary arteries.

In conclusion, the inhibitory effects of cocoa polyphenols on 5-LOX and 15-LOX-1 may contribute to their beneficial actions [57].

6.2. Effects on NO Bioactivity. Cocoa phenols also promote the bioactivity of NO *in vivo*, which is critical for protecting the cardiovascular system. Sies et al. [57] compared the effects of cocoa beverages containing different contents of flavanols among selected volunteers, concluding that ingestion of flavanol-rich cocoa by individuals with diminished endothelial function led to an increase in the plasma NO concentration approaching the mean concentrations of healthy control subjects and doubled flow-mediated dilation after 2 h. Plasma concentrations of NO were correlated with flow-mediated dilation, which suggests that the improvement of endothelial function might be attributable to increased bioavailability of NO. In contrast, these changes did not occur among individuals who received either water or a low-flavanol cocoa drink, which excluded effects of beverage constituents other than flavanols. The NO-promoting effects of cocoa polyphenols may also contribute to a decrease in blood pressure because they interfere with NO metabolism at several levels. They especially favor the synthesis of NO

and suppress deleterious metabolites such as superoxide, peroxynitrite, and NO_2 [57].

As Corti et al. [54] cite, these compounds also induce NO-dependent vasodilation in the rat aorta and in the finger or forearm circulation of healthy humans or patients with cardiovascular risk factors. Cocoa lowers vascular arginase activity in human endothelial cells, augmenting local levels of L-arginine, which is then transformed into NO by endothelial nitric oxide synthase (eNOS). Once released, NO increases intracellular cyclic guanosine monophosphate (cGMP) concentrations, inducing a relaxation of vascular smooth muscle cells. NO not only leads to vasodilatation, but also prevents leukocyte adhesion and migration, smooth muscle cell proliferation, and platelet adhesion and aggregation. In patients with cardiovascular risk factors, including smoking, a cocoa drink high in flavonol content rapidly enhances the circulating pool of bioactive NO and augments flow-mediated vasodilation. Moreover, chronic consumption of a high-flavanol diet is associated with high urinary excretion of NO metabolites, which is consistent with augmented NO production or diminished degradation. Moreover, flavonoid-enriched cocoa results in regional changes in cerebral blood flow and an overall increased blood flow. In elderly humans on a high-flavanol diet, cerebral blood flow velocity in the middle cerebral artery has been shown to increase, suggesting that cocoa flavanols may protect against dementia and stroke [54]. The improvement of endothelial function is probably one of the mechanisms by which polyphenols confer cardiovascular protection. Indeed, polyphenols are able to induce NO-mediated, endothelium-dependent relaxations in a large number of arteries, including the coronary artery. In some arteries they can also induce endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxations. Taken together, these mechanisms may help explain the antihypertensive and cardioprotective effects of polyphenols *in vivo* [58].

6.3. Effects of Cocoa Phenolics on Other Mediators Implicated in Vascular Properties. Cocoa procyanidins are potent inhibitors of mitogen-activated protein kinase kinase (MEK) and membrane type-1 (MT1)-matrix metalloproteinase (MMP). They subsequently inhibit the expression and activation of pro-MMP-2 as well as the invasion and migration of human vascular smooth muscle cells (VSMCs). In fact, both cocoa procyanidin fraction (3 μg/mL) and procyanidin B2 (5 μM) were found to strongly inhibit the thrombin-induced activation and expression of pro-MMP-2 in VSMCs, the thrombin-induced invasion and migration of VSMCs, MT1-MMP activity, and the thrombin-induced phosphorylation of extracellular signal-regulated protein kinase (ERK) (but not that of MEK) in VSMCs. Kinase and pull-down data show that cocoa procyanidin fraction and procyanidin B2 inhibit MEK1 activity, binding directly with glutathione-S-transferase-MEK1. These results may partially explain the molecular action of cocoa's antiatherosclerotic effects [59].

There is evidence of the structure-activity relationship of endothelium-dependent vasodilator responses to procyanidins in isolated vessels. This is consistent with the hypothesis that these responses are mediated by a procyanidin receptor, which could represent an important target for new agents to treat endothelial dysfunction. Because this response involves signaling through superoxide or hydrogen peroxide, it could even be considered prooxidant. Flow-mediated dilatation also involves signaling through hydrogen peroxide. Hence, these findings bolster the hypothesis that procyanidins trigger an endothelial response similar to that of laminar shear stress [60].

In a controlled trial, Monagas et al. [32] evaluated the effects of chronic cocoa consumption on cellular and serum biomarkers related to atherosclerosis in 42 high-risk patients in a randomized crossover feeding trial. All subjects received 40 g cocoa powder with 500 mL skim milk per day or only 500 mL skim milk per day for 4 weeks. No significant changes in the expression of adhesion molecules on T lymphocyte surfaces were observed. However, in monocytes, the expression of VLA-4, CD40, and CD36 was significantly lower in the cocoa intake group compared to the control group. In addition, serum concentrations of the soluble endothelium-derived adhesion molecule P-selectin and intercellular adhesion molecule (ICAM)-1 were significantly lower after cocoa intake than after milk intake. The authors concluded that the intake of cocoa polyphenols may modulate inflammatory mediators in patients at high risk for cardiovascular disease; indeed, these anti-inflammatory effects may contribute to the overall benefits of cocoa consumption against atherosclerosis.

The vascular actions associated with transforming growth factor (TGF)-β_1 and homeostatic modulation of its production by flavan-3-ols and procyanidins from cocoa may constitute another mechanism by which phenolics from cocoa can potentially benefit cardiovascular health. In this context, Mao et al. [61] studied the effect of flavan-3-ols and procyanidins fractions (monomers through decamers) isolated from cocoa on the secretion of the cytokine TGF-β_1 in resting human peripheral blood mononuclear cells (PBMCs) from 13 healthy subjects. When cells of individuals with low baseline levels of TGF-β_1 were stimulated by individual flavan-3-ol and procyanidin fractions (25 μg/mL), TGF-β_1 release was enhanced in the range of 16–66%. The low-molecular-weight fractions ($\leq$pentamers) were more effective at augmenting TGF-β_1 secretion than their larger counterparts (hexamer or higher), with the monomer and dimer inducing the greatest increases (66% and 68%, resp.). In contrast, in the case high TGF-β_1 baseline subjects, the TGF-β_1 secretion was inhibited, being the inhibition most pronounced for trimmers through decamers (28–42%, resp.) and moderate for monomers to dimers (17–23%, resp.). Given the vascular actions associated with TGF-β_1 release, this homeostatic modulation by cocoa polyphenols offers an additional mechanism by which polyphenol-rich foods can benefit cardiovascular health.

6.4. Cocoa Phenols and Cardiovascular Disease. Ding et al. [62] carried out a systematic review of experimental, observational, and clinical studies of the relationship between chocolate and cocoa and the risk of cardiovascular disease. The data from a total of 136 publications suggests that cocoa and chocolate may exert beneficial effects with regard

to cardiovascular risk via various mechanisms, including lowering blood pressure, anti-inflammation, anti-platelet function, increasing HDL level while decreasing LDL oxidation. In addition, prospective studies of flavonoids suggest that the phenolic content of chocolate may reduce the risk of cardiovascular mortality.

Phenolics from cocoa also decrease the tendency of blood to clot. For example, an acute dose of flavanols and oligomeric procyanidins from cocoa powder was found to inhibit platelet activation and function for over 6 h in humans. In a blinded parallel-design study, 32 healthy subjects were assigned to consume 234 mg cocoa phenolics a day [63].

6.5. Effects on Platelet Function. Platelet function was determined by measuring platelet aggregation, ATP release, and the expression of activation-dependent platelet antigens. After 28 days of treatment, cocoa polyphenol supplementation increased plasma epicatechin and catechin concentrations by 81% and 28%, respectively, while decreasing platelet function. These findings are in agreement with results from studies that used higher doses of cocoa flavanols and procyanidins [63]. Moreover, cocoa decreases not only platelet aggregation, but also adhesion. The effects of cocoa polyphenols could thus be explained in part by their ability to reduce the ADP-induced expression of the activated conformation of glycoprotein IIb/IIIa surface proteins [54]. Various studies have indicated that dark chocolate induces coronary vasodilation, improves coronary vascular function, and decreases platelet adhesion. These immediate beneficial effects are paralleled by a significant reduction in serum oxidative stress and have been positively correlated with changes in serum epicatechin concentration. Flammer et al. [64] studied these effects on coronary vascular and platelet function in 22 heart transplant recipients in a double-blind, randomized study. The ingestion of 40 g of flavonoid-rich dark chocolate (containing 0.27 mg/g of catechin and 0.9 mg/g of epicatechin, with a total polyphenol content of 15.6 mg of epicatechin equivalents per gram) increased coronary artery diameter, improved endothelium-dependent coronary vasomotion, and decreased platelet adhesion, all of which remained unchanged in the control group.

Carnevale et al. [65] carried out a crossover, single blind study with 20 healthy subjects and 20 smokers who received 40 g of dark (cocoa > 85%) or milk (cocoa < 35%) chocolate. Compared to healthy subjects, smokers showed enhancements in platelet recruitment, platelet formation of reactive oxygen species (ROS) and eicosanoids, and nicotinamide adenine dinucleotide phosphate (NADPH)-reduced oxidase (NOX)2 activation. In the dark chocolate group, platelet ROS, 8-iso-prostaglandin (PG)$F_{2\alpha}$, and NOX2 activation decreased significantly, but in the subgroup of healthy subjects taking dark chocolate, platelet variables did not change. No changes were detected in either of the groups treated with milk chocolate. The authors concluded that dark chocolate inhibits platelet function by lowering oxidative stress only in smokers; this effect seems to be dependent on its polyphenol content.

6.6. Effects on Arterial Hypertension. As discussed above, animal and human studies suggest that cocoa and chocolate could regulate NO bioavailability, affecting endothelial function and, in turn, blood pressure. Although these studies are not definitive and many questions remain, the body of evidence suggests that it is worth considering diets that can provide benefits to patients with high blood pressure; indeed, these benefits will be more significant in subjects that do not have their hypertension under control [66].

As mentioned above, significant reductions in diastolic blood pressure and mean arterial pressure after chronic intake were observed in Hooper's review [48].

Cienfuegos-Jovellanos et al. [67] evaluated the effects of various doses of a natural flavonoid-enriched cocoa powder (50–600 mg/kg) on spontaneously hypertensive rats. The polyphenol extract produced an antihypertensive effect in hypertensive rats without modifying the arterial blood pressure in normotensive rats. However, no dose-response effect was observed: the maximum effect on the systolic blood pressure of hypertensive rats was reached with 300 mg/kg of extract, while the maximum effect on the diastolic blood pressure, was achieved with a dose of 100 mg/kg. In a complementary study, Almoosawi et al. [53] found that the 500 mg polyphenol dose was equally effective in reducing systolic blood pressure, and diastolic blood pressure as the 1000 mg polyphenol dose, which suggests that a saturation effect might occur with increased doses of polyphenols.

Akita et al. [68] studied the effect of dietary intake of cocoa polyphenols on heart rate, blood pressure, and plasma lipid concentrations. The area of atherosclerotic lesions in the aortas of the polyphenol-treated group was significantly smaller than in the control group. In addition, whereas the high-frequency power of heart rate variability of the rabbits in the standard diet group decreased significantly with ageing, there were no differences between long- and short-term treatments in the polyphenol group. Moreover, cocoa liquor polyphenols preserved parasympathetic nervous tone, although this decreased significantly with ageing in the standard diet group. No differences in the plasma lipid concentrations were observed. The authors concluded that cocoa liquor polyphenols may play an important role in protecting cardiovascular function.

To date, epidemiological data on cocoa polyphenols has been scarce. Rimbach et al. [69] reviewed different relevant clinical trials and selected the results of five of them in which the following conclusions were established: in a cohort of elderly men, cocoa intake was inversely associated with blood pressure as well as with 15-year cardiovascular and all-cause mortality; a prospective study examined flavonoid intake and cardiovascular disease mortality in postmenopausal women; a meta-analysis of 133 studies on flavonoids, flavonoid-rich foods, and cardiovascular disease risk found a correlation between chocolate intake and increased flow-mediated dilation along with reductions in both systolic and diastolic blood pressure; a cohort study involving over 1100 nondiabetic patients assessed the long-term effects of chocolate consumption amongst patients with established coronary heart disease; finally, a study conducted with patients surviving their first acute myocardial infarction

found that chocolate consumption was associated with significant reductions in cardiac mortality and beneficial effects on vascular health. However, these studies raise many doubts. For example, the products used in controlled studies often contain much higher polyphenol contents than most commercially available products. It thus needs to be established whether the consumption of products with lower polyphenol content is associated with any health benefits in humans. Moreover, many of these studies have mainly been funded by industrial sponsors, and it must be remembered that the food industry is encouraged to label the flavonoid content on cocoa-derived products [69].

In a 6-month clinical trial carried out by Ried et al. [70] with 36 prehypertensive healthy adult volunteers, at content levels of 120–139 and 80–89 mmHg, no significant differences were observed in the blood pressures of the treated and control groups. However, a marked difference in acceptability between the two treatment forms (chocolate or capsule) was observed due to the amount of chocolate that had to be eaten (50 g of dark chocolate every day). In contrast, other studies [71] have reported on blood pressure changes in response to flavanol-rich cocoa in healthy subjects as well as in prehypertensive and hypertensive patients, suggesting that the inclusion of moderate amounts of flavanol-rich cocoa or chocolate in the daily diet may potentially ameliorate or delay the onset of hypertension. Indeed, it has been estimated that a 3 mmHg reduction in systolic blood pressure reduces the relative risk of stroke mortality by 8%, of coronary artery disease mortality by 5%, and of all-cause mortality by 4%.

Actis-Goretta et al. [72] examined the effects of different isolated polyphenols from cocoa on angiotensin converting enzyme activity; several procyanidins (dimer and hexamer) and epigallocatechin significantly inhibited the enzyme activity, whereas monomers, flavonols, phenolic acids, and resveratrol at similar concentrations showed no activity. Authors established three levels of IC_{50}: values in the mM range for monomeric flavan-3-ols, in the 100 μM range for dimer and trimer, and in the 10 μM range for larger procyanidins, although these concentrations should be interpreted with caution as they were estimated using nonphysiological *in vitro* conditions. These *in vitro* effects were correlated with the *in vivo* reductions in blood pressure observed in several experimental models [72].

Cherniack [13] reviewed the effects of polyphenols on metabolic syndrome and noted that eight patients with coronary artery disease who were given cocoa drinks containing a 375 mg dose of cocoa polyphenols twice a day for 1 month had improved vasodilatory capacity in their brachial arteries and were able to produce larger numbers of endothelial progenitor cells. Moreover, the systolic blood pressure of subjects taking the larger dose cocoa decreased 4.2 mmHg, an effect which was not observed in the group that consumed only 9 mg twice a day. In addition, meta-analyses of human trials have concluded that cocoa can lower systolic and diastolic blood pressure by 4.5 and 2.5, respectively [13].

A meta-analysis with 10 randomized controlled trials comprising 297 individuals confirmed the blood pressure-lowering capacity of flavanol-rich cocoa products in a larger set of trials than previously reported. Both the systolic and diastolic blood pressures decreased in either healthy normotensive adults or patients with prehypertension/stage 1 hypertension. However, significant statistical heterogeneity across studies was described [73].

In summary, critical attention must be paid to the flavanol content of cocoa foods since manufacturing processes significantly reduce its concentration in the final product. The high fat and sugar content of many cocoa snacks and confectionaries must also be taken into account. However, even with these limitations, the latest findings on the effects of flavonoid-rich cocoa are of clinical relevance and hold promise for the development of new, low calorie cocoa foods and beverages. Further research is needed to characterize the mechanisms of action leading to the final effects of cocoa. Also, before recommending cocoa products as a treatment option in hypertensive and at-risk patients, well-designed, long-term clinical trials are needed to definitively confirm the putative beneficial role of long-term consumption of flavanol-rich foods. Specific attention should be paid to clarifying questions concerning the most appropriate dose, the best targets, and eventual long-term and high-dose side effect profiles [69].

7. Effects of Cocoa Phenolics on the Immune System

Kenny et al. [21] demonstrated that cocoa oligomers are potent stimulators of both the innate immune system and early events in adaptive immunity. They supported their hypothesis with evidence that the chain length of flavanol fractions showed a significant effect on cytokine release from both unstimulated and LPS-stimulated PBMCs after 16 h treatment (20 μg/mL). For example, there was a marked increase in LPS-induced synthesis of interleukin (IL)-1β, IL-6, IL-10, and TNF-α in the presence of long-chain flavanol fractions (hexamers to decamers). Both long- and short-chain flavanol fractions stimulated the production of granulocyte macrophage colony-stimulating factor (GM-CSF) in the absence of LPS and increased the expression of the B cell markers CD69 and CD83. However, different results were obtained by other authors using phytohemagglutinin A (PHA)-stimulated PBMCs, in which the production of these cytokines depended on the degree of polymerization. For example, IL-1β was suppressed by monomers to tetramers, but stimulated by pentamers to decamers, and TNF-α was clearly increased only by decamers [25]. These results indicate that some effects of cocoa polyphenols depend on their degree of polymerization as well as the agent used for cell stimulation.

8. Anti-Inflammatory Properties of Phenolics from Cocoa

Sies et al. [57] reviewed the effects of cocoa phenolics on inflammatory mediators such as leukotrienes. Some of these effects have been previously cited for their implication in cardiovascular diseases. They reported that (−)-epicatechin

inhibits the dioxygenase and LTA_2 synthase activities of 5-LOX, the first two consecutive steps in the conversion of arachidonic acid into various proinflammatory agents, including leukotrienes, which are catalyzed by the same enzyme. (−)-Epicatechin has also been shown to inhibit 15-LOX-1. When other cocoa products (e.g., oligomeric procyanidins) were tested as potential inhibitors, the authors observed that only small procyanidins (dimers to pentamers) showed effects similar to those of monomers. However, the pattern against 12-LOX-1 was quite different. The inhibitory potency first decreased from monomers to tetramers but then increased with the oligomer size, with the decamer being the most potent. These effects were also demonstrated *in vivo*. The subjects with a diet including polyphenol-rich cocoa products had lower levels of plasma leukotrienes LTC_4, LTD_4, and LTE_4, along with increased levels of prostacyclin 2 h after treatment [25].

8.1. Effects on Proinflammatory Cytokine Release. Ramiro et al. [74] studied the effects of a cocoa extract on the secretion and RNA expression of various proinflammatory mediators by macrophages. Of these, monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)-α were significantly and dose-dependently diminished by the extract. All cocoa flavonoids tested were capable of reducing MCP-1 secretion after 6 h of LPS activation. Thus, epicatechin (100−200 μM, corresponding to 29−58 μg/mL) reduced MCP-1 secretion, exhibiting an inhibition of up to 28%; cocoa extract (50 μg/mL) also diminished MCP-1 secretion, and this effect was significantly stronger than that produced by epicatechin (58 μg/mL). Isoquercitrin (5−40 μM, which represents 2.32−18.6 μg/mL) was the most effective in lowering MCP-1 levels, causing a 28−65% dose-dependent decrease. As for TNF-α, both epicatechin (58 μg/mL) and isoquercitrin (18.6 μg/mL) significantly reduced TNF-α secretion, achieving an inhibition of approximately 35% and 20%, respectively. Cocoa extract (50 μg/mL) also significantly decreased TNF-α secretion by about 15%. These effects may be produced, at least in part, at the transcriptional level. A 58 μg/mL concentration of epicatechin reduced TNF-α and IL-6 mRNA levels, having a higher effect on IL-1α mRNA expression, and one which was very close to basal levels. A 25 μg/mL concentration of cocoa extract produced a greater downregulation of TNF-α, IL-1α, and IL-6 mRNA. Following cocoa extract treatment, IL-1α mRNA remained at basal levels and LPS-induced TNF-α mRNA overexpression was reduced. In addition, the extract decreased NO secretion in a dose-dependent manner and with a greater effect than that produced by epicatechin.

There is experimental and clinical evidence that polyphenols from cocoa may reduce the production of more specific proinflammatory cytokines than those listed above, such as IL-1β and IL-2, while stimulating the production of anti-inflammatory cytokines such as TGF-β and IL-4. Selmi et al. [25] reviewed the studies carried out in resting or phytohemagglutinin A (PHA)-stimulated PBMCs. As they recall, IL-2 production is inhibited by pentamers to heptamers in PHA-stimulated PBMCs at mRNA level. In the case of IL-1β, they describe the biphasic effect of flavonol fractions from monomers to tetramers, which suppressed its mRNA expression and protein secretion, while fractions from pentamers to decamers stimulated IL-1β production. Similar effects were observed in the case of TNF-α, which was increased 4-fold by decameric fractions, while monomer and dimer fractions increased TNF-α production only slightly. In the case of the anti-inflammatory cytokine IL-4, large cocoa flavanols fractions (pentamer through decamer) enhance protein levels from resting PBMCs, whereas the monomers to tetramers were not stimulatory. However, in PHA-stimulated cells, the monomer was the only flavanol fraction able to induce IL-4.

8.2. Effects on Nuclear Factor Activation and Signal Transcription. The regulatory effects of cocoa flavanols on nuclear factor-κB (NF-κB) activation have also been studied. (−)-epicatechin, (+)-catechin, and their dimeric forms were found to inhibit the NF-κB activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in T cells, with a clear reduction of NF-κB-DNA binding activity that leads to a reduction in IL-2 production. These effects were related to direct interaction with the inhibitor of κB (IκB) to prevent its phosphorylation, thereby preventing NF-κB activation [25]. In a complementary study, Zhang et al. [75] investigated the effects of procyanidin dimer B2 on COX-2 expression and demonstrated that pretreatment for 30 min of differentiated human monocytic cells (THP-1) in culture with procyanidin B2 (50 μM) reduced the LPS-induced expression of this enzyme. This effect was correlated to the decreased activation of ERK, Jun-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK), as well as to the suppression of NF-κB activation through stabilization of IκB proteins, which suggests that these signal-transducing enzymes could be potential targets for procyanidin B2.

8.3. Inflammatory Bowel Disease (IBD). Various studies conducted in both *in vivo* and *in vitro* models have provided evidence that pure polyphenols and natural polyphenol plant extracts can modulate intestinal inflammation. These studies indicate that pure polyphenols and plant polyphenolic extracts may play an anti-inflammatory role via the modulation of intracellular signaling cascades in intestinal cells. However, the number of studies on this subject is limited and the results have been contradictory [76].

Romier-Crouzet et al. [77] studied different polyphenol extracts, including those from cocoa, and tested their ability to modulate intestinal inflammation using human intestinal Caco-2 cells treated for 4 h and stimulated with IL-1β for 24 or 48 h. Surprisingly, they found that in the absence of any proinflammatory stimulus, the polyphenol extract of cocoa (50 μmol of gallic acid/L) induced a basal PGE_2 synthesis in Caco-2 cells. They attributed this effect to cyclooxygenase (COX)-1 induction. However, the same extract decreased PGE_2 synthesis 4.6- and 2.2-fold in Caco-2 cells stimulated for 24 or 48 h, respectively. As COX-1 seems to be implicated in maintaining mucosal integrity, the induction of COX-1 activity could be a new beneficial property of cocoa polyphenol extract.

A recent study performed by Andújar et al. [27] demonstrated the anti-inflammatory effects of a cocoa polyphenol-enriched extract (500 mg/kg) on an experimental model of IBD. The extract was shown to reduce weight loss, improve normal stool consistency, and reduce or eliminate visible blood in feces in a dextran sulfate sodium (DSS)-induced model of colitis. In parallel, it reduced neutrophil infiltration, NO production, COX-2 expression, and expression of the signal transducers and activators of transcription (STAT)-3 and STAT1α phosphorylation; however, the authors could not demonstrate a clear inhibitory effect on NF-κB in the nuclear extract of the mid and distal colon. The activation of STAT1 triggers an important signaling pathway for many cytokine and growth factor receptors. STAT1 plays a relevant role in IBD and especially in ulcerative colitis, because there is an increased expression and activation of this transcription factor in the neutrophils and monocytes present in the intestinal lamina propria. In addition, STAT3 can be activated by different mediators and after dimerization and migration to the nucleus; it can induce the activation of various genes, with the consequent induction of other proinflammatory mediators. The inhibition of these transcription factors decreases the production of COX-2, IL-6, IL-1β, and TNF-α, which may explain the effect of cocoa polyphenol on experimental models of ulcerative colitis. In isolated peritoneal macrophages, the authors observed reductions in the proinflammatory cytokines IL-1β, IL-6, and TNF-α, probably as a consequence of the inhibition of the transcriptional factors STAT3 and STAT1α. However, interferon (IFN)-γ was not modified. Finally, working with LPS-stimulated RAW 264.7 cells, a reduction of nuclear p65 was observed in the NF-κB reporter gene assay.

The controversy on the effects of cocoa consumption was clarified somewhat by Vázquez-Agell et al. [78], who demonstrated that cocoa polyphenols reduce NF-κB activation in peripheral blood mononuclear cells in humans. They evaluated the effects of 40 g of cocoa powder with either milk or water against those of milk alone in 18 healthy volunteers. NF-κB activation in leukocytes and adhesion molecules was measured before and 6 h after each intervention. The results established that consumption of cocoa with water significantly decreased NF-κB activation from the baseline value. Cocoa with milk produced no change, and milk alone led to a significant increase in NF-κB activation. The concentration of soluble intercellular adhesion molecule (sICAM)-1 significantly decreased 6 h after cocoa-water and cocoa-milk interventions, with E-selectin decreasing only after cocoa-water intervention. No significant changes were observed in the concentration of soluble vascular cell adhesion molecule (sVCAM)-1. The authors concluded that cocoa consumption may confer beneficial anti-inflammatory effects mediated by inhibition of the NF-κB-dependent transcription pathway or by direct interaction with certain cytokines and that the food matrix probably plays a crucial role in the modulation of this effect.

Ingestion or systemic administration of polyphenols may inhibit gene expression and induce phase II antioxidant and detoxifying proteins, which may help them prevent and/or ameliorate inflammatory colitis. Because enteral nutrition plays an important role in the management of IBD, artificial nutrition attempts to safely administer the essential and otherwise beneficial constituents of food to patients with an impaired ability to ingest or digest food; yet polyphenols are not included in the formulae. The addition of these compounds to artificial nutritional formulae could improve the outcome of patients with IBD in need of enteral nutrition [79].

9. Phenolics from Cocoa in Cancer Prevention

The antioxidant properties of cocoa or cocoa-derived products enriched in flavonoids may help protect against diseases in which oxidative stress is implicated as a causal or contributing factor. The most relevant antioxidant properties have been described above; the following sections are a compilation of these effects and their implications for cancer.

9.1. Cell Protection against Active Oxygen Radicals. Cocoa liquor polyphenols could thus be therapeutically beneficial, as they effectively inhibit mitomycin C-induced DNA damage and exert anticlastogenic effects. Because oxidative DNA damage seems to be implicated in mutagenesis and carcinogenesis, the anticlastogenic activities of cocoa extract, both through the inhibitory effect of cocoa polyphenols on the DNA strand cleavage induced by mitomycin C *in vitro* and through their effect against the formation of micronuclei induced by mitomycin C in bone marrow cells and peripheral blood cells of mice, may explain cocoa's role in the prevention of DNA damage, which probably occurs through the scavenging of active oxygen radicals generated in reactions initiated by mitomycin C [80].

Cho et al. [81] studied the protective effects of a cocoa procyanidin fraction and procyanidin B2 against the apoptosis of PC12 rat pheochromocytoma cells induced by hydrogen peroxide. In this case, the fraction (1 and 5 μg/mL) and procyanidin B2 (1 and 5 μM) reduced cell death by attenuating the hydrogen peroxide-induced fragmentation of the nucleus and DNA in PC12 cells. Hydrogen peroxide acts in part by inducing cleavage of poly(ADP-ribose)polymerase (PARP) and downregulating Bcl-XL and Bcl-2 in PC12 cells, but pretreatment with cocoa procyanidin fraction or procyanidin B2 before hydrogen peroxide treatment diminished PARP cleavage, increased Bcl-XL and Bcl-2 expression, and also inhibited activation of caspase-3 by hydrogen peroxide while attenuating the phosphorylation of JNK and MAPK. Cocoa procyanidin fraction and procyanidin B2 thus have protective effects against both hydrogen peroxide and the oxidative stress induced by ROS, which has been strongly associated with the pathogenesis of neurodegenerative disorders, including Alzheimer's disease. A cocoa-rich diet also exerts antiproliferative effects in azoxymethane-induced colon cancer, decreasing ERK, protein kinase B (Akt), and cyclin D1 levels, as demonstrated in Wistar rats. In addition, it has proapoptotic effects, as evidenced by reduced Bcl-XL levels and increased levels of Bax and caspase-3 activity [82].

Martín et al. [83] studied the potential protective effect of a polyphenolic extract from cocoa on cell viability and

the antioxidant defenses of cultured human HepG2 cells subjected to oxidative stress by *t*-butyl hydroperoxide. Pretreatment of cells with 0.05–50 μg/mL of cocoa polyphenol extract for 2 or 20 h completely prevented cell damage and enhanced the activity of antioxidant enzymes. Moreover, the lower levels of GSH caused by *t*-butyl hydroperoxide in HepG2 cells were partly recovered through pretreatment with cocoa-enriched extract, which also prevented in a dose-dependent fashion the increase in ROS induced by *t*-butyl hydroperoxide. Cocoa polyphenols thus seem to confer significant protection against oxidation in these cells.

Cocoa polyphenol extracts dose-dependently (10–100 μM) attenuated hydrogen peroxide-induced inhibition of gap-junction intercellular communication (GJIC) in rat liver epithelial (RLE) cells and inhibited the hydrogen peroxide-induced phosphorylation and internalization of connexin 43, which is a regulating protein of GJIC in RLE cells. It also inhibited hydrogen peroxide-induced accumulation of ROS and activation of ERK. However, cocoa polyphenol extract did not block hydrogen peroxide-induced phosphorylation of MAPK. Similar results were observed *ex vivo* in RLE cell lysates in which cocoa polyphenol extract inhibited hydrogen peroxide-induced MAPK/MEK1 activity. In these assays cocoa polyphenol bound directly with MEK1 to inhibit its activity. Inhibition of GJIC is strongly involved in carcinogenesis, particularly the tumor promotion process. Abnormal GJIC is attributable to phosphorylation of connexin 43, which is a major protein constituting gap-junction channels. The activation of ERK and MAPK signaling pathways is strongly related to both the phosphorylation of connexin 43 and the inhibition of GJIC in RLE cells. The authors thus concluded that cocoa polyphenols protect against hydrogen peroxide-induced inhibition of GJIC through antioxidant activity and direct inhibition of MEK activity, which may contribute to their chemopreventive potential [84].

9.2. Antiproliferative Effects. Treatment of cells with 50 μg/mL of procyanidin-enriched extracts inhibited cell growth by 70%, blocking the cell cycle in the G_2/M phase. The extracts also caused a significant decrease in the activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase, two key enzymes of polyamine biosynthesis, thus leading to a decrease in the intracellular pool of polyamines. This could prove to be an important target in the antiproliferative effects of cocoa polyphenols [85].

9.3. Antimutagenic and Chemoprotective Effects. Ohno et al. [86] found that cocoa extracts have an antimutagenic effect on chemical mutagens (benzo[a]pyrene) that require metabolic activation by cytochrome P450 (CYP), specifically CYP1A, *in vitro*. Cocoa products may thus prevent initiation of cancer by inhibiting the metabolic activation of carcinogens by CYP1A. The authors used the Ames test to study the effects of various enriched extracts of whole cocoa products on the mutagenicity of benzo[a]pyrene in *Salmonella typhimurium* strain TA 98 and *t*-butyl hydroperoxide in *S. typhimurium* strain TA 102. Benzo[a]pyrene induces mutagenicity through metabolic activation, whereas *t*-butyl hydroperoxide induces it through generation of free radicals. While white chocolate did not modulate the number of revertant colonies produced by benzo[a]pyrene treatment, milk chocolate and cocoa powder extracts did. Surprisingly, none of the cocoa products tested affected the number of revertant colonies produced by *t*-butyl hydroperoxide. Nevertheless, at maximum concentration (13.25 mg cocoa powder/mL), the crude cocoa powder extract reduced ethoxyresorufin *O*-deethylase activity to 17.4%, suggesting that whole cocoa products inhibit CYP1A activity, which may prevent DNA damage by reducing the metabolic activation of carcinogens.

In another study, Oleaga et al. [87] evaluated the effect of polyphenol cocoa extract in breast cancer cells (MCF-7 and SKBR3). After incubation with 250 ng/μL of cocoa extract, 7 genes out of 84 were overexpressed and 1 was underexpressed in MCF-7 cells, whereas 9 genes were overexpressed in SKBR3 cells. Because CYP1A1 was expressed in both cell lines, it was selected for further study. CYP1A1 mRNA, protein levels, and enzymatic activity all increased upon incubation with polyphenol cocoa extract. The combination of polyphenol cocoa extract with tamoxifen caused a synergistic cytotoxicity in both cell lines due to increased apoptosis of MCF-7 cells. The authors concluded that the interaction between estrogen receptor (ER)-α and aryl hydrocarbon receptor (AhR) upon incubation with polyphenol cocoa extract leads to the induction of CYP1A1 in breast cancer cells. The synergy observed between polyphenol cocoa extract and noncytotoxic tamoxifen concentrations opens the possibility for a combination therapy in which polyphenols from cocoa are used to boost the efficacy of tamoxifen.

A cocoa procyanidin fraction and procyanidin B2 at 5 μg/mL and 40 μM, respectively, inhibited TPA-induced neoplastic cell transformation of (JB6 P^+) mouse epidermal (JB6 P^+) cells by 47 and 93%, respectively, through the suppression of the kinase activity of MEK. They also inhibited the expression of COX-2, the TPA-induced activation of activator protein (AP)-1 and NF-κB, and the TPA-induced phosphorylation of MEK, ERK, and p90 ribosomal s6 kinase, all of which are implicated in inflammation and cancer [88].

Kim et al. [89] studied a cocoa polyphenol extract and its effect on vascular endothelial growth factor (VEGF) expression JB6 P^+ cells as well as its biological consequences, including its potential effects against proinflammatory cytokine-mediated skin cancer and inflammation. The cocoa polyphenol extract (5–20 μg/mL) inhibited TNF-α-induced upregulation of VEGF by reducing TNF-α-induced activation of the nuclear transcription factors AP-1 and NF-κB, which are key regulators of VEGF expression. The extract also inhibited TNF-α-induced phosphorylation of Akt and ERK, blocked activation of their downstream kinases (p70 ribosomal protein S6 kinase and p90 ribosomal protein S6 kinase), and suppressed phosphoinositide 3-kinase (PI3 K) activity by binding PI3 K directly. Moreover, although it did not affect the TNF-α-induced phosphorylation of MEK1, it suppressed TNF-α-induced MEK1 activity. The direct inhibition of PI3 K and MEK1 activities though the reduction of TNF-α-induced upregulation of VEGF may contribute to the chemopreventive potential of cocoa polyphenol. Indeed, the expression of VEGF, a multifunctional cytokine that is

a key regulator of angiogenesis, is not only associated with tumorigenesis, but also with cardiovascular disease, rheumatoid arthritis, and psoriasis. Because cancerous cells require blood vessels to increase the flow of nutrients and oxygen vital for their development and survival via angiogenesis, the inhibition of VEGF expression may be a promising approach for protecting against carcinogenesis. Cocoa polyphenols could thus be considered potential chemopreventive agents against cancer and inflammation.

10. Phenols from Cocoa in Tooth Health

The production of caries and consumption of chocolate are often cited in the literature, with the intake of chocolate considered detrimental for tooth health. However, this negative effect is due to high concentrations of compounds present in chocolate other than cocoa, such as sugar. Ferrazzano et al. [90] were of the first to note the possible protective effect of cocoa on dental caries. Cocoa products contain inhibitors of the dextransucrase enzyme, which is responsible for the formation of the plaque extracellular polysaccharides from sucrose. Moreover, phenolic substances may be responsible for the observed anticaries effect of cocoa powder. Indeed, a water-soluble extract, of cocoa powder was shown to significantly reduce caries scores in rats infected with *Streptococcus sobrinus*, a potent cariogenic α-haemolytic streptococcus. Complementary studies have demonstrated that cocoa polyphenols inhibit the growth of *S. sanguinis*, but not that of *S. mutans*. Although *S. mutans* appears to be refractory to the growth-inhibitory or lethal effects of the cocoa polyphenol pentamer in routine studies, the compound still managed to significantly inhibit acid production from sucrose. Indeed, a recent study reports on the use of the ground husk of cocoa beans, a by-product of cocoa manufacture, to prepare a mouth rinse for children that was even more effective in decreasing plaque scores.

Tomofuji et al. [91] studied the potential effects of a cocoa-enriched diet (10% of the total food intake) on gingival oxidative stress in a rat-periodontitis model and concluded that a diet rich in cocoa could diminish periodontitis-induced oxidative stress, which, in turn, might suppress the progression of periodontitis. In fact, they observed that rats with induced-experimental periodontitis which were fed a cocoa-enriched diet did not show impairments in serum reactive oxygen metabolite levels or gingival levels for 8-hydroxydeoxyguanosine, nor did they exhibit a reduced/oxidized glutathione ratio, as did the control group. They proposed further studies to definitively establish the bacterial pathogens in the periodontium and the optimum dose of cocoa in the diet, along with new experiments to develop an inflammatory process equivalent to chronic periodontitis in humans.

Mao et al. [92] suggested that the consumption of some cocoas and chocolates could reduce the risk for dental caries and periodontal disease, justifying their hypothesis on the basis of purified cocoa flavanol oligomers' immunomodulatory effects on the *in vitro* production of cytokines IL-1β, IL-2, IL-4, and IL-5. These authors established a biphasic-type effect in which the larger (hexamer through decamer) procyanidin fractions showed more activity in regulating cytokine production. In the case of IL-5, the smaller procyanidin fractions (monomer through trimer) can augment IL-5 secretion. This cytokine is implicated in the differentiation of B cells to IgA-producing plasma cells. IgA is considered to be protective in periodontal diseases; therefore in this enhanced effect of certain cocoa oligomer fractions could be therapeutic for periodontal disease.

11. Other Pharmacological Effects

11.1. Antiobesity Effects. Rats fed a cocoa-enriched diet experienced a reduction in body weight, most likely due to lower adipose tissue synthesis. Ferrazzano et al. hypothesized that the polyphenols contained in cocoa may have antiobesity effects due to their ability to suppress fatty acid synthesis while stimulating cell energy expenditure in the mitochondria [90].

Moreover, cocoa consumption may also have beneficial effects on satiety, cognitive function, and mood [93].

11.2. Chronic Fatigue Syndrome. Sathyapalan et al. [94] studied a polyphenol-rich chocolate and observed that it reduced the symptoms of chronic fatigue syndrome. In fact, in a double blind, randomized, clinical pilot crossover study with 10 subjects, the authors compared the effect of consuming either high cocoa liquor/polyphenol-rich chocolate or simulated isocalorific chocolate (cocoa liquor free/low polyphenol content) on fatigue and residual function in subjects with chronic fatigue syndrome. After 8 weeks, subjects in the high cocoa liquor/polyphenol-rich chocolate group showed significant improvement as measured by different scoring methods, including the Chalder Fatigue Scale and Hospital Anxiety and Depression scores, whereas subjects taking isocalorific chocolate deteriorated.

11.3. Neurodegenerative Diseases. Bisson et al. [95] studied and demonstrated that a cocoa polyphenol extract (Acticoa powder) may help delay age-related brain impairments, including cognitive deficits in normal ageing and perhaps neurodegenerative diseases. In experiments using rats, administration of Acticoa powder (24 mg/kg/day, orally, between 15 and 27 months of age) did not show any influence on weight or food and water consumption throughout the study period; the benefits observed with the cocoa extract are thus not due to dietary restriction. While the results of this study suggest that Acticoa powder may be beneficial in retarding age-related brain impairments, further studies are needed to elucidate the mechanisms of cocoa polyphenols in neuroprotection and to explore their effects in man [95]. Moreover, cocoa consumption protects nerves from injury and inflammation [93].

11.4. Antimalarial Effects. Numerous anecdotal reports of reduced episodic malaria in people from Ghana who drink a natural, unsweetened cocoa beverage daily prompted a search for scientific mechanisms to account for cocoa's possible antimalarial effects. In a review of the data from

Table 1: Effects of cocoa phenolics on metabolism, cardiovascular diseases, inflammation, and cancer prevention.

(a)

Metabolic and endocrine disorders		
	Assays	Effects
In vitro	Pancreatic α-amylase, pancreatic lipase, and secreted PLA_2 inhibitions [45]	(i) Cocoa extracts and procyanidins dose-dependently inhibited pancreatic α-amylase, pancreatic lipase, and secreted PLA_2, showing greater inhibitory activity against PLA_2. The inhibitory potency increases with the degree of polymerization
	HepG2 and Caco2 [50]	(i) (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 (10 μM): ↑ ApoA1 ↓ ApoB due to upregulation of SREBPs and increased LDL receptor activity
In vivo	Randomized crossover trial with 19 hypertensive patients with impaired glucose tolerance [47]	(i) Flavanol-rich dark chocolate (100 g/day for 15 days): (a) ↓ insulin resistance, systolic and diastolic blood pressure, total cholesterol, and LDL-cholesterol (b) ↑ insulin sensitivity, β-cell function, and flow-mediated dilation
	Randomized, crossover feeding trial in 42 high-risk volunteers [51]	(i) Chronic cocoa consumption (42 g/day for 4 weeks): (a) significant increases of phase II metabolites (glucuronide, sulfate conjugates) in 24 h urine (b) ↑ HDL-cholesterol (c) ↓ LDL-cholesterol
	Meta-analysis of 8 randomized controlled trials involving 215 participants [52]	(i) Short-term cocoa consumption: (a) ↓ LDL and total cholesterol (changes depended on the amount of cocoa consumed and the health status of participants)
	Randomized, single-blind, crossover study with 14 overweight/obese subjects [53]	(i) 20 g of dark chocolate with 500 mg of polyphenols and then 20 g of dark chocolate with 1000 mg of polyphenols or vice versa for 2 weeks separated by a 1-week washout period: (a) both doses were equally effective in reducing fasting blood glucose levels, systolic blood pressure, and diastolic blood pressure
	Randomized, placebo-controlled double-blind crossover trial [31]	(i) High polyphenol chocolate (16.6 mg of epicatechins in 45 g): (a) ↑ HDL-cholesterol (b) ↓ LDL-cholesterol, total cholesterol/HDL ratio High-polyphenol chocolate is effective in improving the atherosclerotic cholesterol profile in patients with diabetes

(b)

Cardiovascular diseases	
Assays	Effects
Forty-two high-risk patients in a randomized crossover feeding trial for 4 weeks [32]	(i) 40 g of cocoa powder with 500 mL skim milk/day: (a) ↓VLA-4, CD40, and CD36 in monocytes (b) ↓ serum concentrations of P-selectin and ICAM-1
Resting human PBMCs from 13 healthy subjects treated with 25 μg/mL of procyanidin fractions isolated from cocoa [61]	(i) Individuals with low baseline levels of TGF-β_1: TGF-β_1 release was enhanced in the range of 16–66%. Low-molecular-weight fractions (≤ pentamers) were more effective than their larger counterparts (hexamer or higher), with the monomer and dimer inducing the greatest increases (66% and 68%, resp.) (ii) Individuals with high baseline levels of TGF-β_1: TGF-β_1 secretion was inhibited, being the inhibition most pronounced for trimmers through decamers (28–42%, resp.) and moderate for monomers to dimers (17–23%, resp.)
Twenty healthy subjects followed a balanced diet for 4 weeks; since day 14 to 27, they introduced daily 45 g of dark chocolate (860 mg of polyphenols, of which 58 mg were epicatechin) or white chocolate (5 mg polyphenols, undetectable epicatechin) [44]	(i) 2 h after dark chocolate intake (860 mg of polyphenols, of which 58 mg were epicatechin): (a) detectable epicatechin levels were observed (b) less DNA damage to mononuclear blood cells (c) no effect on plasma total antioxidant activity (ii) Effects were no longer evident after 22 h: dark chocolate

(b) Continued.

Cardiovascular diseases	
Assays	Effects
Blinded parallel-design study with 32 healthy subjects consuming 234 mg cocoa phenolics a day for 28 days [63]	(i) ↑ Plasma epicatechin and catechin concentrations by 81% and 28%, respectively (ii) ↓ Platelet function
Double-blind, randomized study with 22 heart transplant recipients [64]	(i) 2 h after ingestion of 40 g of flavonoid-rich dark chocolate (0.27 mg/g of catechin and 0.9 mg/g of epicatechin, with a total polyphenol content of 15.6 mg of epicatechin equivalents per gram): (a) ↑ coronary artery diameter (b) improved endothelium-dependent coronary vasomotion (c) ↓ platelet adhesion
Crossover, single blind study with 20 healthy subjects and 20 smokers who received 40 g of dark (cocoa > 85%) or milk (cocoa < 35%) chocolate [65]	(i) Smokers: (a) ↑ platelet recruitment, platelet formation of ROS and eicosanoids, and NOX2 activation (ii) Smokers + dark chocolate group: (a) ↓ platelet ROS, $PGF_{2\alpha}$, and NOX2 activation decreased significantly (iii) Healthy + dark chocolate: (a) platelet variables did not change (iv) Milk chocolate (smokers and healthy): (a) no changes detected in either of the groups treated with milk chocolate
Single oral administration of a natural flavonoid-enriched cocoa powder (50–600 mg/kg) in spontaneously hypertensive rats [67]	Antihypertensive effect in hypertensive rats without modifying the arterial blood pressure in normotensive rats. No dose-response effect was observed
Fifty male Kurosawa and Kusanagi-hypercholesterolemic rabbits received 100 g/day of standard diet or cacao liquor polyphenol diet [68]	(i) Polyphenol-treated group: (a)↓ area of atherosclerotic lesions in the aortas of the polyphenol-treated group was significantly smaller than in the control group (b) preserved parasympathetic nervous tone (c) no differences in the plasma lipid concentrations
Six-month clinical trial with 36 prehypertensive healthy adult volunteers, at content levels of 120–139 and 80–89 mmHg [70]	(i) 50 g of dark chocolate/day: (a) no significant differences were observed in the blood pressures of the treated and control groups
In vitro measurement of the kinetics of inhibition of purified angiotensin I converting enzyme from rabbit lung [72]	(i) Significantly inhibited the angiotensin I converting enzyme activity (ii) Monomeric flavan-3-ols: IC_{50} in the mM range (iii) Dimer and trimer: IC_{50} in the 100 μM range (iv) Larger procyanidins: IC_{50} in the 10 μM range

(c)

Anti-inflammatory properties		
In vitro assays	Polyphenol	Effect
Unstimulated and LPS-stimulated PBMCs [20]	Different flavanol fractions (20 μg/mL)	(i) Monomers-pentamers: ↑ of LPS-induced synthesis of IL-1β, IL-6, IL-10, and TNF-α (ii) Long- and short-chain flavanol fractions: ↑ the production of GM-CSF in the absence of LPS and the expression of CD69 and CD83
Macrophages [73]	(i) Epicatechin (58 μg/mL) (ii) Cocoa extract (50 μg/mL) (iii) Isoquercitrin (18.6 μg/mL)	(i) ↓ MCP-1 and TNF-α at the transcriptional level (ii) ↓ TNF-α, IL-1α, and IL-6 mRNA levels (iii) ↓ NO secretion
THP-1 [72]	Procyanidin dimer B2: pretreatment for 30 min with 50 μM	(i) ↓ Expression of COX-2 (ii) ↓ Activation of ERK, JNK, and p38 MAPK (iii) Suppresses NF-κB activation through stabilization of IκB proteins
Caco-2 IL-1β-stimulated [77]	Polyphenol extract of cocoa (50 μmol of gallic acid/L); 4 h treatment	(i) In the absence of proinflammatory stimuli, the polyphenol extract of cocoa induces a basal PGE_2 synthesis due to COX-1 induction (ii) After 24 or 48 h stimulation: it decreased PGE_2 synthesis 4.6- and 2.2-fold, respectively

(c) Continued.

Anti-inflammatory properties		
In vivo/Ex vivo	Polyphenol	Effect
DSS-induced ulcerative colitis in Balb/C mice [27]	Cocoa polyphenol-enriched extract (500 mg/kg)	(i) *In vivo*: ↓ weight loss, improves normal stool consistency, ↓ visible blood in feces, ↓ neutrophil infiltration, ↓ NO production, ↓ COX-2 expression, ↓ STAT3 and STAT1α phosphorylation (ii) *Ex vivo* in peritoneal macrophages: ↓ IL-6, IL-1β, and TNF-α (iii) *In vitro* in LPS-stimulated RAW 264.7 cells: inhibits NF-κB
Human PBMCs from 18 healthy volunteers [78]	Cocoa powder (40 g) with either milk or water	(i) Cocoa + water: (a) ↓ NF-κB activation in leukocytes and adhesion molecules (b) ↓ sICAM-1 (c)↓ E-selectin (ii) Cocoa + milk: (a) no change in NF-κB activation (b) ↓ sICAM-1 (iii) Milk: (a) ↑ NF-κB activation
MCF-7 and SKBR3 [87]	(i) After incubation with 250 ng/μL of cocoa extract, 7 genes out of 84 were overexpressed and 1 was underexpressed in MCF-7 cells, whereas 9 genes were overexpressed in SKBR3 cells (ii) CYP1A1 mRNA, protein levels, and enzymatic activity increased (iii) The combination of polyphenol cocoa extract + tamoxifen caused a synergistic cytotoxicity	
RLE cells *in vitro* and *ex vivo* [84]	(i) Cocoa polyphenol extracts dose-dependently (10–100 μM) attenuated *in vitro* hydrogen peroxide-induced (a) inhibition of GJIC (b) phosphorylation and internalization of connexin 43 (c) accumulation of ROS and activation of ERK (ii) *Ex vivo* in RLE cell lysates (a) inhibits hydrogen peroxide-induced MAPK/ MEK1 activity	

In vivo

Animals	Effects
Wistar Han rats (5 weeks old) pretreated for 2 weeks with a cocoa-rich diet and injected with azoxymethane once a week for 2 weeks [82]	(i) The cocoa-rich diet (1 g of polyphenol/kg of diet): (a) antiproliferative effects in azoxymethane-induced colon cancer: ↓ ERK, Akt, and cyclin D1 (b) proapoptotic effects: ↓ Bcl-X_L levels and ↑ levels of Bax and caspase-3 activity

(d)

Cancer prevention	
In vitro	
Cell line	Effects
Caco-2 cells [85]	(i) Treatment of cells with 50 μg/mL of procyanidin-enriched extracts: (a) inhibits cell growth by 70%, blocking the cell cycle in the G_2/M phase (b) ↓ activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase, and therefore, ↓ the intracellular pool of polyamines
PC12 rat pheochromocytoma cells [81]	(i) Cocoa procyanidin fraction (1 and 5 μg/mL) and procyanidin B2 (1 and 5 μM): (a) ↓ cell death attenuating the hydrogen peroxide-induced fragmentation of the nucleus and DNA (b) ↓ PARP cleavage, increased Bcl-X_L and Bcl-2 expression, and also inhibited activation of caspase-3 by hydrogen peroxide while attenuating the phosphorylation of JNK and MAPK
HepG2 cells [83]	(i) Pretreatment of cells subjected to oxidative stress with 0.05–50 μg/mL of cocoa polyphenol extract for 2 or 20 h: (a) completely prevented cell damage and enhanced the activity of antioxidant enzymes (b) recovered levels of GSH (c) prevented in a dose-dependent fashion the increase in ROS

(d) Continued.

Cancer prevention	
In vitro	
Salmonella typhimurium strain TA 98 and TA 102 [86]	(i) Treatment with benzo[a]pyrene (a) white chocolate did not modulate the number of revertant colonies produced by treatment (b) milk chocolate and cocoa powder extracts did (ii) Treatment with *t*-butyl hydroperoxide (a) none of the cocoa products tested affected the number of revertant colonies (b) 13.25 mg cocoa powder/mL reduced ethoxyresorufin *O*-deethylase activity to 17.4% suggesting that whole cocoa products inhibit CYP1A activity
JB6 P+ cells [88, 89]	(i) Cocoa procyanidin fraction (5 μg/mL) and procyanidin B2 (40 μM) inhibit (a) TPA-induced neoplastic cell transformation at 47 and 93%, respectively (b) phosphorylation of MEK, ERK, and p90 ribosomal s6 kinase (c) COX-2 expression (d) AP-1 and NF-κB activation, and the TPA induced (ii) Cocoa polyphenol extract (5–20 μg/mL) (a) inhibits TNF-α-induced upregulation of VEGF by reducing TNF-α-induced activation of AP-1 and NF-κB (b) inhibits TNF-α-induced phosphorylation of Akt and ERK (c) suppresses PI3K activity by binding PI3K directly (d) suppresses TNF-α-induced MEK1 activity
MCF-7 and SKBR3 [87]	(i) After incubation with 250 ng/μL of cocoa extract, 7 genes out of 84 were overexpressed and 1 was underexpressed in MCF-7 cells, whereas 9 genes were overexpressed in SKBR3 cells (ii) CYP1A1 mRNA, protein levels, and enzymatic activity increased (iii) The combination of polyphenol cocoa extract + tamoxifen caused a synergistic cytotoxicity
RLE cells *in vitro* and *ex vivo* [84]	(i) Cocoa polyphenol extracts dose-dependently (10–100 μM) attenuated *in vitro* hydrogen peroxide-induced (a) inhibition of GJIC (b) phosphorylation and internalization of connexin 43 (c) accumulation of ROS and activation of ERK (ii) *Ex vivo* in RLE cell lysates (a) inhibits hydrogen peroxide-induced MAPK/MEK1 activity
In vivo	
Animals	Effects
Wistar Han rats (5 weeks old) pretreated for 2 weeks with a cocoa-rich diet and injected with azoxymethane once a week for 2 weeks [82]	(i) The cocoa-rich diet (1 g of polyphenol/kg of diet): (a) antiproliferative effects in azoxymethane-induced colon cancer: ↓ ERK, Akt, and cyclin D1 (b) proapoptotic effects: ↓ Bcl-X_L levels and ↑ levels of Bax and caspase-3 activity

the literature on this subject, Addai [96] established five possible antimalarial mechanisms for cocoa. Thus, the effects could be due to the increased availability of antioxidants in plasma, membrane effects in general and in the erythrocyte membrane in particular, increased plasma levels of NO, specific antimalarial activity of cocoa flavanoids and their derivatives, and enhancement of the immune system mediated by components of cocoa including cocoa polyphenols.

11.5. Effects on Skin Tone and Elasticity. Cocoa polyphenols have a positive effect on skin structure when applied for at least 5 days. Studying these effects *ex vivo*, Gasser et al. [97] observed that cocoa polyphenols exhibit a positive action on the parameters assessed, including glycosaminoglycans and collagens I, III, and IV, associated with skin tone and elasticity. The doses at which they improve each of these parameters were determined to be 0.75% and 0.50%.

Mukai et al. [98] studied the suppression by a cocoa polyphenol extract (100 mg/kg, orally) of aryl hydrocarbon receptor transformation in C57BL/6 mice. Three hours after administration of the aryl hydrocarbon receptor agonist 3-methylcholanthrene (10 mg/kg, i.p.), the cocoa extract suppressed the induced transformation to control levels by inhibiting the formation of a heterodimer between the aryl hydrocarbon receptor and an aryl hydrocarbon receptor nuclear translocator in the liver. The extract also suppressed 3-methylcholanthrene-induced CYP1A1 expression and NAD(P)H: quinone-oxidoreductase activity while increasing glutathione *S*-transferase activity after 25 h. The authors concluded that the intake of cocoa polyphenol extract suppresses the toxicological effects of dioxins in the body [98]. Moreover, in topical preparations, protect the skin from oxidative damage caused by ultraviolet (UV) radiation [93].

12. Conclusions

Dark chocolate and cocoa enriched-polyphenol extracts may be beneficial for human health in different ways. Cocoa

polyphenols have antioxidant properties, which endow them with various positive effects against several pathological disorders, including cardiovascular disease, inflammatory processes, and cancer. Indeed, cocoa polyphenols induce coronary vasodilatation, increase endothelial NO concentrations to induce vascular relaxation, improve vascular function, and decrease platelet adhesion. Moreover, they decrease levels of LDL-cholesterol and its oxidation while increasing HDL-cholesterol. Polyphenols also have anti-inflammatory activity, especially against IBD, through the inhibition of different transcription factor and cytokines. These effects also produce chemopreventive effects on other chronic diseases such as cancer by inhibiting the growth of various cancer cell lines. Of special interest are the effects of polyphenols on colon cancer. Indeed, the protective effects of polyphenols against IBD prevent it from evolving into cancer. In addition, it has been demonstrated that polyphenols cause nonapoptotic cell death, blocking the cell cycle in the G_2/M phase, which potentiates their antiproliferative effects. To summarize these effects, Table 1 lists the most relevant studies on cocoa phenolics and their findings, including the principal effects on metabolism, cardiovascular diseases, inflammation, and cancer prevention.

Abbreviations, Symbols, and Cell Lines

ABTS: 2, 2′-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
Akt: Protein kinase B
AhR: Aryl hydrocarbon receptor
AP: Activator protein
Apo: Apolipoprotein
Caco-2: Human epithelial colorectal adenocarcinoma cells
CD: Cluster of differentiation
CD36: Thrombospondin receptor
CD40: TNF receptor superfamily member 5
cGMP: Cyclic guanosine monophosphate
COX: Cyclooxygenase
CYP: Cytochrome P450
DNA: Deoxyribonucleic acid
DPPH: 2,2-Diphenyl-1-pycrylhydrazyl
DSS: Dextran sulfate sodium
EDHF: Endothelium-derived hyperpolarizing factor
eNOS: Endothelial nitric oxide synthase
ER-α: Estrogen receptor-α
ERK: Extracellular signal-regulated protein kinase
FRAP: Ferric reducing-antioxidant power
GJIC: Gap-junction intercellular communication
GM-CSF: Granulocyte macrophage colony-stimulating factor
HDL: High-density lipoprotein
HepG2: Human hepatocellular carcinoma
IBD: Inflammatory bowel disease
IC_{50}: Inhibitory concentration-50
ICAM: Intercellular adhesion molecule
IFN-γ: Interferon-γ
Ig: Immunoglobulin
IκB: Inhibitor of κB
IL: Interleukin
i.p.: Intraperitoneally
JNK: Jun-terminal kinase
LDL: Low-density lipoprotein
LOX: Lipoxygenase
LPS: Lipopolysaccharide
LT: Leukotrienes
MAPK: Mitogen-activated protein kinase
MCF-7: Breast cancer cells (Michigan Cancer Foundation-7)
MCP: Monocyte chemoattractant protein
MEK: Mitogen-activated protein kinase kinase
MMP: Matrix metalloproteinase
MPO: Myeloperoxidase
MT1: Membrane type-1
NADPH: Nicotinamide adenine dinucleotide phosphate
NF-κB: Nuclear factor-κB
NO: Nitric oxide
NOX: NADPH oxidase
PARP: Poly(ADP-ribose)polymerase
PBMCs: Human peripheral blood mononuclear cells
PC12: Rat pheochromocytoma cells
PG: Prostaglandin
PHA: Phytohemagglutinin
PI3 K: Phosphoinositide 3-kinase
PLA_2: Phospholipase A_2
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RLE: Rat liver epithelial
sICAM: Soluble intercellular adhesion molecule; CD54
SKBR3: Human breast carcinoma cells
SREBP: Sterol regulatory element binding protein
STAT: Signal transducers and activators of transcription
sVCAM: Soluble vascular cell adhesion molecule; CD106
TGF-β_1: Transforming growth factor-β_1
THP-1: Human monocytic cells
TNF-α: Tumor necrosis factor-α
TPA: 12-*O*-Tetradecanoylphorbol-13-acetate
UV: Ultraviolet
VEGF: Vascular endothelial growth factor
VLA-4: Very late antigen-4
VSMC: Vascular smooth muscle cell.

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Research Article

Coffee Polyphenols Change the Expression of STAT5B and ATF-2 Modifying Cyclin D1 Levels in Cancer Cells

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Background. Epidemiological studies suggest that coffee consumption reduces the risk of cancer, but the molecular mechanisms of its chemopreventive effects remain unknown. *Objective*. To identify differentially expressed genes upon incubation of HT29 colon cancer cells with instant caffeinated coffee (ICC) or caffeic acid (CA) using whole-genome microarrays. *Results*. ICC incubation of HT29 cells caused the overexpression of 57 genes and the underexpression of 161, while CA incubation induced the overexpression of 12 genes and the underexpression of 32. Using Venn-Diagrams, we built a list of five overexpressed genes and twelve underexpressed genes in common between the two experimental conditions. This list was used to generate a biological association network in which STAT5B and ATF-2 appeared as highly interconnected nodes. STAT5B overexpression was confirmed at the mRNA and protein levels. For ATF-2, the changes in mRNA levels were confirmed for both ICC and CA, whereas the decrease in protein levels was only observed in CA-treated cells. The levels of cyclin D1, a target gene for both STAT5B and ATF-2, were downregulated by CA in colon cancer cells and by ICC and CA in breast cancer cells. *Conclusions*. Coffee polyphenols are able to affect cyclin D1 expression in cancer cells through the modulation of STAT5B and ATF-2.

1. Introduction

Polyphenols are the most abundant antioxidants in the diet. Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis suggesting a role of these antioxidants in the prevention of neurodegenerative diseases and diabetes mellitus [1].

It is well established that polyphenol ingestion results in an increase of the plasma-antioxidant capacity. However, there is still some uncertainties about their efficiency to enhance the protection of cellular components, such as lipids or DNA, against oxidative stress in humans [2]. Polyphenols and other antioxidants were thought to protect cell constituents against oxidative damage by scavenging free radicals. However, this concept now appears to be an oversimplified view of their mode of action [3]. More likely, cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions [4]. This could also apply to the anticarcinogenic effects of polyphenols, which properties may be explained by many different mechanisms.

Hydroxycinnamic acids are a major class of polyphenols found in almost every plant [2]. The major representative of hydroxycinnamic acids is caffeic acid, which occurs in food mainly as an ester with quinic acid named chlorogenic acid (5-caffeoylquinic acid). Coffee is a major source of chlorogenic acid in the human diet; the daily intake in coffee drinkers is 0.5–1 g whereas coffee abstainers will usually ingest <100 mg/day. Studies have shown that approximately the 33% of ingested chlorogenic acid and the 95% of caffeic acid are absorbed intestinally [5]. Thus, about two-thirds of ingested chlorogenic acid reach the colon where it is probably metabolized to caffeic acid [6].

Bioavailability data suggest that the biological effects of chlorogenic acid would become apparent after its metabolism to caffeic acid, and hence the need of studying the effects of this acid. Chlorogenic acid and caffeic acid are

antioxidants *in vitro* [7], and they might inhibit the formation of mutagenic and carcinogenic N-nitroso compounds since they are inhibitors of the N-nitrosation reaction *in vivo* [8]. Furthermore, chlorogenic acid can inhibit DNA damage *in vitro* [9] as it inhibits lipid peroxidation-induced DNA adduct formation [10] and suppresses reactive oxygen species-mediated nuclear factor (NF-κB), activator protein-1 (AP-1), and mitogen-activated protein kinase activation by upregulating antioxidant enzymes [11]. These studies suggested that coffee polyphenols are potent chemopreventive agents.

Recent meta-analyses demonstrate inverse associations between coffee intake and the risk of colon, liver, breast, and endometrial cancer [12–15]. Moreover, in prospective population-based cohort studies, the inverse association between coffee consumption and risk of cancer has been shown. The group of Naganuma [16] found that the consumption of at least one cup of coffee per day was associated with a 49% lower risk of upper gastrointestinal cancer in a Japanese population, while Wilson and collaborators [17] found that men who regularly drink coffee appeared to have a lower risk of developing a lethal form of prostate cancer. The lower risk was evident when consuming either regular or decaffeinated coffee. It has been proposed that the inverse association between coffee intake and colon cancer could be explained, at least in part, by the presence of chlorogenic acid in coffee [18]. Ganmaa et al. [19] observed a general protective effect of caffeine intake on breast cancer risk for both ER subtypes, but the effect was only found to be significant for ER-positive breast cancers. In this study, the association between caffeine and breast cancer was stronger among postmenopausal women with estrogen-receptor and progesterone-receptor-positive breast cancer than those with estrogen-receptor and progesterone-receptor negative breast cancer [19]. In another study, coffee drinking specifically reduced the risk of developing ER-negative breast cancer but not ER-positive breast cancer [20].

Although there is enough evidence from epidemiological data supporting that coffee seems to reduce the risk of certain cancers, the molecular mechanisms underlying the chemopreventive effects of coffee remain unknown. For this reason, the aim of our study was to determine the effect at the molecular level of coffee polyphenols at low concentrations equivalent to one cup of coffee, using as a model a human colon cancer cell line HT29 in a nutrigenomic approach. Furthermore, the effect of coffee polyphenols was also evaluated in breast cancer cells.

2. Materials and Methods

2.1. Materials and Chemicals. Cells were incubated with Instant Caffeinated Coffee (ICC) (regular lyophilized instant coffee) and Caffeic acid (CA, Sigma). Compounds were dissolved either in DMSO (CA), or sterile water (ICC), and stored at -20°C.

2.2. Cell Culture. Colon adenocarcinoma HT29 and breast cancer MCF-7 cell lines were routinely grown in Ham's F12 medium supplemented with 7% fetal bovine serum (FBS, both from Gibco) at 37°C in a 5% CO_2 humidified atmosphere in 10 cm dish, or in 33 mm plate.

Cells were incubated with ICC or CA at concentrations equivalent to one cup of coffee. The concentrations used in cell incubations, 7 μg/mL in H_2O mQ for ICC and 1.68 μg/mL in DMSO for CA, respectively, took into account the amount of these compounds in one cup of coffee and their distribution in a regular human body with 75% water content. These concentrations did not cause any cytotoxic effect in the cell incubations as determined by the MTT assay [21].

2.3. Microarrays. Gene expression was analyzed by hybridization to The GeneChip Human Genome U133A plus 2.0 microarrays from Affymetrix, containing 47,000 transcripts and variants. HT29 cells were incubated with ICC and CA for 24 h. Total RNA was prepared from triplicate samples using Speedtools Total RNA Extraction Kit (Biotools) following the recommendations of the manufacturer. RNA quality was tested by 2100 Bioanalyzer Eukaryote Total RNA Nano Series II (Agilent Technologies). Labeling, hybridization, and detection were carried out following the manufacturer's specifications at the IDIBAPS Genomic Service (Hospital Clínic, Barcelona).

2.4. Microarray Data Analyses. Quantification was carried out with GeneSpring GX v.11.5.1 software (Agilent Technologies), which allows multifilter comparisons using data from different experiments to perform the normalization, generation of lists, and the functional classification of the differentially expressed genes. The input data was subjected to preprocess baseline transformation using the Robust Multiarray Average summarization algorithm using the median of control samples. After grouping the triplicate of each experimental condition, list of differentially expressed genes could be generated by using volcano plot analysis. The expression of each gene is reported as the ratio of the value obtained after each condition relative to control condition after normalization and statistical analysis of the data. The corrected P value cutoff applied was of <0.05; then the output of this statistical analysis was filtered by fold expression, selecting specifically those genes that had a differential expression of at least 1.3-fold. Gene classification was established by the Gene Ontology database.

2.5. Common Genes between ICC and CA Treatments. Common genes were selected from the lists of differentially expressed genes for each treatment using Venn-Diagrams. The newly generated list contained both over and underexpressed genes.

2.6. Generation of Biological Association Networks. BANs were constructed with the aid of the Pathway Analysis within the GeneSpring v.11.5.1 (Agilent) as described in Selga et al. [22] with the list of common genes differentially expressed in both treatments. A filtered screening was processed by the program between our data and bibliographic interaction

databases up to a total of 100 related genes. Network associations were confirmed in the literature.

2.7. RT Real-Time PCR. Total RNA was extracted from HT29 cells using Ultraspec (Biotex) in accordance with the manufacturer's instructions.

Complementary DNA was synthesized as described in Selga et al. [23] and the cDNA product was used for amplification by real time PCR. STAT5B and ATF-2 mRNA levels were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 3 μL of the cDNA reaction and the assays-on-demand Hs00560035_m1 for STAT5B, Hs00153179_ml for ATF-2, and Hs00356991_m1 for APRT (all from Applied Biosystems). APRT mRNA was used as an endogenous control. The reaction was performed following the manufacturers recommendations. Fold changes in gene expression were calculated using the standard $\Delta\Delta$Ct method.

2.8. Western Blot. Whole extracts were obtained from 2.5×10^6 control or treated cells according to Selga et al. [23]. Five μL of the extract was used to determine protein concentration by the Bradford assay (Bio-Rad). The extracts were frozen in liquid N_2 and stored at -80°C. Total extracts (50 μg) were resolved on SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon P, Millipore) using a semidry electroblotter.

The SNAP i.d. protein detection system technology (Millipore) was used to probe the membranes. This system applies vacuum through the membrane to actively drive reagents to protein locations, unlike the traditional technique of diffusion over the membrane as a reagent transport. Table 1 compiles the antibodies used in the different determinations.

Signals were detected by secondary horseradish peroxidase-conjugated antibody, either anti-rabbit (1 : 5000 or 1 : 10000 dilution; Dako) or anti-mouse (1 : 2500 dilution, Amersham NIF 824) and enhanced chemiluminescence using the ECL method, as recommended by the manufacturer (Amersham). Chemiluminescence was detected with ImageQuant LAS 4000 Mini technology (GE Healthcare).

2.9. Statistical Methods. For the RT-PCR and Western blot analyses, values are expressed as the mean ± SE of three different experiments. Data were evaluated by unpaired Student's *t* test, and analyses were performed using the PASW Statistics v. 18.0.0. software.

3. Results

3.1. Effect of ICC and CA Incubations in HT29 Gene Expression. The expression profile of over 47,000 transcripts and variants included in the microarray HG U133 plus 2.0 from Affymetrix was compared between HT29 control cells and cells incubated with either CA or ICC, at nontoxic concentrations for 24 h. GeneSpring GX software v.11.5.1 was used to analyze the results. A list of differentially expressed genes by 1.3-fold with a *P* value cutoff of <0.05 was generated as described in Methods. When HT29 cells were incubated with ICC, 57 genes were overexpressed whereas 161 genes were underexpressed. Among the overexpressed genes, 24% belonged to the Transcription factors category and 19% to Cell cycle or to Biosynthetic processes. Within the underexpressed genes, the category corresponding to cell cycle was the most affected (53% of the genes) followed by Transcription factors (19%) and Biosynthetic processes (12%). Upon incubation with CA, 12 genes were overexpressed whereas 32 genes were underexpressed. Among the overexpressed genes, 33% belonged to the Transcription factors category, 25% to Cell cycle, and 16,7% to Biosynthetic processes or immune response. Within the underexpressed genes, again the category corresponding to Cell cycle was the most affected (30% of the genes) followed by Biosynthetic processes (15%) and Transcription factors (12%). The lists of differentially expressed genes are presented as Tables 2, 3, 4, and 5. The data presented in this work have been deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number [GSM867162].

3.2. Generation of Biological Association Networks. A Biological Association Network (BAN) was constructed using the Pathway Analysis within GeneSpring v.11.5.1 as described in Methods using as the starting list the common genes differentially expressed upon incubation with CA and ICC. This list included five overexpressed genes and twelve underexpressed genes (Table 6). In the generated network, signal transducer and activator of transcription 5B (STAT5B) and activating transcription factor 2 (ATF-2) appeared as highly interconnected nodes (Figure 1). These two main nodes were selected for further validations. STAT5B was overexpressed with respect to the control by 23.8% in cells treated with ICC and by 33.4% in cells treated with CA, whereas ATF-2 was found underexpressed in HT29 incubated with ICC (32.5% decrease compared to the control) and with CA (26% decrease).

3.3. Validation of STAT5B and ATF-2 Changes at the mRNA and Protein Levels. STAT5B overexpression in HT29 cells upon incubation with CA and ICC was confirmed at the mRNA (1.16- and 1.3-fold compared to the control, respectively) and protein levels (1.5- and 1.2-fold compared to the control, respectively) (Figures 2(a) and 2(c)). In the case of ATF-2, the changes in mRNA levels were confirmed for both CA and ICC (0.88- and 0.86-fold compared to the control, respectively), whereas the decrease in protein levels was only observed in CA-treated cells (0.62-fold compared to the control) (Figures 2(b) and 2(d)).

3.4. Expression of Cyclin D1 upon Incubation with ICC and CA. Cyclin D1 is overexpressed at the mRNA and protein level in over 50% of the breast cancers either in the presence or absence of gene amplification, and it is one of the most commonly overexpressed proteins in breast cancer [24, 25]. Cyclin D1 transcription is regulated by STAT5 [26–29] and ATF-2 [30–32].

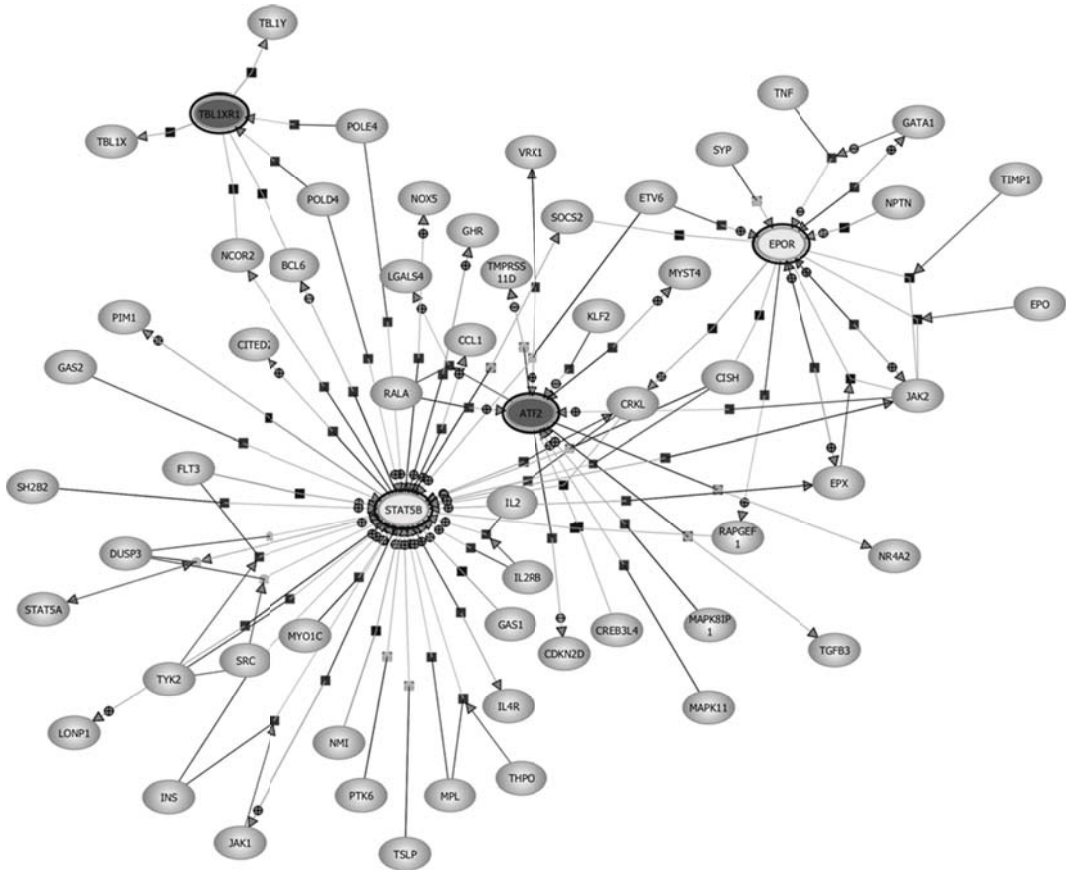

Figure 1: Biological association network (BAN) of differentially expressed genes in common between CA and ICC. The list of common genes between both treatments was used to construct a BAN with the Pathway Analysis software within GeneSpring v.11.5.1. An expanded network was constructed by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification, and regulation. Only proteins are represented. The BAN shows the node genes STAT5B and ATF-2 that were further studied.

Table 1

Antibody	Molecular weight (KDa)	Dilution used	Supplier
STAT5B	95	1: 200	sc-835, Santa Cruz Biotechnology Inc.
ATF-2	72	1: 200	sc-6233, Santa Cruz Biotechnology Inc.
Cyclin D1	38	1: 200	sc-8396, Santa Cruz Biotechnology Inc.
β-actin	42	1: 200	A2066, Sigma
Tubulin	60	1: 100	CP06, Calbiochem

We analyzed the levels of cyclin D1 by western blot in MCF-7 and HT29 cells upon incubation with ICC and CA. As shown in Figure 3(a), incubation of MCF-7 cells with either CA and ICC led to a drastic decrease in the levels of cyclin D1 protein, together with an increase in the levels of STAT5B, but not to a decrease in the levels of ATF-2. In HT29 cells, incubation with CA did not affect cyclin D1 levels, whereas the presence of ICC led to an increase in cyclin D1 levels 3 (b).

4. Discussion

In this work we analyzed the gene expression profile of human cancer cells treated with either ICC or CA. Caffeic acid was chosen since it is the main representative of hydroxycinnamic acids. Using microarrays we identified the differential expression of specific genes involved in several biological pathways. The changes in mRNA expression of two outlier genes, STAT5B and ATF-2, observed in the microarrays were confirmed by RT real-time PCR, and the changes in protein levels were also analyzed by Western blot. The selection of STAT5B and ATF-2 was made according to the results obtained in the construction of a biological association network. Finally, the modulation of cyclin D1, a target of STAT5B and ATF-2 transcription factors, upon incubation with coffee polyphenols was also established.

We show that ICC and the amount of CA of one cup of coffee are able to induce STAT5B mRNA and protein

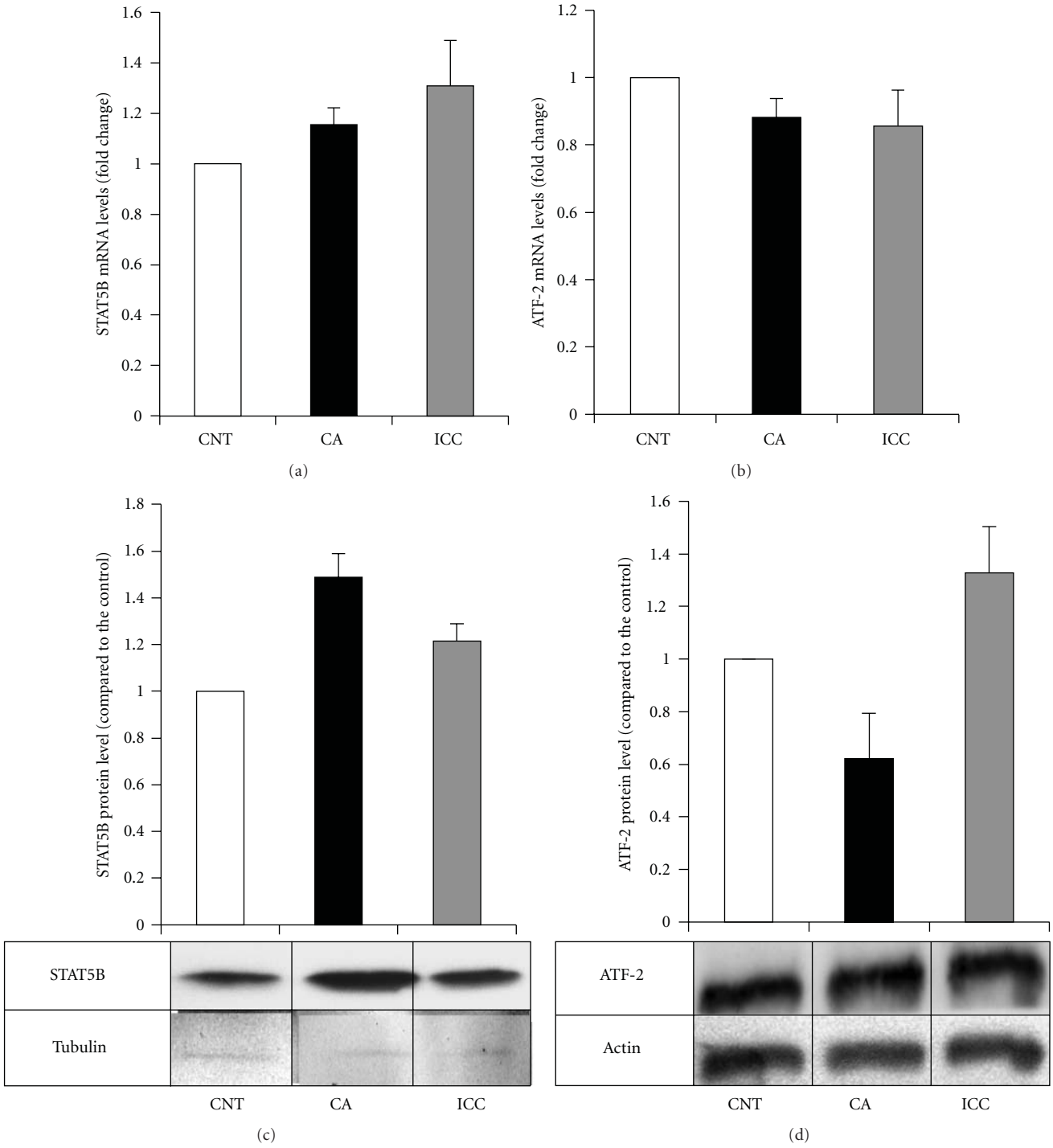

Figure 2: Quantitation of mRNA and protein levels for STAT5B and ATF-2 in HT29 cells. The mRNA levels of STAT5B (a) and ATF-2 (b) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) and instant caffeinated coffee (ICC, grey bars) by RT real-time PCR as described in Methods. Results are expressed in fold changes compared to the control and are the mean ± SE of 3 different experiments. $^{*}P < 0.05$ compared with the corresponding control. The protein levels of STAT5B (c) and ATF-2 (d) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) and instant caffeinated coffee (ICC, grey bars) by Western blot. Blots were reprobed with an antibody against β-actin or tubulin to normalize the results. Results represent the mean ± SE of 3 different experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the corresponding control.

Table 2: List of overexpressed genes in HT29 cells upon incubation with instant caffeinated coffee.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
CALM3	Calmodulin 3 (phosphorylase kinase, delta)	0.016	1.3	Up
CDC42EP1	CDC42 effector protein (Rho GTPase binding) 1	0.027	1.3	Up
FOXN3	Forkhead box N3	0.022	1.3	Up
KIR2DL1	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 1	0.023	1.3	Up
ORAI2	ORAI calcium release-activated calcium modulator 2	0.011	1.3	Up
RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1	0.022	1.3	Up
STH	Saitohin	0.031	1.3	Up
SLC39A3	Solute carrier family 39 (zinc transporter), member 3	0.028	1.3	Up
ZNF397OS	Zinc finger protein 397 opposite strand	0.024	1.3	Up
ZP4	Zona pellucida glycoprotein 4	0.046	1.3	Up
FGFRL1	Fibroblast growth factor receptor-like 1	0.035	1.31	Up
ITGA9	Integrin, alpha 9	0.002	1.31	Up
IRAK1	Interleukin-1 receptor-associated kinase 1	0.038	1.31	Up
OBSL1	Obscurin-like 1	0.008	1.31	Up
RPS17L4	Ribosomal protein S17-like 4	0.026	1.31	Up
STAT5B	Signal transducer and activator of transcription 5B	0.007	1.31	Up
TRABD	TraB domain containing	0.043	1.31	Up
MYO9B	Myosin IXB	0.041	1.32	Up
NME7	Nonmetastatic cells 7, protein expressed in (nucleoside-diphosphate kinase)	0.037	1.32	Up
RPS6KA4	Ribosomal protein S6 kinase, 90 kDa, polypeptide 4	0.014	1.32	Up
SIRPA	Signal-regulatory protein alpha	0.019	1.32	Up
TBX20	T-box 20	0.035	1.32	Up
TCF20	Transcription factor 20 (AR1)	0.022	1.32	Up
ALDH3B1	Aldehyde dehydrogenase 3 family, member B1	0.005	1.33	Up
BGN	Biglycan	0.029	1.33	Up
GNB4	Guanine nucleotide binding-protein (G protein), b-polypeptide 4	0.044	1.33	Up
IFNA17	Interferon, alpha 17	0.026	1.33	Up
KY	Kyphoscoliosis peptidase	0.013	1.33	Up
SCARF1	Scavenger receptor class F, member 1	0.025	1.33	Up
SERPINB8	Serpin peptidase inhibitor, clade B (ovalbumin), member 8	0.01	1.33	Up
FST	Follistatin	0.025	1.34	Up
MOGAT1	Monoacylglycerol O-acyltransferase 1	0.009	1.34	Up
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	0.015	1.34	Up
SUCLG2	Succinate-CoA ligase, GDP-forming, beta subunit	0.011	1.34	Up
SULT1B1	Sulfotransferase family, cytosolic, 1B, member 1	0.018	1.34	Up
TBX10	T-box 10	0.011	1.34	Up
ZNF503	Zinc finger protein 503	0.022	1.34	Up
HBA1	Hemoglobin, alpha 1	0.04	1.35	Up
MEPE	Matrix, extracellular phosphoglycoprotein with ASARM motif	0.001	1.35	Up
PPP1CB	Protein phosphatase 1, catalytic subunit, beta isoform	0.03	1.35	Up
ARV1	ARV1 homolog (*S. cerevisiae*)	0.011	1.36	Up
BCL3	B-cell CLL/lymphoma 3	0.034	1.36	Up
CTRC	Chymotrypsin C (caldecrin)	0.045	1.36	Up
EPOR	Erythropoietin receptor	0.008	1.37	Up
HMGA1	High-mobility group AT-hook 1	0.039	1.37	Up
IL19	Interleukin 19	0.018	1.38	Up
ABCC12	ATP-binding cassette, subfamily C (CFTR/MRP), member 12	6.00E-04	1.39	Up
RAI1	Retinoic acid induced 1	0.017	1.39	Up

Table 2: Continued.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
KLF5	Kruppel-like factor 5 (intestinal)	0.028	1.4	Up
CBWD1	COBW domain containing 1	0.044	1.41	Up
ASAH3	N-acylsphingosine amidohydrolase (alkaline ceramidase) 3	0.039	1.43	Up
ABHD14B	Abhydrolase domain containing 14B	0.03	1.45	Up
TLN1	Talin 1	0.049	1.45	Up
ARHGAP23	Rho GTPase-activating protein 23	0.024	1.65	Up
HINT3	Histidine triad nucleotide binding protein 3	0.002	1.77	Up
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	0.034	1.83	Up
CALR	Calreticulin	0.007	1.93	Up

The table shows the list of overexpressed genes by 1.3-fold with a P value < 0.05 obtained in cells treated with instant caffeinated coffee and includes the gene symbol for all genes, and their associated description. The ratio columns correspond to the absolute fold change in expression relative to the control group and the type of regulation (up: upregulation).

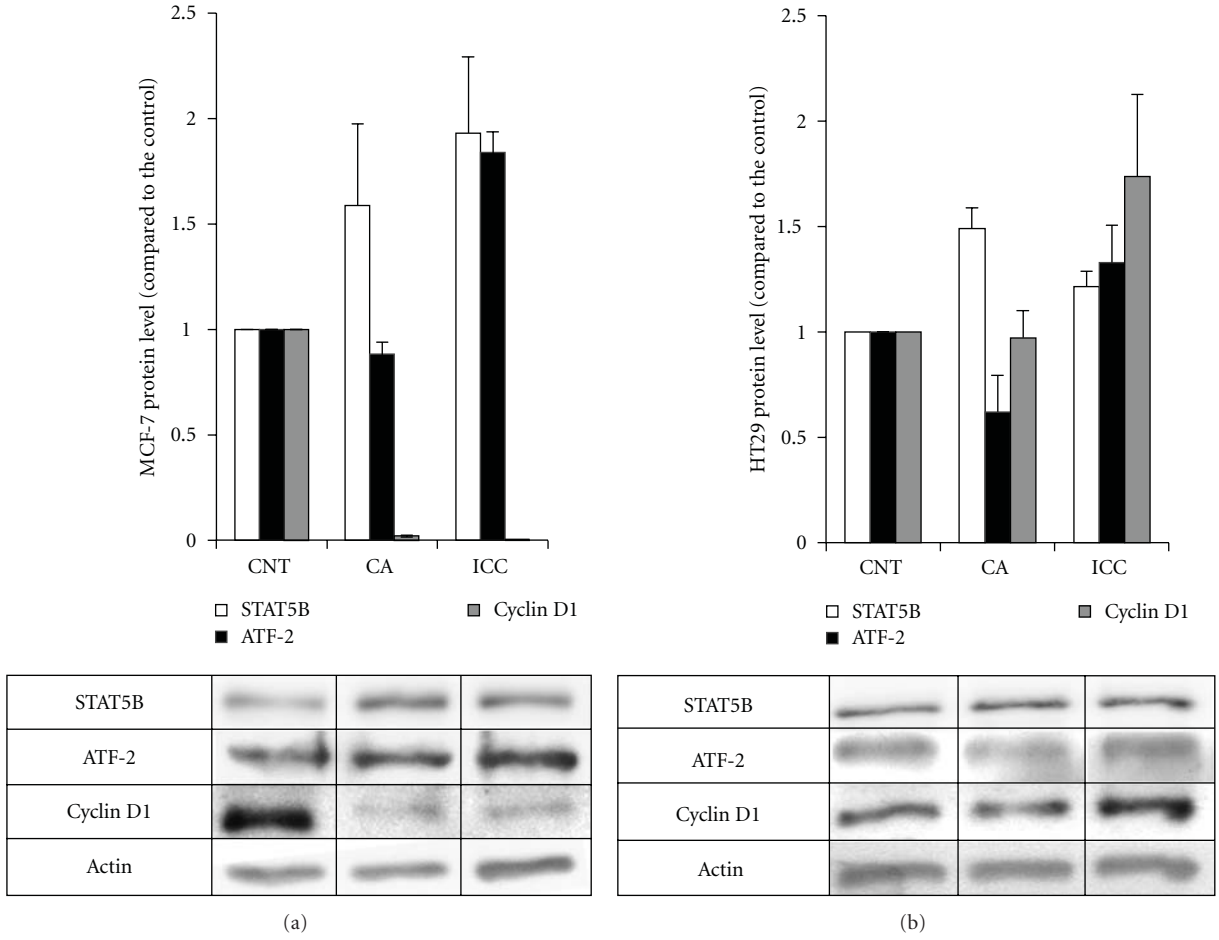

Figure 3: Expression of cyclin D1 upon incubation with ICC and CA in HT29 and MCF-7 cells. (a) Quantitation of STAT5b (empty bars), ATF-2 (filled bars), and cyclin D1 (grey bars) protein levels in MCF-7 cells. The protein levels were determined in control MCF-7 cells (CNT) and cells treated with caffeic acid (CA) and instant coffee (ICC) by Western blot. Blots were reprobed with an antibody against β-actin to normalize the results. Results represent the mean ± SE of 3 different experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the corresponding control. (b) Quantitation of STAT5b (empty bars), ATF-2 (filled bars), and cyclin D1 (grey bars) protein levels in HT29 cells. The protein levels were determined in control HT29 cells (CNT) and cells treated with caffeic acid (CA) and instant coffee (ICC) by Western blot. Blots were reprobed with an antibody against β-actin to normalize the results. Results represent the mean ± SE of 3 different experiments.$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the corresponding control.

Table 3: List of underexpressed genes in HT29 cells upon incubation with instant coffee.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
ACBD5	Acyl-coenzyme A binding domain containing 5	0.017	1.3	Down
CXADR	Coxsackie virus and adenovirus receptor	0.015	1.3	Down
FANCD2	Fanconi anemia, complementation group D2	0.047	1.3	Down
FRYL	FRY-like	0.039	1.3	Down
NUB1	Negative regulator of ubiquitin-like proteins 1	0.029	1.3	Down
PBRM1	Polybromo 1	0.004	1.3	Down
PRKACB	Protein kinase, cAMP-dependent, catalytic, beta	0.033	1.3	Down
RIF1	RAP1 interacting factor homolog (yeast)	0.012	1.3	Down
SLC39A6	Solute carrier family 39 (zinc transporter), member 6	0.022	1.3	Down
TMEM170	Transmembrane protein 170	0.032	1.3	Down
WDR26	WD repeat domain 26	0.028	1.3	Down
RNGTT	RNA guanylyltransferase and 5′-phosphatase	0.04	1.3	Down
CTDSPL2	CTD small phosphatase like 2	0.03	1.3	Down
ZC3H11A	Zinc finger CCCH-type containing 11A	0.014	1.3	Down
TMOD3	Tropomodulin 3 (ubiquitous)	0.0171	1.3	Down
CPD	Carboxypeptidase D	0.002	1.31	Down
CBL	Cas-Br-M ecotropic retroviral transforming sequence	0.008	1.31	Down
CDC42SE2	CDC42 small effector 2	0.022	1.31	Down
CLN5	Ceroid-lipofuscinosis, neuronal 5	0.001	1.31	Down
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	0.027	1.31	Down
FGFR1OP2	FGFR1 oncogene partner 2	0.049	1.31	Down
LRRFIP1	Leucine-rich repeat (in FLII) interacting protein 1	0.026	1.31	Down
PDCD4	Programmed cell death 4	0.005	1.31	Down
REPS2	RALBP1-associated Eps domain containing 2	0.046	1.31	Down
SLC7A6	Solute carrier family 7, member 6	0.002	1.31	Down
TFRC	Transferrin receptor (p90, CD71)	0.038	1.31	Down
TMEM19	Transmembrane protein 19	0.024	1.31	Down
AGPS	Alkylglycerone phosphate synthase	0.001	1.31	Down
SLC4A7	Solute carrier family 4, member 7	0.028	1.31	Down
SPTAN1	Spectrin, alpha, nonerythrocytic 1 (alpha-fodrin)	0.02	1.31	Down
GPD2	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	0.033	1.31	Down
BICD1	Bicaudal D homolog 1 (*Drosophila*)	0.008	1.31	Down
FBXW11	F-box and WD repeat domain containing 11	0.025	1.31	Down
BCLAF1	BCL2-associated transcription factor 1	0.025	1.32	Down
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	0.011	1.32	Down
CLK4	CDC-like kinase 4	0.049	1.32	Down
PTAR1	Protein prenyltransferase alpha subunit repeat containing 1	0.027	1.32	Down
SMEK2	SMEK homolog 2, suppressor of mek1 (*Dictyostelium*)	0.012	1.32	Down
CEPT1	Choline/ethanolamine phosphotransferase 1	0.038	1.32	Down
SAR1A	SAR1 gene homolog A (*S. cerevisiae*)	0.033	1.32	Down
PDGFC	Platelet-derived growth factor C	0.02	1.32	Down
NFAT5	Nuclear factor of activated T-cells 5, tonicity responsive	0.045	1.32	Down
FRS2	Fibroblast growth factor receptor substrate 2	0.03	1.32	Down
BMS1P5	BMS1 pseudogene 5	0.036	1.33	Down
GLS	Glutaminase	5.00E-04	1.33	Down
LMAN1	Lectin, mannose binding, 1	7.00E-04	1.33	Down
ARHGAP18	Rho GTPase-activating protein 18	8.00E-04	1.33	Down

Table 3: Continued.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
ARHGAP5	Rho GTPase-activating protein 5	0.006	1.33	Down
CCNE2	Cyclin E2	0.036	1.33	Down
SPCS3	Signal peptidase complex subunit 3 homolog (*S. cerevisiae*)	0.008	1.33	Down
NCOA2	Nuclear receptor coactivator 2	0.005	1.33	Down
SRPRB	Signal recognition particle receptor, B subunit	0.018	1.33	Down
TLK1	Tousled-like kinase 1	0.04	1.33	Down
NCOA3	Nuclear receptor coactivator 3	0.048	1.33	Down
STRN3	Striatin, calmodulin-binding protein 3	2.00E-04	1.33	Down
AP1G1	Adaptor-related protein complex 1, gamma 1 subunit	0.004	1.34	Down
B3GALNT2	Beta-1,3-N-acetylgalactosaminyltransferase 2	0.034	1.34	Down
PPHLN1	Periphilin 1	2.00E-04	1.34	Down
SNX13	Sorting nexin 13	0.001	1.34	Down
TMED2	Transmembrane emp24 domain-trafficking protein 2	0.041	1.34	Down
BRWD1	Bromodomain and WD repeat domain containing 1	0.011	1.34	Down
HLA-B	Major histocompatibility complex, class I, B	0.028	1.34	Down
CHP	Calcium-binding protein P22	0.002	1.34	Down
MTMR9	Myotubularin-related protein 9	0.026	1.34	Down
DCUN1D4	DCN1, defective in cullin neddylation 1, domain containing 4	0.031	1.34	Down
ARL6IP2	ADP-ribosylation factor-like 6 interacting protein 2	0.02	1.35	Down
GLIS3	GLIS family zinc finger 3	0.01	1.35	Down
LARP4	La ribonucleoprotein domain family, member 4	0.019	1.35	Down
PTPLB	Protein tyrosine phosphatase-like member b	0.036	1.35	Down
TRAM1	Translocation-associated membrane protein 1	0.002	1.35	Down
TMEM64	Transmembrane protein 64	0.001	1.35	Down
CBFB	Core-binding factor, beta subunit	0.005	1.35	Down
SELT	Selenoprotein T	0.002	1.35	Down
PEX13	Peroxisome biogenesis factor 13	0.011	1.35	Down
TNKS2	TRF1-interacting ankyrin-related ADP-ribose polymerase 2	0.034	1.35	Down
TMPO	Thymopoietin	0.001	1.35	Down
LIN7C	Lin-7 homolog C (*C. elegans*)	0.007	1.35	Down
MTA2	Metastasis-associated 1 family, member 2	0.013	1.36	Down
TMEM168	Transmembrane protein 168	0.035	1.36	Down
CREBZF	CREB/ATF bZIP transcription factor	0.016	1.36	Down
OSTF1	Osteoclast-stimulating factor 1	0.002	1.36	Down
WDR57	WD repeat domain 57 (U5 snRNP specific)	0.001	1.36	Down
GLT25D1	Glycosyltransferase 25 domain containing 1	0.008	1.36	Down
NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma	0.015	1.36	Down
CCDC126	Coiled-coil domain containing 126	0.039	1.37	Down
LASS6	LAG1 homolog, ceramide synthase 6	0.005	1.37	Down
MYSM1	Myb-like, SWIRM and MPN domains 1	0.021	1.37	Down
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	0.007	1.37	Down
PDE4DIP	Phosphodiesterase 4D interacting protein (myomegalin)	0.024	1.37	Down
SAP30L	SAP30-like	0.012	1.37	Down
PTPRJ	Protein tyrosine phosphatase, receptor type, J	0.011	1.37	Down
PGGT1B	Protein geranylgeranyltransferase type I, beta subunit	9.00E-04	1.37	Down
ASPH	Aspartate beta-hydroxylase	0.011	1.37	Down
SEMA3C	Sema domain, (semaphorin) 3C	0.036	1.38	Down
WDR76	WD repeat domain 76	0.016	1.38	Down

Table 3: Continued.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
ATP13A3	ATPase-type 13A3	0.002	1.38	Down
LMBR1	Limb region 1 homolog (mouse)	0.014	1.38	Down
GLUD1	Glutamate dehydrogenase 1	0.001	1.39	Down
GSTCD	Glutathione S-transferase, C-terminal domain containing	0.029	1.39	Down
SPTLC1	Serine palmitoyltransferase, subunit 1	0.02	1.39	Down
U2AF1	U2 small nuclear RNA auxiliary factor 1	9.00E-04	1.39	Down
UHMK1	U2AF homology motif (UHM) kinase 1	0.007	1.39	Down
ARGLU1	Arginine and glutamate-rich 1	6.00E-04	1.39	Down
ANKRD12	Ankyrin repeat domain 12	0.03	1.39	Down
PPP3R1	Protein phosphatase 3, regulatory subunit B, alpha isoform	0.023	1.39	Down
XRN1	5′-3′ exoribonuclease 1	0.019	1.4	Down
CLSPN	Claspin homolog (*Xenopus laevis*)	0.013	1.4	Down
CXADRP1	Coxsackie virus and adenovirus receptor pseudogene 1	0.034	1.4	Down
G3BP1	GTPase-activating protein- (SH3 domain) binding protein 1	0.002	1.4	Down
TMEM30A	Transmembrane protein 30A	0.01	1.4	Down
CLCN3	Chloride channel 3	0.035	1.41	Down
STK4	Serine/threonine kinase 4	0.039	1.41	Down
ZNF644	Zinc finger protein 644	0.02	1.41	Down
TCP11L1	T-complex 11 (mouse)-like 1	0.014	1.41	Down
SFRS6	Splicing factor, arginine/serine-rich 6	0.031	1.41	Down
NPL	N-acetylneuraminate pyruvate lyase	0.006	1.41	Down
G3BP2	GTPase-activating protein- (SH3 domain) binding protein 2	0.001	1.42	Down
HNRNPU	Heterogeneous nuclear ribonucleoprotein U	0.01	1.42	Down
TBL1XR1	Transducin (beta)-like 1 X-linked receptor 1	0.001	1.42	Down
PHTF2	Putative homeodomain transcription factor 2	0.002	1.42	Down
ADAM10	ADAM metallopeptidase domain 10	0.011	1.43	Down
ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)	0.01	1.43	Down
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1	0.04	1.43	Down
SCARB2	Scavenger receptor class B, member 2	0.001	1.43	Down
CANX	Calnexin	0.043	1.43	Down
CASP2	Caspase 2, apoptosis-related cysteine peptidase	0.033	1.43	Down
TRPS1	Trichorhinophalangeal syndrome I	0.005	1.44	Down
ZFX	Zinc finger protein, X-linked	0.033	1.44	Down
SGPL1	Sphingosine-1-phosphate lyase 1	0.04	1.44	Down
PTPN11	Protein tyrosine phosphatase, nonreceptor type 11	0.045	1.44	Down
SFRS11	Splicing factor, arginine/serine-rich 11	0.045	1.45	Down
B3GNT5	Beta-1,3-N-acetylglucosaminyltransferase 5	0.021	1.45	Down
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	0.019	1.45	Down
SNHG4	Small nucleolar RNA host gene (nonprotein coding) 4	0.004	1.46	Down
PARD6B	Par-6 partitioning defective 6 homolog beta (*C. elegans*)	0.04	1.46	Down
ROD1	ROD1 regulator of differentiation 1 (*S. pombe*)	0.001	1.46	Down
SPTBN1	Spectrin, beta, nonerythrocytic 1	0.02	1.48	Down
TXNDC1	Thioredoxin domain containing 1	0.013	1.48	Down
ATF2	Activating transcription factor 2	0.005	1.48	Down
RDX	Radixin	0.043	1.48	Down
SCAMP1	Secretory carrier membrane protein 1	0.009	1.48	Down
PTAR1	Protein prenyltransferase alpha subunit repeat containing 1	0.018	1.49	Down
RC3H2	Ring finger and CCCH-type zinc finger domains 2	0.0037	1.49	Down

Table 3: Continued.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
ADAM17	ADAM metallopeptidase domain 17	0.007	1.49	Down
FAM76B	Family with sequence similarity 76, member B	0.014	1.5	Down
ITGB8	Integrin, beta 8	1.00E-04	1.5	Down
TRIM23	Tripartite motif-containing 23	0.005	1.5	Down
CASC5	Cancer susceptibility candidate 5	0.019	1.52	Down
SLC16A1	Solute carrier family 16, member 1	0.002	1.52	Down
FNBP1	Formin-binding protein 1	0.037	1.53	Down
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha	9.00E-04	1.53	Down
B4GALT1	Beta 1,4-galactosyltransferase, polypeptide 1	0.035	1.55	Down
MDM4	Mdm4 p53-binding protein homolog (mouse)	0.011	1.58	Down
FGD4	FYVE, RhoGEF, and PH domain containing 4	0.001	1.59	Down
UBA6	Ubiquitin-like modifier activating enzyme 6	8.00E-04	1.62	Down
ZDHHC21	Zinc finger, DHHC-type containing 21	0.036	1.64	Down
REEP3	Receptor accessory protein 3	7.00E-04	1.65	Down
SSR3	Signal sequence receptor, gamma	0.014	1.65	Down
ZDHHC20	Zinc finger, DHHC-type containing 20	0.003	1.66	Down
EIF2S3	Eukaryotic translation initiation factor 2, subunit 3 gamma	0.001	1.7	Down
HNRNPH1	Heterogeneous nuclear ribonucleoprotein H1	0.011	1.79	Down
ATL3	Atlastin 3	0.001	2.02	Down

The table shows the list of underexpressed genes by 1.3-fold with a P value < 0.05 obtained in cells treated with instant caffeinated coffee and includes the gene symbol for all genes, and their associated description. The ratio columns correspond to the absolute fold change in expression relative to the control group and the type of regulation (down: downregulation).

Table 4: List of overexpressed genes in HT29 cells upon incubation with caffeic acid.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
SULT1B1	Sulfotransferase family, cytosolic, 1B, member 1	0.02	1.3	Up
BCL6B	B-cell CLL/lymphoma 6, member B (zinc finger protein)	3.00E-04	1.3	Up
KCNJ5	Potassium inwardly-rectifying channel, subfamily J, member 5	0.01	1.31	Up
EPOR	Erythropoietin receptor	0.02	1.32	Up
DNAJC21	DnaJ (Hsp40) homolog, subfamily C, member 21	0.049	1.33	Up
STAT5B	Signal transducer and activator of transcription 5B	0.012	1.33	Up
FST	Follistatin	0.021	1.37	Up
CD84	CD84 molecule	0.033	1.37	Up
THRA	Thyroid hormone receptor, alpha	0.017	1.37	Up
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3	0.028	1.4	Up
SIAE	Sialic acid acetylesterase	0.01	2.42	Up
HINT3	Histidine triad nucleotide-binding protein 3	0.033	2.6	Up

The table shows the list of overexpressed genes by 1.3-fold with a P value < 0.05 obtained in cells treated with caffeic acid and includes the gene symbol for all genes, their associated description. The ratio columns correspond to the absolute fold change in expression relative to the control group and the type of regulation (up: upregulation).

levels in HT29 cells. STAT5 was originally described as a prolactin-induced mammary gland factor [33]. The cloning of two closely related STAT5 cDNAs, from both mouse and human cDNA libraries, showed two distinct genes, STAT5A and STAT5B that encoded two STAT5 proteins [34–37].

In addition to prolactin, STAT5 proteins are activated by a wide variety of cytokines and growth factors, including IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, granulocyte-macrophage colony-stimulating factor, erythropoietin, growth hormone, thrombopoietin, epidermal growth factor, and platelet-derived growth factor. The key function of STAT5B is to mediate the effects of growth hormone [38, 39]. Modulation of STAT5 levels or transcriptional activity has already been described in cells treated with natural compounds such as nobiletin, a citrus flavonoid [40], thea flavins [41], and silibinin, a natural polyphenolic flavonoid which is a major bioactive component of silymarin isolated from *Silybum marianum* [42]. Furthermore, it has been reported that

Table 5: List of underexpressed genes in HT29 cells upon incubation with caffeic acid.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
MFSD7	Major facilitator superfamily domain containing 7	1.00E-04	1.3	Down
MSI2	Musashi homolog 2 (*Drosophila*)	0.027	1.3	Down
CDA	Cytidine deaminase	2.00E-04	1.31	Down
DEFB1	Defensin, beta 1	0.026	1.31	Down
PIP5K1A	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	0.027	1.31	Down
ZDHHC20	Zinc finger, DHHC-type containing 20	0.005	1.31	Down
ZDHHC21	Zinc finger, DHHC-type containing 21	0.016	1.31	Down
SLC4A7	Solute carrier family 4, member 7	0.0249	1.32	Down
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	0.0459	1.32	Down
PDZRN3	PDZ domain containing RING finger 3	0.002	1.32	Down
WDR62	WD repeat domain 62	0.005	1.32	Down
FAM76B	Family with sequence similarity 76, member B	0.036	1.32	Down
TCF21	Transcription factor 21	0.029	1.33	Down
TBL1XR1	Transducin (beta)-like 1 X-linked receptor 1	6.00E-04	1.33	Down
CLK4	CDC-like kinase 4	0.021	1.33	Down
CYP2A13	Cytochrome P450, family 2, subfamily A, polypeptide 13	0.009	1.34	Down
CXCR4	Chemokine (C-X-C motif) receptor 4	0.0488	1.34	Down
ATF2	Activating transcription factor 2	0.0158	1.35	Down
PDE10A	Phosphodiesterase 10A	0.03	1.35	Down
METT10D	Methyltransferase 10 domain containing	0.003	1.35	Down
PRMT2	Protein arginine methyltransferase 2	7.00E-04	1.36	Down
GLS	Glutaminase	5.70E-04	1.37	Down
SLC38A5	Solute carrier family 38, member 5	0.043	1.37	Down
TINAG	Tubulointerstitial nephritis antigen	0.043	1.38	Down
AQP1	Aquaporin 1 (Colton blood group)	0.0221	1.4	Down
JMJD6	Jumonji domain containing 6	0.004	1.4	Down
SAP30L	SAP30-like	0.021	1.4	Down
FGD4	FYVE, RhoGEF, and PH domain containing 4	0.026	1.52	Down
S100A2	S100 calcium-binding protein A2	0.005	1.53	Down
CTSZ	Cathepsin Z	0.045	1.53	Down
SLC4A4	Solute carrier family 4, member 4	9.00E-04	1.54	Down
AGR3	Anterior gradient homolog 3 (*Xenopus laevis*)	0.011	1.69	Down

The table shows the list of underexpressed genes by 1.3-fold with a P value < 0.05 obtained in cells treated with caffeic acid and includes the gene symbol for all genes, their associated description. The ratio columns correspond to the absolute fold change in expression relative to the control group and the type of regulation (down: downregulation).

butein, the major biologically active polyphenolic component of the stems of *Rhus verniciflua*, downregulated the expression of STAT3-regulated gene products such as Bcl-xL, Bcl-2, cyclin D1, and Mcl-1 [43].

STAT5B participates in diverse biological processes, such as growth development, immunoregulation, apoptosis, reproduction, prolactin pathway, and lipid metabolism. STAT5B deficiency is a recently identified disease entity that involves both severe growth hormone-resistant growth failure and severe immunodeficiency [44–46]. The induction of STAT5B expression upon incubation with CA and ICC could represent a nutritional tool to upregulate this transcription factor and suggests novel research strategies for natural therapies in Crohn's disease and inflammatory bowel disease in which STAT5B appears to maintain the mucosal barrier integrity and tolerance [47, 48]. In colorectal cancer both STAT5a and STAT5b play important roles in progression and downregulation of both STAT5A and STAT5B results in a gradual decrease in cell viability, predominantly attributed to G1 cell cycle arrest, and apoptotic cell death [49]. In this context the increase in STAT5B caused by ICC and CA would have a negative effect on colorectal cancer patients, as it would trigger cell proliferation and survival.

In human breast cancer, STAT5A/B has been shown a dual role in the mammary gland as an initiator of tumor formation as well as a promoter of differentiation of established tumors. STAT3, STAT5A, and STAT5B are overexpressed or constitutively activated in breast cancer [50–52] and active STAT5A/B in human breast cancer predicted favorable clinical outcome [53]. Prolactin receptor signal

Table 6: Common differentially expressed genes in HT29 treated-cells.

Gene symbol	FC absolute ICC	*P* value	Regulation	FC absolute CA	*P* value	Regulation
FST	1.343	0.025	Up	1.375	0.022	Up
SULT1B1	1.349	0.018	Up	1.304	0.020	Up
EPOR	1.372	0.008	Up	1.321	0.021	Up
HINT3	2.410	0.040	Up	2.607	0.033	Up
STAT5B	**1.312**	**0.007**	**Up**	**1.334**	**0.012**	**Up**
GLS	1.335	0.001	Down	1.370	0.001	Down
PPP3R1	1.397	0.023	Down	1.423	0.026	Down
ATF2	**1.481**	**0.005**	**Down**	**1.354**	**0.016**	**Down**
SLC4A7	1.314	0.029	Down	1.322	0.025	Down
MARCH3	1.330	0.016	Down	1.319	0.005	Down
TBL1XR1	1.426	0.001	Down	1.332	0.001	Down
SAP30L	1.375	0.013	Down	1.405	0.021	Down
FGD4	1.593	0.001	Down	1.523	0.027	Down
ZDHHC20	1.665	0.004	Down	1.314	0.005	Down
ZDHHC21	1.642	0.037	Down	1.318	0.016	Down
FAM76B	1.506	0.014	Down	1.325	0.037	Down
CLK4	1.326	0.049	Down	1.339	0.021	Down

Common differentially expressed genes in HT29 treated-cells with a P value < 0.05 and a minimum fold of 1.3. Column ICC correspond to cells treated with instant caffeinated coffee and column CA corresponds to cells treated with caffeic acid. Overexpressed genes are indicated on the upper part of the table, whereas underexpressed genes are depicted in the lower part. The genes in bold, STAT5B and ATF-2, were chosen for further analysis.

transduction through the Jak2-STAT5 pathway has been considered to be essential for proliferation and differentiation of normal mammary epithelial cells [54–56]. It has been shown that the levels of NUC-pYSTAT5 decreased as breast cancer progressed from normal to *in situ*, to invasive, and then to nodal metastases [57]. Additionally Peck et al. [57] found that the absence of detectable NUC-pYStat5 in tumors of patients how where under antiestrogen therapy was associated with poor breast cancer-specific survival. We analyzed STAT5B modulation through the PRL pathway in response to coffee polyphenols in a breast cancer cell line. The MCF-7 cell line was chosen because expression of the prolactin receptor is more often found in estrogen receptor-positive breast tumors [58]. In our conditions, incubation with CA and ICC led to an increase in STAT5B protein levels in MCF-7 cells, and this result could be the basis for a possible inclusion of coffee polyphenols in the diet of breast cancer patients.

ATF-2 is a member of the ATF-cAMP response element-binding protein (CREB) family of transcription factors that can bind to the cAMP response element (CRE) found in many mammalian gene promoters [59, 60]. ATF-2 exhibits both oncogenic and tumor suppressor functions [61]. CREs are found in several genes involved in the control of the cell cycle, for example, the cyclin D1 gene, and ATF-2 binding to this sequence stimulates the transcription of cyclin D1 [30, 31]. ATF-2 mediated cyclin D1 promoter induction can be stimulated by a number of growth-promoting agents, such as estrogen [31], hepatocyte growth factor [62], and regenerating gene product [63]. ATF-2 has been correlated with proliferation, invasion, migration, and resistance to DNA-damaging agents in breast cancer cell lines.

The downregulation of ATF-2 expression after CA and ICC incubation in HT29 cells reported here is in accordance with the observed decrease in activity of ATF-2 in gastric cells when incubating with chlorogenic acid, the precursor of caffeic acid [64]. Surprisingly, the validation of the protein levels showed the upregulation of ATF-2 protein with ICC, but not with CA, both in HT29 and MCF-7 cells. This differential behavior could be due to other ICC components besides CA. In this direction Rubach et al. [64] reported a different response in ATF-2 activity after incubation of a gastric cell line with different coffee compounds. The presence of pyrogallol, catechol, βN-alkanoylhydroxytryptamides, and N-methylpyridinium increased ATF-2 activity, whereas chlorogenic acid and caffeine decrease it [64]. In our conditions incubation of HT29 cells with ICC caused a modest decrease in ATF-2 mRNA levels. However this effect was not translated at the protein level. We hypothesize that ICC contains other polyphenols in addition to caffeic acid that are able to increase ATF-2 protein levels through an increase of the translation of its mRNA, the increase of stability of the protein or an inhibition of its degradation. In this direction several plant polyphenols such as (-)-epigallocatechins-3-gallate (EGCG), genistein, luteolin, apigenin, chrysin, quercetin, curcumin, and tannic acid have been described to possess proteasome-inhibitory activity [65, 66].

The regulation of ATF-2 transcriptional activity, mostly at the level of its phosphorylation status, has been described upon treatment of cancer cells with several natural compounds. In MCF-7 cells, the anticancer agent 3,30-Diindolylmethane, derived from *Brassica* vegetables, activates both JNK and p38 pathways, resulting in c-Jun and ATF-2 phosphorylation, and the increase of binding of the

c-Jun–ATF-2 homodimers and heterodimers to the proximal regulatory element of IFN-γ promoter [67]. Biochanin-A, an isoflavone, existing in red clover, cabbage and alfalfa, has an inhibitory and apoptogenic effect on certain cancer cells by blocking the phosphorylation of p38 MAPK and ATF-2 in a dose-dependent fashion [68]. The JNK stress-activated pathway is one of the major intracellular signal transduction cascades involved in intestinal inflammation [69, 70], and upregulation of ATF-2 has been shown in Crohn's disease [71, 72]. Thus CA could represent potential therapeutical properties in different states of intestinal inflammation due to its combined effects on STAT5B and ATF-2 in HT29 cells.

Finally, the modulation of cyclin D1, a target of STAT5B and ATF-2 transcription factors, upon incubation with coffee polyphenols was established in colon and breast cancer cells. Cyclin D1 overexpression is common in colorectal cancer, but the findings regarding its prognostic value are conflicting. In a recent study, positive expression of cyclin D1 protein was detected in 95 of 169 colonic adenocarcinoma specimens, and increased cyclin D1 levels were associated with poorer prognosis [73]. Furthermore, there was a significant correlation between the positive expression of p-Stat5 and cyclin D1 in patients with colonic adenocarcinoma. However, in a second study, cyclin D1 overexpression was associated with improved outcome in a total of 386 patients who underwent surgical resection for colon cancer, classified as TNM stage II or III. Belt et al. [74] showed that low p21, high p53, low cyclin D1, and high AURKA were associated with disease recurrence in stage II and III colon cancer patients. In this context the effect of ICC on cyclin D1 levels could represent either a positive or a negative effect in colon cancer cells, depending on tumor progression. The increase in cyclin D1 levels could represent a marker of better outcome since it has been recently established that cyclin D1 expression is strongly associated with prolonged survival in male colorectal cancer and that lack of cyclin D1 is associated with a more aggressive phenotype in male patients [75]. However, several natural compounds such as anthocyanins, anthocyanidins, apigenin, luteolin, and fisetin have all been described to induce experimentally cell-cycle arrest and apoptosis through the decrease of cyclin D1 levels in HT29 cells [76–80]. In accordance to these data, the increase observed in cyclin D1 levels in HT29 cells upon incubation with ICC could probably be the consequence of the presence of different compounds other than polyphenols in ICC.

In MCF-7 breast cancer cells, cyclin D1 was down-regulated upon incubation with coffee polyphenols. The rationale for the choice of MCF-7 cell line was based on the observation that although cyclin D1 overexpression is present across multiple histologic subtypes of breast cancer, it has been shown that the large majority of cyclin D1–overexpressing breast cancers are ER positive [24, 25, 81]. Cyclin D1 overexpression has been reported between 40 and 90% of cases of invasive breast cancer, while gene amplification is seen in about 5–20% of tumors [24, 81–83]. In cyclin D1-driven cancers, blocking cyclin D1 expression by targeting the cyclin D1 gene, RNA, or protein should increase the chances for therapeutic success. Cell culture studies have raised the possibility that certain compounds might act in this way [84, 85] and approaches to blocking cyclin D1 expression using antisense, siRNA, or related molecules specifically target the driving molecular lesion itself [86–88]. It is believed that compounds that modulate cyclin D1 expression could have a role in the prevention and treatment of human neoplasias. For instance, flavopiridol, a synthetic flavonoid based on an extract from an Indian plant for the potential treatment of cancer, induces a rapid decline in cyclin D1 steady-state protein levels [89]. Taking all these results together, inhibition of cyclin D1 expression appears to be a good approach for cancer treatment. In this direction our observation that coffee and caffeic acid are able to drastically reduce the expression of cyclin D1 in breast cancer cells could suggest that some coffee components could be used as a coadjuvant therapeutic tool in the treatment of breast cancer.

Abbreviations

APRT: Adenine phosphoribosyltransferase
ATF-2: Activating transcription factor
BAN: Biological association network
CA: Caffeic acid
DMSO: Dimethyl sulfoxide
DEPC: Diethyl pyrocarbonate
ICC: Instant caffeinated coffee
RT-PCR: Reverse transcription-polymerase chain reaction
STAT5B: Signal transducer and activator of transcription 5B.

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Research Article

Erythrocyte Antioxidant Protection of Rose Hips (*Rosa* spp.)

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Rose hips are popular in health promoting products as the fruits contain high content of bioactive compounds. The aim of this study was to investigate whether health benefits are attributable to ascorbic acid, phenols, or other rose-hip-derived compounds. Freeze-dried powder of rose hips was preextracted with metaphosphoric acid and the sample was then sequentially eluted on a C_{18} column. The degree of amelioration of oxidative damage was determined in an erythrocyte *in vitro* bioassay by comparing the effects of a reducing agent on erythrocytes alone or on erythrocytes pretreated with berry extracts. The maximum protection against oxidative stress, 59.4 ± 4.0% (mean ± standard deviation), was achieved when incubating the cells with the first eluted meta-phosphoric extract. Removal of ascorbic acid from this extract increased the protection against oxidative stress to 67.9 ± 1.9%. The protection from the 20% and 100% methanol extracts was 20.8 ± 8.2% and 5.0 ± 3.2%, respectively. Antioxidant uptake was confirmed by measurement of catechin by HPLC-ESI-MS in the 20% methanol extract. The fact that all sequentially eluted extracts studied contributed to protective effects on the erythrocytes indicates that rose hips contain a promising level of clinically relevant antioxidant protection.

1. Introduction

Oxidative stress is associated with many different diseases such as heart- and cardiovascular disease, diabetes, obesity, and cancer. Epidemiological studies show that diets rich in fruits promote good health, at least partly through delaying the onset of diseases associated with oxidative stress [1]. These beneficial effects may be mediated by different phytochemicals with high antioxidant capacity, of which the polyphenols is a large group abundantly found in berries [2, 3]. Measurement of antioxidant capacity can be performed with many different methods. The relevance of general, chemical methods and their relationship to actual human health benefits is, however, controversial [4–6]. Human cell-based systems may provide more biological relevance than simple chemical assessments and they also allow the opportunity to consider interactions between added nutrients and functionally complete cellular enzyme systems, as well as with the intact membranes of living cells.

Erythrocytes can serve as a relevant human cell model in the investigation of bioavailability and antioxidant protection by natural products against oxidative stress. The antioxidant potential of plant phytochemicals against oxidative stress has previously been assessed using different erythrocyte methods. The degree of lipid peroxidation of the cell membrane has been investigated, using either intact erythrocytes or erythrocyte membranes for measuring malondialdehyde, an indicator of lipid peroxidation [7, 8]. Coleman [9] used methaemoglobin generation as a model for oxidative stress. Other laboratories have focused on the levels of redox enzymes [10–13] as well as the use of free radical generators to induce erythrocyte lysis [12, 14, 15]. Strategies for the study of dietary antioxidant protection using erythrocytes have also been published [16–19]. Erythrocytes have been used to test for oxidative stress in several different diseases [20–22] and for *in vitro* testing of cell-permeating therapeutic antioxidants. Honzel et al. [23] and Blasa et al. [24] have developed erythrocyte models

in order to identify natural products, which may provide sustained intracellular protection from oxidative damage.

The suitability of the erythrocyte as an assay for oxidative stress measurements lies in its adaptation to the prevention of oxidative stress-mediated perturbation of the structure and therefore the function of haemoglobin. Erythrocytes contain over 90% of their weight as haemoglobin, and the cells stabilize this protein with the second highest intracellular GSH level (after the liver) in the body [9]. The high GSH concentration quenches reactive oxygen species-mediated structural haemoglobin damage, which can occur as a result of superoxide formation, which in turn arises from normal oxygen carriage [9]. The GSH maintenance system also provides the reducing power to maintain other cellular antioxidants in their reduced states. Various phytochemicals with different chemical properties may combat oxidative stress within the erythrocyte and potentially preserve intracellular thiols [9].

There is a growing interest in fruits and berries as rich sources of many bioactive compounds that may promote health. Many studies have investigated artificial substances and/or pure compounds in high concentrations rather than the joint effect of phytochemicals in complex foods. During the last decade, rose hips have gained increased interest since they contain compounds that are known to possess several antioxidant, antimutagenic, and anticarcinogenic effects [25, 26]. Rose hips have been used to treat conditions such as arthritis [27–29], rheumatism, and diabetes. The major bioactive compounds within rose hips are phenols, ascorbic acid, tocopherols, β-carotene, lycopene, tannins, pectins, sugars, organic acids, amino acids and essential fatty acids [30, 31]. Other rose-hip-derived compounds reported include galactolipids [31] and triterpenic acids [32]. Interestingly, the antioxidant effects of these compounds cannot fully account for the clinical effects of rose hip powder [33] and the efficacy of the phenolic compounds in the rose hips has yet to be evaluated in controlled clinical trials [30]. Indeed, it still remains unclear whether the protective effects of polyphenols to human health are attributable to the phenols or other agents in the diet [30], such as phytochemicals, that may enhance total oxidant-scavenging capacities by binding to erythrocytes [34]. The main purpose of this study was to investigate different aspects of the antioxidant protection of rose hips on oxidative stress in an erythrocyte *in vitro* test system.

2. Materials and Methods

2.1. Chemicals and Cells. Methanol, formic acid, acetonitrile, 85% orthophosphoric acid, and meta-phosphoric were obtained from Merck (Darmstadt, Germany). Ascorbic acid, ascorbate oxidase, dimethyl sulfoxide (DMSO), KH_2PO_4, Na_2PO_4, EDTA, and H_2O_2 were purchased from Sigma-Aldrich (Seelze, Germany). Phosphate-buffered saline (PBS), without calcium or magnesium, and dichlorofluorescein diacetate (DCF-DA) were obtained from Invitrogen (Lidingo, Sweden). The standards used for HPLC-ESI-MS analysis (catechin, proanthocyanidin, rutin, quercetin galactoside, cyanidin glucoside) were purchased from Extrasynthese (Genay, France).

2.2. Plant Materials. To evaluate the antioxidant uptake in erythrocytes rose hips from three advanced selections ("BRo30173," "BRo30289," and "BRo05035") were sampled at full maturity. The seeds were removed and the remaining flesh with skin was lyophilized and ground to a fine powder in a laboratory mill (Yellow line, A10, IKA-Werke, Staufen, Germany) before extraction.

2.3. Preparation of Polyphenol Rich Extracts. The freeze-dried powders of rose hips from the three selections were blended in equal proportions and 2.5 g of the powder was added to 50 mM metaphosphoric acid (50 mL) for preextraction. The rose hip preextract (PE) was kept in an ultrasonic bath for 15 min before centrifugation at 4500 rpm for 10 min. The supernatant was applied to a C_{18} (EC) column (Isolute SPE Columns, Biotage, Sorbent AB) that had been pre-equilibrated with 100% methanol and washed with dH_2O. A sequential elution was performed and the first obtained extract (E1A) consisted of the metaphosphoric acid eluent of the pre-extract. Extract two (E2A) and three (E3A) consisted of the eluents with 50 mL 20% aqueous methanol and 50 mL 100% methanol, respectively. Metaphosphoric acid and methanol were used as they preferentially extract different bioactive compounds according to their physicochemical properties. The solvents of the extracts were removed using a rotary evaporator at 45°C. The concentrated extracts were then dissolved in 50 mM metaphosphoric acid.

2.4. Enzymatic Removal of Ascorbic Acid. Ascorbate oxidase (AO, Sigma Aldrich) was used for enzymatic removal of ascorbic acid by reducing ascorbic acid to dehydroascorbic acid. Ascorbate oxidase was dissolved in a phosphate buffer consisting of 100 mM KH_2PO_4, 4 mM Na_2PO_4, and 5 mM EDTA, and pH was set to 5.6. Aliquots of the extracts (E1A–E3A) were taken, and pH adjusted to 5.6. These extracts were then treated with ascorbate oxidase to provide ascorbate-depleted extracts (E1B—E3B). For this purpose 100 units of ascorbate oxidase was added to the test tubes containing the samples and left in the dark at room temperature for 24 hours.

2.5. Analysis of Ascorbic Acid Content in the Extracts. The ascorbic acid content of the extract was determined on a Shimadzu HPLC system (SIL-10A autosampler, SCL-10AVP control unit, LC-10AD pump, SPD-10AV VP UV-Vis detector unit, BergmanLabora, Sweden). The pre-extract (PE) and the metaphosphoric acid extract (E1A) were diluted with 2% meta-phosphoric acid, 20 and 30, times respectively, before analysis. No dilution was performed with the other extracts. The isocratic eluent consisted of 0.05M NaH_2PO_4 and orthophosphoric acid (8.5%), pH of the eluent was adjusted to 2.8. The separation was performed using a Restek, 150 × 4.6 mm, column kept at 30°C (Column Chiller, Sorbent AB) and a guard column. Detection was carried

out at 254 nm. Evaluation of data was done with Shimadzu Class-VP software (version 6.13 SP2) using retention times and spectral data as compared with an external standard of ascorbic acid. Each sample was analyzed in triplicate.

2.6. Analysis of Phenols by HPLC-ESI-MS. The content of major single phenols in the different extracts was measured on a Perkin Elmer Sciex API 150EX Single Quadrupole mass spectrometer (with a Turbo Ion Spray interface) according to a modified method from that described by Salminen et al. [35]. The HPLC system consisted of two Perkin Elmer pumps connected to a Perkin Elmer autosampler (Serie 200). The compounds were separated using a Phenomenex Synergi 4μ Hydro-RP 80A, 250 $\times$ 4.6 mm column, and a C_{18} precolumn. The mobile phase consisted of 0.4% formic acid (Buffer A) and acetonitrile (Buffer B). The eluent was run at a flow rate of 1.0 mL min^{-1} and the gradient elution was as follows: 0% B (0–3 min), 30% B (3–30 min), 40% B (30–35 min), 40% B (35–38 min), and 0% B (38–42.5 min). The injection volume was for all samples 8 μL. The eluent was split to 0.3 mL min^{-1} before being introduced to the ESI-MS system. The electrospray chamber was operated at 4.0 kV in the negative ion mode and mass ions obtained by acquiring data in peak jump and scan mode. Catechin was detected at m/z 289.3 $(M\text{-}H)^-$, proanthocyanidin monomer 577.5 $(M\text{-}H)^-$, proanthocyanidin dimer 577.5 $(M\text{-}H)^-$, rutin 609.5 $(M\text{-}H)^-$, quercetin galactoside 463.4 $(M\text{-}H)^-$, quercetin glucoside 463.4 $(M\text{-}H)^-$, and cyanidin glucoside 477.1 $(M\text{-}H)^-$. The general conditions for the quantitative HPLC-ESI-MS analyses were the following: nebulizer gas 9.0 L min^{-1}, curtain gas 12.0 L min^{-1}, and dry gas temperature 300°C.

2.7. Determination of Total Phenols. The content of total phenols was measured using the Folin-Ciocalteu method [36]. In brief, five μL of the different extracts were mixed with 100 μL 5% ethanol, 200 μL Folin-Ciocalteu reagent, 2 mL of 15% Na_2CO_3, and 1 mL of dH_2O. The absorbance was measured at 765 nm after 2 h incubation at room temperature. Gallic acid was used as a standard and the total content of phenols was expressed as mg gallic acid equivalents (GAE)/g dry weight (dw).

2.8. Ferric Reducing Ability of Plasma. The ferric reducing ability of plasma (FRAP) of the extracts was measured according to the method developed by Benzie and Strain [37], but modified to fit a 96-well format [38]. The different extracts were diluted 100-fold. Ten μL of these preparations were incubated at 37°C and then mixed with 260 μL of ferric-TPTZ reagent (prepared by mixing 300 mM acetate buffer, pH 3.6; 10 mM of 2,4,6-tripyridyl-s-triazine in 40 mM HCl; and 20 mM $FeCI_3$ in the ratio of 4 : 1 : 1; the solution was kept at 37°C). The absorbance was measured at 595 nm after 4 min on a plate reader (Sunrise, Tecan Nordic AB, Sweden). Fe^{2+} was used as a standard and L-ascorbic acid was used as a control where one mole of ascorbic acid corresponds approximately to two moles of FRAP (we obtained and used the value 2.02).

2.9. Preparation of Erythrocytes. The preparation of erythrocytes was performed as described by Honzel et al. [23]. Briefly, a healthy human volunteer served as blood donor. Peripheral venous blood samples were drawn into sodium K_2-EDTA vials (BD Vacutainer, UK). The vials were centrifuged for 5 min at 2400 rpm. Plasma and leukocytes were removed and the erythrocytes were harvested by pipettes and transferred into new vials. The erythrocytes were washed three times with PBS in a centrifuge at 2400 rpm for 5 min.

2.10. Analysis of Antioxidant Protection in the Erythrocyte Model. The protocol for the erythrocyte bioassay was based on the cellular antioxidant protection assay using erythrocytes (CAP-e) [23] modified to a microplate-based assay, but using H_2O_2, as the free radical generator. Briefly, from the remaining packed erythrocytes, 0.12 mL was added to 12 mL of PBS. The erythrocytes were then treated with serial dilutions of the previously obtained extracts (PE, E1A–E3A and E1B–E3B). The erythrocyte suspension was incubated in the dark on a rocker at room temperature for 120 min. The erythrocytes were then washed twice in PBS and any extracellular potential antioxidants were thereby removed. The cell pellet was then lysed through the addition of dH_2O and the lysed sample treated with the fluorescent dye 5-(and-6)-carboxy-2′,7′-dichlorofluorescein (DCF-DA), which becomes fluorescent as a result of oxidative damage. After this the sample was exposed to free radicals by addition of 167 mM hydrogen peroxide (hydroxyl free radical generator). The degree of antioxidant damage was recorded after 10 min by measuring the fluorescence intensity of each sample. The mean DCF-DA fluorescence intensity was compared between untreated erythrocytes (negative controls), hydrogen-peroxide-treated erythrocytes (positive controls), and erythrocytes pretreated with extracts from rose hips on three separate plates.

2.11. Analysis of Erythrocyte Uptake of Phenols. From the remaining packed erythrocytes, pure erythrocytes were treated with extract E2A. All measurements were performed with three true replicates. For this purpose, the erythrocyte cell suspension (1/3 sample and 2/3 purified erythrocytes) was incubated in the dark on a rocker at room temperature for 60 min. The erythrocytes were washed twice in PBS to remove the antioxidants not able to enter the cells after the 60 min incubation. The supernatants following the wash were retained for analysis. The cell pellet was lysed with absolute ethanol, vortexed and placed in an ultrasonic bath for 5 min, and then centrifuged at 13000 rpm for 5 min. The supernatant was then kept for analysis using HPLC-ESI-MS as described in Section 2.6.

2.12. Statistical Analysis. The results of the oxidative stress experiments were expressed as the mean ± standard deviation.Each observation was repeated in triplicate in different 96-well plates. Statistical analyses were carried out using the Minitab 16 software (Minitab, State College, PA, USA). Paired *t*-test analyses were performed to reveal any significant difference between treatments (extracts).

Table 1: Content of ascorbic acid and its contribution to antioxidant capacity of different extracts (E1–E3, without (A) or with (B) ascorbate oxidase treatment) obtained from sequential elution of a metaphosphoric preextract (PE) of rose hips. Data are presented as mg ascorbic acid/g dw rose hip powder, mg ascorbic acid/mL extract, and FRAP antioxidant capacity mmol Fe^{2+}/L extract (mean ± standard deviation).

Fraction	Ascorbic acid (mg/g dw)		Ascorbic acid (mg/mL)		FRAP ascorbic acid (mmol Fe^{2+}/L)	
	A	B	A	B	A	B
PE (HPO_3)	80.18 ± 1.02		2.74 ± 0.05		31.40 ± 0.62	
E1 (Eluent of PE)	42.03 ± 1.60	0.03 ± 0.06	5.97 ± 0.23	0.00 ± 0.01	68.41 ± 2.60	0.06 ± 0.09
E2 (20% MeOH)	1.41 ± 1.72	0.00 ± 0.00	0.59 ± 0.72	0.00 ± 0.00	6.72 ± 8.23	0.00 ± 0.00
E3 (100% MeOH)	0.04 ± 0.01	0.00 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.21 ± 0.05	0.00 ± 0.00

3. Results

In this study, the protection against oxidative stress of human erythrocytes was measured *in vitro*. Experiments were performed with a rose hip metaphosphoric acid preextract and three extracts of different polarity obtained through sequential elution of the pre-extract on a C_{18} solid phase column.

The content of ascorbic acid in the extracts and in the different dilutions before and after enzymatic treatment is presented in Table 1. From these data, it is clear that treatment with ascorbate oxidase efficiently reduced the content of ascorbate in all samples, although minute quantities remained in extract E1B.

The Folin-Ciocalteu assay was used for measurement of the content of total phenols. A high content of phenols was found in the metaphosphoric acid extracts (PE and E1A). Although this value decreased after enzyme treatment (E1B), it was still higher than in extracts two (E2A) and three (E3A) (Table 2).

The extracts were also analysed for content of single phenolic compounds using mass spectrometry. We found significant amounts of catechin, a proanthocyanidin monomer, a proanthocyanidin dimer, rutin, quercetin galactoside, quercetin glucoside, and cyanidin-glucoside (Table 3). The metaphosphoric acid extract (E1A) contained only low levels of different phenols, whereas the second (20% methanol) extract (E2A) in particular, but also the third (100% methanol) extract (E3A) contained considerably more phenols. Of the quantified single phenolic compounds catechin and proanthocyanidins were present in highest amounts.

Using the chemical FRAP assay to measure antioxidant capacity, the metaphosphoric extract (E1A) showed the highest antioxidant capacity, 464.4 ± 84.2 μmol Fe^{2+}/g dry weight (dw). This value decreased four-fold after enzymatic treatment to 98.0 ± 10.6 μmol Fe^{2+}/g dw (E1B) and was then lower than in the second (20% methanol) extract (E2A) (Table 4).

This is in contrast to the biological model using erythrocytes. In Figure 1, the protection against oxidative stress on erythrocytes is shown for all extracts, both with ascorbic acid (a) and without ascorbic acid (b). Interestingly, there was a significant ($P = 0.023$) increase in protection after removal of ascorbic acid, with a protection of 59.4% and 67.9% for E1A and E1B, respectively, at an extract concentration of 3 mg rose hip powder per mL. E2A with the highest contents of polyphenols was the most protective of the polyphenol rich extracts with 20.8% inhibition of oxidative damage. The corresponding protection from the third extract (E3A) was 5.0%.

Table 2: Content of total phenols in different extracts (E1–E3, without (A) or with (B) ascorbate oxidase treatment) obtained from sequential elution of a metaphosphoric acid pre-extract (PE) of rose hips, measured by the Folin-Ciocalteu method. The bias from the ascorbic acid content in the extracts to the phenol estimates is obvious. Data are presented as mg GAE/g dw rose hip powder and mg GAE/mL extract (mean ± standard deviation).

Extract	Total phenols (mg GAE/mL)		Total phenols (mg GAE/g dw)	
	A	B	A	B
PE (HPO_3)	3.0 ± 0.3		86.4 ± 8.9	
E1 (Eluent of PE)	3.4 ± 0.1	2.1 ± 0.2	24.2 ± 1.4	15.0 ± 1.3
E2 (20% MeOH)	5.2 ± 0.8	4.2 ± 0.9	12.4 ± 1.8	10.0 ± 2.0
E3 (100% MeOH)	2.0 ± 0.1	1.8 ± 0.4	4.8 ± 0.9	4.4 ± 0.8

To confirm uptake of antioxidants by the erythrocytes, the content of polyphenols was measured in erythrocytes treated with the polyphenol rich (20% methanol) extract (E2A) using HPLC-MS. Of the studied polyphenols only catechin and proanthocyanidins entered the erythrocytes in detectable amounts (Figure 2). The uptake in the erythrocytes was very low, 3.1% of the total amount of catechin supplied (Table 5). The supernatant contained 50.7%, thus 46.2% of the added catechin remained in the pellet consisting of the erythrocyte membranes.

4. Discussion

Human erythrocytes are carriers of oxygen and may be exposed to reactive oxygen species which could lead to oxidative damage. Several micronutrients may protect erythrocytes against oxidative stress. In this study, we used human erythrocytes to investigate cellular protection and uptake of bioactive compounds in sequential extracts eluted from an acid water extract of rose hips. The fact that all extracts studied contributed to protective effects on the erythrocytes indicate that rose hips contain a variety of effective antioxidant compounds.

Table 3: Content of major single phenols in different extracts (E1A–E3A) obtained from sequential elution of a metaphosphoric acid pre-extract of rose hips. Data are presented as μg/mL extract (mean ± standard deviation).

Polyphenol (μg/mL)	PE (HPO_3)	E1A (Eluent of PE)	E2A (20% MeOH)	E3A (100% MeOH)
Catechin	436.1 ± 77.7	7.6 ± 1.2	335.4 ± 22.8	33.8 ± 10.7
Proanthocyanidin monomer	774.4 ± 87.2	5.6 ± 2.4	625.1 ± 38.5	40.8 ± 10.4
Proanthocyanidin dimer	379.0 ± 53.8	0.0 ± 0.0	296.4 ± 26.8	10.9 ± 2.2
Rutin	44.6 ± 11.6	0.0 ± 0.0	0.0 ± 0.0	35.9 ± 1.1
Q-galactoside + Q-glucoside	158.5 ± 29.4	0.0 ± 0.0	0.0 ± 0.0	116.3 ± 1.9
Cyanidin-glucoside	44.1 ± 5.1	0.0 ± 0.0	32.0 ± 6.4	23.9 ± 3.7

Table 4: Antioxidant capacity of different extracts (E1–E3, without (A) or with (B) ascorbate oxidase treatment) obtained from elution of a metaphosphoric pre-extract (PE) of rose hips, measured by ferric reducing ability (FRAP). Data are presented as mmol Fe^{2+}/L extract and μmol Fe^{2+}/g dw rose hip powder (mean ± standard deviation).

Fraction	FRAP (mmol Fe^{2+}/L)		FRAP (μmol Fe^{2+}/g dw)	
	A	B	A	B
PE (HPO_3)	58.51 ± 2.47		1712.18 ± 80.60	
E1 (Eluent of PE)	65.97 ± 11.97	13.91 ± 1.50	464.40 ± 84.25	97.95 ± 10.56
E2 (20% MeOH)	74.91 ± 12.20	59.25 ± 12.24	179.78 ± 29.28	142.20 ± 29.37
E3 (100% MeOH)	13.12 ± 2.30	9.36 ± 3.12	31.49 ± 5.51	22.47 ± 7.49

The content of ascorbic acid was highest in the pre-extract and the eluted metaphosphoric acid extract (PE and E1A). In this study, the metaphosphoric acid aqueous extracts showed superior protection against oxidative damage. In a previous study [40], we showed that the protective effect (~75%) of rose hips could not be due to the ascorbic acid content alone as the rose hips extract only contained 0.4 mg ascorbic acid/mL in 5.0 mg dry weight rose hip powder/mL compared with a control that contained 5.0 mg ascorbic acid/mL and offered ~65% protection. This implies protective capacity of notably the ascorbic acid but also of other novel compounds.

The Folin-Ciocalteu assay for measurement of total phenols has previously been found to be biased due to the interference of nonphenolic reducing compounds such as ascorbic acid and sugars [41, 42] which results in an overestimation. The contribution of ascorbic acid to total antioxidant activity can be determined, since one mole of ascorbic acid corresponds approximately to two moles of FRAP (in our assay we obtained and used the value 2.02). The contribution to the FRAP value of the ascorbic acid content of the rose hip metaphosphoric acid pre-extract was thus calculated to be 31 mmol Fe^{2+}/L (Table 1), which should be compared with the obtained total FRAP value of the same extract which was 59 mmol Fe^{2+}/L (Table 4). In extract E1A, the FRAP value of the ascorbic acid content was similar to the total FRAP value, indicating the contribution of ascorbic acid, but in extract E2A, the FRAP contribution of the ascorbic acid was only 6 mmol Fe^{2+}/L compared with the total FRAP value of 75 mmol Fe^{2+}/L. The remaining extracts made almost no contribution of FRAP ascorbic acid; hence, there were significant amounts of other compounds that contributed to the antioxidant capacity of these extracts.

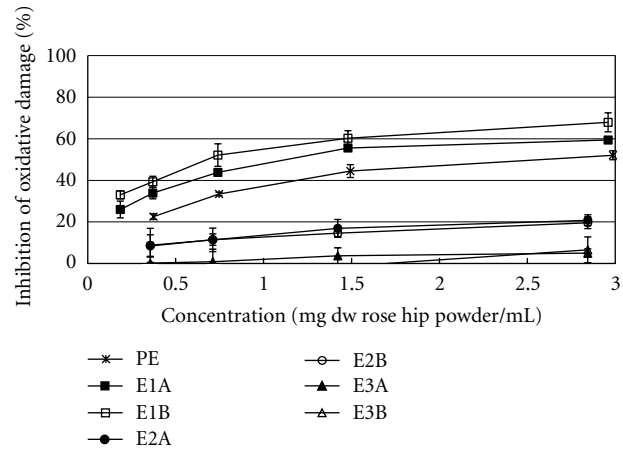

Figure 1: The protective effect against oxidative stress of different rose hip extracts (PE = pre-extract, E1 = HPO_3 eluent, E2 = 20% MeOH eluent, E3 = 100% MeOH eluent; without (A) or with (B) ascorbate oxidase treatment) as measured on erythrocytes *in vitro*. Vertical bars represent standard deviation.

In the FRAP assay, the metaphosphoric acid extract (E1A) showed the highest ferric reducing ability, but this value decreased four-fold after enzyme treatment. The activity of the enzyme-treated extract (E1B) was lower than the secondly eluted (20% methanol) extract (E2A).

Pandey and Rizvi [13] investigated the protective effect of resveratrol on markers of oxidative stress in human erythrocytes. Human erythrocytes are able to take up resveratrol and quercetin, and once inside the cell, these compounds can donate electrons to extracellular electron acceptors through the erythrocyte plasma membrane redox

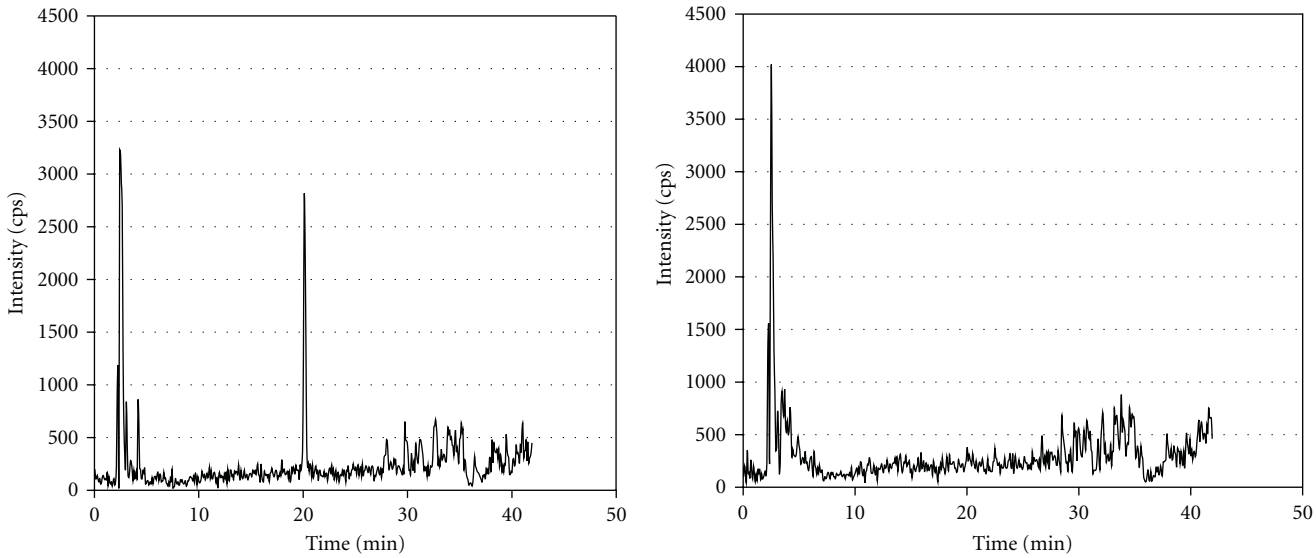

Figure 2: Chromatograms of erythrocytes incubated with a polyphenol rich extract (E2A) eluted from a rose hip extract (a) showing presence of catechin and control sample consisting of pure erythrocytes (b).

system. Fiorani and Accorsi [11] showed uptake of a variety of flavonoids such as quercetin, luteolin, kaempferol, fisetin, isorhamnetin, acacetin, chrysin, apigenin, galangin, and tamarixetin. Most of these agents accumulate within the cells because of their ability to bind to hemoglobin. In another study, Fiorani et al. [43] investigated flavonoids in aqueous and ether extracts and showed that polyphenols in ether extracts elicited their antioxidant effects by activation of plasma membrane oxidoreductase but that flavonoids in aqueous extracts were ineffective in the cell-based assay. The authors accounted for these observations through the inability of aqueous components to cross the erythrocyte membrane [43]. In our study, the protective capacity was most obvious in the aqueous extracts. The highest degree of protection was obtained with the first eluted metaphosphoric acid extract (E1A), and, interestingly, there was an increase in protection of erythrocytes after removal of ascorbic acid from this eluent (E1B). Ascorbate oxidase efficiently reduced the content of ascorbate in this extract, E1B, with only minute quantities remaining. However, extract E1B most likely contained DHA, which has equivalent biological activity to ascorbic acid as erythrocytes have a high capacity to regenerate ascorbic acid. Erythrocytes lack an active transporter for ascorbate, whereas DHA is rapidly taken up by facilitated diffusion by a glucose transport protein, GLUT1. Intracellular DHA is rapidly reduced to ascorbate by GSH in a direct chemical reaction and trapped within the cell [44]. Enzyme-dependent mechanisms involving both glutaredoxin and thioredoxin reductase have also been demonstrated [44]. The increased protection obtained with the enzyme-treated extract (E1B) may thus be explained by reactivated ascorbate and GSH. Haemoglobin is a reactive protein, and erythrocytes stabilize and protect it with intracellular GSH levels. The GSH maintenance system provides the reducing power to maintain other cellular antioxidants in their reduced states. The uptake of DHA into the cells may have temporarily depleted intracellular thiol levels, although any significant reduction in GSH levels and subsequent rise in GSSG will immediately stimulate NADPH formation by the hexose monophosphate shunt (HMP) [9]. The HMP activity will then restore GSH levels, and the cells will have reactivated ascorbate and GSH, which might account for the increased protection of the enzyme-treated extract. It may also be that the major contribution to total antioxidant activity could have come from a combination of phytochemicals, not from ascorbic acid alone, as previously shown by Sun et al. [45].

To confirm antioxidant uptake, the content of catechin was measured by HPLC-MS. The uptake was only 3.1% of the total amount of catechin supplied. The supernatant contained 50.7%, thus 46.2%, of the added catechin remained in the pellet consisting of the erythrocyte membranes. This is in agreement with Koren et al. [34], who showed that human erythrocytes not only carry oxygen but also have the ability to bind polyphenol antioxidants.

5. Conclusions

The fact that all sequentially eluted extracts studied contributed to protective effects on the erythrocytes indicate that rose hips contain many different antioxidant compounds. The aqueous metaphosphoric acid extracts showed the highest protection against oxidative damage, this implies protective capacity of the ascorbic acid as well as other unidentified compounds.

Table 5: Mean content of major polyphenols μg/mL (mean ± standard deviation) in erythrocytes incubated with the polyphenol rich rose hip extract E2A (eluted with 20% methanol).

Polyphenol	Polyphenols (μg/mL)
Catechin	0.321 ± 0.011
Proanthocyanidin monomer	0.002 ± 0.003
Proanthocyanidin dimer	0.125 ± 0.190

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Research Article

Preconditioning and Acute Effects of Flavonoids in Protecting Cardiomyocytes from Oxidative Cell Death

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While flavonoids can reportedly protect against cardiac ischemia-reperfusion injury, the relative effectiveness of different flavonoids and the mechanisms involved are unclear. We compared protection by different flavonoids using rat embryonic ventricular H9c2 cells subjected to simulated ischemia-reperfusion (IR) and to *tert*-butyl hydroperoxide (*t*-buOOH). Characterization of the IR model showed the relative contributions of glucose, serum, and oxygen deprivation to cell death. With long-term (2-3 day) pretreatment before IR the best protection was given by catechin, epigallocatechin gallate, proanthocyanidins, and ascorbate, which protected at all doses. Quercetin protected (34%) at 5 μM but was cytotoxic at higher doses. Cyanidin protected mildly (10–15%) at 5 and 20 μM, while delphinidin had no effect at 5 μM and was cytotoxic at higher doses. Comparing long-term and acute protection by catechin, a higher concentration was needed for benefit with acute (1 hr) pretreatment. With a pure oxidative stress (*t*-buOOH) only quercetin significantly protected with 3-day pretreatment, while with short-term (1 h) pretreatments protection was best with quercetin and epigallocatechin gallate. The results suggest catechins to be especially useful as IR preconditioning agents, while quercetin and epigallocatechin gallate may be the most protective acutely in situations of oxidative stress.

1. Introduction

Ischemia and reperfusion in the myocardium initiates metabolic and ionic perturbations, mostly as a result of oxidative stress, ultimately leading to cell demise and tissue necrosis. Although recent notions have posed myocardial cell renewal as a part of normal cardiac homeostasis [1], cardiac cells likely have limited ability to proliferate. Hence, reducing cell death during ischemia-reperfusion is of great importance in preventing the irreversible injury.

Polyphenols and especially flavonoids are well acknowledged for their antioxidant and protective effects in circumstances of oxidative stress [2, 3]. They have also been recognized to interact with cell death-survival signaling pathways which depending on the dose may promote or inhibit apoptosis, exhibiting chemopreventive or cytoprotective effects, respectively [4, 5].

A large body of evidence points to protective effects of flavonoids against cardiac ischemia-reperfusion injury (e.g., reviewed in Akhlaghi and Bandy [6]). However, in most studies the cardioprotective effect of a single flavonoid has been investigated. Only one in vitro report has compared effects of a few flavonoids from two classes on ischemia-reperfusion-induced cell death [7].

One goal of the current study therefore, using cultured rat embryonic ventricular H9c2 cells, was to compare a broader variety of flavonoids. In this study, we compared flavonoids from flavonol (catechin and proanthocyanidins), catechin gallate (epigallocatechin gallate), flavonol (quercetin), and anthocyanin (cyanidin, delphinidin) classes (Figure 1). Such a comparison could help distinguish which types of flavonoids might be most effective in protecting against ischemia-reperfusion injury. Ascorbate was also compared as another factor in fruit and vegetables that may help protect [8].

A second goal was to distinguish indirect (preconditioning) effects versus direct effects. Because flavonoids may produce protection indirectly, for instance, through inducing

phase 2 enzymes and endogenous antioxidant systems [9], we compared short-term (1 hr) with long-term (2-3 day) exposure prior to the oxidative stress. The short-term pretreatment was designed to allow enough time for the flavonoids to be taken up into the cells and provide direct antioxidant or other effects during ischemia and reperfusion, but not enough time for preconditioning requiring protein induction and synthesis. The long-term pretreatment would additionally allow such preconditioning. Also, to evaluate different oxidative stimuli which may provoke different signaling cascades [10], we compared simulated ischemia-reperfusion with exposure of the cells to *tert*-butyl hydroperoxide (*t*-buOOH).

2. Materials and Methods

2.1. Materials. Quercetin, (+)-catechin, epigallocatechin gallate, gallic acid, caffeic acid, resveratrol and sodium ascorbate were purchased from Sigma. Delphinidin and cyanidin were from Extrasynthèse SA (Genay Cedex, France). Activin Grape Seed Extract (95% proanthocyanidins) was from Trophic Canada Ltd.

2.2. Cell Culture. Rat embryonic ventricular cells, H9c2 (American Tissue Culture Collection, Manassas, VA), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1.5 g/L $NaHCO_3$ and 1.0 g/L glucose, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. Tissue culture flasks were kept at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every 2-3 days and the cells were subcultured regularly. For experiments, cells were cultured on 96-well plates, kept in DMEM with 10% FBS until confluency, and then were switched to DMEM with 2% FBS.

2.3. Treatment with Flavonoids. Confluent cells were treated with various concentrations of flavonoids (in dimethyl sulfoxide) for different time courses prior to induction of ischemia or incubation with t-buOOH. The concentrations were those that could be achieved in plasma with acute or long-term dietary supplementation (5–10 μM at high levels) [11–13] and supraphysiologic concentrations that could be administered intravenously or added to cardioplegic solutions in situations such as coronary bypass surgery or heart transplant. The concentration of dimethyl sulfoxide in DMEM was kept equal to or less than 0.05% v/v. The concentrations of ascorbate used were those normally in plasma ($\sim$100 μM) and those that can be achieved with supplementation (up to 250 μM) or intravenous administration [14].

2.4. Ischemia-Reperfusion. To induce ischemia, after removing DMEM, cells were washed twice with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.3) and covered with 100 μL of ischemia-mimetic solution containing 140 mM NaCl, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 8 mM KCl, and 6 mM HEPES, pH 6 [15]. The ischemia solution was bubbled with 100% nitrogen gas for at least 30 min before adding to the cells. The cells were placed in a 37°C modular hypoxia chamber, and the chamber was flushed with nitrogen gas for the initial 1.5 h of ischemia and sealed for the rest of the ischemic period. The time course of flushing (1.5 h) was chosen according to the initial experiments when the atmosphere of the chamber was tested with the oxygen indicator methylene blue. The ischemia was established for 5 h followed by reperfusion for 18 h. For reperfusion, the ischemia solution was replaced with DMEM (with 2% FBS) containing 300 mg/L $NaHCO_3$ and flavonoids (if any), and the plates were placed in the same chamber as the control plates. In preliminary experiments, we determined that 300 mg/L $NaHCO_3$ was sufficient to keep the pH at 7.4 during the 18 hr reperfusion in room air (with <0.1% CO_2).

Cells in control (nonischemic) groups experienced the same procedures including washing steps, and the DMEM was replaced with fresh DMEM containing 300 mg/L $NaHCO_3$. Control plates were kept at 37°C in a humidified chamber containing ambient air throughout the experiment. Those wells in the control plate which had been treated with flavonoids were treated the same during the experiment.

2.5. Treatment with Tert-Butyl Hydroperoxide. In hydroperoxide experiments, after treatment with flavonoids or ascorbate, cells were incubated with 400 μM *t*-buOOH for 24 h before cell viability assessment.

2.6. MTT (3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyltetrazolium Bromide) Assay. The method used was a modification of the method used by Denizot and Lang [16]. After reperfusion, the medium was removed and the cells were washed twice with PBS to remove residual flavonoids. Then, 100 μL of solution containing 140 mM NaCl, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM KCl, 10 mM glucose, and 6 mM HEPES, pH 7.4 was added to each well, and the cells were incubated at 37°C with final concentration of 0.5 mg/mL MTT (in PBS). After 1 h, the solution was removed and purple formazan crystals were solubilized in 100 μL dimethyl sulfoxide for 15 min, and the absorbance was determined at 570 nm using an absorbance spectrophotometer.

2.7. Microscopic Determination of Cell Viability. To facilitate counting, the cells were incubated with a final concentration of 4 μg/mL acridine orange (in PBS) added to the medium [17]. After 1 h, the medium was aspirated, the cells were washed with PBS, 100 μL of the above-mentioned solution containing 140 mM NaCl, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM KCl, 10 mM glucose, and 6 mM HEPES (pH 7.4) was added to each well to maintain the cells alive, and the cells were visualized using a fluorescence microscope. As dead cells were washed away during the washing process, the only cells observed on the microscope were live cells. A specific area from each well with a magnification of 10x was chosen for imaging.

2.8. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). The activity of GAPDH was used as an indicator of cell death. The activity of GAPDH released into the medium was measured using aCella-TOX kit according to the manufacturer's instructions.

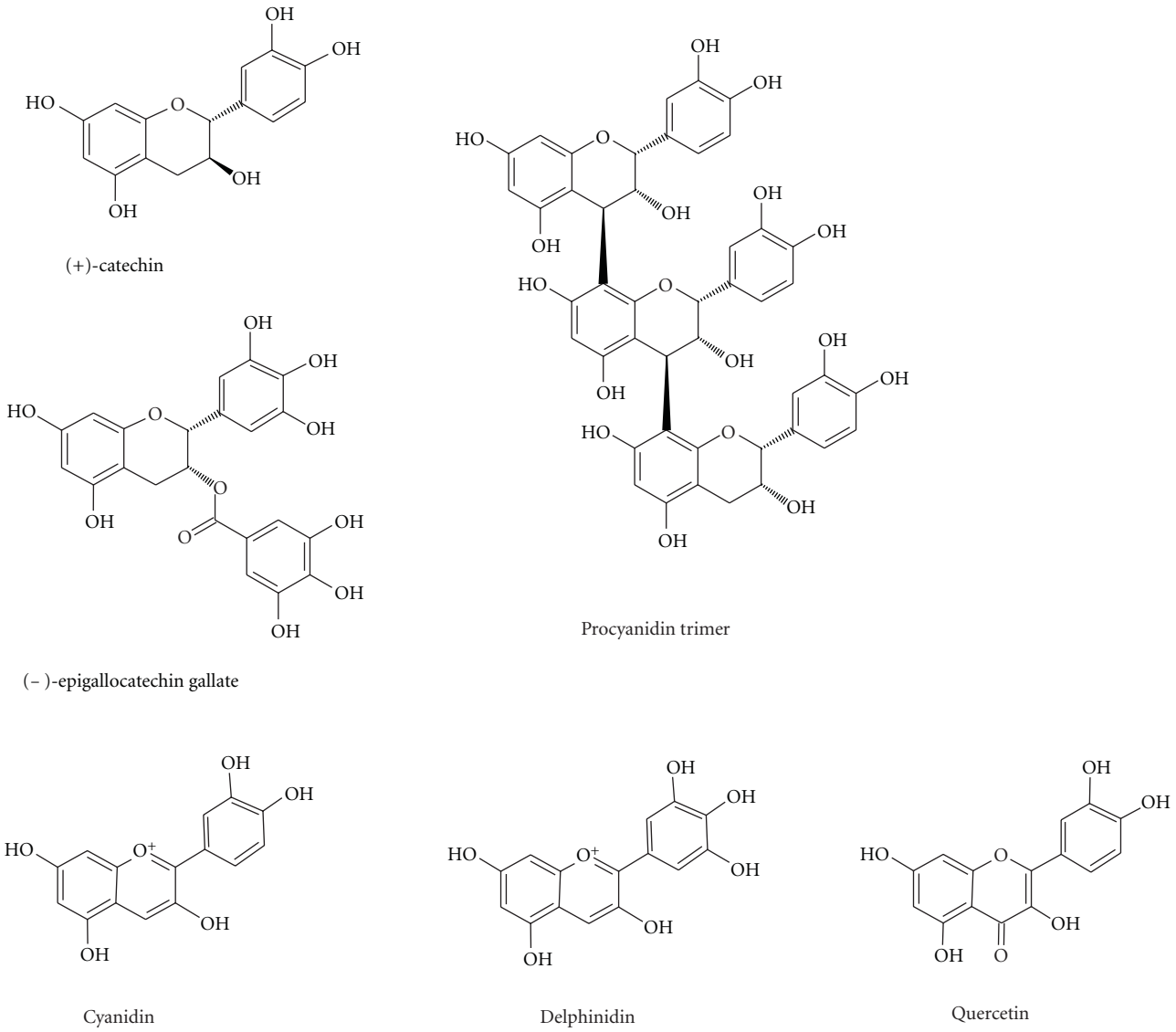

Figure 1: Flavonoids studied. The flavonoids studied were flavonols (catechin and a proanthocyanidin mixture including procyanidin trimer), a catechin gallate (epigallocatechin gallate), a flavonol (quercetin), and anthocyanins (cyanidin and delphinidin).

2.9. Statistics. Data represent means ± SEM. Statistical analyses were performed with SPSS software using one-way analysis of variance (ANOVA). As a post hoc test, 2-sided Dunnett was used to compare treatments with either nonischemic control (named as control in graphs) or ischemic-reperfused control (named as IR in graphs). In nonischemia conditions, flavonoid treatments were compared with the nonischemia control, while in ischemia-reperfusion conditions, flavonoid-treated groups were compared with the IR control. A P value of less than 0.05 was considered significant.

3. Results

3.1. Ischemia-Reperfusion Experiments. Cell death in this model occurred as a result of hypoxia, serum removal, and glucose deprivation (Figure 2). Each of these factors contributed to cell death, with lack of oxygen contributing 34% ($100 \times (\text{MR/R} - \text{I/R})/(\text{Control} - \text{I/R})$), lack of glucose contributing 24% ($100 \times (\text{HSR/R} - \text{I/R})/(\text{Control} - \text{I/R})$), and the lack of FBS contributing 42% (the remaining proportion of cell death).

Three-day pretreatment with different concentrations of flavonoids showed little or no protection by quercetin, cyanidin, or delphinidin (Figure 3) and notable protection by catechin (Figure 3). Concentrations of 5 to 50 μM catechin improved cell viability by 21 to 58 percent, while cyanidin gave significant mild protection (10–15%) at 5 and 20 μM.

Because catechin gave the strongest protection, we conducted additional experiments comparing different catechins (epigallocatechin gallate and a proanthocyanidin mixture) (Figure 4). Like catechin, concentrations of 5 to 100 μM

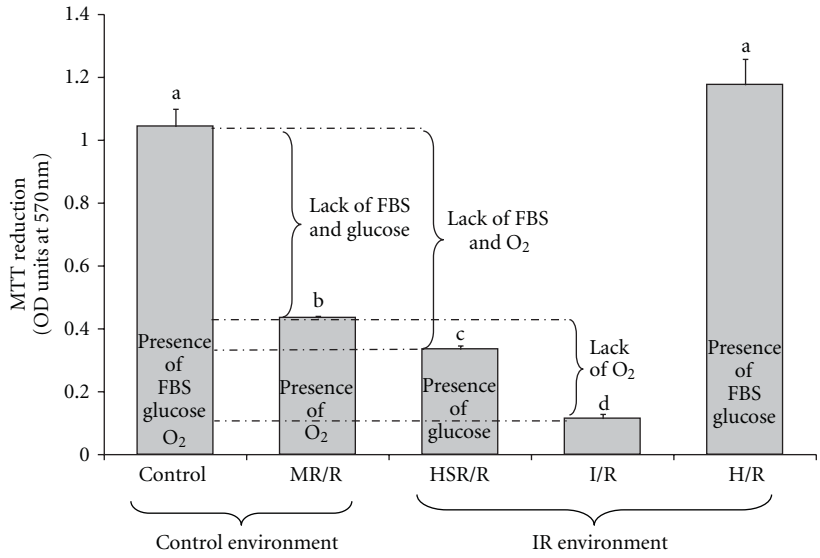

Figure 2: Contributions of oxygen, glucose, and serum removal on ischemia-reperfusion-induced cell death in H9c2 cells. Cell viability was assessed by the MTT assay as described in Section 2. The condition of the experiment for different groups was as follows: *Control,* cells were kept in the control environment (presence of oxygen) and received DMEM plus FBS throughout the experiment. *MR/R (medium restriction/reperfusion)*, cells were placed in the control environment (presence of oxygen) and received ischemia solution during the time of ischemia and DMEM plus FBS during the time of reperfusion. *HSR/R (hypoxia-serum restriction/reperfusion),* cells were placed in the ischemia environment (absence of oxygen) and received ischemia solution plus glucose (N_2-saturated) during hypoxia and transferred to the control environment (presence of oxygen) and treated with DMEM plus FBS during reperfusion. *I/R (ischemia/reperfusion),* cells were placed in the ischemia environment (absence of oxygen) and received ischemia solution during ischemia and transferred to the control plate (presence of oxygen) where they received DMEM plus FBS during reperfusion. *H/R (hypoxia/reperfusion),* cells were kept in the ischemia environment (absence of oxygen) during ischemia and transferred to the control plate (presence of oxygen) during reperfusion but received DMEM plus FBS throughout the experiment. Bars represent means of 3–6 wells ± SEM. $^{a–d}$Letters that are different indicate a significant difference at $P < 0.05$.

epigallocatechin gallate and proanthocyanidins also gave 28 to 49 and 22 to 44 percent protection, respectively, which in some concentrations was statistically significant (Figure 4).

To see if the length of pretreatment is effective in the protection observed, catechin was tested in short-(1 h) and long-(2-3 days) term pretreatments. Whereas in long-term (2-3 days) applications concentrations of catechin as low as 5 to 10 μM exhibited almost 50% protection (Figures 4 and 5), in short-term (1 h) pretreatment 50 μM catechin gave a 98% improvement in cell viability, while 10 μM catechin showed nonsignificant protection (Figure 5).

Sodium ascorbate also gave significant protection (57–92%) at all concentrations tested (Figure 6).

3.2. Hydroperoxide Experiments. To investigate any differences between protection against ischemia-reperfusion and a pure oxidative stress, experiments were conducted with *t*-buOOH. The cells were pretreated for 3 days with a concentration of 25 μM of flavonoids. Except quercetin which exhibited 46% protection against cell death caused by *t*-buOOH, none of the other compounds protected cells at this concentration and time course of treatment (Figure 7). Conversely, some compounds, that is, cyanidin, delphinidin, epigallocatechin gallate, and proanthocyanidins worsened cytotoxicity in the presence of *t*-buOOH after 3-day pretreatment. Short-term (1 h) pretreatment however did not produce cytotoxicity even when a higher concentration of flavonoids was used (Figure 7). With short-term incubation, epigallocatechin gallate gave cytoprotection (66%) rather than cytotoxicity, and it along with quercetin was the only compound that inhibited *t*-buOOH-induced cell death, increasing cell survival by 66 and 95 percent (Figure 7).

The protective effect of epigallocatechin gallate and quercetin on H9c2 cells against *t*-buOOH was confirmed when the medium was tested for released glyceraldehyde-3-phosphate dehydrogenase (Figure 8).

As short-term exposure to catechin protected at a higher concentration in the ischemia-reperfusion model (Figure 5) but did not protect at 25 μM in the *t*-buOOH experiments (Figure 7), an experiment was performed using different concentrations of catechin along with a comparison between catechin and epigallocatechin gallate. Catechin did show significant protection against *t*-buOOH but only at 100 μM (Figure 9). Epigallocatechin gallate showed stronger protection and protected significantly at 50 μM and 100 μM (Figure 9).

4. Discussion

The main objective of the ischemia-reperfusion study was to compare the protective effects of flavonoids against cell death caused by ischemia-reperfusion. An in vitro model of ischemia-reperfusion [15] was used to mimic the in vivo situation. An evaluation of the components of this model

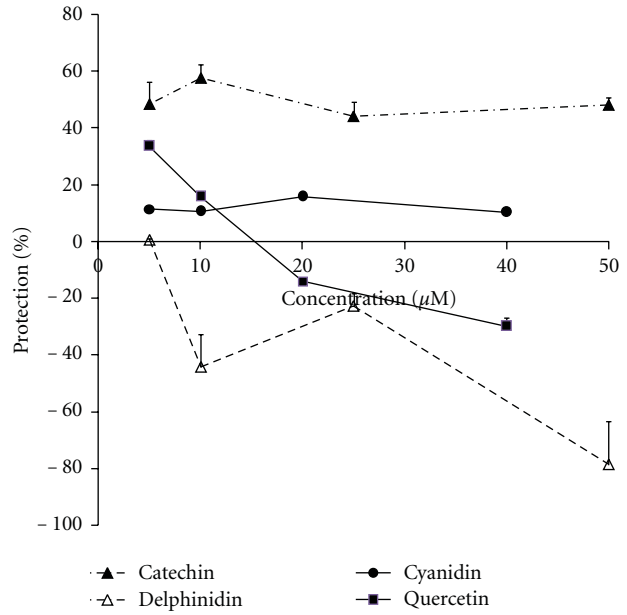

Figure 3: Protection compared to IR control in H9c2 cells pre-treated for 3 days with catechin, quercetin, cyanidin, and delphinidin before exposure to ischemia-reperfusion. Confluent cells were kept in 2% FBS-DMEM for 6 days including 3-day pretreatment with different concentrations of the flavonoids, and then ischemia and reperfusion were established according to the procedure described in Section 2. The flavonoids were also present during the reperfusion time. For the experiments with catechin and delphinidin, the number of live cells was counted after staining cells with acridine orange. For the experiments with quercetin and cyanidin, viability was measured by the MTT method. Data are expressed as the percent protection compared to the nonischemic and IR controls. Points are means of 4-5 wells ± SEM. *Represents $P < 0.05$ *versus* the control ischemic-reperfused condition. In the control condition (4 days total treatment without ischemia) 40–50 μM quercetin or delphinidin showed significant cytotoxicity (inhibiting cell growth by 25–55%), while lower concentrations did not (results not shown).

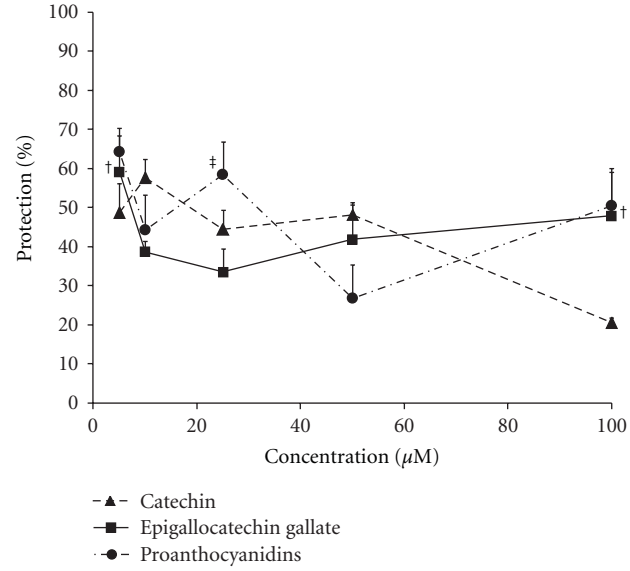

Figure 4: Protection by 3-day pretreatment with catechin, epigallocatechin gallate, or proanthocyanidins of H9c2 cells exposed to ischemia-reperfusion. Confluent cells were kept in 2% FBS-DMEM for 6 days including 3-day pretreatment with various concentrations of catechin (results from Figure 3 but with an additional point at 100 μM), epigallocatechin gallate, or proanthocyanidins, and then ischemia-reperfusion groups were exposed to 5 h ischemia followed by 18 h reperfusion. Catechin, epigallocatechin gallate, or proanthocyanidins were present during the reperfusion time. The number of live cells was counted after staining cells with acridine orange. Data were expressed as percent protection against cell death in treatment groups compared to the control nonischemic and ischemia-reperfusion conditions. Each point represents means of 3-4 wells ± SEM. *, †, and ‡ Show $P < 0.05$ *versus* nontreated ischemic-reperfused condition for catechin, epigallocatechin gallate, and proanthocyanidins, respectively. There were no significant effects of the flavonoids on growth and viability of the cells under the control (non-IR) condition (data not shown).

(Figure 2) showed that death of H9c2 cells occurred as a consequence of serum removal, hypoxia, and glucose deprivation. The process of cell death caused by serum removal reportedly involves mitochondrial dysfunction [18] that leads to increased production of reactive oxygen species [18, 19]. Thus, up to 76% of the death which occurred after FBS and oxygen deprivation may have been related in some way to oxidative stress, with the remaining 24% from glucose (energy) deprivation. In addition, serum removal causes release of Ca^{2+} from endoplasmic reticulum [20], resulting in cellular Ca^{2+} overload, one of the prominent consequences of ischemia-reperfusion [21]. Thus, the serum withdrawal-induced changes in reactive oxygen species and cellular Ca^{2+} resemble those that happen in *in vivo* conditions of ischemia-reperfusion. Interestingly, hypoxia reoxygenation alone (without serum and glucose deprivation) did not decrease cell number (last bar in Figure 2). However, this does not necessarily mean that no cell death occurred in this group, as both apoptosis and cell proliferation have been reported to be stimulated by hypoxia reoxygenation [22].

Among the tested flavonoids, those with a catechin-type structure (catechin, epigallocatechin gallate, and proanthocyanidins) inhibited cell death induced by simulated ischemia-reperfusion. Notably, the catechins were present for 3 days prior to ischemia-reperfusion, allowing adaptations such as induction of phase 2 and antioxidant enzymes as previously observed [23, 24], as well as during ischemia and reperfusion, allowing direct antioxidant effects. These results support previous findings of protection by catechins in models of ischemia-reperfusion in vitro and in vivo [25–29]. It has also been reported that epigallocatechin gallate can inhibit apoptosis induced by serum removal in cultured neurons [30], suggesting that this flavonoid is capable of inhibiting at least this component of ischemia-reperfusion injury in cultured cells.

Interestingly, in long-term exposure prior to ischemia-reperfusion dose response measurements did not show concentration as a factor (at least above 5 μM) in protection

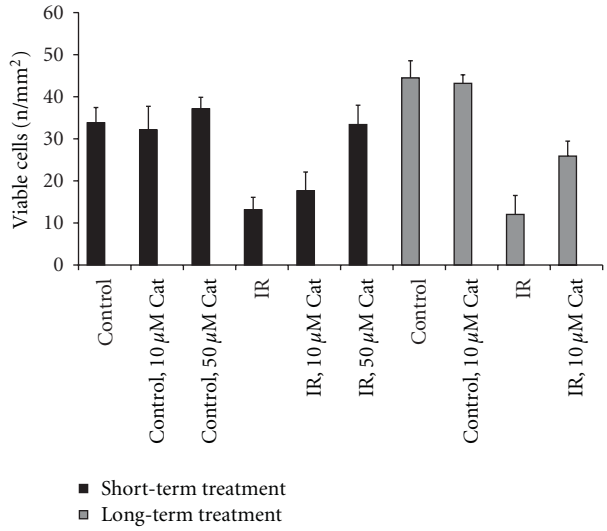

Figure 5: Effects of short-(1 h) and long-(2 days) term pretreatment with catechin (Cat) in H9c2 cells subjected to ischemia-reperfusion. Cells were kept in DMEM with 2% FBS for 2 days and treated with either 10 or 50 μM catechin for either 1 h (short-term) or 2 days (long-term) before induction of ischemia and reperfusion. Catechin was also present during the reperfusion. Live cells were counted with a microscope after staining cells with acridine orange. Columns are means of 8 wells ± SEM. *Shows $P < 0.05$ compared to the corresponding ischemia-reperfusion group (IR).

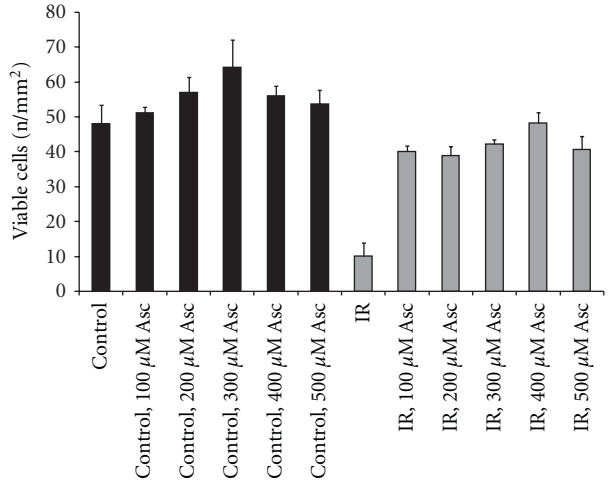

Figure 6: Effects of 3-day pretreatment with sodium ascorbate (Asc) against ischemia-reperfusion-induced cell death in H9c2 cells. Confluent cells were maintained in DMEM with 2% FBS for 6 days including 3-day pretreatment with different concentrations of ascorbate, and then ischemia-reperfusion groups were exposed to ischemia and reperfusion. Ascorbate was also present during the reperfusion time. Cell viability was determined using a microscope after staining the cells with acridine orange. Bars are means of 4-5 wells ± SEM. *Indicates $P < 0.05$ compared to the nontreated ischemic-reperfused group (IR). There were no significant effects of ascorbate on growth and viability of the cells under the control (non-IR) condition.

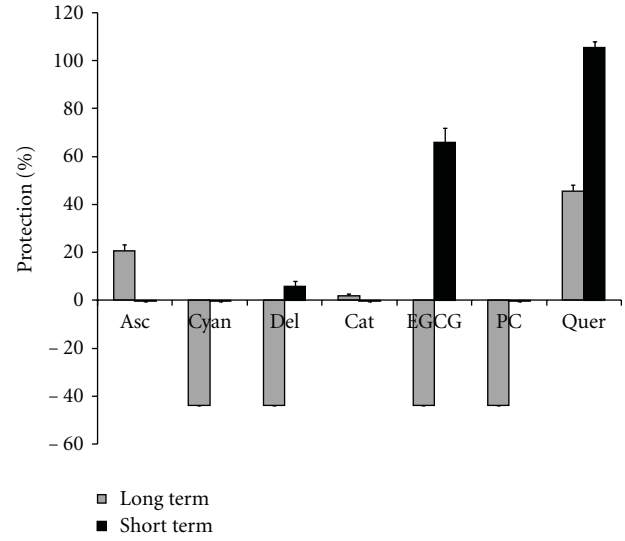

Figure 7: Protection by short- and long-term pretreatment with flavonoids against cell death caused by *t*-buOOH in H9c2 cells. *Long-term pretreatment:* cells were kept in DMEM with 2% FBS for 3-day treatment with 25 μM of different flavonoids or 100 μM sodium ascorbate prior to incubating with 400 μM *t*-buOOH for 24 hr. Live cells were then counted after staining with acridine orange. *Short-term pretreatment:* cells were kept in DMEM with 2% FBS for 3 days, and treated with 50 μM of different flavonoids or 100 μM sodium ascorbate for 1 h. After the treatment periods, cells were incubated with 400 μM *t*-buOOH for 24 h, and live cells were counted after staining with acridine orange. Bars show the percent protection upon treatment with flavonoids or ascorbate after incubation with *t*-buOOH. Asc: ascorbate; Cyan: cyanidin; Del: delphinidin; Cat: catechin; EGCG: epigallocatechin gallate; PC: proanthocyanidins; Quer: quercetin. *Indicates $P < 0.05$ *versus* non-treated *t*-buOOH group.

exhibited by the catechins (Figure 4). In contrast, 50 μM but not 10 μM catechin was able to protect the cells with short-term pretreatment (Figure 5), suggesting that higher levels are required for direct antioxidant effects. It seems likely that direct antioxidant effects are dose dependent in this range because the antioxidant is competing with cellular components for the reactive species. With adaptive protection due to long-term exposure the response may be amplified through induction of antioxidant enzymes and thus occurred with lower levels of flavonoid. That the protection was already maximal at 5 μM with long-term preexposure suggests that indirect adaptive responses have predominated in this condition.

Quercetin and one of the two examined anthocyanins (cyanidin) showed protection at a relatively low dose (5 μM) against ischemia-reperfusion with long-term pre-exposure, but at higher concentrations quercetin (and delphinidin) exhibited cytotoxicity in control (data not shown) and IR conditions. Quercetin [31, 32] and anthocyanins [33–35] have previously been reported to provide protection in models of ischemia-reperfusion. However in the current study they were not as effective as catechins, and with quercetin and delphinidin in particular they showed

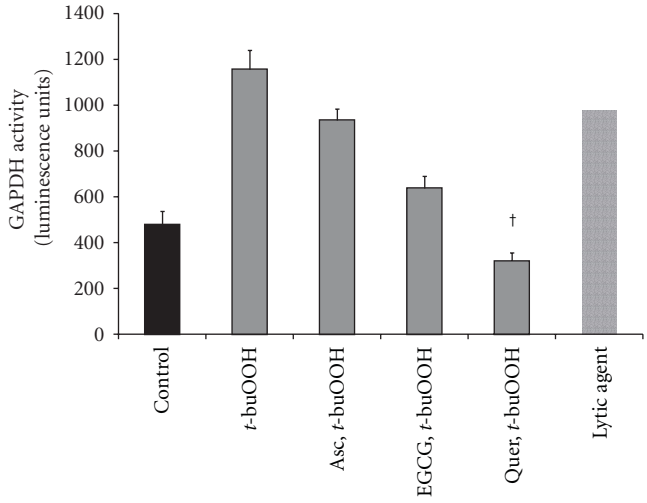

Figure 8: Effects of epigallocatechin gallate (EGCG), quercetin (Quer), or ascorbate (Asc) on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) released to medium. Cells were treated with 50 μM EGCG or Quer or 100 μM sodium ascorbate for 1 h prior to *t*-buOOH. Cell death was tested by measuring GAPDH activity in the medium 24 h after treating cells with 400 μM *t*-buOOH. Bars are means of 3-4 wells ± SEM. *Shows $P < 0.05$ *versus t*-buOOH. †Shows $P < 0.05$ *versus* EGCG.

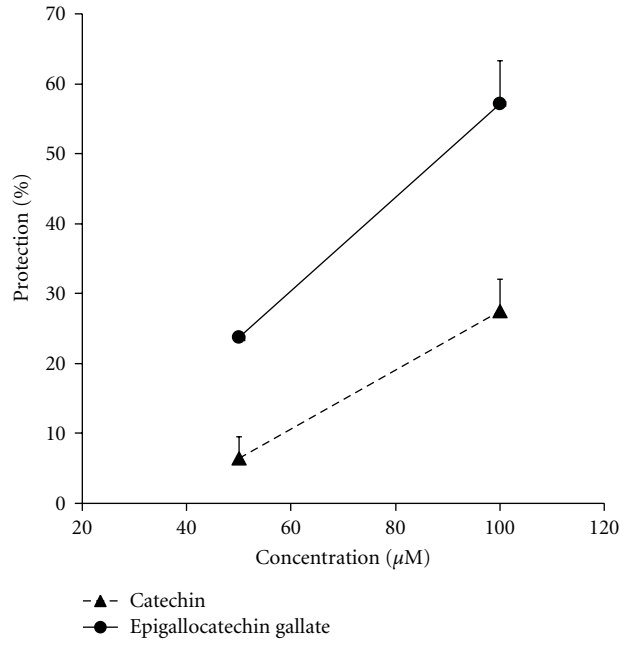

Figure 9: Protection by pretreatment with two concentrations of catechin and epigallocatechin gallate 1 h prior to incubation with *t*-buOOH. Cells were treated with 50 or 100 μM catechin or epigallocatechin gallate for 1 h. Then, cells were treated with 400 μM *t*-buOOH for 24 h before counting live cells. Data were expressed as the percent protection. *Shows $P < 0.05$ *versus* non-treated *t*-buOOH group.

a potential for cytotoxicity in the absence of ischemia-reperfusion.

Ascorbate also showed strong protection against ischemia-reperfusion in this model with long-term exposure. Part of this protection may have been through pre-conditioning, as ascorbate oxidizes in cell culture media to produce H_2O_2 [36] and thereby may induce antioxidant enzymes in cultured cells. This result with ascorbate in the current model is consistent with previous findings of protection by ascorbate against other types of ischemia-reperfusion in vitro [37] and in vivo [38, 39].

In this ischemia-reperfusion model we tested whether catechin showed protection in short-term as well as long-term pretreatment. A low concentration (i.e., 10 μM) of catechin protected if left with the cells for prolonged preincubation times (in the day range), while a higher concentration (i.e., 50 μM) was needed to protect with short-term (1 h) pretreatment. The reason for protection in the short-term pretreatment could be a direct antioxidant effect of catechin. In long-term pretreatments most of the catechin may have been oxidized during regular cellular metabolism and due to factors such as transition metals present in the medium [36], which thereby may provide pre-conditioning. These results are in keeping with those of Chang et al. [7] where protection of chick embryo cardiomyocytes by 25 μM catechin or procyanidin B2 was much more effective with long-term (72 hr) pretreatment than with treatment during ischemia or reperfusion.

If not much antioxidant activity from catechin remained after 2-3-day pretreatment, why was protection observed? One possibility is that oxidized forms of catechins generated during long-term pretreatment activated specific signaling pathways involved in induction of endogenous antioxidants [40]. Consistent with this supposition, Du et al. [23] found that 24 h incubation of H9c2 cells with 50–100 μM (but not 25 μM) catechin or proanthocyanidin B_4 increased endogenous cellular antioxidants (glutathione) and antioxidant enzyme activities (superoxide dismutase, glutathione S-transferase, and catalase) and mitigated cell death caused by the xanthine oxidase system [23]. Consistent with such a preconditioning effect, we observed that feeding rats green tea catechins for 10 days prior to subjecting the isolated hearts to ischemia-reperfusion helped to protect the hearts from oxidative cell death along with maintaining relatively higher levels of phase 2 enzyme activities [24].

Using *t*-buOOH we compared the extent to which the different flavonoids protected against a pure oxidative stress. In the ischemia-reperfusion model, long-term treatment with the catechins protected, as did short-term treatment with a higher concentration of catechin. However, in *t*-buOOH-induced cell death the protection was only observed with short-term (1 h) pre-incubation with epigallocatechin gallate, while long-term (3 days) preincubation with epigallocatechin gallate or proanthocyanidins intensified the cytotoxicity of *t*-buOOH. This paradox may be related to the oxidation products of catechins, which include unstable dimer quinones [41], and would accumulate during long-term treatment. If such products become cytotoxic in reactions with *t*-buOOH it could account for the detrimental effects.

With quercetin, while significant improvement in cell viability was observed in ischemia-reperfusion experiments only at 5 μM, it displayed the best protection against *t*-buOOH. This result suggests that quercetin may be the most effective as a direct-acting antioxidant, while the catechins may invoke other pathways.

Contrary to quercetin, ascorbate protected against ischemia-reperfusion but not against *t*-buOOH. One possible explanation for the lack of protection of ascorbate against *t*-buOOH is that ascorbate causes decomposition of organic hydroperoxides to cytotoxic species [42]. This diverse behaviour by flavonoids or ascorbate in different conditions of oxidative stress suggests that flavonoids and in general antioxidants may target an expanded number of signaling pathways within the cell. Different stimuli may engage different signaling pathways. As flavonoids differ in their chemical structure they probably vary in their interaction with these signaling pathways. If due to their structure some flavonoids work in broader signaling pathways, then they may have a capacity to show more extensive biological properties. In our study such a capacity was observed with epigallocatechin gallate which revealed to be effective against both ischemia-reperfusion and *t*-buOOH (although in the latter only with short-term pretreatment). As catechin and proanthocyanidins were also helpful for alleviation of ischemia-reperfusion damage but failed to protect as well as epigallocatechin gallate, it is likely that the catechol ring can provide protection against ischemia-reperfusion, while the gallic acid moiety was important to combat against *t*-buOOH. More flavonoids exist in nature or can be synthesized which may have expansive capacities. Additionally, it is always possible to benefit from cotreatments. For instance, one might achieve better protection upon coadministration of quercetin with catechins in uncertain conditions of stress, especially in in vivo situations where cells are exposed to a variety of oxidants and stress stimuli.

In conclusion, the results support the possibility that catechins, which are richly present in teas and seeds, have the capacity to act as pre-conditioning agents to help protect heart cells from ischemia-reperfusion injury. Such preconditioning effects can occur at relatively low concentrations, while higher levels, which may be difficult to achieve through diet, are needed for direct antioxidant effects. In acute situations of oxidative stress quercetin and epigallocatechin gallate were the most potent antioxidants among the flavonoids tested. Directions for future research include investigations of the mechanisms involved, the roles of flavonoid metabolites, and potential synergisms with different flavonoids and with other antioxidants.

Acknowledgments

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Research Article

Schizandrin, an Antioxidant Lignan from *Schisandra chinensis*, Ameliorates Aβ_{1-42}-Induced Memory Impairment in Mice

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In the present study, we examined the effect of schisandrin (SCH) of *Schisandra chinensis* on the amyloid-beta$_{1-42}$- (Aβ_{1-42}-) induced memory impairment in mice and elucidated the possible antioxidative mechanism. Mice were intracerebroventricular (i.c.v.) injected with the aggregated Aβ_{1-42} and then treated with SCH (4, 12, and 36 mg/kg body weight) or donepezil (DPZ), a reference drug (0.65 mg/kg) by intragastric infusion for 14 days. Noncognitive disturbances and cognitive performance were evaluated by locomotor activity test, Y-maze test, and water maze test. Antioxidative enzyme activities including superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) and levels of malondialdehyde (MDA), glutathione (GSH), and oxidized glutathione (GSSG) within the cerebral cortex and hippocampus of mice were measured to elucidate the mechanism. Our results showed that SCH significantly improved Aβ_{1-42}-induced short-term and spatial reference memory impairments in Y-maze test and water maze test. Furthermore, in the cerebral cortex and hippocampus of mice, SOD and GSH-px activities, GSH level, and GSH/GSSG ratio were increased, and levels of MDA and GSSG were decreased by the treatment of SCH. These results suggest that SCH is a potential cognitive enhancer against Alzheimer's disease through antioxidative action.

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder with cognitive function impairment due to the presence of senile plaques and neurofibrillary tangles in the brain, is the most common form of dementia [1]. The main component of these plaques is amyloid-beta (Aβ), which plays an important role in the pathogenesis of AD [2]. Numerous studies suggest that oxidative stresses are involved in the mechanism of Aβ-induced neurotoxicity and AD pathogenesis [3, 4]. Many attempts have been made to reverse cognitive deficits by using tacrine, donepezil (DPZ), galantamine and so on; however, the use of these medicines is not always well accepted due to their severe side effects and high cost [5, 6]. Therefore, searching for safe, better tolerated, and powerful drugs is necessary.

The fruit of *Schisandra chinensis* (Turcz.) Baill. (Schisandrae Fructus) has been used as a tonic for kidney and brain in traditional Chinese medicine for thousands of years. It was used to improve cognitive function by recent clinical physicians, and it has been reported to reverse cycloheximide-induced amnesia in rats [7–9]. Schisandrin (SCH, molecular structure shown in Figure 1) is a main effective compound of this herb. Previous report indicated that SCH significantly reversed the scopolamine-induced impairment of the eight-arm radial maze and the passive avoidance response test in rats [10]. We observed that SCH significantly inhibited LPS-induced activation of microglia and production of NO [11]. It is widely recognized that activation of microglia by Aβ generates elevation of several markers of oxidative stress. Therefore, in the present study, we investigated the effect of SCH on the Aβ_{1-42}-induced memory impairment with behavioral studies. Besides, we performed an antioxidant assay in the cerebral cortex and hippocampus of mice to elucidate the mechanism of cognitive-enhancing activity.

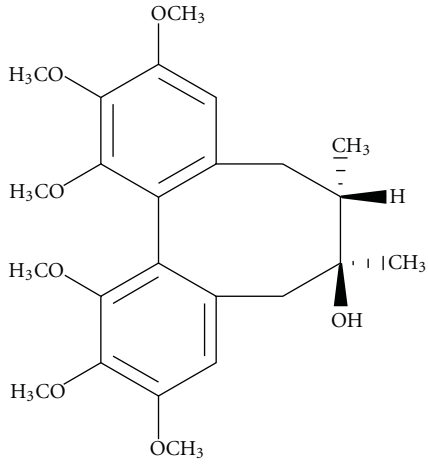

Figure 1: Chemical structure of SCH.

2. Materials and Methods

2.1. Plant Material. Schisandrae Fructus was collected in Liaoning province in China and identified by Professor Jun Yin (Department of Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, China) according to the guidelines of the Chinese Pharmacopoeia (2010). A voucher specimen (SPU 1201) was deposited in the Herbarium of Shenyang Pharmaceutical University.

2.2. Isolation of SCH. Schisandrae Fructus (4000 g) was macerated with EtOAc (12 L × 3) at room temperature for 24 h and then concentrated under reduced pressure to yield an extract (643 g). The extract was applied to a column of silica gel column chromatography (10 × 115 cm, 200–300 mesh, 2000 g) and eluted with a gradient of petroleum ether-EtOAc (25 : 1, 11 L; 10 : 1, 25 L; 5 : 1, 29 L; 2 : 1, 7 L) to obtain 6 fractions. Fraction 5 (56 g) was further separated by silica gel column (10 × 115 cm, 200–300 mesh, 2000 g) eluted with petroleum ether-EtOAc (7 : 1, 20 L; 5 : 1, 31 L) to afford SCH (5 g) which was verified by comparison of the NMR data (Bruker ARX-300, Bruker AV-600) with those in previous reports [12].

2.3. Animals. Male Kunming mice (18–22 g) were purchased from the animal experiment centre of Shenyang Pharmaceutical University and housed 4-5 per cage, allowed access to water and food *ad libitum*, and maintained under a constant temperature (23 ± 1°C) and relative humidity (50 ± 10%) under a 12 h light/dark cycle (light on 07.00–19.00 h). All the studies were carried out in accordance with the principles approved by the Animal Ethical Committee of Shenyang Pharmaceutical University (no.: 2009-0004, date: September 28, 2009).

2.4. Intracerebroventricular (i.c.v.) Injection and Drug Administration. SCH (greater than 95% purity) was suspended in a 0.5% carboxyl-methylcellulose (CMC) saline solution according to the desired concentration. DPZ (greater than 98% purity, Weicai Pharmaceuticals Co., Beijing, China) was dissolved in distilled water with the concentration (20 mL/kg for dose of 0.65 mg/kg body weigh). Aβ_{1-42} peptide (0.1 mg) was dissolved in DMSO (1.2 μL), then the solution was diluted with sterile physiological saline (161 μL) in the tube, which was then sealed and incubated for 120 h at 37°C to cause the peptide to aggregate.

Mice were randomly assigned to six groups of 8–10 individuals each: sham, control, positive, and SCH (4, 12, 36 mg/kg). All the mice were anesthetized with 3.5% chloral hydrate (0.1 mL/10 g). Then, mice in the control, positive, and SCH (4, 12, 36 mg/kg) groups were injected with aggregated Aβ_{1-42} peptide (410 pmol/3 μL) into the right lateral ventricle within 3 s by means of a stereotaxic apparatus (AP, −0.2 mm, ML, ±1.0 mm, DV, −2.5 mm) [13]. The needle was removed with 1 min delay to allow diffusion. Mice in the sham-operated group were injected in an identical manner with the same amount of 0.7% DMSO-physiological saline (3 μL). After surgery, animals were returned to their cages.

From the next day, mice in the SCH (4, 12, 36 mg/kg) groups were administered SCH (20 mL/kg for doses of 4, 12, and 36 mg/kg body weight) daily for 14 consecutive days by intragastric infusion (i.g.). Mice in positive group were administered with DPZ (0.65 mg/kg, i.g.), which is clinically used for symptomatic treatment of patients with AD [14, 15]. Mice in sham-operated and control groups were treated with distilled water (20 mL/kg, i.g.) in the same period.

2.5. Locomotor Activity Test. 7 days after the surgical operation, locomotor activity of each animal was observed in a locomotor cage (50 × 50 × 40 cm) using video-recorded analytical system (Shanghai Jiliang Software Technology Co.Ltd., Shanghai, China). The total distance of movements was evaluated over a 10 min period.

2.6. Y-Maze Task. The Y-maze task was carried out as previously described on day 8 after surgical operation [16]. The Y-maze is a three-arm maze with all arms at equal angles (labelled A, B, and C). Each arm was 40 cm long, 13 cm high, 5 cm wide at the bottom, 10 cm wide at the top. Each mouse was placed on one end of an arm and allowed to move freely through the maze during an 8 min session. The sequence and number of arm entries were recorded manually. A spontaneous alternation behavior, which is regarded as a measure of working memory, was defined as entries into all three arms on consecutive occasions (i.e., ACB, CBC, BCA, CBA). The percentage alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two), multiplied by 100. The number of arm entries was used as an indicator of locomotor activity.

2.7. Water Maze Task. The water maze task was carried out as previously described on day 9 after surgical operation [17–20]. The water maze apparatus consisted of a brown organic glass water tank (63 cm long, 36 cm wide, and 20 cm high), which is crisscross divided into 5 conjoint parts by brown plastic clapboards to form a circuitous waterway containing 10 cm depth of water at 23 ± 1°C. A hidden platform was

Table 1: Effects of SCH on locomotor activity of $A\beta_{1-42}$-induced memory impairment in mice (n = 9-10).

Group	Dose (mg/kg)	Locomotor activity (cm/10 min, mean ± S.E.M.)
Sham	—	4923.37 ± 399.171
Control	—	5205.88 ± 213.25
DPZ	0.65	5293.63 ± 457.79
SCH	4	4685.69 ± 406.90
SCH	12	5035.32 ± 757.24
SCH	36	5082.48 ± 281.53

Mice in sham-operated and control groups were treated with distilled water orally (20 mL/kg). Values were mean ± S.E.M. and analyzed by one-way ANOVA followed by LSD test for multiple comparisons.

set inside one corner of the tank, which was called safety area. Mice were placed in the diagonal area of the safety area, allowed to swim for a maximum of 60 s. In early experiments, they can be induced to safety area. When a mouse arrived at the platform, it can get a rest. Such training trials were repeated for 5 days (10 trials/day). The time required to reach the safety area (the escape latency time) was recorded.

2.8. Antioxidant Assay. On day 14 after surgical operation, mice were anesthetized by pentobarbital (50 mg/kg) and then decapitated. The skulls were opened, and the brains were quickly harvested and placed on ice. The cerebral cortexes were excised, then the hippocampus below them were also carefully dissected, and the tissues were stored at −80°C until analysis. Samples were homogenized in a glass Teflon homogenizer containing 9-fold volumes of precooling physiological saline and then centrifuged at 3000 × g for 15 min at 4°C. The supernatant obtained was used to measure antioxidative enzyme activities including superoxide dismutase (SOD) and glutathione peroxidase (GSH-px), and contents of protein, malondialdehyde (MDA), glutathione (GSH), and oxidized glutathione (GGSG) by means of the assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.9. Statistical Analysis. All statistical analyses were performed using the SPSS software, version 13.0. Data of escape latency values in water maze test were analyzed by a two-way analysis of variance (ANOVA) with the day as one variable and the treatment as a second, followed by the Bonferroni post hoc test. The other data were analyzed using one-way ANOVA followed by LSD test in the condition of variance homogeneity or Tamhane test in variance heterogeneity to detect intergroup differences. Data were expressed as mean ± S.E.M. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Locomotor Activity. Locomotor activity was evaluated to exclude the possibility that the improved learning and memory effects were caused by the increased locomotor activity of the animals. As summarized in Table 1, there was no significant difference in the locomotor activity among the sham, control, DPZ (0.65 mg/kg), and SCH (4, 12, 36 mg/kg) groups (F (5, 51) = 2.036, $P > 0.05$).

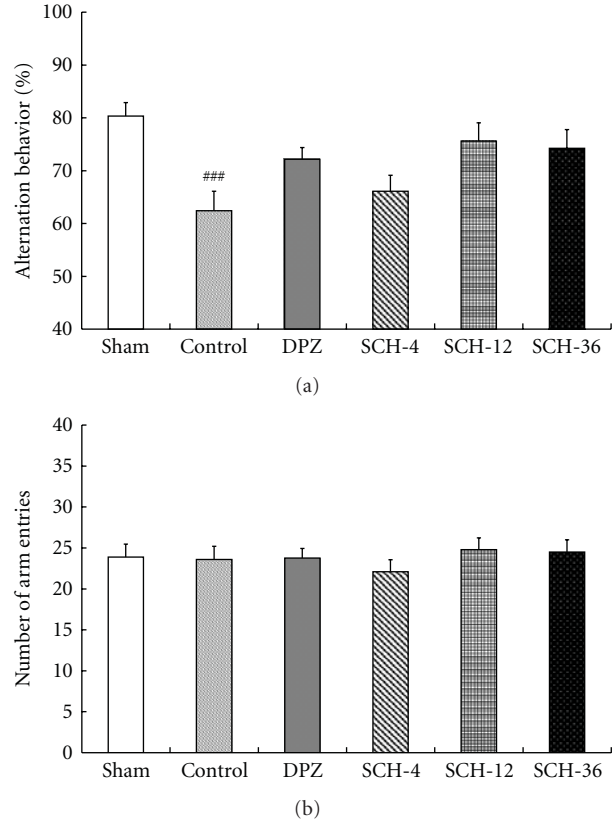

Figure 2: Effects of SCH against $A\beta_{1-42}$-induced memory impairment in Y-maze test. Spontaneous alternation behavior (a) and total number of arm entries (b) were measured during an 8-min session. Sham-operated and control groups: mice were given distilled water orally (20 mL/kg). Positive control: mice were given DPZ 0.65 mg/kg, i.g. SCH-4, SCH-12, and SCH-36: mice were given 4, 12, and 36 mg/kg of SCH, i.g., respectively. Data in the bar graphs were represented as mean ± S.E.M. and analyzed by one-way ANOVA followed by LSD test for multiple comparisons (n = 8–10). $^{###}P < 0.001$ compared with the sham-operated group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

3.2. Y-Maze Task. In the Y-maze test, the spontaneous alternation behavior in the control group was significantly less than that in the sham group (22.30% decrease in alternation behavior, $P < 0.001$). However, this decreased spontaneous alternation behaviors induced by $A\beta_{1-42}$ that were significantly improved by DPZ (0.65 mg/kg, $P = 0.037$, Figure 2(a)) and SCH (12, 36 mg/kg, $P = 0.007$ and $P = 0.011$, resp., Figure 2(a)). In the SCH (4, 12, 36 mg/kg) and DPZ (0.65 mg/kg) groups, the spontaneous alternation behaviors were increased to 82.26, 94.12, 92.38, and 89.82% of the sham group, respectively. The total number of arm entries was not significantly different among all groups (F (5, 57) = 0.421, $P > 0.05$, Figure 2(b)), suggesting that

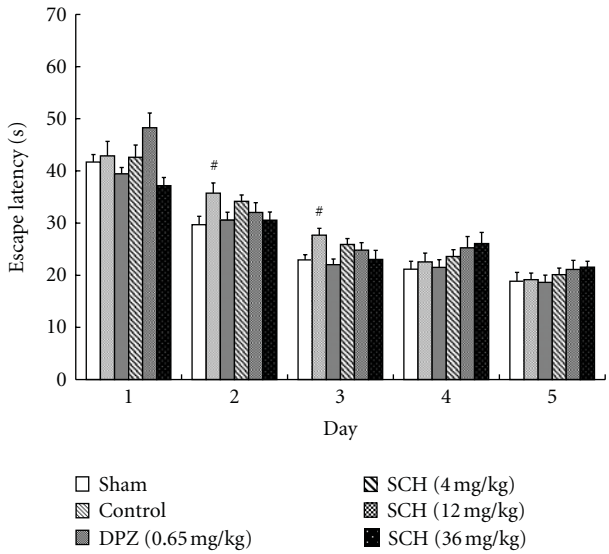

Figure 3: Effects of SCH against Aβ_{1-42}-induced memory impairment on average escape latency time of the water maze test. Sham-operated and control groups: mice were given distilled water orally (20 mL/kg). Positive control: mice were given DPZ 0.65 mg/kg, i.g. SCH-4, SCH-12, and SCH-36: mice were given 4, 12, and 36 mg/kg of SCH, i.g., respectively. Values indicated mean ± S.E.M. and were analyzed by a two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test (n = 8–10). $^{\#}P < 0.05$ compared with the sham-operated group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

the changes in spontaneous alternation behavior were not due to locomotor deficits.

3.3. Water Maze Task. In water maze task, the changes in escape latency to find the safety area after training trials in each group were shown in Figure 3. In all groups, escape latencies decreased with training (F (4, 288) = 160.97, $P < 0.001$) and escape latencies were significantly different among these groups (F (5, 288) = 4.237, P = 0.001). On day 1, the escape latencies of the sham-operated and control group were 41.71 s and 42.87 s. On day 2, the escape latencies of the sham, control, DPZ (0.65 mg/kg), and SCH (4, 12, 36 mg/kg) groups were 29.67 s, 35.73 s, 30.54 s, 34.16 s, 32.03 s, and 30.56 s, respectively. On day 3, they were 22.93 s, 27.71 s, 22.04 s, 25.88 s, 24.82 s, and 23.02 s, respectively. The sham-operated group rapidly lessened the escape latency time to find the location of platform from day 2 and reached stable escape latency time from day 3 to day 5. In contrast, the escape latencies of the control group were significantly delayed compared to that of the sham group on day 2 and day 3, DPZ (0.65 mg/kg) and SCH (36 mg/kg) significantly reduced the escape latencies prolonged by Aβ_{1-42} on day 2 and day 3.

3.4. Antioxidant Assay. In order to elucidate the biochemical mechanism of antidementia effects of SCH in brain tissue, the antioxidative enzyme activities including SOD and GSH-px and levels of MDA, GSH, and GSSG were determined. When compared with the sham group, the Aβ_{1-42} injection generated a dramatic decrease in SOD (−34.33%) and GSH-px (−31.22%) activities as well as a significant increase in MDA (49.49%) production in the cerebral cortex or hippocampus of mice, while DPZ (0.65 mg/kg) preserved the activities of SOD (88.09%) and GSH-px (89.72%) caused by Aβ_{1-42}, and SCH (36 mg/kg) preserved 82.18% SOD and 87.02% GSH-px activities (Table 2), respectively. The increase in MDA production was also significantly attenuated by the treatment with DPZ (0.65 mg/kg) and SCH (36 mg/kg), and the increase was 9.09% and 14.29%, respectively, by comparison with the sham group.

In the sham group, the levels of GSH and GSSG and GSH/GSSG ratio were 260.63 ± 54.56 μmol/L, 94.88 ± 15.19 μmol/L, and 2.79 ± 0.65, respectively. In the Aβ_{1-42}-injected control group, the GSH level (161.04 ± 26.74 μmol/L) and the GSH/GSSG ratio (1.38 ± 0.40) were significantly decreased with a concomitant increase of GSSG level (122.36 ± 32.67 μmol/L) which was not significant changed compared with the sham-operated group. In DPZ (0.65 mg/kg) and SCH (4, 12, 36 mg/kg) groups, the levels of GSH and GSSG (μmol/L) and GSH/GSSG ratio were 220.63 ± 47.36 μmol/L, 136.25 ± 40.00 μmol/L, 167.3 ± 32.67 μmol/L, and 202.71 ± 30.52 μmol/L; 95.76 ± 6.79 μmol/L, 114.08 ± 19.36 μmol/L, 104.54 ± 11.55 μmol/L, and 98.52 ± 3.81 μmol/L; 2.31 ± 0.5, 1.21 ± 0.38, 1.64 ± 0.46, and 2.06 ± 0.34, respectively.

4. Discussion

At present, there is no animal model which could mimic all the cognitive, behavioral, biochemical, and histopathological abnormalities observed in patients with AD. However, partial reproductions of AD neuropathology and cognitive deficits have been achieved by pharmacological and genetic approaches [21]. Iβ hinders memory and cognitive function in both animals and humans, specially short-term and spatial reference memory. As such, continuous infusion or acute injection of *Aβ* into the brain has been used to an experimental model for AD [22–24]. The anti-inflammatory effect of SCH was observed *in vitro* in our previous work [11], but its role in the dementia model is unclear. For these reasons, we used Aβ_{1-42}-injection mice model in this research to clarify the role of SCH.

In this research, a single i.c.v. injection of Aβ_{1-42}-induced significant impairment of working memory in the Y-maze and a deficit of short-term spatial learning and memory in the water maze on day 2-3 (Figures 2(a) and 3), which partially simulated the earlier stage characteristics of learning and memory obstacles of AD.

The blood-brain barrier (BBB) is a major obstacle to the effective delivery of drugs to the brain. It is generally accepted that only small molecules with low molecular mass (<450 Da) and high lipid solubility permeate the healthy BBB by a passive transcellular process [25]. The molecular mass of SCH is 432 Da, and it is liposoluble and can distribute widely in various areas of the rat's brain [26]. Schisandrae Fructus has been used as a tonic in traditional Chinese medicine and a health food product in China for thousands of years

Table 2: Effects of SCH on SOD, GSH-px activities; MDA, GSH, GSSG levels and GSH/GSSG ratio in the cerebral cortex or hippocampus of Aβ_{1-42}-induced memory impairment in mice.

Group	Dose (mg/kg)	SOD (U/mg protein) Cerebral cortex	MDA (nmol/mg protein) Cerebral cortex	GSH-px (U/mg protein) Hippocampus	GSH (μmol/L) Hippocampus	GSSG (μmol/L) Hippocampus	GSH/GSSG Hippocampus
Sham	—	9.99 ± 0.71	6.93 ± 0.77	20.34 ± 1.37	260.63 ± 19.29	94.88 ± 5.37	2.79 ± 0.23
Control	—	6.56 ± 0.55$^{\#\#\#}$	10.36 ± 0.8$^{\#\#}$	13.99 ± 1.46$^{\#\#\#}$	161.04 ± 9.45$^{\#}$	122.36 ± 11.55	1.38 ± 0.14$^{\#\#\#}$
DPZ	0.65	8.80 ± 0.32**	7.56 ± 0.62*	18.25 ± 0.95*	220.63 ± 16.74	95.76 ± 2.40	2.31 ± 0.18***
SCH	4	6.93 ± 0.36	10.81 ± 0.83	13.94 ± 1.36	136.25 ± 14.14	114.08 ± 6.84	1.21 ± 0.14
SCH	12	7.65 ± 0.47	9.66 ± 1.18	13.61 ± 1.00	167.3 ± 11.557	104.54 ± 4.08	1.64 ± 0.16
SCH	36	8.21 ± 0.48*	7.92 ± 0.57*	17.70 ± 0.49*	202.71 ± 10.79	98.52 ± 1.35	2.06 ± 0.12**

Mice in sham-operated and control groups were treated with distilled water orally (20 mL/kg). Values represented mean ± S.E.M. and analyzed by one-way ANOVA followed by LSD test or Tamhane test for multiple comparisons ($n = 8$–10). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the sham-operated group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ compared with the control group.

and currently is being used as a dietary supplement in the United States. To determine whether SCH can be a potential candidate for preventing and/or delaying progression of the learning and memory impairments of AD, it was tested *in vivo* experiments by oral administration due to its liposoluble property. The administration doses of SCH were chosen according to the preliminary study (data were not shown here), which were much lower than the most tolerant dosage 1000 mg/kg measured in the acute toxicity experiment [27]. DPZ which has been approved for treatment of AD was used to evaluate whether the model was successfully established and the treatment effect of SCH on memory impairment [14, 15]. Dose of DPZ was calculated based on the clinical dose (5 mg per day) [28] according to the formula recorded in the pharmacology experimental methodology edited by Xu et al. [29].

Our results demonstrated total distance of movements of locomotor activity test, and the total number of arm entries in Y-maze test was not significantly different among all groups (Table 1, Figure 2(b)), suggesting that there were no potential drug effects on nonspecific processes, such as arousal, attention, or sensory motor functions [30].

Previous report has demonstrated that SCH significantly reversed the scopolamine-induced impairment in rats using the passive avoidance response test which evaluates long-term memory [10]. In the present study, we measured spontaneous alternation behaviors in the Y-maze test to appraise working memory, which is one of the short-term memories impaired at an early stage of AD. The spontaneous alternation behaviors were significantly improved by SCH (12, 36 mg/kg), and the effects of them on improving working memory were better than DPZ (0.65 mg/kg) (Figure 2(a)).

Spatial memory was assessed using water maze task. In all groups, training had a significant effect on spatial learning performance. This effect was more evident than the treatment effect. These results showed that, in spite of the Aβ_{1-42}-induced memory impairment in mice, all the animals were able to learn to find the platform when guided by extra-maze cues on day 4 and 5. Therefore, escape latencies were not significantly different on day 4 and 5. SCH (36 mg/kg) could significantly improve spatial learning and memory impairment in mice on day 2 and day 3 (Figure 3). As a single i.c.v. injection of Aβ_{1-42} only induced short-term impairment of spatial learning and memory on day 2-3, other long-term impairment models of AD should be investigated to find if they can be rescued by SCH in further study.

The etiology of AD has not been revealed clearly. Nevertheless, lots of clinical studies have reported that oxidative stress is involved in cognitive and functional decline in AD [31–33]. Lignans of Schisandrae Fructus have antioxidative effects for example, eliminating reactive oxygen species by increasing SOD and catalase activities and inhibiting lipid peroxidation in cell membranes [34–37]. So we performed an antioxidant assay in the cerebral cortex and hippocampus of mice to elucidate the mechanism of cognitive-enhancing activity.

Lipid peroxidation is one of the main outcomes of free radical-mediated tissue injury that directly damages membranes and, in turn, generates a number of secondary products, such as MDA [38]. In the present research, it was showed that Aβ_{1-42}-peptide generated significant increased MDA in mouse cerebral cortex and SCH (36 mg/kg) could significantly decrease the lipid peroxidation caused by Aβ_{1-42} (Table 2). SOD and GSH-px are important antioxidant enzymes involved in cellular protection against damage caused by oxygen-derived free radicals, by means of removing harmful peroxide metabolites and blocking lipid peroxidation chain reaction. SCH (36 mg/kg) significantly increased the activities of SOD and GSH-px enzyme caused by Aβ_{1-42} (Table 2).

GSH, which is a water-soluble endogenous antioxidant in cells, can transform to an oxidation form of GSSG by catalysis of GSH-px and removing oxygen free radicals. So it plays important roles in protection from oxidative stress in the brain. Our results showed that SCH prevented the reduction of GSH and reduced GSSG level, but this improvement was not notable, while GSH/GSSG ratios were significantly increased in SCH (36 mg/kg) group by comparison with the control group (Table 2). The reason may be that there are other mechanisms associated with the inhibiting oxidative stress and the subsequent amelioration in cognitive function in SCH-treated mice. Previous report has suggested that

SCH reverses scopolamine-induced memory impairment by enhancing cholinergic function [10].

It was reported that absolute bioavailability of SCH was 14.8% after i.v. administration (1 mg/kg) and i.g. administration (10 mg/kg) in rat. Orally administered SCH can be quickly absorbed into the blood and may then express its pharmacological effects within 1 h [39]. It was shown that the major metabolite of SCH, 7, 8-dihydroxy-schisandrin had the almost same sedative effect with SCH [40]. Therefore, in future, it is necessary to investigate whether the metabolites of SCH are active compounds to improve memory impairment.

In conclusion, the present study revealed that SCH could ameliorate Aβ_{1-42}-induced memory impairment in mice at least in part by enhancing the activity of the antioxidative defense system and free radical-scavenging activity. Further study is required to figure out the detailed mechanism of SCH in the treatment of AD, such as measurement of neurodegeneration, oxidative stress in Aβ_{1-42}-induced mice, and other models in brain tissue sections with immunohistochemistry.

Abbreviations

Aβ: Amyloid-beta
AD: Alzheimer's disease
BBB: Blood-brain barrier
DPZ: Donepezil
GGSG: Oxidized glutathione
GSH: Glutathione
GSH-px: Glutathione peroxidase
i.c.v.: Intracerebroventricular
i.g.: Intragastric
MDA: Malondialdehyde
SCH: Schisandrin
SOD: Superoxide dismutase.

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Review Article

Wine Polyphenols: Potential Agents in Neuroprotection

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There are numerous studies indicating that a moderate consumption of red wine provides certain health benefits, such as the protection against neurodegenerative diseases. This protective effect is most likely due to the presence of phenolic compounds in wine. Wine polyphenolic compounds are well known for the antioxidant properties. Oxidative stress is involved in many forms of cellular and molecular deterioration. This damage can lead to cell death and various neurodegenerative disorders, such as Parkinson's or Alzheimer's diseases. Extensive investigations have been undertaken to determine the neuroprotective effects of wine-related polyphenols. In this review we present the neuroprotective abilities of the major classes of wine-related polyphenols.

1. Introduction

Aging is a risk factor common to a number of neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease. Moreover, associated with the population aging, the occurrence of these neurodegenerative disorders is also likely to augment [1]. In addition to the possible involvement in aging, the common characteristic of most degenerative diseases is that they result from neuronal death. Oxidative stress may play a crucial role in progressive neuronal death [2].

Free radicals are oxidative molecules that occur naturally in the environment but can also be generated *in vivo*. Reactive oxygen species (ROS) are produced by immune cells in order to sustain their antibacterial and antifungal functions [3]. When ROS are overproduced, they are taken in charge by various enzymatic pathways for inactivation (superoxide dismutase, catalase, cytochromes, etc.) [4, 5]. Although these enzymatic pathways can be overstepped, ROS accumulate and can react with the different cell molecules such as lipids, proteins, carbohydrates, and nucleic acids. These interactions with the ROS apply an oxidative stress to cells [6]. Some tissues, particularly the brain, are highly exposed to oxidative damages because of their elevated oxygen consumption and the induced generation of large amounts of reactive oxygen species [7, 8].

Oxidative stress resulting in ROS generation and inflammation is responsible for many forms of cellular and molecular deterioration such as mitochondrial collapsing, DNA damage, and protein, carbohydrate, and lipid oxidation [9]. This damage can lead to early cell aging, cell death, and various chronic pathologies like neurodegenerative disorders, cardiovascular illnesses, cancers, or type 2 diabetes [2, 6, 10, 11]. Difficulty in treating these diseases and better understanding of their development and causes highlight the usefulness of antioxidants as prevention treatments.

A number of epidemiological studies have shown that the consumption of a diet rich in antioxidants can influence the incidence of neurodegenerative disorders [12]. Orgogozo et al. have shown a positive correlation between a moderate consumption of red wine and a decreased incidence of dementia [13, 14]. This protective effect is most likely due to the presence of phenolic compounds in wine. Wine and grape vine polyphenols are mainly flavonoids (flavanols,

flavonols, and anthocyanins) and nonflavonoids (phenolic acids, hydrolysable tannins, and stilbenes) [15]. Extensive investigations have been undertaken to determine the neuroprotective effects of wine polyphenols [16–19]. These polyphenols have displayed neuroprotective capacities in numerous *in vitro* and animal models of neurotoxicities [19]. Several neuroprotective mechanisms of action have been proposed, suggesting that polyphenols exert their activities by reducing the production and the accumulation of ROS, whose accumulation is likely to play a crucial pathological role in brain aging, reducing oxidative stress and inflammation and modulating the activity of intracellular signal transduction molecules [19–21].

In this review we investigate the neuroprotective abilities of the major classes of polyphenols in wine: flavanols, proanthocyanins, flavonols, anthocyanins, phenolic acids, tannins, and stilbenes. Each specific class of polyphenol has shown neuroprotective effects against neurodegenerative diseases. Their neuroprotective activity has been documented, and we underline the evidence suggesting that their mechanism of action involves their antioxidant activity.

2. Wine Polyphenols

Products such as wine extract, grape seed, and grape skin extracts are all known to contain a large variety of potent antioxidants in the form of polyphenols. Plant phenolic constituents are produced through two metabolic pathways, the main being that of shikimic acid which leads to cinnamic acids, whereas the polyacetate pathway induces the linkage of a second aromatic ring to the first pathway molecules [22–24].

Wines contain various water-soluble polyphenols including phenolic acids, stilbenes, tannins, flavanols, flavonols, and anthocyanins (see Figure 1). Wine phenolics are divided into two groups: flavonoid and nonflavonoid. The amounts of phenolic compounds in wines are highly variable due to varietal differences and process diversities. Indicative levels of phenolic components in wine are shown in Table 1 [15]. Due to wine processing, red wines contain more polyphenols than white wines. Red wine has more antioxidant capacity than white wine due to its phenolic content [25–27]. Phenolic red wines are mainly composed by flavonoids with 1450 mg/L for young wines and 1285 mg/L for aged ones. Phenolic white wines are composed principally of nonflavonoids with 164 mg/L for young wines and 245 mg/L for aged ones [15, 24].

3. Wine Polyphenols and Neuroprotection

3.1. Flavonoids and Neuroprotection

3.1.1. Flavanols. The flavanols, also called flavan-3-ols or catechins, are the most reduced form of flavonoids. They are present in various plants and are associated with the health benefits of green tea [28, 29]. The levels in wine depend on the different grape cultivars and are typically in the range of 20–100 mg/L. Catechin is usually the major flavanol in wine [15, 30]. The condensations of flavanol in wine induce the formation of oligomers (proanthocyanidins and condensed tannins).

Flavanol intake has been associated with various beneficial health effects [31, 32], and flavanols are known to be brain-permeable substances [33]. Their transport is stereoselective involving one or more stereoselective entities and metabolizing with glucuronic acid, for example [34]. Numerous studies indicate that flavanols are of benefit for neuronal health. Catechin may protect against the brain injuries produced by endogenous neurotoxins involved in the onset of Parkinson's disease [35]. Catechin and epicatechin gallate have also shown an ability to suppress neuroinflammation and can attenuate and inhibit activation of microglia and/or astrocytes associated with the release of the mediators linked to the apoptotic death of neurons [36]. In addition, numerous studies indicate that catechin derivatives may delay the onset of neurodegenerative disorders such as Alzheimer's disease through a numerous different mechanisms such as iron chelators, radical scavengers, and modulators of pro-survival genes [31, 37–40].

3.1.2. Proanthocyanidins. Proanthocyanidins and condensed tannins are complex flavonoid polymers naturally present in cereals, legumes, and fruits [41]. They are mainly formed by the condensation of flavanol units to generate oligomers (proanthocyanidins) and polymers (condensed tannins). Their levels in wine depend on pressing techniques and grape varieties. Typically they range from 5 mg/L in white wines to 1 g/L or even higher levels in old red wines [15, 41]. They are associated with a change in wine quality such as a modification of the hue and a decrease in astringency.

Very few studies have concerned the bioavailability of proanthocyanidins. Condensed tannins should be degraded in monomeric phenols, absorbed and metabolized, as has been shown for other flavonoids [1, 2]. Numerous studies indicate that proanthocyanidins and condensed tannins might prevent both cancers and cardiovascular diseases [42, 43]. Some reports demonstrate that its biological abilities to scavenge the reactive oxygen species are associated to the degree of polyphenol oligomerization. Some of these polyphenols might have specific structures that exhibit neuroprotective effects by interacting with putative neuron-specific receptors [44]. Takahashi et al. have shown that procyanidin oligomers from grape seed exhibit higher growth-promoting activity than the monomers toward mouse hair epithelial cells *in vitro* and *in vivo*, these results indicating that the specific effect might be correlated with their structure [45]. Other research on rat brain suggests that grape seed extract enriched in proanthocyanidins might protect against pathology age-related oxidative brain damage [46].

3.1.3. Flavonols. Flavonols occur in a wide range of vegetables. There polyphenols are always found in glycoside forms in plants including grape berries, where they are present in the skin. Flavonol glycosides and aglycones are found in grape wine from trace amounts up to 200 mg/L in some

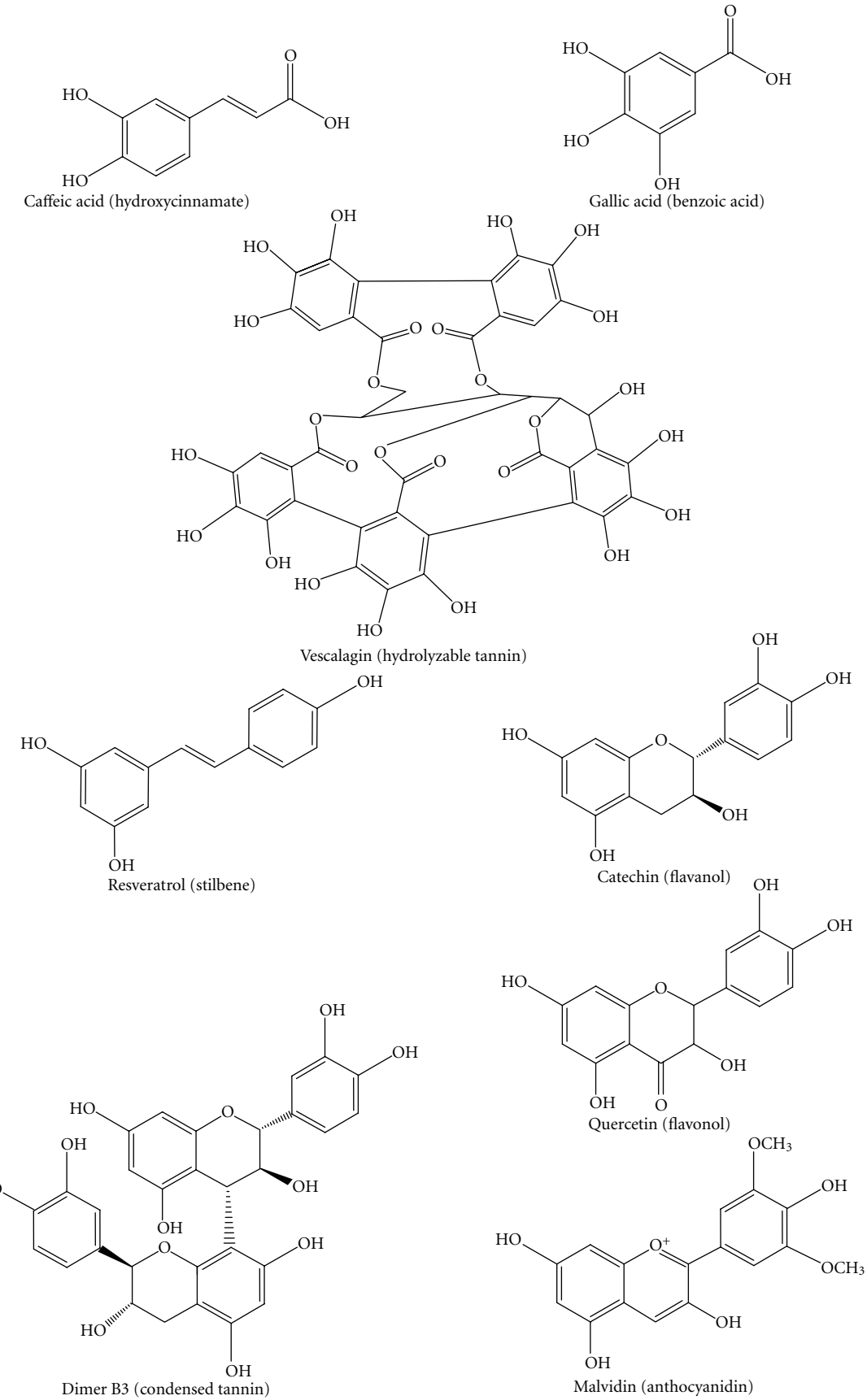

Figure 1: Chemical structures of some phenolic compounds from wine.

Table 1: The levels of principal phenolic classes (mg/L) in red and white table wine [15].

Phenol class	White wine		Red wine	
	Young	Aged	Young	Aged
nonflavonoids				
hydroxycinnamates	154	130	165	60
benzoic acids	10	15	60	60
hydrolyzable tannins	0	100	0	250
stilbenes (resveratrol)	0.5	0.5	7	7
Total mg/L	164.5	245.5	232	377
flavonoids				
flavanol monomers	25	15	200	100
Condensed tannins	20	25	750	1000
flavonols	—	—	100	100
anthocyanins	—	—	400	90
Total mg/L	45	40	1450	1285

red wines [15, 47, 48]. Myricetin, quercetin, and kaempferol conjugates are the major flavonols in wine [48, 49].

Primary results indicate that flavonols can pass the blood-brain barrier [50, 51]. Moreover, numerous studies indicated that flavonols, in addition to many other health benefits, contribute significantly to the protection of neuronal cells against oxidative-stress-induced neurotoxicty [52, 53]. In Alzheimer's disease, neuronal loss is preceded by the extracellular accumulation of amyloid-β peptide (Aβ). It has been shown that pretreatment of primary hippocampal cultures with quercetin significantly attenuates Aβ-induced toxicity, lipid peroxidation, protein oxidation and apoptosis [54]. A dose-response study indicated that quercetin exhibited protective capacities against Aβ-induced toxicity by modulating oxidative stress at lower doses [54]. In cerebral ischemia, calcium dysregulation is one of the main instigators of neuronal cell death and brain damage. Quercetin has been shown to exert significant protection against ischemic injury. Indeed, treatment with quercetin reduced the spectrin breakdown products caused by ischemic activation of calcium-dependent protease calpoin and inhibited the acid-mediated intracellular calcium level [55].

3.1.4. Anthocyanins. Anthocyanins act as guard systems in plants and protect them from UV damage. They form complex molecules with other phenolic molecules and strongly contribute to the color and the aging of wine [56–58]. The aglycone ring of these flavonoids is called anthocyanidin. However, nonconjugated anthocyanidins are never found in grapes or wine, except in trace quantities. In wine there are five anthocyanidins: malvidin, cyanidin, delphinidin, peonidin, and petunidin. Malvidin is the most abundant anthocyanidin in red wines [15].

Among the wine flavonoids, anthocyanins constitute one of the higher potent antioxidants correlated to their capacity to delocalize electrons and form resonating structures [59–62]. Anthocyanins present numerous health benefits such as anticarcinogenic, anti-inflammatory, or antidiabetic effects [61, 63–66]. Anthocyanins also possess beneficial neuroprotective abilities. Some of them have the ability to cross the blood-brain barrier and diffuse through the central nervous system [67, 68]. Anthocyanins have neuroprotective benefits in reducing age-associated oxidative stress and improving cognitive brain function [61, 69–72]. They induce significant neuroprotective effects against oxidative stress, DNA fragmentation and lipid peroxidation in mouse brain [73, 74]. Thus, it appears that the antioxidant and anti-inflammatory effects of anthocyanins contribute to its neuroprotective effect.

3.2. Nonflavonoids and Neuroprotection [73, 74]

3.2.1. Phenolic Acids. The benzoic acids are a minor component in wines. Whereas the hydroxycinnamates are the most important class of nonflavonoid phenols in grape vine and the major class of phenolics in white wine [15, 75]. The three important ones in wine are coumaric acid, caffeic acid, and ferulic acid. Amount of total hydroxycinnamates in wine are typically about 60 mg/L in reds and 130 mg/L in whites [15].

Hydroxycinnamates have an antioxidant activity by scavenging free radicals [76, 77]. Their strong antioxidant properties help to explain their beneficial role on health and in reducing disease risk. Hydroxycinnamates and other phenolic acids have received less attention. It has been shown that p-coumaric acid, hydroxycinnamates caffeic acid, and a Champagne wine extract rich in these compounds have neuroprotective effects against injury induced by 5-S-cysteinyl-dopamine *in vitro* [78]. Caffeic acid has been reported to have neuroprotective effects against Aβ-induced neurotoxicity *in vitro* and to inhibit peroxynitrite-induced neuronal injury [78–80]. Ferulic acid has been showed to protect primary neuronal cell cultures against hydroxyl- and peroxyl-radical-mediated oxidative damage [81, 82].

3.2.2. Hydrolyzable Tannins. Tannins are water-soluble polyphenols. One of the major properties of these molecules is their capacity to precipitate proteins such as gelatin from solution [83–85]. In wine, hydrolyzable tannins arise during maturation and ageing of wines in oak barrels [86]. Castalagin and vescalagine are the main representative compounds of ellagic tannins [87]. Their levels are about 100 mg/L in aged white wines, while red wine levels are about 250 mg/L after aging in oak barrels for two or more years [15, 88]. They are mainly ellagic acid and gallic acid ester derivatives with glucose or other sugars. Due to the presence of the ester linkage, they are described as being hydrolyzable. Hydrolyzable tannins are not present in *Vitis vinifera* but are present in other fruits such as muscadine grapes and raspberries [89]. These polyphenols are excellentantioxidantsand natural preservatives, also helping give the wine structure and texture. However, recent research on tannins has focused on their potential to impact positively on human health. Tannins have demonstrated a host of potent biological activities, antiperoxidation properties, inhibition of mutagenicity of carcinogens and tumor promotion, specific antitumor abilities in relation with tannin structures, antibacterial activity,

and antiviral activity [89–91]. *In vivo*, ellagitannins are mainly transformed into ellagic acid and its metabolites. In fact, they could be the agent responsible for the effects of dietary ellagitannins observed *in vivo* [92, 93].

There are few studies whose objective has been the neuroprotective activity of hydrolyzable tannins. Ellagic acid has been reported to promote the formation of βA fibril and significant oligomer loss, in contradiction to previous results indicating that polyphenols inhibited Aβ fibril formation [94]. Nevertheless, ellagic acid reduces significantly Aβ-induced neurotoxicity in human SH-SY5Y neuroblastoma cells. These results are in agreement with the hypothesis that Aβ fibril formation may represent a protective mechanism of local Aβ clearance. Thus, ellagic acid may have therapeutic value in Alzheimer's disease.

3.2.3. Resveratrol and Other Stilbenes. Stilbenes are secondary metabolites described as phytoalexins. Stilbenes are found in grape vine and wine [95–97]. The main characteristic of stilbenes consist of diary groups on either end of an active double bond that generates the stilbene skeleton, the so-called resveratrol. Stilbenes can also be found in oligomeric and polymeric forms in wine [98]. Resveratrol is found in wine from trace amounts up to 10 mg/L typically 0.1 mg/L in white wines and 2.0 mg/L in red wines [15, 99]. It is a substance with great potential that is being investigated intensively, and its derivatives exhibit a wide range of pharmacological and biological properties [100].

Resveratrol and its derivatives have also been reported to be active against neuron cell dysfunction and death in animal models [20, 101–104]. Resveratrol can cross the blood–brain barrier and exhibit neuroprotective properties against cerebral injury [105]. Numerous mechanisms may underline resveratrol neuroprotective effects against Aβ-induced neurotoxicity [106]. Resveratrol can act by reducing the intracellular Aβ level by inducing protease degradation of the peptide in Alzheimer's disease. Resveratrol and other stilbenes have been shown to inhibit Aβ fibril formation *in vitro* [107, 108]. Furthermore, resveratrol has been shown to exhibit significant free-radical scavenging abilities in numerous cellular models [109–112]. Thus, overall scientific data tend to show that among stilbenoids resveratrol has effects against brain injuries in reducing brain damage in complex manner including antioxidant properties, regulation on neurovascular system, or ability to inhibit known neuropathological processes.

4. Mechanisms

4.1. Bioavailability of Wine-Related Polyphenol. It is now well established that wine polyphenols exhibit some beneficial activities on health, particularly on neurodegenerative diseases [113]. Biological activities are often measured on cultured cells or isolated tissues using polyphenols in their form present in wine (as aglycone or their sugar derivatives). However, the question of their achievable concentration after ingestion as well as the possibility of conjugate formation has been ignored by many studies [114]. These data are though crucial for understanding polyphenol bioactivity. Several studies indicate that the antioxidant effect *in vitro* of some polyphenols may not indicate its activity *in vivo*. Indeed, its alteration into metabolites and other derivative constituents constitute the true bioactive molecules [113, 115–118]. Polyphenols are extensively metabolized in different tissues such as colon, small intestine, and liver [119]. Polyphenols are absorbed through the gut barrier. Some of them who are not absorbed pass to the large intestine and undergo colonic biotransformation by the enzymes of the colonic microflora [118]. Polyphenols metabolized in the gastrointestinal tract undergo conjugation in the liver after absorption. Then, polyphenols are present in circulation as sulfated, glucuronidated, methylated, and as mixed forms. Moreover, a large proportion of polyphenols ingested are subjected to hydrolyses and degradation by colonic microflora to simple phenolic compounds. Wine polyphenols are grouped into two categories: flavonoids and nonflavonoids as described before. Chemical structure of polyphenol is a factor involved in the gut absorption and the metabolism. We report here data on the absorption and the metabolism of the main polyphenols found in wine.

Concerning flavonoids, flavanols were absorbed and eliminated at low micromolar amounts of their direct conjugates (methylated, sulfated, and glucuronidated derivatives) [120]. However, the degradation of flavan structure in colon leads to the formation of phenolic compounds [114, 121]. Because of their hydrosolubility and high molecular weight, proanthocyanidins are not absorbed in the gut. The large majority of proanthocyanidins cross without alteration through the small intestine after which they are transformed by the colonic microflora to produce simple phenolic acids such as phenylacetic and phenylpropionic derivatives [122]. However, Tsang et al. reported that administration of grape-seed procyanidins induce the formation of catechin glucuronide derivatives in rat plasma [123]. Flavonols are naturally occurred as glycosides. Results indicate that flavonols uptake induce a cleavage of the glycoside part in the small intestine followed by absorption and metabolism of the aglycone [124]. Aglycones are then conjugated by sulfation and glucuronidation as well as methylation of the catechol group [118]. A large number of colonic metabolites identified are simple phenolic acids [125]. Anthocyanins was absorbed and excreted at a low proportion of the intact glycosides after injection of wine extract [126, 127]. The anthocyanins degradation at the pH of the intestine in addition to the microflora activity in the colon are at least in part involved in the degradation of anthocyanins into more stable compound such as phenolic acids [113].

Concerning nonflavonoids, ellagitannins are not absorbed due to their large molecular size [128]. They are principally hydrolysed to ellagic acid under physiological conditions in small intestine [113]. Ellagic acid and ellagitannins reach the distal part of the small intestine and the colon, they are mainly transformated by gut microflora into urolithin derivatives [129]. The major stilbene compound found in wine is resveratrol; thus, bioavailability of resveratrol was investigated. Many investigations in animal models and humans have indicated that a low bioavailability of

unconjugated resveratrol. More than 70% of the resveratrol uptaked is absorbed and readily transformed to produce essentially sulphate and glucuronide derivatives [99].

The conjugation of polyphenols has been recognized for many years, most of the biological studies have only been carried with polyphenol aglycones, and very little is known about the biological properties of conjugated derivatives. Numerous studies indicate that metabolic transformations of phenolic compounds reduce their antioxidant properties leading to less active antioxidants than the original compounds [130–132]. The formation of conjugated polyphenols and degradation products such as simple phenolic compounds will modify the properties observed *in vivo* in comparison to their unconjugated forms [114]. Much research effort is still needed to evaluate the biological effects of the conjugated derivatives and microbial metabolites of wine polyphenols.

4.2. Neurodegenerative Disorders and Oxidative Stress. Reactive oxygen species (ROS) are generated in living organisms due to various metabolic processes [108]. The narrow definition of ROS refers to oxygen free radicals, including superoxide radical anion, hydroxyl radical, hydroperoxyl radical and nonfree radicals, which can induce the generation of free radicals through divers chemical reactions. ROS produced in the human body can cause oxidative damage. Under oxidative stress, the excessive production of ROS may directly damage proteins, lipids, carbohydrates, DNA, and even cellular molecules involved in antioxidant defense systems. The over production of free radicals is implied in the progress of numerous diseases such as cardiovascular diseases, cancer, and neurodegenerative disorders [133–137]. To control, the levels of free radicals various defense mechanisms have been promoted in living organisms such as endogenous enzymes glutathione peroxidase, catalase, or superoxide dismutase. In addition to these endogenous mechanisms, much attention has been focused on the antioxidant role of some dietary compounds like polyphenols [27, 138, 139].

4.3. Antioxidants and Neuroprotection. The brain is characterized by its high susceptibility to oxidative stress due to its high oxygen consumption, its high fatty acids levels, and low antioxidant enzyme levels. Numerous works in the literature indicate that wine related-phenolic compounds exhibit a positive effect on nerve cells [18, 19, 80]. The mechanism proposed as explaining the effect on wine polyphenolic compounds on health can be principally summarized as scavenging intracellular ROS and inhibition of LDL oxidation [110, 140–142]. In recent years, studies on the activity of wine polyphenols have been extended to animal models of CNS disorders and injury [18, 143]. These effects are principally associated to their strong antioxidant capacities, since they can act as free-radical scavengers and hydrogen or electron, to preventing DNA damage and lipid peroxidation [144, 145]. Antioxidant polyphenols protect cell constituents from oxidative alteration and thus limit the risk of developing degenerative disorders induced by oxidative stress, such as in ischemia, Parkinson's disease or Alzheimer's disease. For example, an increasing number of reports has shown that acute chronic treatment of resveratrol exhibits neuroprotective effects against colchicine andnitropropionic acid [146] or motor impairment as well as hippocampal neuron loss [147, 148]. These properties are mainly associated to the antioxidant activity of resveratrol. Resveratrol decreases the oxidative damages, in reducing the levels of malondialdehyde, lipid peroxidation, xanthine oxidase, and nitric oxide, and in increasing the depleted glutathione levels and succinate dehydrogenase activity in rat brain [149, 150].

4.4. Effect of Wine Polyphenols on Redox Imbalance. In the living organism, free radicals are generated both enzymatically and nonenzymatically, inducing the generation of reactive oxygen species, which have a crucial role in neurodegenerative disorders. Thus, in neurodegenerative pathologies and aging, the neuronal cells of specific brain regions may be exposed to ROS attack, and apoptotic cell death occurs and progressively worsens until malfunctioning of the neural network and manifestations of neurodegenerative disorders ensue [151].

Compelling evidence supports that oxidative stress plays key role in the physiopathology of neurodegenerative disorders. ROS level augmentation induces oxidation of cellular components leading to a neurodegenerative signaling cascade, which generates cellular damages and induces cell death [152–155]. To prevent oxidative damage, mammalian cells have developed a complex antioxidant defense system converting ROS to less harmful species [156, 157]. Thus, a potential approach in the treatment of neurodegenerative disorders is the use of antioxidants. They have the capacity to scavenge ROS and to upmodulate endogenous antioxidant defenses. In brain, such compounds should have the capacity to cross the blood-brain barrier.

In cells, oxidative stress is associated to sugar, lipid, DNA, and protein damages. The imbalance between antioxidant defense mechanisms and the intracellular production of free radicals induces oxidative stress [158]. Neurons in their ability to regulate for redox imbalance have an age-related decrease, even minor cellular stresses can lead to irreversible disorders and, as such, participate to the causes of neurodegenerative pathologies [158]. The accumulation of free radicals may activate β-secretase, resulting in formation of β-amyloid, which is believed to be responsible for synaptic dysfunction and neuronal cell death in Alzheimer's disease [159, 160]. The wine polyphenols are powerful anti-oxidants that inhibit the production of free radicals [161, 162], preventing cells from free radical and cellular DNA damages [163–165]. Indeed, the overproduction of reactive nitrogen and oxygen species by phagocytes induces oxidative damage to proteins, lipoproteins, and DNA. These reactions may be harmful to cells and tissues and lead to inflammation [166, 167]. Thus, to reduce many inflammatory disorders inhibition of reactive nitrogen and oxygen species production is a popular target. Wine related polyphenols with their antioxidative capacities may have therapeutic value in the prevention of oxidative stress [168, 169]. Furthermore, results indicate

that polyphenols from wine have both the antioxidative and anti-inflammatory properties [170, 171] and that they can prevent cardiovascular diseases [172, 173]. It is also thought that polyphenols act to modulate free radical-mediated lipid peroxidation of low-density lipoproteins (LDL), which is correlated to chronic diseases such as atherosclerosis [138, 174, 175].

4.5. Effect of Wine Polyphenols on NO Production. In complement to the antioxidant abilities of polyphenols, they might exert protective effects by improving endothelial function as indicated by both experimental and clinical studies [176]. As known for a long time, the endothelium have a crucial function in vascular health by regulating several vasorelaxing factors such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor [177–180]. Indeed, the beneficial mechanisms of red wine polyphenolic compounds mainly involve the activation of endothelial NO, release through an increase in calcium levels and activation of the phosphoinositide-3 kinase/Akt pathway in endothelial cells [178, 179, 181]. Red wine polyphenolic compounds may also regulate NO activity at the level of endothelial nitric oxide synthase (eNOS) protein expression in endothelial cells [182] and blood vessels [183]. Chronic upregulation of eNOS by red wine polyphenolic compounds might constitute a preventive approach to reduce tissue injury associated with the risk of cerebral ischemia. For example, results indicate that resveratrol protects the spinal cord from ischemia-reperfusion injury by decreasing oxidative stress and increasing NO release. Resveratrol-induced neuroprotection is thus mediated by both antioxidant- and NO-promoting properties [184]. There is substantial evidence that polyphenols in red wine can exhibit anti-inflammatory abilities. This could be due to their capacities to scavenge NO or to decrease the NO synthase activity [110, 164, 185]. Resveratrol could also inhibit the neuronal NO synthase and the inducible NO synthase isoforms [164, 186]. Therefore, resveratrol could induce the activity of vasodilator-inducing enzyme such as the endothelial isoform eNOS. This effect may be associated to the anti-inflammatory property of resveratrol [187]. Additionally, Han et al. indicated that resveratrol analogues exert neuroprotective effects by the activation of some receptor binding sites localized at the level of the cellular plasma membrane in rat brain [188]. The polyphenol binding to this specific receptor may induce nitric oxide synthase activity in the brain [189]. However, the polyphenol prolonged action could modulate the sensitivity and the tolerance of its receptor. Similarly, low and high doses of red wine polyphenolic compounds have, respectively, pro- and antiangiogenic properties on postischemic neovascularization *in vivo*. Finally, results indicate that resveratrol uptake, in association with a moderate intake of wine, induces an NO upregulation effect on human platelets [190]. In addition, wine related-polyphenols may exert other effects such as decrease LDL oxidation and increase HDL to generate [190–192]. This unique dual effect of red wine polyphenolic compounds offers important perspectives for the treatment and prevention of different diseases.

4.6. Other Potent Effects of Wine Polyphenols. Because oxidative and nitrosative stress have a crucial impact in the causes of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases and because antioxidant activity is the most studied effect of wine polyphenols, this paper was mainly focused on oxidative damage to neuronal molecules. Nevertheless, studies have indicated that polyphenols could exert neuronal regulation at different levels such antiamyloidogenic effects, neuroprotection through modulation of neural mediators and enzymes, and interaction with signaling pathways.

Resveratrol has been identified as a potential antiaging agent and several studies have been realized on protective abilities of stilbenes (resveratrol derivatives) against aging and specific neurodegenerative disorders [19, 107, 115, 164]. The neuronal regulation of resveratrol derivatives molecules could be defined through a number of complex biological processes involving, as previously discussed upregulation of brain-redox imbalance, interactions with signaling pathways crucial in inducing neuronal function and survival, regulation on neurovascular system, and ability to inhibit known neuropathological processes. In APP695-transfected cell lines, resveratrol, could reduce the level of secreted Aβ peptide without directly affecting any other components of the Aβ metabolism tested [193]. The decrease of the production of Aβ peptide could be related to the increase of its degradation. Also, resveratrol did not promote the Aβ peptide clearance by metalloendopeptidases. The treatment of cells with proteasome inhibitors reduced the Aβ decrease induced by resveratrol. Thus, resveratrol could affect the proteasome involved in the degradation of the Aβ peptide. It has been shown that resveratrol could have beneficial effects on cognitive function mediated by regulation on neurovascular system. A higher microvascular density in association with the increase of cerebral blood flow might ameliorate performance by direct increase of glucose and oxygen supply in brain [194]. Furthermore, a recent study reports that resveratrol quickly enhances blood flow into the brain, followed by increased brain oxygenation which has been correlated to the increased memory capacity and improved cognition [195]. Studies revealed that resveratrol and its derivatives identified in wine such as piceid and ε-viniferin glucoside inhibited *in vitro* the Aβ fibrils formation [107, 108]. Examination of the inhibitory data for the stilbene monomers suggests specific structure-activity relationships [196]. ε-viniferin glucoside has been shown to inhibit fibrillization of Aβ peptide and to protect PC12 cells against Aβ-induced toxicity [197]. These results together suggest that neuroprotective action of resveratrol could protect neurons against brain injuries in reducing brain damage in complex manner. In addition to resveratrol, various polyphenols present in wine protective effects against neurodegenerative diseases by regulation at different levels [115, 198, 199].

5. Conclusion

Wine polyphenols appear to be potentially neuroprotective agents by their capacity to inhibit and/or modulate several

neurodegenerative processes. Their neuroprotective effects in *in vitro* and *in vivo* models of neurodegenerative disorders have been documented, and our own findings suggest that their mechanism of action involves their antioxidant activity, principally as scavenging intracellular ROS and inhibition of LDL oxidation, and also their activating effect on endothelial and inhibitory action on both neuronal and inducible nitric oxide synthase activity and subsequent NO production. On the other hand, as indicated by Singh et al., it would be unwise to extrapolate these results to human without conducting proper clinical trials in patients suffering from irreversible and extensive neuronal loss [200].

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Research Article

Detailed Analysis of Apoptosis and Delayed Luminescence of Human Leukemia Jurkat T Cells after Proton Irradiation and Treatments with Oxidant Agents and Flavonoids

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Following previous work, we investigated in more detail the relationship between apoptosis and delayed luminescence (DL) in human leukemia Jurkat T cells under a wide variety of treatments. We used menadione and hydrogen peroxide to induce oxidative stress and two flavonoids, quercetin, and epigallocatechin gallate, applied alone or in combination with menadione or H_2O_2. 62 MeV proton beams were used to irradiate cells under a uniform dose of 2 or 10 Gy, respectively. We assessed apoptosis, cell cycle distributions, and DL. Menadione, H_2O_2 and quercetin were potent inducers of apoptosis and DL inhibitors. Quercetin decreased clonogenic survival and the NAD(P)H level in a dose-dependent manner. Proton irradiation with 2 Gy but not 10 Gy increased the apoptotic rate. However, both doses induced a substantial G_2/M arrest. Quercetin reduced apoptosis and prolonged the G_2/M arrest induced by radiation. DL spectroscopy indicated that proton irradiation disrupted the electron flow within Complex I of the mitochondrial respiratory chain, thus explaining the massive necrosis induced by 10 Gy of protons and also suggested an equivalent action of menadione and quercetin at the level of the Fe/S center N2, which may be mediated by their binding to a common site within Complex I, probably the rotenone-binding site.

1. Introduction

During the past decades there has been a steadily growing interest in the benefits of natural flavonoids. These compounds which are ubiquitously occurring in fruits, vegetables, and tea possess chemopreventive, cardioprotective, anticancer, anti-inflammatory, antiallergenic, and anti-microbial properties. Epigallocatechine-3-gallate (EGCG) and quercetin (QC; 3,5,7,3′,4′-pentahydroxyflavone) are two well-investigated flavonoids which inhibit cell proliferation and induce apoptosis in various cancer cell types [1–9]. Both EGCG and QC can exert a dual, pro- and antioxidant effect, depending on dosage and time of treatment, and numerous studies have indicated that malignant cells are more susceptible than normal cells to the cytotoxicity of these two flavonoids [2, 7–9]. At present, only a few agents are known to possess such potential for selective/preferential elimination of cancer cells while exerting cytoprotective effects on normal cells [2]. Therefore, this property could be exploited to prevent leukemia or to increase the efficiency of leukemia chemotherapies.

At the moment, the antiproliferative effects of EGCG and QC and their dose dependence in human acute lymphoblastoid leukemia Jurkat T cells are largely unknown. It has been shown that QC can accumulate in large quantities inside the mitochondria, where it is stored in a biologically active form bound to mitochondrial proteins [10]. QC can also act as an activator or inhibitor of the mitochondrial permeability transition pore, depending on its pro- or antioxidant character, respectively [11]. QC is able to inhibit Complexes I and III of the mitochondrial electron transport chain (ETC) [12] and participate in quinone redox cycling [8, 13]. At high doses, QC enhances the cellular production of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$) [9, 11, 14]. $O_2^{-\bullet}$ can be then dismutated to H_2O_2 by cytosolic or mitochondrial superoxide dismutases. Furthermore, additional $OH^\bullet$ can be produced from H_2O_2 through Fe/Cu-dependent Fenton reactions. At low doses ($\sim 10\,\mu$M) QC exercises protective effects against H_2O_2 but not MD in Jurkat cells [15]. When applied for short periods, quercetin can decrease the cellular H_2O_2 content, whereas in long-term administration it depresses the level of the endogenous antioxidant GSH (reduced glutathione). This in turn may lead to accelerated production of reactive oxygen species (ROS) and toxic metabolites of quercetin [9, 11, 14, 16, 17]. Recent studies have shown that in Jurkat cells low EGCG concentrations ($\sim 10\,\mu$M) exhibit a protective, antioxidant effect, whereas high concentrations ($\sim 100\,\mu$M) have a prooxidant, cyto, and genotoxic effect, inducing DNA lesions even in the absence of exogenous oxidant agents [1]. In Jurkat cells, EGCG ($50\,\mu$M) produces intracellular H_2O_2, which induces apoptosis by a Fe^{2+}-dependent mechanism, through generation of hydroxyl radicals via Fenton reactions [5]. In a different cell type, EGCG has been shown to associate with mitochondria and other yet unidentified cytoplasmic organelles [18].

A clinically important chemotherapeutic agent used in the treatment of leukemia is menadione (vitamin K_3) [6, 19]. Early studies have revealed that menadione (MD) reduction at Complex I of the mitochondrial ETC [20, 21] accounts for $\sim$50% of MD metabolism [21]. As a consequence, MD can increase superoxide production by disrupting the electron flow within Complex I [21]. MD, H_2O_2, EGCG, and QC can activate the apoptotic program in various cell types via a Ca^{2+}-dependent mitochondrial pathway, which is mediated by elevation of cytosolic Ca^{2+} levels and dissipation of the mitochondrial membrane potential [7, 11, 12, 19, 22–26]. However, the current available data on the effects of these compounds on the cell cycle or apoptosis/necrosis in Jurkat cells are extremely limited.

In this work we have extended our previous studies [15] and carried out a thorough investigation of the relationship between apoptosis and delayed luminescence (DL) under a vast range of conditions induced by oxidative stress, flavonoid treatments, and irradiation with 62 MeV proton beams (some preliminary data were reported in [27]). We have performed a detailed analysis of the kinetics of apoptosis induction and cell cycle progression following various treatments. Moreover, we have investigated for the first time the effects of high-energy protons on these cells and found that this type of radiation preferentially induces necrosis, not apoptosis in Jurkat cells, as well as arrest of the cell cycle in the G_2/M phase. This may have relevant implications in radiotherapy, as it is generally known that the radiosensitivity of human leukemia Jurkat T cells is relatively high [25, 28–30]. In addition, it has been reported that high doses (≥ 10 Gy) of X or γ radiation induce significant apoptosis in Jurkat cells, in a time- and dose-dependent manner [25, 29, 30]. Thus, our investigations suggest a differential effect on cell death induction depending on the type of radiation. Moreover, quercetin was able to reduce apoptosis and prolong the G_2/M arrest induced by proton irradiation.

In addition, our current data obtained by DL spectroscopy provide novel insights into the effects of MD, H_2O_2, EGCG, QC, and high-energy protons at the level of mitochondrial Complex I. Delayed luminescence, which is also called "delayed fluorescence", represents a very weak light emission following exposure to pulsed light or UV radiation [31–43]. Its main characteristics are the multicomponent decay pattern of photoemission and the long-time scale of the process. In this work, DL spectroscopy indicated that proton irradiation disrupted the electron flow within Complex I of the mitochondrial respiratory chain and also suggested an equivalent action of menadione and quercetin at the level of Complex I.

2. Materials and Methods

The experiments and methodologies described in this study were generally conducted as described earlier [15, 27, 44].

2.1. Cell Cultures. Human leukemia Jurkat T-cell lymphoblasts were cultured in suspension in MegaCell RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and $100\,\mu$g/mL streptomycin, at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Exponentially growing cells were adjusted to a density of 0.2×10^6 cells/mL the day before the experiment. We used hydrogen peroxide 30% solution and stock solutions of menadione sodium bisulphite dissolved in phosphate buffer saline (PBS) or dehydrated quercetin and epigallocatechin gallate dissolved in dimethyl sulfoxide (DMSO). In combined treatments, the oxidant agent was added directly to the cell cultures after preincubation with QC or EGCG as specified, without intermediary wash out. Unless specified otherwise, all chemicals were from Sigma-Aldrich. After each treatment, cells were washed thoroughly with PBS and resuspended in PBS (for DL samples, $\sim 40 \times 10^6$ cells/mL) or in complete medium for apoptosis assessment ($\sim 0.2 \times 10^6$ cells/mL). DL samples were analyzed immediately by DL spectroscopy. Cell density, viability, and morphology were examined with a CCD camera Logitech QuickCam Pro 4000, connected to an Olympus CK30 phase contrast microscope. For cell density assessment, $25\,\mu$L aliquots of the DL samples was diluted in PBS, stained with 0.4% trypan blue solution and $\sim$1500–2000 cells were imaged on a Bürker haemocytometer at the time of the DL assay. Cell count evaluation was performed both during DL experiments, directly by visual inspection under the

microscope, and later on, by analyzing the micrographs with the use of the software ImageJ.

2.2. Proton Irradiation. Cell suspensions (7 mL) were irradiated in 50 mL centrifuge tubes fixed in a vertical position. 62 MeV proton beams accelerated by the superconducting cyclotron at LNS-INFN, Catania (Italy) were used for proton irradiation at a dose rate of 11.76 Gy/min. The proton beams were modulated in the wide-spread Bragg peak configuration, to produce a uniform distribution of the absorbed dose in the entire cell suspension. A plane-parallel advanced PTW 34045 Markus ionization chamber was adopted as a reference dosimeter. The dose measurements were performed in a water phantom, according to International Atomic Energy Agency (IAEA) TRS 398. The absorbed dose to water per monitor unit (cGy/M.U.) was measured at the isocenter, at the depth corresponding to the middle of the modulated beam, with the reference circular collimator (diameter = 25 mm).

2.3. Clonogenic Survival Assay. After the treatment, cells were washed thoroughly with warm PBS and plated in 96 well plates at a plating density of 3, 4, or 10 cells/well in 100 μL of complete medium per well. After 4 weeks of incubation, the plates were inspected by microscopy and the wells containing colonies with >50 cells were counted. The plating efficiency was calculated as $\ln[96/(\text{no. of negative wells})]/(\text{plating density}) \times 100$. Clonogenic survival was calculated as the ratio between the plating efficiency of treated and control cells, respectively.

2.4. Flow Cytometry. At specified times after the treatment, samples containing 10^6 cells were fixed in 70% ethanol and frozen at -20°C. For flow-cytometer determinations, the ethanol-fixed samples were washed with PBS, incubated with a propidium iodide PI/RNAse staining buffer (PHARMINGEN 550825) for 15 min, in the dark at room temperature and analyzed with a Becton Dickinson FACS Calibur flow cytometer. For data acquisition and analysis we used the software CellQuest, WinMDI 2.9 and Cylchred, together with a Gaussian deconvolution algorithm as described [15]. Apoptosis was evaluated as the fraction of hypodiploid cell fragments (the sub-G_0/G_1 cell fraction). The G_0/G_1, S, and G_2/M cell fractions were calculated for the nonapoptotic cell population, by excluding the hypodiploid events from the cell cycle analysis.

2.5. Delayed Luminescence Spectroscopy. We used an improved version of the ARETUSA set-up [39], a highly sensitive equipment able to detect single photons. The cell samples were excited by a Nitrogen Laser source (Laser Photonics LN 230C; wavelength 337 nm, pulse-width 5 ns, energy 100 ± 5 μJ/pulse). A multialkali photomultiplier tube (Hamamatsu R-7602-1/Q) was used as a detector for photoemission signals with wavelengths in the visible range (VIS, 400–800 nm), in single photon counting mode. In some determinations, two broad band (about 80 nm FWHM) Lot-Oriel interferential filters, disposed in a wheel between the sample and the photomultiplier, were used to select photons with wavelength of 460 nm and 645 nm, respectively. The detected signals were acquired by a Multichannel Scaler (Ortec MCS PCI) with a minimum dwell time of 200 ns. DL measurements were done on at least 3 different drops from each cell sample (drop volume 15–25 μL) at room temperature (20 ± 1°C). PBS luminescence was subtracted from all recordings. Photoemission was recorded between 11 μs and 100 ms after laser excitation. DL intensity (I) was obtained as the number of photons recorded within a certain time interval divided to that time interval and to the number of living cells in the drop. The quantum yield was calculated in three time domains of the DL emission: 11–100 μs (DL-I), 100 μs–1 ms (DL-II), and 1–10 ms (DL-III), as the ratio between the I-integral and the energy of the laser. This analysis could not be performed in consistent manner in the time domain 10–100 ms, as in some cases the signal-to-noise ratio was too high within this region.

2.6. Spectrofluorimetry. For determination of the relative level of intracellular nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate in their reduced form (NADH and NADPH, resp.), denoted generically as NAD(P)H, exponentially growing cells were washed twice in a standard saline solution (SS) containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM glucose, pH 7.2/NaOH, resuspended in SS at $\sim 10^6$ cells/mL, and transferred into a 2 mL quartz cuvette maintained at 37°C under continuous stirring in a Horiba Jobin Yvon spectrofluorimeter. Every 22 s the cell sample was excited at 340 nm and emission was collected at 450 nm. After stabilization of the fluorescence signal, QC at the indicated dose was added directly to the cuvette, and the kinetic recording was carried on for an additional 45–60 min. The resting value of NAD(P)H fluorescence was obtained as the average intensity recorded over the final 10 min. before the addition of QC. To express the relative variation in the cellular NAD(P)H level, NAD(P)H fluorescence was normalized to the resting value. The background fluorescence obtained in a cuvette containing equivalent amounts of SS or SS and DMSO was correspondingly subtracted from all data.

2.7. Statistics. Unless indicated otherwise, the data are presented as median ± s.e.m. of at least three different measurements. Statistically significant differences were determined using Student's t-test. A level of $P < 0.05$ was considered significant in all statistical tests.

3. Results

3.1. Effects of Proton Radiation, MD, H_2O_2, QC, and EGCG on Apoptosis and Cell Cycle. First we assessed apoptosis and cell cycle distributions of Jurkat cells undergoing various treatments. The results are collected in Figure 1.

Some of our preliminary determinations indicated that high doses (10 Gy) of accelerated protons induce necrosis, not apoptosis in Jurkat cells [27]. Consistent with this, in the present study we found that irradiation with 2 Gy but not

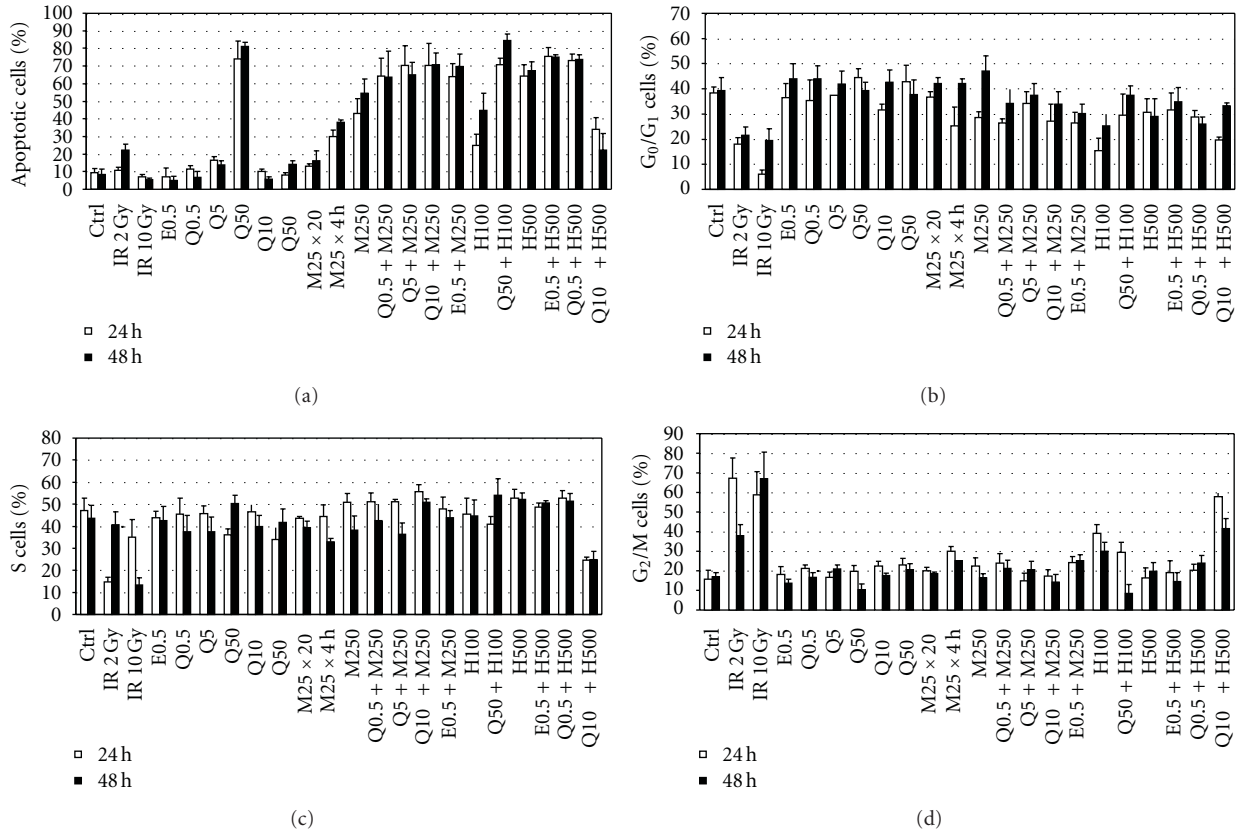

Figure 1: Apoptosis and cell-cycle distributions assessed at 24 and 48 h after treatment of Jurkat cells with the vehicle (Ctrl), with 0.5, 5 or 50 μM QC for 24 h (Q0.5, Q5, Q50), 10 or 50 μM QC for 1 h (Q10*, Q50*), 0.5 μM EGCG for 24 h (E0.5), 250 μM MD for 20 min. (M250), 100, or 500 μM H_2O_2 for 20 min. (H100 or H500) or after combined treatments (QC or EGCG preincubation followed by addition of 250 μM MD or 100/500 μM H_2O_2 for 20 min.) and after irradiation with 2 Gy or 10 Gy of protons (IR 2 Gy, IR 10 Gy). Apoptotic rates (a), G_0/G_1 (b), S-phase (c), and G_2/M (d) cell fractions are indicated.

10 Gy of high-energy protons produced a significant increase in the apoptotic rate at 48 h after irradiation (Figure 1(a), see Figure S1A,B in Supplementary Material available online at doi:10.1155/2012/498914). However, both doses consistently blocked the cell cycle at the G_2/M phase within 24 h after irradiation, suggesting the presence of severe DNA damage in irradiated cells (Figure 1(d)). There was a consistent decline in the G_2/M cell fraction at 48 h after irradiation with 2 Gy but not 10 Gy (Figure 1(d)), which was associated with a reduction in the S-cell pool (Figure 1(c)), suggesting that a part of the cells receiving 2 Gy of protons were able to initiate apoptosis from the G_2/M phase arrest whereas those receiving 10 Gy were too damaged to trigger apoptosis and to repair the DNA lesions. Indeed, clonogenic survival after irradiation with 10 Gy of protons was below the resolution of our assay (i.e., <0.1%), indicating that this dose of protons induced massive necrosis in this cell system, similar to other reports for X- or γ-irradiation [25, 29, 30]. Trypan blue exclusion tests confirmed high rates of cell death, namely, 18.4 ± 3.2% and 46.6 ± 6.8% at 24 h and 48 h after irradiation with 10 Gy of protons, respectively.

0.5 μM EGCG applied for 24 h did not affect the apoptotic rate or the cell cycle distribution (Figure 1) but could enhance apoptosis induced by MD or H_2O_2 (discussed below).

In agreement with our previous investigations [15, 27], quercetin induced apoptosis in Jurkat cells in a dose- and time-dependent manner (Figure 1(a), Figure S1C). Thus, 50 μM QC delivered for 1 h or 24 h produced at 48 h after the treatment an apoptotic rate of 14.1 ± 2.1% (Figure S1C) or 81.5 ± 2.5%, respectively, as compared with the corresponding rate of 8.4 ± 3.2% in control cells (Figure S1A). From kinetic measurements it appeared that quercetin can arrest Jurkat cells in the G_2/M phase (Figure 2). Moreover, the G_2/M fraction of cells treated with 50 μM QC for 24 h decreased from 39.8 ± 6.4% at 9 h to 10.3 ± 3.0% at 48 h after the treatment (Figure 2(d)). The G_2/M block was associated with a significant reduction in the G_0/G_1 cell fraction (Figure 2(b)), whereas the S-phase distribution was unaltered (Figure 2(c)). The cells also displayed a consistent apoptotic rate (52.2 ± 7.3%) 9 h after drug removal, which then increased gradually up to 81.5% during the probing interval (Figure 2(a)). This value is in agreement with the clonogenic survival of these cells, which was determined to be 35.9 ± 10.4% ($n = 4$). In addition, the consistent depletion of the G_2/M cell pool in the absence of significant changes in the G_0/G_1 and S-phase distributions at 48 h after the treatment suggests that a part of the G_2/M-arrested cells most likely initiated apoptosis after 1 day from the removal of quercetin.

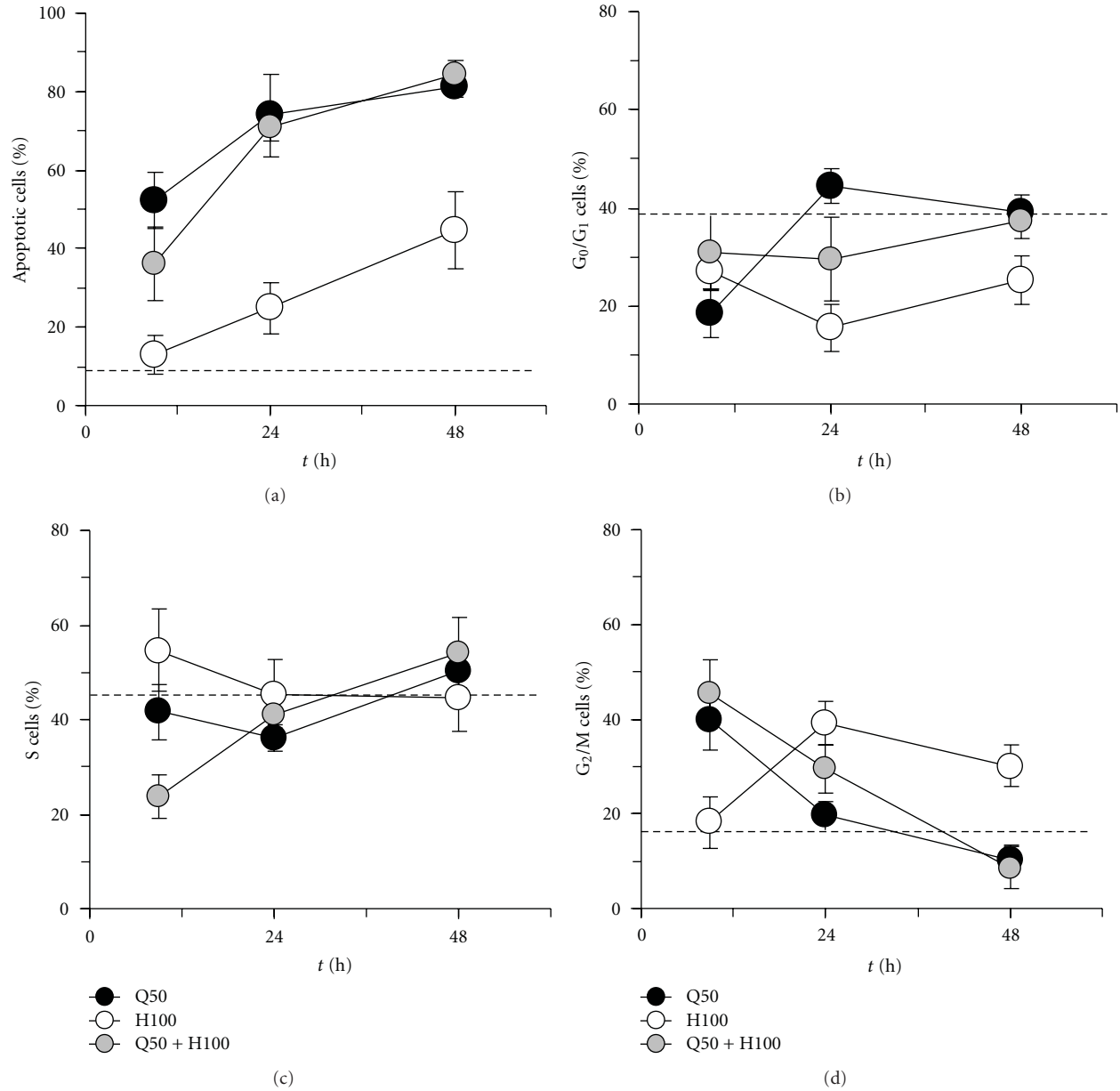

Figure 2: Time course of the apoptotic rate and cell-cycle distribution after treatment of Jurkat cells with 50 μM QC for 24 h (Q50, solid circles), 100 μM H_2O_2 for 20 min. (H100, open circles), or combination of the two (50 μM QC preincubation followed by addition of 100 μM H_2O_2 for 20 min.; treatment denoted as Q50 + H100, gray circles). Apoptotic rates (a), G_0/G_1 (b), S phase (c), and G_2/M (d) cell fractions are indicated. The dashed line represents the average obtained from control cell samples.

Menadione also induced apoptosis dose and time dependently (Figure 1(a), Figure S1D). A relatively low dose of 25 μM MD produced a consistent apoptotic rate when delivered for a long time (4 h, treatment "M25 × 4 h") but not for a short interval (20 min., treatment "M25"). The highest dose used here, 250 μM MD delivered for 20 min. (treatment "M250"), produced large apoptotic cell fractions at 24 and 48 h after the treatment (Figure S1D) and decreased clonogenic survival of Jurkat cells to 12.3 ± 3.6% ($n = 4$). A significant enhancement of apoptosis induced by 250 μM MD was obtained by preincubating Jurkat cells with 5 μM QC or 0.5 μM EGCG for 24 h, as well as with 10 μM QC for 1 h but not with 0.5 μM QC for 24 h. In general, MD, alone or in combination with QC or EGCG, decreased the G_0/G_1 cell fraction and elevated to some extent the S cell fraction in the first 24 h after the treatment (Figures 1(b) and 1(c)). The G_2/M cell fraction increased significantly in the treatments M25 × 4 h and M250, as well as in combination with a long preincubation (24 h) with very low doses of 0.5 μM QC or EGCG (Figure 1(d)). 250 μM MD applied alone for 20 min. increased significantly the G_2/M cell fraction from the control baseline of 15.7 ± 4.5% to 22.5 ± 4.3% in the first 24 h after the treatment (Figure 1(d)). The parallel decrease in the G_0/G_1 cell fraction (Figure 1(b)) and conservation of the S-phase distribution (Figure 1(c)) suggest that MD did not affect G_0/G_1 and S-phase

progression. However, during the subsequent 24 h the G_2/M block was removed and the cell cycle distribution became similar to that of control cells. At variance with this result, a persistent G_2/M arrest could be observed in cells treated with the low dose of 25 μM MD for 4 h. Furthermore, the cell cycle dynamics following this treatment suggest also a G_0/G_1 arrest that could lead to an apparently normal G_0/G_1 cell fraction and to a decline in the S-cell fraction at 48 h after the treatment. Similarly, in the EGCG-MD combination the G_2/M blockage persisted even after 48 h following the treatment, when the G_2/M cell fraction increased to 25.2 ± 2.9%. However, in this case the reduction of the G_0/G_1 cell pool and the conservation of the S-cell fraction indicate that the G_0/G_1 and S phases were not affected by this treatment.

Hydrogen peroxide acted as a potent inducer of apoptosis in Jurkat cells (Figures 1 and 2, Figure S1E). Interestingly, 100 μM H_2O_2 but not 500 μM H_2O_2 applied for 20 min. could increase significantly the G_2/M cell fraction (Figure 1(d)), suggesting that the highest dose produced severe cell damage that inhibited the activation and/or the maintenance of the G_2/M checkpoint. This conclusion was further supported by results obtained with cells exposed to 500 μM H_2O_2 after a 1 h preincubation with 10 μM QC. These cells presented a greatly reduced apoptotic rate and a considerable blockage in the G_2/M phase, as compared with the cells exposed to 500 μM H_2O_2 in the absence of QC, indicating that QC exerted an antioxidant effect and protected cells against H_2O_2. Application of 100 μM H_2O_2 for 20 min. after preincubation with 50 μM QC for 24 h induced apoptosis in a manner that was closely similar to that produced by the quercetin treatment alone (Figure 2(a)). This effect was also observed for the G_2/M cell fraction (Figure 2(d)). However, a significant difference was visible in the G_0/G_1 and S cell pools 9 h after the treatment (Figures 2(b) and 2(c)), when the S-cell fraction exhibited a marked reduction, while the G_0/G_1 fraction did not change significantly. Together, these findings suggest that, beside the observed G_2/M arrest, the combination of QC and H_2O_2 produced an additional blockage of the cell cycle in the G_0/G_1 phase as well, whereas the S phase progressed normally. However, after 48 h the cell cycle distribution became similar to that produced by quercetin alone. A significant enhancement of apoptosis induced by H_2O_2 was obtained by preincubation with 0.5 μM EGCG for 24 h (Figure 1), whereas a short incubation with 10 μM QC for 1 h exerted protective effects against H_2O_2, consistent with earlier findings [15, 27]. Thus, the apoptotic cell fraction produced by 500 μM H_2O_2 decreased 3 times, and this process was correlated with a consistent G_2/M block (Figure 1), indicating that the short preincubation with quercetin can protect cells against the deleterious effects of H_2O_2 and improve the cell capacity of repair.

In treatments with duration of 1 h, QC decreased clonogenic survival in an exponential manner, with an estimated dose for reduction of clonogenicity to 50%, $D_{50\%}$ = 109.8 μM (Figure 3(a)). In separate spectrofluorimetry experiments, QC also decreased the cellular content of NAD(P)H in a dose-dependent manner, with an effective dose for half-maximal effect IC_{50} = 39.5 μM (Figure 3(b)). In Figure 3(c) we present some examples of NAD(P)H fluorescence recordings in Jurkat cell suspensions exposed to different concentrations of QC. After addition of QC, the NAD(P)H fluorescence signal decreased slowly (in up to ~15 min.) to a steady value which appeared to be dose dependent. Figure 3(b) summarizes the steady state data obtained from recordings like those in Figure 3(c).

In a different set of experiments, we investigated the effects of preincubating Jurkat cells with 50 μM QC for 1 h on apoptosis and cell cycle distribution after irradiation with 2 Gy of protons (Figure 4, Figure S1F). Quercetin exercised an inhibitory effect on apoptosis (Figure 4(a), Figure S1F) and appeared to prolong significantly the G_2/M arrest induced by proton irradiation (Figure 4(d)), which may indicate an enhanced capacity for DNA repair and maintenance of the G_2/M checkpoint active. The parallel reduction in the S-cell pool (Figure 4(c)) and conservation of the G_0/G_1 cell fraction (Figure 4(b)) suggests that cells surviving irradiation may experience an additional G_0/G_1 but not S-phase arrest after 48 h from irradiation.

3.2. Effects of Proton Radiation, MD, H_2O_2, QC, and EGCG on Delayed Luminescence. Delayed luminescence of control cells presented a multiscale kinetics (Figure 5(a)) which could be fitted very well by a linear combination of seven exponential components (not shown). In addition to collecting all photons emitted in the entire visible domain, DL could be also measured at emission wavelengths of 460 nm and 645 nm, respectively, by using selective filters. Photoemission at these two wavelengths exhibited kinetic profiles that were qualitatively similar to that obtained in the visible domain. The intensity of emitted red light was consistently higher than the intensity of emitted blue light in the time domain 10–100 μs and was closely similar to that of blue light in the time domain 100 μs–100 ms.

DL of Jurkat cells irradiated with 10 Gy of high-energy protons exhibited different characteristics when probed at 1 h or 24 h after irradiation. Hence, a reduction of 34.1 ± 9.6% in the DL-III relative quantum yield in VIS was observed after 1 h from irradiation, whereas the cell samples probed at 24 h after irradiation exhibited an increase of 27.3 ± 8.5% in the DL-II relative quantum yield and an increase of 41.8 ± 14.3% in the time domain 10–100 ms, while all the other components of the DL emission in VIS were not significantly different from the resting DL emission (Figure 5(b)). Shortly after irradiation, DL emitted at 460 nm was similar to that of control cells; however, the surviving cells exhibited 24 h later a significant overall DL enhancement with about 35% of the control intensity (Figure 5(c)). A remarkable augmentation, up to ~1.7-fold, of blue light emission was detected for a DL component with an established time constant of 178 μs (value derived from fitting analysis, not shown; however, the distinctive peak centered on ~180 μs is clearly visible in Figure 5(c)). 1 h after irradiation, delayed emission of red light presented a significant reduction of the DL-III component, with 38.3 ± 11.5%, whereas 24 h later DL-I decreased to 76.1 ± 13.8% of control emission, and there was a significant increase of a DL component with an estimated time constant of 379 μs (Figure 5(d), and data analysis not shown).

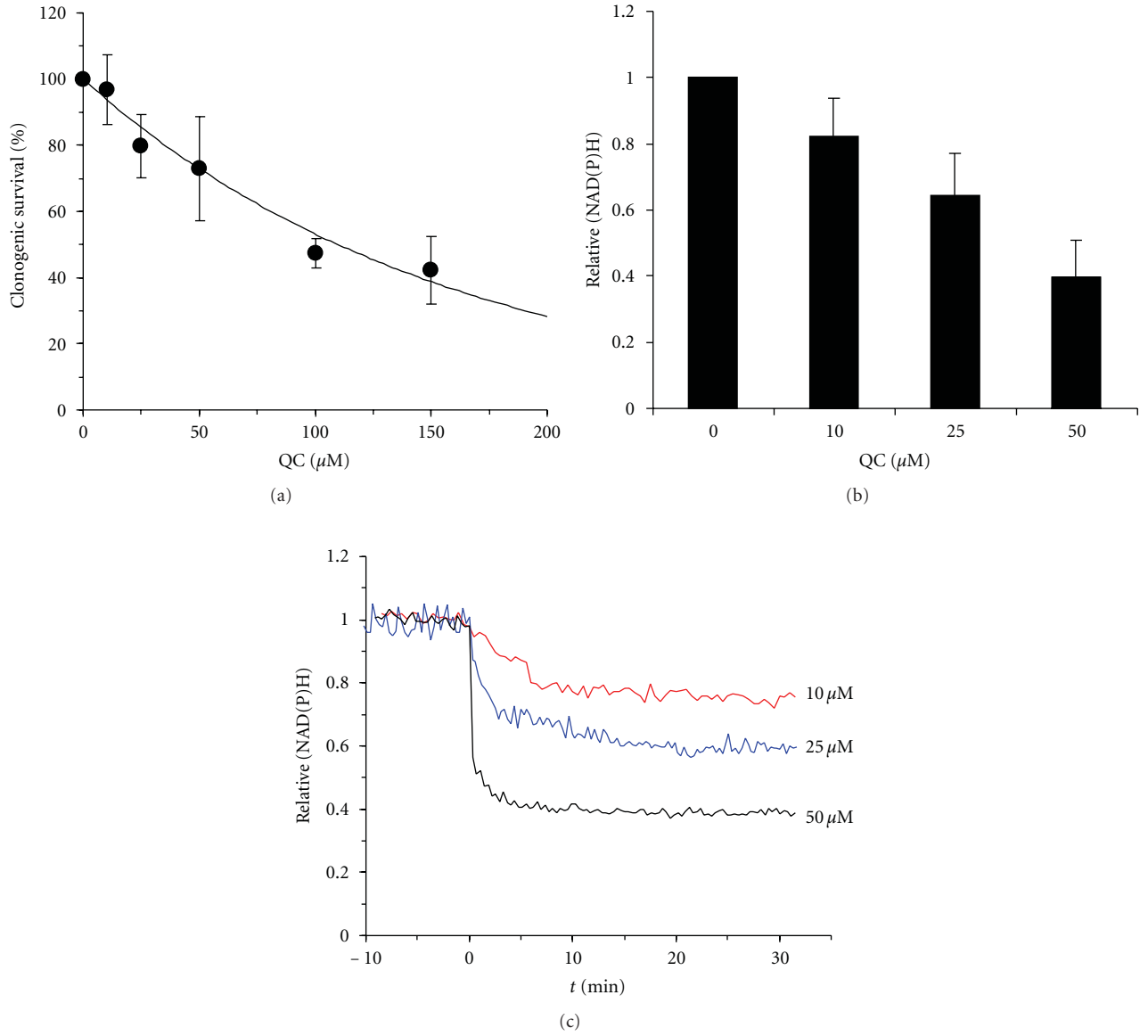

Figure 3: Quercetin decreases clonogenic survival and the cellular content of NAD(P)H in Jurkat cells. (a) Dose response of clonogenicity (S) was fitted to an exponential function (curve) of the form $S(\%) = 100 \times \exp(-D/D_0)$, where D represents the dose of QC applied for 1 h and the characteristic dose derived from the fit was $D_0 = 158.5\,\mu$M. Data are expressed as mean ± standard deviation of 4–6 separate determinations. (b) The ratio between NAD(P)H fluorescence of treated versus control cells (relative NAD(P)H) obtained in steady state after addition of QC to cell suspensions decreases with the level of QC. (c) Representative recordings of NAD(P)H fluorescence relative to the resting value in cell suspensions before and after addition of QC at various levels indicated near each trace.

At increasing doses, quercetin inhibited DL progressively (Figure 6(a)). The most sensitive DL region was DL-III, which decreased by one order of magnitude after the treatment with 50 μM QC for 24 h, whereas DL-I was only slightly affected by QC. EGCG exerted a qualitatively different effect on DL by producing a fairly uniform reduction of the photoemission intensity along the entire timescale.

500 μM H_2O_2 applied for 20 min. reduced DL significantly over the regions DL-I and DL-II (Figures 6(b), 7(a) and 7(b)). Pretreatment with 0.5 μM EGCG for 24 h was able to induce a significant recovery of DL-II emission, whereas preincubation with 10 μM QC for 1 h further reduced the DL-III intensity. The lower dose of 100 μM H_2O_2 had a modest effect on DL and inhibited photoemission by ≈22% over the entire timescale (Figures 7(a)–7(c)). Preincubation with 50 μM QC for 24 h restored DL-I emission but inhibited substantially DL-II and DL-III.

Menadione also inhibited DL in a dose-dependent manner. In addition, at variance with the modest effect of QC on DL-I, MD reduced substantially photoemission in the DL-I region (Figure 7(a)). This inhibition was strong even at the lowest dose of 25 μM menadione. DL-II was inhibited to a similar extent by high doses of MD (Figure 7(b)), whereas DL-III exhibited a drastic reduction and thus, in the M250 treatment, the DL-III quantum yield reached 15.5 ± 6.1% of its resting value (Figure 7(c)). Preincubation with the two

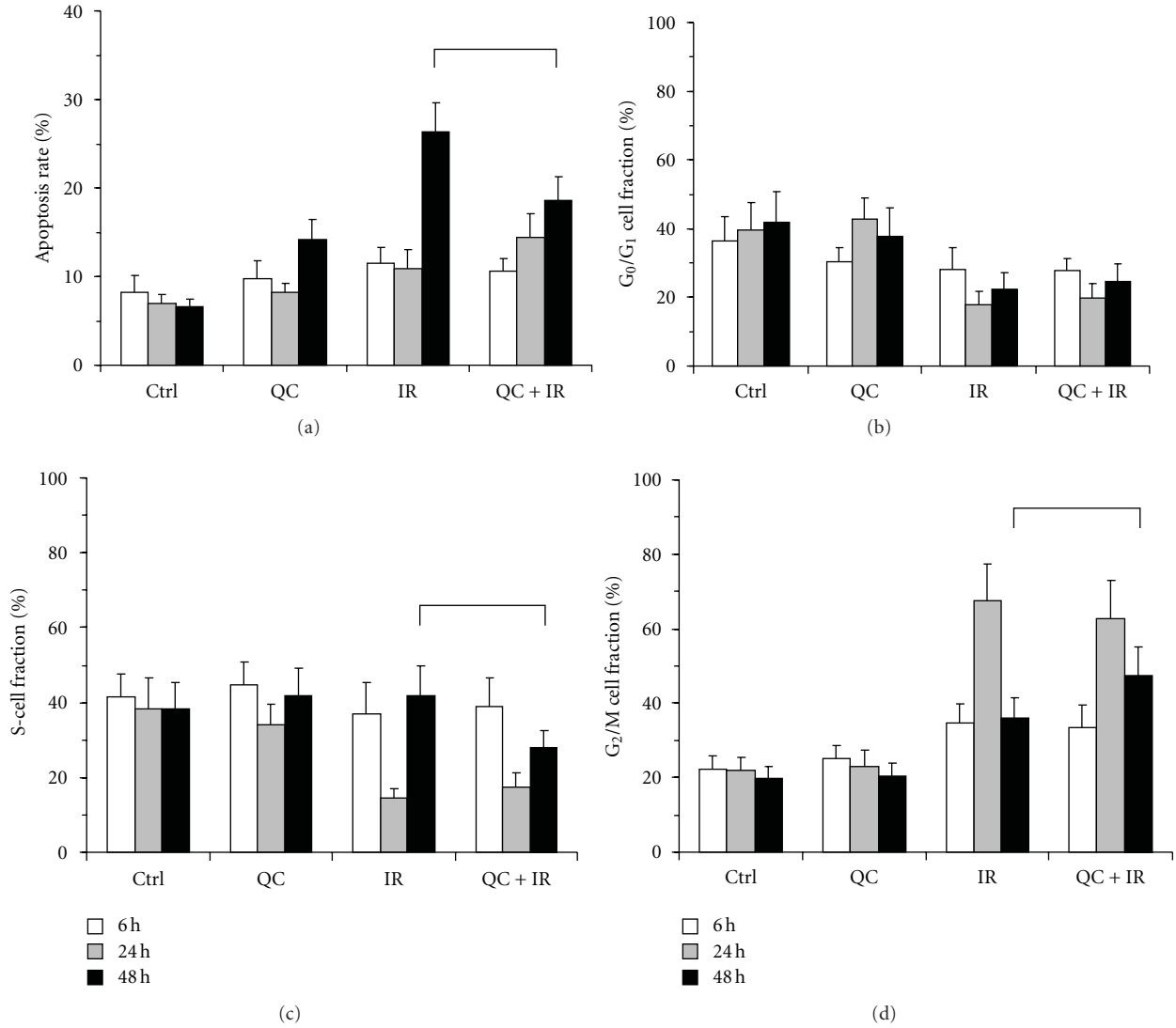

Figure 4: Apoptosis and cell-cycle distributions assessed at 6, 24, and 48 h after treatment of Jurkat cells with the vehicle (Ctrl), with 50 μM QC for 1 h (QC), with 2 Gy of proton radiation (IR), or with 2 Gy of proton radiation after preincubation with 50 μM QC for 1 h (QC + IR). Apoptotic rates (a), G_0/G_1 (b), S-phase (c), and G_2/M (d) cell fractions are illustrated. The star denotes significant difference between the treatments: IR and QC + IR.

flavonoids generally induced partial recovery of DL-III up to ~25% of the resting value, except in the case of pretreatment with 5 μM QC for 24 h, when a further reduction to 9.2 ± 3.8% was recorded.

With treatments of varying time and dosage of two oxidative stress inducers, MD and H_2O_2, and two flavonoids, QC and EGCG, as well as irradiation with high-energy protons, we obtained a significant anticorrelation ($r_{\text{all}} = -0.61$) between apoptosis and DL-II (Figure 7(e)). Notably, all MD treatments (with MD applied alone or in combination with QC or EGCG) alongside the radiation treatment were associated with a strong anticorrelation between DL-I, DL-II or DL-III, and apoptosis ($r_{\text{M/MQ/ME/IR}} = -0.76$, -0.98 and -0.84, resp.) (Figures 7(d)–7(f)). Furthermore, by selecting only treatments with MD, QC, and combinations of the two, we obtained a very strong anticorrelation between DL-II, or DL-III and apoptosis ($r_{\text{M/Q/MQ}} = -0.91$ and -0.82, resp.).

4. Discussion

At the moment, the effects of QC or EGCG on apoptosis induced in Jurkat T cells by the flavonoids themselves or in conjunction with menadione and hydrogen peroxide are poorly known. It is widely recognized that after intake, flavonoids like QC and EGCG exhibit an overall poor bioavailability, as they are rapidly metabolized and their levels in the plasma remain below 10 μM [8, 45]. However, the content of active flavonoids can increase considerably in human tissues, in particular at the inflammatory sites. In vitro studies have shown that in human normal lymphocytes, high levels of 50 μM QC can increase considerably the cellular content of $O_2^{-\bullet}$ and $OH^{\bullet}$ within 30 min. of treatment [9]. Here we found that a 24-hour treatment with physiological levels (0.5–5 μM) of QC and EGCG can potentiate the antiproliferative activity of menadione by enhancing

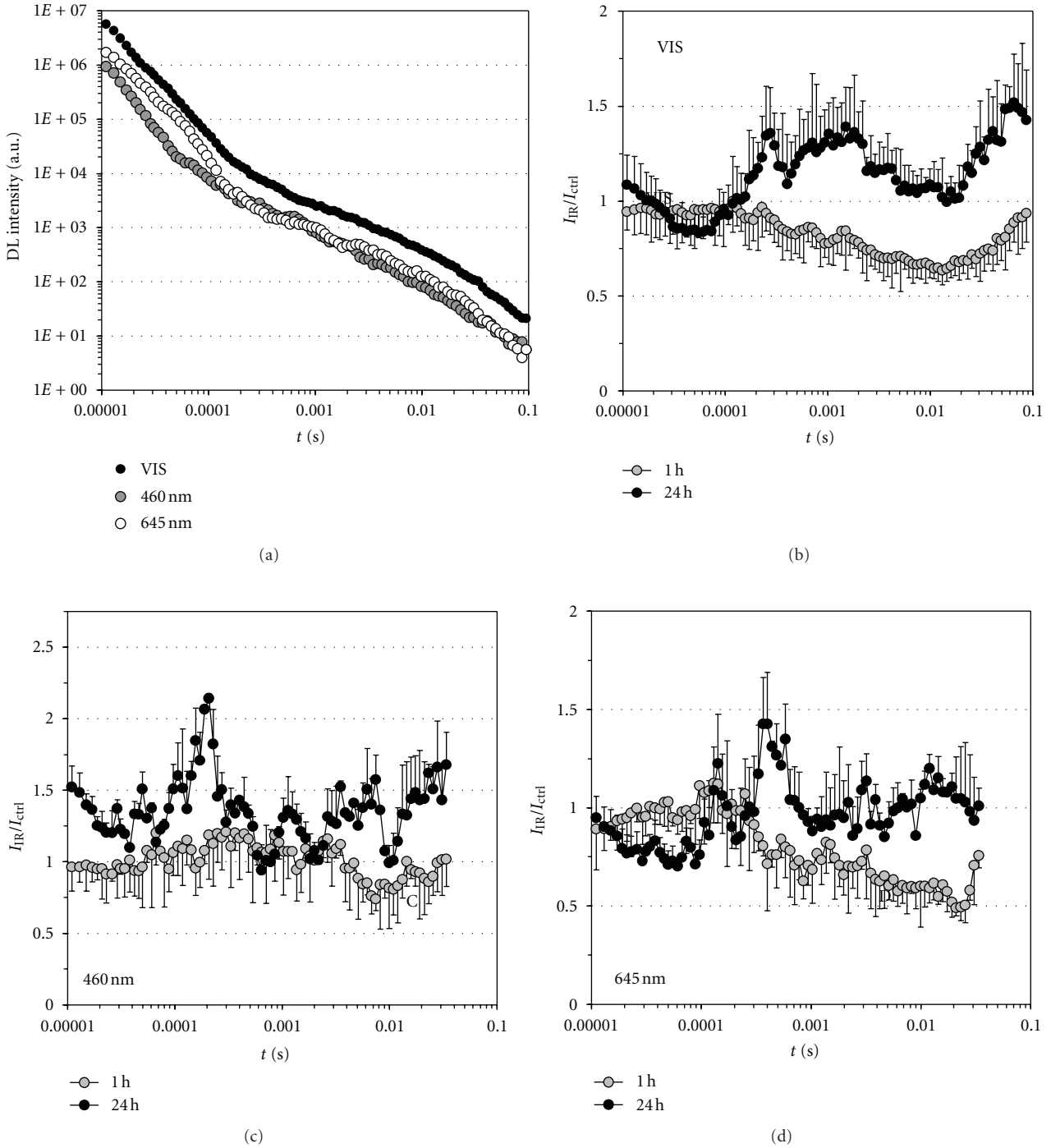

Figure 5: Kinetics of DL emission of Jurkat cells under control conditions (a) or after irradiation with 10 Gy of protons (b–d). In (a) some representative photoemission curves are shown for the entire visible domain (VIS), as well as for detection of 460 nm and 645 nm light emitted by the same cell sample. In (b–d) the intensity of light emission of irradiated cells (I_{IR}) is normalized to the DL intensity of sham-irradiated cultures (I_{Ctrl}). Measurements were done after 1 h and 24 h from irradiation, as indicated. Results are presented for VIS (b), 460 nm (c), and 645 nm (d) emitted light.

drug-induced apoptosis in human leukemia Jurkat T cells. In agreement with previous reports that QC is a more potent inhibitor of hydroxyl radical formation than a scavenger of superoxide anions [46], none of the quercetin-based treatments used in the present work exercised protective effects against MD, whereas a short incubation with 10 μM QC for 1 h offered consistent protection against H_2O_2 and induced G_2/M cell cycle arrest, hence allowing time for repair of H_2O_2-induced damage. In addition, preincubation for 24 h with a very low level (0.5 μM) of EGCG increased significantly the G_2/M cell fraction after exposure to 250 μM MD. Nevertheless, albeit long-term administration of QC

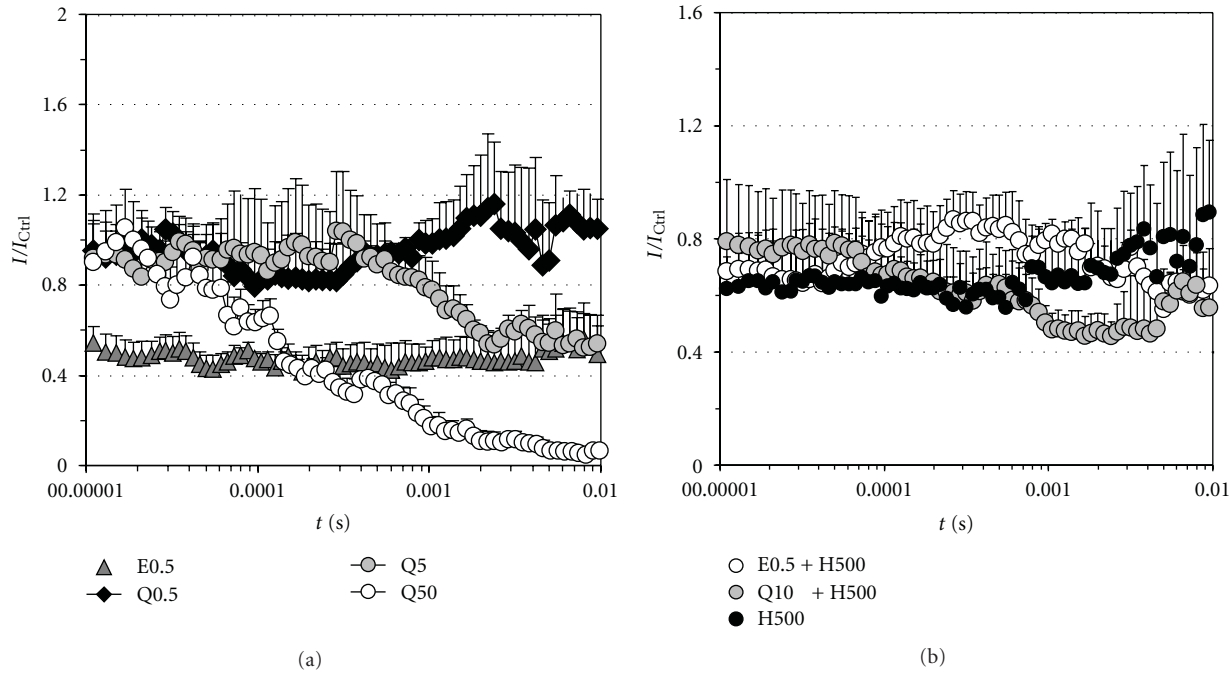

Figure 6: Kinetics of DL emission of Jurkat cells after various treatments with flavonoids (a) or with H_2O_2 alone or in combination with EGCG or QC (b). Treatments are labeled as in Figure 1. The intensity of light emission of treated cells (I) is normalized to the DL intensity of control cells (I_{Ctrl}).

or EGCG may improve significantly the menadione-based treatment of leukemia, it is important to establish the critical level of flavonoid that is no longer beneficial to normal cells.

To our knowledge, there are only few reports (e.g., [47]) regarding the effects of quercetin on clonogenic survival, which may be a critical indicator for the antiproliferative efficiency of anti-cancer drugs as well as for the likelihood of relapse after chemotherapy. While short-term treatments (1 h here) with up to 50 μM QC appeared to be effective in protecting Jurkat cells against H_2O_2 or proton irradiation, clonogenicity decreased considerably at higher doses of the flavonoid, with an estimated dose $D_{50\%}$ = 109.8 μM. Our investigations suggest a connection between the ability of quercetin to decrease the level of NAD(P)H and the induction of apoptosis, which is probably mediated by the failure to maintain the ATP-dependent electrochemical gradient across the inner mitochondrial membrane and the consequent dissipation of the mitochondrial membrane potential. The mechanism by which QC decreases the cellular content of NAD(P)H is unclear, since some early studies reported that QC can inhibit mitochondrial respiration [12], so we expected to observe an increase rather than a decrease in the NAD(P)H level. However, recent investigations based on different assays have indicated that QC can bind with high affinity to Complex I without inhibiting it [48] and can also stimulate mitochondrial respiration [10, 11]. So, it is likely that under our experimental conditions, QC actually stimulated the activity of Complex I in Jurkat cells and thus led to an increased rate of NADH consumption as a substrate for Complex I.

Interestingly, we found that 100 μM H_2O_2 but not 500 μM H_2O_2 applied for 20 min. could increase significantly the G_2/M cell fraction, suggesting that the higher dose of the oxidant agent produced more severe cell damage that inhibited the activation and/or the maintenance of the G_2/M checkpoint. A similar interesting outcome of our studies regarding the cellular effects of high-energy protons is that a higher dose (10 Gy here) of radiation could produce more damage to the apoptotic apparatus and could also reduce the capacity for DNA repair as compared with a lower dose (2 Gy here). At variance with this interpretation, an increase of ~12% in the apoptotic rate at 24 h after irradiation with 10 Gy of gamma rays was reported [29]. Moreover, it has been found that high doses (≥10 Gy) of X or γ radiation can induce significant apoptosis in Jurkat cells, in a time- and dose-dependent manner [25, 29, 30], which is in marked contrast with our findings. We then addressed this issue in a different cell type. Remarkably, in a B-lymphocyte cell line irradiated with 2 and 5 Gy of protons, respectively, the apoptotic rate and the cell-cycle distribution obtained after irradiation of these cells were qualitatively similar to those observed with Jurkat cells (not shown). Taken together, all these findings support the notion that, in comparison with the X or γ radiation, larger doses of high-energy protons produce more clusters of ionizations, which lead to more severe damage to the apoptotic or the cell cycle machinery.

Similarly to the protective effect of QC against H_2O_2 discussed above, our results suggest that short treatments with quercetin could be able to improve cell survival after proton irradiation, most likely by inhibiting hydroxyl radical formation after irradiation and protecting against cellular oxidative DNA damage. However, the fact that high-energy protons produce cellular lesions predominantly via direct ionizations, not ROS formation, together with our findings

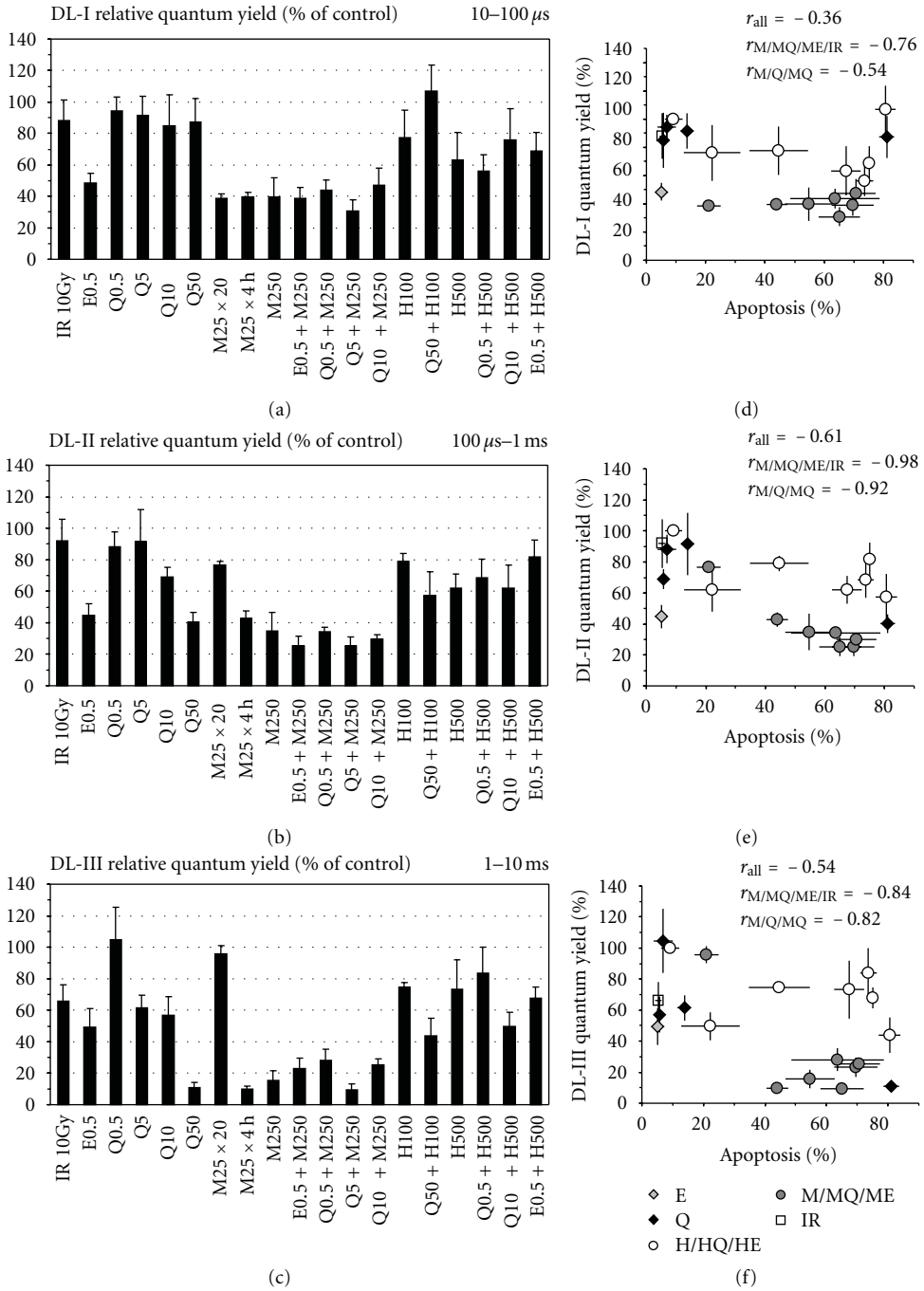

Figure 7: DL-quantum yield relative to control (a–c) and its correlation to the apoptotic cell fraction (d–f) under various treatments indicated in Figure 1. Q, E, M/MQ/ME, H/HQ/HE, and IR denote single QC or EGCG treatments, MD treatments with or without QC or EGCG preincubation, H_2O_2-treatments with or without QC or EGCG preincubation, and irradiation with 10 Gy of protons, respectively. Pearson correlation coefficients are shown for all treatments (r_{all}), for the M/MQ/ME/IR treatments ($r_{M/MQ/ME/IR}$) and for the M/Q/MQ treatments ($r_{M/Q/MQ}$). Results obtained for separate DL time domains indicated inside boxes are displayed individually for DL-I (a, d), DL-II (b, e), and DL-III (c, f).

that the protective effects of quercetin against proton-irradiation were relatively modest, suggests that quercetin may not be able to protect against the formation of the more severe lesions induced by clustered ionizations.

Our studies offer novel insights into the relationship between the cell status and delayed luminescence. In biological systems, DL may be generated by direct emitters like flavins, carbonyl derivatives, and aromatic compounds, by molecular oxygen and its various species, by the DNA or the cytoskeleton, as well as by collective molecular interactions, for example, triplet-triplet annihilation, electric field effects in membranes [31–40]. Previous data from our laboratories have indicated an important role of the mitochondrial Complex I in DL [15], consistent with earlier findings that

some electron transport inhibitors can reduce light emission by mitochondria and chloroplasts [41–43]. In Complex I, the two electrons delivered by reduced nicotinamide adenine dinucleotide (NADH) to flavine mononucleotide (FMN) are transferred between eight consecutive iron-sulfur clusters, eventually reaching the ubiquinone. Our earlier findings [15] suggested that upon UV irradiation, FMN can produce excited singlet states that may either decay to the ground state by prompt fluorescence [49] or undergo intersystem crossing to long-lived triplet states [50] which can subsequently relax to some metastable intermediate states [50]. These triplet- or metastable-state species exhibit an intrinsically long lifetime, allowing a series of photochemical reactions to occur in Complex I via charge recombination in the Fe/S redox centers and then produce secondary excitations, hence giving rise to delayed luminescence.

The data presented here indicate that DL of proton-irradiated cells probed shortly after irradiation was dominated by light emission in the red region of the spectrum and was characterized by a significant reduction in the millisecond DL-III region. On the contrary, DL emission of irradiated cells that survived the subsequent 24 h was dominated by light emission in the blue region of the spectrum and exhibited a significant increase in the submillisecond DL-II region. Moreover, cells that survived 1 day postirradiation revealed two distinctive DL states, namely, a blue-light emitting state with a characteristic lifetime of 178 μs and a red-light emitting state with a characteristic lifetime of 379 μs. In agreement with our previous results [15] and a series of data we have obtained with rotenone-treated cells (not shown), as well as with established electron transfer rates within Complex I [51], we propose that the red-light emitting state is characteristic to the Fe/S center N2 in reduced form. Given the value of the characteristic lifetime of the blue-light emitting state, it is likely that this state is connected to the other extreme Fe/S center, namely, N1a, which has a similar time constant for its reduction rate [51]. In addition, the DL-III region may reflect the reduction kinetics of the remaining redox centers of Complex I that reside between the two extreme N1a and N2 centers, which have been determined to exhibit a slow reduction on the millisecond scale [51]. Accordingly, our data suggest that immediately after irradiation there is a significant decline in the pool of reduced intermediary Fe/S centers, whereas after 24 h there is a significant increase in the pool of reduced N1a and N2 centers. It means that on short-term high-energy protons can disrupt the electron transfer within Complex I, either by lowering the cellular content of NADH (directly and/or indirectly) and thus decreasing the availability of NADH for binding to Complex I or by directly altering the structure or the functionality of Complex I. However, surviving cells appear to regain functionality of Complex I within one day from irradiation, yet manifesting a partial inhibition at the level of the centers N1a and N2.

We found previously that QC and MD at high doses exhibited virtually identical effects on DL over a wide time interval, from 100 μs to 10 ms after laser excitation [15], probably due to their similar action at the level of Complex I. After measuring superoxide production at Complex I by menadione, Xu and Arriaga concluded that Complex I may accept an electron from menadione at a specific site of Complex I [52]. Our results obtained by DL spectroscopy suggest an equivalent action of MD and QC at the level of the Fe/S center N2, which may be mediated by the binding of each of these molecules to a common site in Complex I. Having in view the structural similarity between QC and rotenone, it is most likely that the QC/MD site is the rotenone-binding site itself [48], which resides near the center N2 and thus allows the bound molecule of rotenone to hinder the electron transfer from N2 to ubiquinone.

The faster DL region, DL-I, exhibited the most variable response to our treatment conditions. QC and proton irradiation did not affect DL-I, while MD and EGCG reduced consistently DL-I even at low doses. Taking into consideration all the results presented here, we suppose that DL-I may be associated with the photoemission characteristics of FMN, not with its availability to donate electrons to the two neighbor centers, N1a and N3, whose reduction takes place on a slower timescale [51] or with its association with NADH. According to this scenario, MD, EGCG, and H_2O_2, but not QC, may directly interact with FMN and alter its electronic configuration, thus reducing DL emission.

Nevertheless, these points need to be further addressed by detailed investigations with specific inhibitors of Complex I. However, it is noteworthy that our previous studies using Jurkat cells [15] and a different cell system [38], the budding yeast *Saccharomyces cerevisiae*, have indicated that DL is correlated with the activity of the Complex I of the mitochondrial respiratory chain but not with the existence of DNA or microtubule damage. Remarkably, these two different cell types, as well as the human glioblastoma U87 cell line produced a closely similar DL emission profile under control conditions (not shown). Taken together, these findings suggest that DL may not be cell-type specific and encourage further studies toward the use of DL spectroscopy in investigating mitochondrial dysfunctions in various diseases or in cancer diagnosis. Having in view the growing interest of using DL spectroscopy in clinical applications [31–35], our results lend further support for the development of this methodology as a valuable tool of investigation and diagnosis.

Acknowledgments

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Research Article

Quercetin and Sesamin Protect Dopaminergic Cells from MPP$^+$-Induced Neuroinflammation in a Microglial (N9)-Neuronal (PC12) Coculture System

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A growing body of evidence indicates that the majority of Parkinson's disease (PD) cases are associated with microglia activation with resultant elevation of various inflammatory mediators and neuroinflammation. In this study, we investigated the effects of 2 natural molecules, quercetin and sesamin, on neuroinflammation induced by the Parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP$^+$) in a glial-neuronal system. We first established that quercetin and sesamin defend microglial cells against MPP$^+$-induced increases in the mRNA or protein levels of 3 pro-inflammatory cytokines (interleukin-6, IL-1β and tumor necrosis factor-alpha), as revealed by real time-quantitative polymerase chain reaction and enzyme-linked immunoabsorbent assay, respectively. Quercetin and sesamin also decrease MPP$^+$-induced oxidative stress in microglial cells by reducing inducible nitric oxide synthase protein expression as well as mitochondrial superoxide radicals. We then measured neuronal cell death and apoptosis after MPP$^+$ activation of microglia, in a microglial (N9)-neuronal (PC12) coculture system. Our results revealed that quercetin and sesamin rescued neuronal PC12 cells from apoptotic death induced by MPP$^+$ activation of microglial cells. Altogether, our data demonstrate that the phytoestrogen quercetin and the lignan sesamin diminish MPP$^+$-evoked microglial activation and suggest that both these molecules may be regarded as potent, natural, anti-inflammatory compounds.

1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disorder characterized by the loss of dopaminergic (DAergic) neurons in the *substantia nigra* (SN) and glial dysfunction. A new flow of information indicates that inflammation-derived oxidative stress and cytokine-dependent toxicity contribute to nigrostriatal pathway degeneration [1–3]. *Postmortem* studies have shown that microglia are activated regionally in the SN of PD patients as well as in PD animal models [4–6]. Microglia, resident immune cells of the brain, are activated in response to initiation factors (i.e., toxins, bacteria or viruses, pesticides, neuronal injury, etc.). These factors may also trigger a self-perpetuating cycle of chronic neuroinflammation, increasing the release of inflammatory chemical substances and promoting microglia activation. Besides, the SN is the brain region with the highest density of microglial cells [7]; thus, the neurons of this region are particularly susceptible to microglial-mediated toxicity *in vitro* and *in vivo* [8].

Proinflammatory cytokines and prostaglandins, identified in the SN, striatum and cerebrospinal fluid of PD patients *postmortem*, include tumor necrosis factor-alpha (TNFα), interleukin-1beta, -2, and -6 (IL-1β, IL-2, IL-6) [9, 10]. TNFα, IL-1α, IL-1β, and IL-6 have also been identified in PD animal models [11–13].

MPP$^+$ (1-methyl-4-phenylpyridinium), the active neurotoxic metabolite of the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), inhibits complex I of the mitochondrial respiratory chain, inducing energy depletion and producing reactive oxygen species (ROS), such as superoxide anion ($^{\bullet}O_2^-$) [14]. The latter can react with nitric oxide (NO) to generate the potent oxidant

peroxynitrite, which has been implicated in the development of several neurological diseases [15, 16]. Accumulation of activated microglia around DAergic neurons has been found in *postmortem* human brains with MPTP-induced parkinsonism [17]. In addition, MPTP primate models confirm that serum TNFα levels are elevated without changes in IL-1β levels after toxin administration [18]. Furthermore, the proinflammatory cytokines TNFα and IL-1β are involved in DAergic neuronal death in MPTP-treated mice [19]. Together, these data indicate a close association between MPP^+-induced microglial activation and the degeneration of DAergic neurons.

Recent investigations have disclosed the powerful properties of various natural polyphenols against oxidative stress in several cellular and *in vivo* paradigms of neurodegenerative diseases [20–23]. In particular, quercetin, a flavonoid possessing free radical scavenging properties, may protect against oxidative injury by its ability to modulate intracellular signals and promote cell survival [24]. Several studies suggest its potential as a cardioprotective, anticarcinogenic, antioxidant, and antiapoptotic molecule (see references in [25]). Quercetin also exerts a protective effect against microglia activation and NO production and defends DAergic cells against inflammatory damage induced by the potent inflammatory molecule lipopolysaccharide (LPS) [26, 27].

Sesamin as well as sesamol and sesaminol, the other 2 primary compounds in sesame seeds, is likely responsible for the increased stability of sesame oil against autooxidation and the development of rancidity caused by free radicals [28]. Sesamin is also recognized for its positive physiological outcomes, such as hypocholesterolemic and antihypertensive actions, regulation of lipid and alcohol metabolism in the liver [29–31], and protection against oxidative stress and inflammation in PC12 cells [25, 32]. Currently, no data on the effects of natural antioxidant molecules against MPP^+-induced neuroinflammation have been reported.

The objective of this study was to investigate the influence of quercetin and sesamin on MPP^+-induced inflammation in a microglial-neuronal coculture system. Our results demonstrate that quercetin and sesamin reduce the gene expression and protein concentrations of 3 proinflammatory cytokines (IL-6, IL-1β, and TNFα) in N9 microglial cells. Also, quercetin and sesamin decrease inducible nitric oxide synthase (iNOS) protein expression and $^{\bullet}O_2^-$ production and rescue neuronal PC12 cells from glial-evoked apoptotic death.

2. Materials and Methods

2.1. Drugs and Chemicals. All reagents and chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

2.2. Cell Culture and Treatments. PC12 cells, obtained from the American Type Culture Collection (Rockville, MD), were maintained in a humidified environment at 37°C and 5% CO_2 atmosphere. They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated horse serum (HS), 5% (v/v) heat-inactivated fetal bovine serum (FBS), and gentamicin (50 μg/mL). Neuronal PC12 cell differentiation was evoked by nerve growth factor-7S (50 ng/mL) in DMEM supplemented with 1% FBS for 5 days, as already described [33]. The microglial cell line N9 (a generous gift from Dr. L. Vallières, Centre de recherche, CHUL, Quebec, QC, Canada) was grown in 10% HS in DMEM nutrient mixture F12-ham (DMEM-F12). To assess the influence of quercetin and sesamin on MPP^+-induced N9 inflammation, the cells were pretreated with quercetin (0.1 μM) or sesamin (1 pM) for 3 h and then exposed to MPP^+ (500 μM) for 12 or 24 h. Quercetin and sesamin concentrations in these experiments were determined by previous dose-response curves and kinetic studies [25, 32, 33]. All experiments were performed in medium with charcoal-stripped serum to remove steroids from the medium.

Neuronal PC12 cells and N9 microglia were cocultured to study the impact of MPP^+-activated microglia on the survival of neuronal PC12 cells. N9 microglial cells were grown in culture inserts (pore size 0.4 μm, BD Falcon, Oakville, ON, Canada); then, MPP^+ was added, and inserts containing N9 cells were transferred on neuronal PC12 cells grown previously on coverslips. In this coculture system, microglial cells communicate with neuronal PC12 cells through the semipermeable membrane, avoiding direct contact between the 2 cell populations [26, 34]. The PC12 supernatant was collected 24 h later for cell death measurement, according to the lactate dehydrogenase (LDH) cytotoxicity test and DNA fragmentation assay described below. To control for possible MPP^+ crossing the insert membrane and causing neuronal PC12 death, we performed control experiments on inserts without microglia and after treatment of the medium with MPP^+.

2.3. Cytotoxicity Measurements. Cytotoxicity was evaluated in control and MPP^+ conditions by colorimetric assay, which is based on the measurement of LDH activity released from damaged cells into the supernatant, as already described [33]. LDH, a stable cytoplasmic enzyme present in all cells, is rapidly released into the cell culture supernatant upon plasma membrane damage. Enzyme activity in the culture supernatant correlates with the proportion of lysed cells [35]. Briefly, 50 μL of cell-free supernatant served to quantify LDH activity by measuring absorbance at a wavelength of 490 nm in a microplate reader (Thermo Lab Systems, Franklin, MA). Total cellular LDH was determined by lysing the cells with 1% Triton X-100 (high control); the assay medium functioned as a low control and was subtracted from all absorbance measurements:

$$\text{Cytotoxicity (\%)} = \frac{(\text{Experimental value} - \text{Low control})}{(\text{High control} - \text{Low control})} \times 100. \quad (1)$$

2.4. DNA Fragmentation Analysis. DNA fragmentation was assessed with the single-stranded DNA (ssDNA) apoptosis

ELISA kit (Chemicon International, Temecula, CA). This procedure is based on selective DNA denaturation by formamide in apoptotic cells but not in necrotic cells or in cells with DNA damage in the absence of apoptosis. The detection of denaturated DNA was performed with a monoclonal antibody to ssDNA. The staining of ssDNA in early apoptosis was undertaken with a mixture of antibody and peroxidase-labelled secondary antibody. The reaction was then stopped, and ssDNA fragmentation was quantified by measuring absorbance at a wavelength of 405 nm in a microplate reader (Thermo Lab Systems). ssDNA was calculated with reference to control conditions. Absorbance of positive and negative controls served as quality control of ELISA.

2.5. Detection of Mitochondrial $^{\bullet}O_2^-$. Microglial cells were grown and treated on collagen-coated circular glass coverslips, and MitoSOX Red (Invitrogen, Burlington, ON, Canada) was deployed to estimate intracellular $^{\bullet}O_2^-$ production. This fluorogenic dye is a highly selective indicator of $^{\bullet}O_2^-$ in the mitochondria of live cells. After treating microglia for 9 h with MPP^+ with or without quercetin or sesamin, the medium was removed and the cells were incubated with MitoSOX Red (5 mM) for 10 min at 37°C (Invitrogen). MitoSOX Red rapidly and selectively targets the mitochondria. Once in the mitochondria, it is oxidized by $^{\bullet}O_2^-$ and exhibits red fluorescence. The cells were washed with Hanks' buffered salt solution and 4′,6′-diamidino-2-phenylindole (DAPI) counterstained all nuclei. Then, the cells were fixed in 4% paraformaldehyde for 6 min at 37°C. Coverslips were mounted with Molecular Probes ProLong Antifade kit (Invitrogen). Images were acquired by Leitz inverted microscope with a high-pressure mercury burner and necessary filters, and analyzed with NIS-Element 2.2 software (Nikon, Mississauga, ON, Canada). To demonstrate MitoSOX Red selectivity, sodium diethyldithiocarbamate (DDC), an inhibitor of superoxide dismutase, served as positive control.

2.6. Electrophoresis and Immunoblot Analysis. N9 cells were grown and treated in 6-well plates. Total proteins were extracted with nuclear extraction kit (Active Motif, Brockville, ON, Canada). Proteins were assessed by bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL), and equal amounts were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel. After electrophoretic separation (180 volts, 45 min), the gels were transferred onto polyvinylidene difluoride membranes (0.22 μm pore size, BioRad, Hercules, CA). The blots were blocked for 1 h at room temperature (RT) in 5% nonfat powder milk. Immunoblotting was performed overnight at RT with anti-iNOS antibody (1 : 50) (StressGen, Biotech, Ann Arbor, MI). The following day, the blots were washed and then incubated with peroxidase-conjugated secondary antibody (1 : 10,000), for 1 h at RT, for development with enhanced chemiluminescence substrate solution.

2.7. Real-Time-Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted with Sigma's GenElute Mammalian Total RNA extraction kit. RNA was spectrophotometrically measured for each condition, and 1 μg of total RNA was reverse-transcribed with 25 U of M-MULV reverse transcriptase, 1.5 μM of dNTP, and 10 μM of random hexamers. RT-qPCR was then performed in a MiniOpticon RT-PCR system (BioRad) in 20 μL-sized reactions containing 4 μL of cDNA mixture, 0.3 μM of each forward and reverse primer and 10 μL of iQ SYBR Green Supermix (BioRad). Incubation at 95°C for 3 min was followed by 40 cycles of 15 s at 95°C and 30 s at 61°C. Primers for TNFα (5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′ and 5′-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3′), IL-1β (5′-GCCCATCCTCTGTGACTCAT-3′ and 5′-AGGCCACAGGTATTTTGTCG-3′), IL-6 (5′-TTCCATCCAGTTGCCTTCTT-3′ and 5′-ATTTCCACGATTTCCCAGAG-3′), ubiquitin C (5′-AGCCCAGTGTTACCACCAAG-3′ and 5′-TCACACCCAAGAACAAGCAC-3′), β-microglobulin (5′-ATGGGAAGCCGAACATACTG-3′ and 5′-CAGTCTCAGTGGGGGTGAAT-3′) were designed by BLAST sequences with PRIMER3 web-based software and synthesized at Sigma Genosys (Oakville, ON, Canada). Reactions were performed in duplicate, and 3 independent preparations of cDNA were studied. A 10-fold dilution series was obtained from a random pool of cDNA ranging from $\times$10 to $\times$100,000 dilution. Mean cycle threshold values (Ct) for each dilution were plotted against log10 of cDNA input to generate efficiency plots. The reaction efficiency of each gene assay was calculated according to the equation $E = 10(-1/\text{slope})$, where E was reaction efficiency and "slope" was the slope of the line generated in efficiency plots. All PCR efficiencies were above 90%. In all PCR experiments, post-PCR DNA-melting curve analysis was undertaken to assess amplification specificity. DNA melting was carried out at a temperature ramping rate of 1°C per step with 1 s rest at each step. Relative gene transcription was calculated by the comparative Ct method, using the real-time efficiency values of each gene. cDNA levels among the samples were normalized by the expression of 2 internal control genes: ubiquitin and β-microglobulin. These housekeeping genes were chosen with the geNorm algorithm [36]. A normalization factor was calculated with the geometric mean of the 2 reference genes. The normalized expression of each gene of interest was calculated by dividing the raw quantities of each sample by the appropriate normalization factor [36].

2.8. ELISAs for IL-6, IL-1β, and TNFα. IL-6, IL-1β, and TNFα were measured by specific ELISAs (BioLegend, San Diego, CA). After incubation with MPP^+, with or without quercetin or sesamin, for 24 h, the supernatants were collected for each respective ELISA. Mouse-specific monoclonal antibody (IL-6, IL-1β, and TNFα) was first coated on 96-well plates. Standards and samples were then added to the wells for 2 h, where IL-6, IL-1β, or TNFα were bound to the immobilized capture antibody. A biotinylated anti-mouse detection antibody was added for 1 h, producing an antibody-antigen-antibody "sandwich" to which an avidin-horseradish peroxidase solution was added for 30 min. Finally, a tetramethylbenzidine solution was included for 15 min in the dark. Reaction with horseradish peroxidase

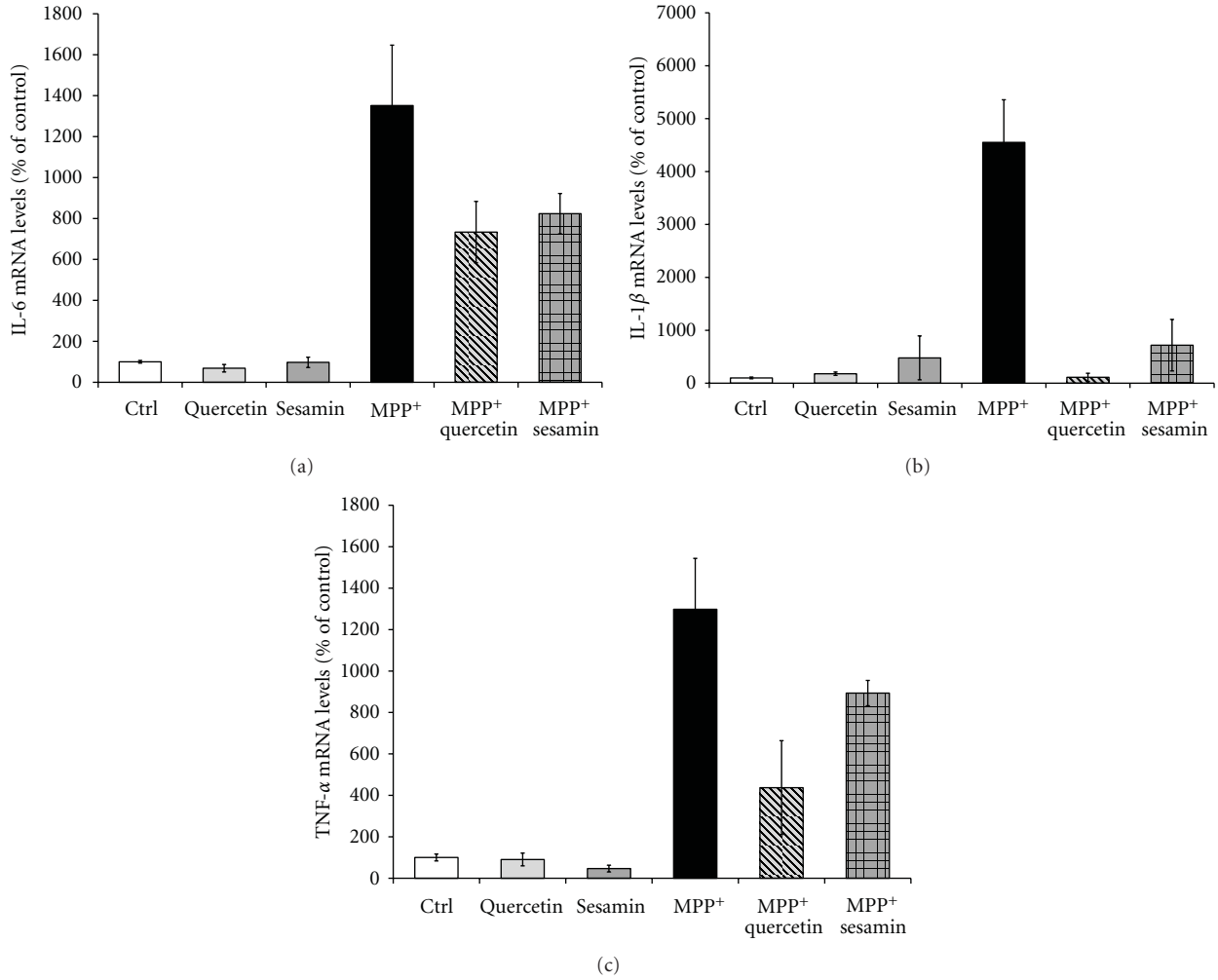

Figure 1: IL-6 (a), IL-1β (b), and TNFα (c) mRNA levels in N9 microglial cells treated first for 3 h with quercetin or sesamin and then with or without MPP$^+$. After 24 h, total RNA was extracted from microglial cells and qRT-PCR was performed. MPP$^+$ clearly induced significant increases of IL-6 (a), IL-1β (b), and TNFα (c) mRNA levels. When N9 microglial cells were treated with quercetin or sesamin 3 h prior to MPP$^+$, we detected significant decreases of IL-6, IL-1β, and TNFα mRNA levels (MPP$^+$ quercetin, MPP$^+$ sesamin). Bars represent the average intensity of the bands ± SEM of 3 independent experiments expressed as % of the controls. $^{***}P < 0.001$ compared to the control (Ctrl), $^{\diamond\diamond}P < 0.01$ and $^{\diamond}P < 0.05$ compared to MPP$^+$, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

resulted in conversion of the substrate to a blue-colored product. Addition of 2 N sulfuric acid stop solution yielded a yellow color. Microwell absorbance was read at 450 nm with a microplate reader (Thermo Lab Systems).

2.9. Statistical Analysis. Significant differences between groups were ascertained by 1-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis with the GraphPad InStat program, version 3.06 for Windows (San Diego, CA; http://www.graphpad.com/). All data, analyzed at the 95% confidence interval, were expressed as means ± SEM from 3 independent experiments. Asterisks indicate statistical differences between the treatment and control condition ($^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$), diamonds denote statistical differences between the treatment and MPP$^+$ condition ($^{\diamond\diamond\diamond}P < 0.001$, $^{\diamond\diamond}P < 0.01$, and $^{\diamond}P < 0.05$), and $^{\circ}$empty circle indicates difference between MPP$^+$-treated cells and their respective control conditions ($^{\circ}P < 0.05$).

3. Results

3.1. Quercetin and Sesamin Decrease MPP$^+$-Induced IL-6, IL-1β, and TNFα mRNA and Protein Concentrations. We measured the expression of the potent proinflammatory cytokines IL-6, IL-1β, and TNFα by RT-qPCR. Figures 1(a), 1(b), and 1(c) show that MPP$^+$ induced N9 microglial cell activation by dramatically increasing these cytokine mRNA levels. No significant difference from the control condition was detected when quercetin and sesamin were administered alone. On the other hand, quercetin or sesamin pretreatment of microglial N9 cells 3 h before MPP$^+$ administration elicited a reduced pattern of IL-6, IL-1β, and TNFα

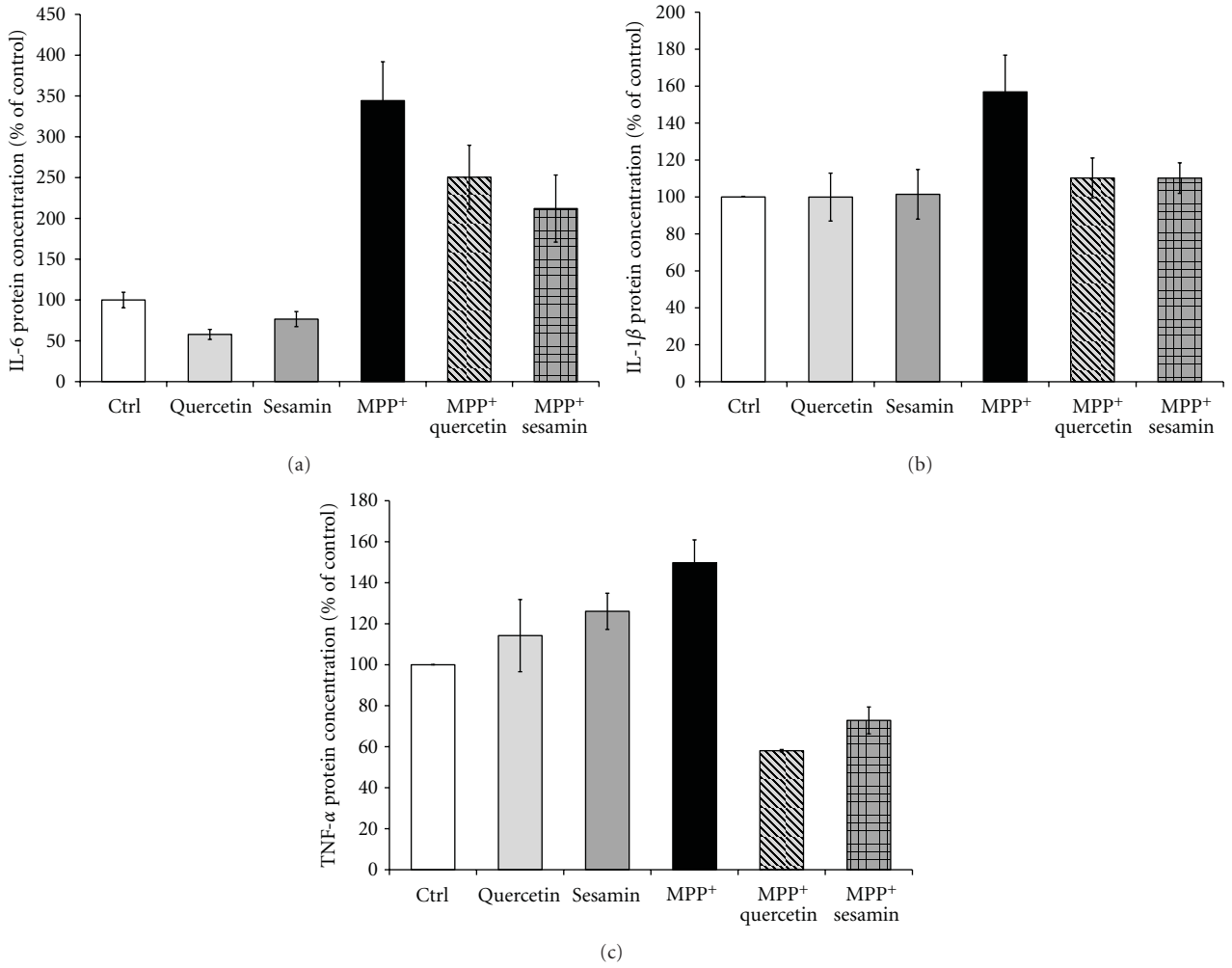

Figure 2: IL-6 (a), IL-1β (b) and TNFα (c) protein concentration in N9 microglial cells treated first for 3 h with quercetin or sesamin and then with or without MPP^+. After 24 h, supernatants were collected and ELISA was performed as described in Materials and Methods. MPP^+ induced marked increases of IL-6 (a), IL-1β (b) and TNFα (c) protein concentration. When N9 microglial cells were treated with quercetin or sesamin 3 h prior to MPP^+, we detected significant declines of IL-6, IL-1β and TNFα protein concentration (MPP^+ Quercetin, MPP^+ Sesamin). $N = 3$, $^{***}P < 0.001$ and $^{*}P < 0.05$ compared to the controls (Ctrl), $^{\diamond\diamond}P < 0.01$ and $^{\diamond}P < 0.05$ compared to MPP^+, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

gene expression, suggesting that quercetin and sesamin are notably involved in the expression of these cytokines. We also evaluated the protein expression of IL-6, IL-1β, and TNFα by specific ELISAs, as described in Section 2. Our results illustrate that the administration of quercetin or sesamin alone does not modulate IL-6, IL-1β, or TNFα protein expression (Figures 2(a), 2(b), and 2(c)). On the other hand, treatment with MPP^+ alone considerably increases the presence of proinflammatory cytokines, indicating that MPP^+ can induce an inflammatory process in microglial cells. The MPP^+-evoked elevation of IL-6, IL1-β, or TNFα protein expression was strongly attenuated to control levels in microglial cells pretreated with quercetin or sesamin (Figures 2(a), 2(b), and 2(c)), suggesting that these natural substances play a role as anti-inflammatory molecules by impacting both the gene and protein expression of proinflammatory cytokines.

3.2. Quercetin and Sesamin Modulate MPP^+-Induced iNOS Protein Expression. Early studies using iNOS inhibitors provided evidence of their potential as neuroprotective agents in the treatment of PD [37]. Here, we observed a very significant rise of iNOS expression by western blotting in microglia cells after only 1 h of MPP^+ administration (Figure 3(a)). Then, iNOS expression decreased during a 24 h period (Figure 3(a)). We thus analyzed whether quercetin or sesamin might modulate iNOS protein expression after 1 h of MPP^+ administration (Figure 3(b)). Our results illustrate that the polyphenol quercetin and the lignan sesamin consistently decreased MPP^+-induced iNOS expression at 1 h.

3.3. Quercetin and Sesamin Counteract MPP^+-Induced $^{\bullet}O_2^-$ Production in N9 Microglial Cells. To investigate the mechanism underlying the protective properties of quercetin and sesamin against MPP^+ treatment in N9 microglial cells,

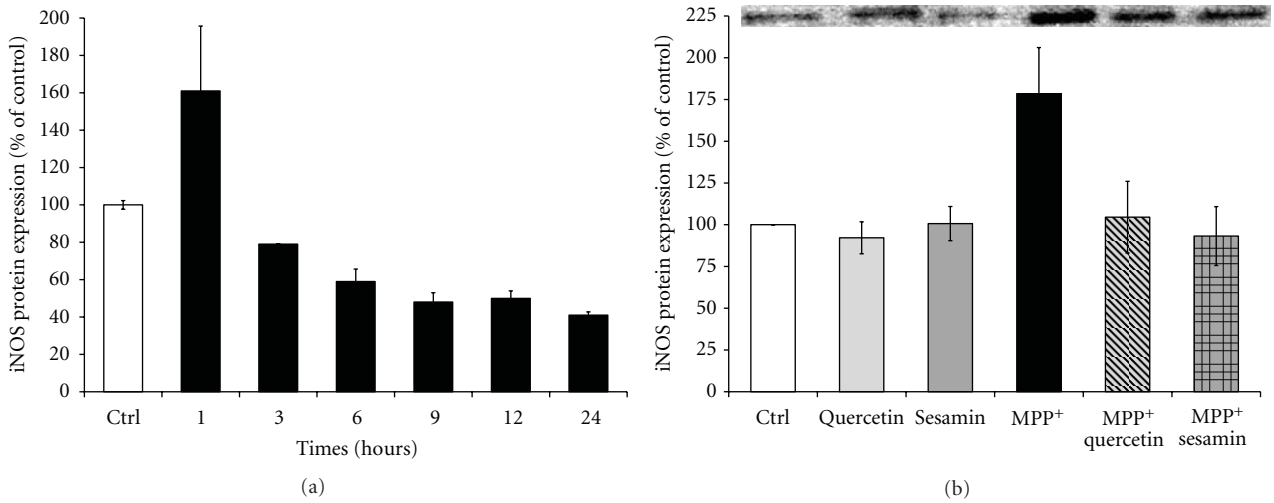

FIGURE 3: (a) Histogram of kinetic studies showing iNOS protein expression for 24 h in neuronal PC12 cells as revealed by western blotting. Ctrl represents 100% of the controls for each time period. (b) iNOS protein expression after MPP^+ administration with or without sesamin or quercetin. Quercetin or sesamin alone did not alter iNOS protein expression, whereas MPP^+ increased iNOS protein levels by 79% in our cellular paradigm. When sesamin or quercetin was administered prior to MPP^+, a significant decline of iNOS was detected. $^{***}P < 0.001$ and $^{*}P < 0.05$ versus Ctrl; $^{\diamond\diamond}P < 0.01$ versus MPP^+, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

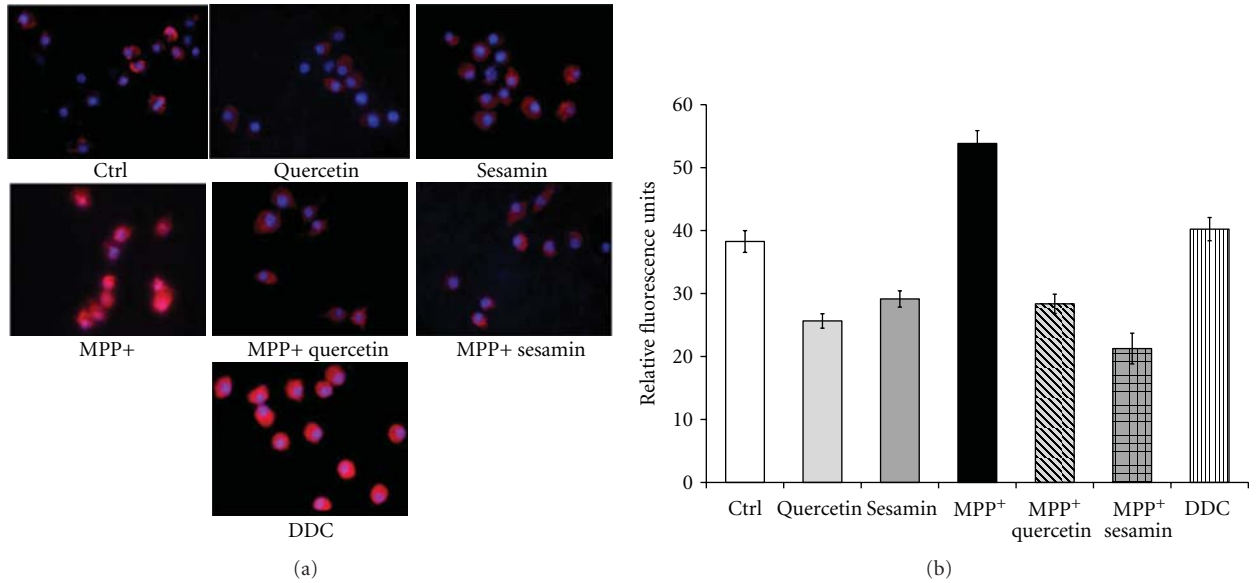

FIGURE 4: Effects of quercetin and sesamin on MPP^+-induced superoxide anion ($^{\bullet}O_2^-$) in N9 microglia cells. (a) Fluorescence photomicrographs. Ctrl: cells were treated with control medium. quercetin, sesamin: cells were treated with quercetin or sesamin in control medium. MPP^+: cells were treated with 500 μM MPP^+. MPP^+quercetin or MPP^+ sesamin: cells were treated with quercetin or sesamin plus MPP^+. A marked red signal was evident only in neuronal PC12 cells treated with MPP^+. Red fluorescence was less intense in cells treated with control medium (Ctrl) or when quercetin or sesamin was added to MPP^+ medium (MPP^+quercetin or MPP^+sesamin). Magnification 400x. $n = 3$. DDC: sodium diethyldithiocarbamate. (b) Semiquantitative image analysis. $^{***}P < 0.001$ and $^{**}P < 0.01$ compared to the control (CTRL), $^{\diamond\diamond\diamond}P < 0.001$ compared to MPP^+, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

we estimated $^{\bullet}O_2^-$ production with MitoSOX Red, a derivative of ethidium bromide, as already described [37], after MPP^+ administration or not, with or without quercetin for 9 h. This time period was considered since ROS, and eventually oxidative stress, are early events in the causative process of cellular death [37]. MitoSOX Red, a fluorogenic dye, was highly selective in detecting $^{\bullet}O_2^-$ in the mitochondria of live cells. Low fluorescence levels were apparent in control microglial cells as well as in cells treated with quercetin or sesamin alone (Figure 4(a): Ctrl, quercetin, sesamin), whereas a marked signal was detected in MPP^+-treated microglial cells (Figure 4(b), MPP^+). Figure 4(b) reports

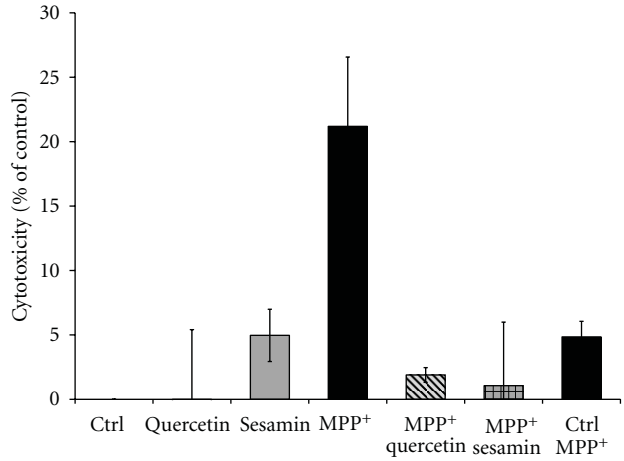

Figure 5: Neuronal PC12 cells were cocultured with MPP^+-activated N9 microglial cells pretreated or not with quercetin or sesamin for 24 h. Then, neuronal PC12 cell supernatants were collected for cell death measurement by the LDH cytotoxicity test. Ctrl: nonactivated microglial cells were placed on neuronal PC12 cells; no neuronal death was detected. MPP^+: MPP^+-activated microglial cells were placed on neuronal PC12 cells; a significant increase of neuronal cell death was apparent, indicating that cytokines produced by microglial cells induce neuronal death. Ctrl MPP^+: MPP^+ was added to the medium of the insert without microglial cells. Pretreatment of N9 cells with quercetin or sesamin prior to MPP^+ clearly reduced neuronal PC12 cell death. Pretreatment of non-MPP^+-activated N9 cells with quercetin or sesamin did not produce cellular death (quercetin, sesamin) $n = 3$. $^{**}P < 0.01$ compared to the control (Ctrl), $^{\circ}P < 0.05$ compared to their respective controls (quercetin, sesamin, or Ctrl), as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

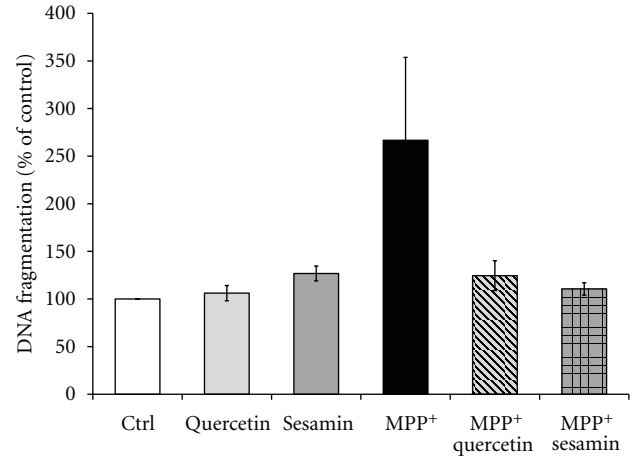

Figure 6: Neuronal PC12 cells were cocultured with MPP^+-activated N9 cells pretreated or not with quercetin or sesamin for 24 h. DNA fragmentation in neuronal cells was detected with a monoclonal antibody to single-stranded DNA (ssDNA). Ctrl: non-activated microglial cells were placed on neuronal PC12 cells; no neuronal death was detected. MPP^+: MPP^+-activated microglial cells were placed on neuronal PC12 cells; a significant increase of neuronal cell death was apparent, indicating that cytokines produced by microglial cells induce neuronal death. Pretreatment of N9 cells with quercetin or sesamin prior to MPP^+ clearly reduced neuronal PC12 cell death. Pretreatment of non-MPP^+-activated N9 cells with quercetin or sesamin did not produce any cellular death. $n = 3$. $^{***}P < 0.01$ compared to the controls (Ctrl), $^{\diamond\diamond}P < 0.01$ compared to quercetin, $^{\diamond\diamond\diamond}P < 0.001$ compared to sesamin, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

on the semi-quantitative analysis of mitochondrial $^{\bullet}O_2^-$ depicted in Figure 4(a), revealing high-level fluorescence in the presence of MPP^+ with a very significant reduction ($P < 0.05$ or $P < 0.001$) when microglia cells were treated with quercetin or sesamin.

3.4. Quercetin and Sesamin Reduce MPP^+-Induced Cytotoxicity and Apoptotic Cell Death in Microglial (N9)-Neuronal (PC12) Coculture. To investigate microglial-activated neuronal cell death, we tested a microglial-neuronal coculture system described elsewhere [26]. N9 microglial cells were cocultured in inserts on neuronal, differentiated PC12 cells to evaluate the effect of MPP^+-induced cytokine secretion from microglial cells on neuronal PC12 cell death. The inserts have a pore size of 0.4 μM; thus, they allow cytokines to pass through but prevent cell contact [38]. Neuronal cells placed beneath non-MPP^+-activated N9 cells did not present any significant cell death (Figure 5: Ctrl), whereas neuronal cells cocultured with MPP^+-activated microglial cells displayed a high level of cell death (Figure 5: MPP^+), demonstrating that MPP^+-activated microglia secrete cytokines transported through the membrane insert inducing neuronal death. Figure 5 also reveals that neuronal cell death is diminished to 1.9% when microglial cells are treated with quercetin, and to 1.1% when they were treated with sesamin prior to MPP^+ administration. In addition, our results show no significant neuronal death of PC12 cells exposed to inserts containing MPP^+ without microglial (Ctrl MPP^+), demonstrating that in our cell coculture system, cytokines secreted by microglial cells cross the membrane and elicit neuroinflammation with consequent neuronal PC12 cell death.

To determine whether quercetin and sesamin can protect neuronal DAergic cells from inflammation-induced apoptosis, we also measured ssDNA fragmentation (Figure 6), a marker of late apoptosis. Microglial cells treated with MPP^+ for 24 h manifested a 167% increase in DNA fragmentation—in comparison to control cells (Figure 6)—that was strongly and significantly prevented by quercetin and sesamin (Figure 6). These results disclose that quercetin or sesamin administration to microglial cells can efficiently reduce the apoptotic death of neuronal PC12 cells induced by microglial activation, thus supporting an anti-inflammatory role of these 2 natural molecules.

4. Discussion

Currently, several studies have described microglia activation in the SN *pars compacta* of PD patients and PD animal models [4–6]. As such, neuroinflammation is considered a feature of PD progression and pathogenesis.

Our present data highlight the neuroprotective properties of quercetin and sesamin, 2 natural molecules that reduce the expression of IL-6, IL-1β, and TNFα, 3 cytokines associated with neuroinflammation. We also show that quercetin and sesamin prevent the production of 2 cellular markers of inflammation, iNOS and $^{\bullet}O_2^-$ as well as the apoptosis of DA-producing neurons provoked by microglia stimulation. Our previous studies have already revealed that quercetin and sesamin have neuroprotective, antiapoptotic and antioxidative properties, reducing MPP^+- and LPS-induced neuronal death [25, 26, 32]. Here, we further demonstrate that quercetin and sesamin can also act as potent anti-inflammatory compounds, restraining microglia activation and oxidative stress.

Activated microglial cells contribute to DAergic cell death by releasing cytotoxic inflammatory compounds, such as the proinflammatory cytokines IL-6, IL-1β, TNFα, and interferon-gamma. Among them, IL-6, IL-1β and TNFα have attracted much attention with regard to neuroinflammatory processes in PD [4]. DAergic degeneration induced by MPTP or MPP^+ is linked with an inflammatory response *in vitro* [39] as well as in mouse and primate models of PD [18, 40]. On the other hand, inhibition of microglia activation is neuroprotective [41, 42] and coupled with the attenuation of TNFα expression [43]. The mechanisms by which microglia are activated are not fully understood. However, very recent data on a mouse MPTP model of PD as well as mesencephalic culture support a role of brain angiotensin II as one of the most potent inducers of inflammation and ROS [44, 45].

In this study, we clearly demonstrated that the flavonoid quercetin and the lignan sesamin strongly reduce the expression of proinflammatory cytokines in N9 microglia cells, indicating an interesting anti-inflammatory role of these natural molecules. We also analyzed the effects of quercetin and sesamin on parameters of oxidative cell distress. Activated microglia and excessive NO production by the high-output NO-synthesizing enzyme iNOS are observed in various neurological diseases, including PD [46]. NO is an apoptosis inducer, and iNOS is a key enzyme that produces large quantities of NO. Quercetin exerts a broad inhibitory effect on iNOS gene expression [27]. Sesamin and sesamin metabolites are known to induce endothelial NOS (eNOS) and thus have a significant antihypertensive function [30]. In this study, we demonstrated that quercetin and sesamin markedly reduced MPP^+-evoked upregulation of iNOS expression in microglial cells, corroborating previous data from our group and others [32, 47].

It should be noted that, in our microglial cellular system, MPP^+ increased iNOS production, after only 1 h of administration, and iNOS protein expression then declined constantly. This might be explained by early iNOS production followed by other intracellular apoptotic mechanisms. Certainly, in-depth studies should be performed to analyze the kinetics of iNOS production in this particular cellular system. Several investigations have determined that quercetin and sesamin can decrease ROS production to near-normal levels in various cellular systems [21, 48]. With MitoSOX Red, a selective indicator of mitochondrial $^{\bullet}O_2^-$ production, we illustrated an increase of fluorescence, when MPP^+ was administered alone, and a substantial reduction with quercetin or sesamin treatment, supporting a potent scavenging role of quercetin and sesamin.

Microglia activation leads to increased production of cytokines that could mediate neuronal apoptosis and precede DAergic nigrostriatal neuron degeneration in a PD mouse model [49]. In this study, we show that quercetin and sesamin reduce the cellular death induced by MPP^+ administration. We also document that quercetin and sesamin are potent modulators of apoptosis, opposing MPP^+-induced DNA fragmentation. Our results demonstrate that when neuronal PC12 cells are cocultured with MPP^+-treated N9 cells, their levels of cellular death increase to 267%. Quercetin or sesamin administration to MPP^+-activated microglia reduces apoptotic DAergic neuronal PC12 cell death to 125% and 112%, respectively.

It should be noted that, although natural polyphenols are being studied intensively *in vitro* and *in vivo* for their neuroprotective properties, our knowledge about their bioavailability and possible target organs is far from being complete ([50] for review). In mammals, flavonoids, such as quercetin, as well as the lignan sesamin, are absorbed in the gut and that their bioavailability is much greater than previously believed [51–53]. Flavonoid concentrations in human plasma vary from 3 to 30 microM and certainly more, so if red blood cell-associated flavonoids are taken into consideration [54]. In addition, polyphenols can cross the blood-brain barrier to varying degrees depending on their chemical structure [55–57]. Recent studies have reported that their bioavailability in the nervous system may be improved by designing specific synergies between orally consumed polyphenols ([58] for review). Recent critical and comprehensive reviews report that quercetin and its metabolite isorhamnetin are found in the brain of rats and pigs in measurable levels ([50, 59] and references within), while nowadays less is known regarding the degree of brain bioavailability of sesame lignans. Sesamin can be converted to the mammalian lignans (enterodiol, enterolactone, and sesamol) by the intestinal microflora and is found in circulating blood. Recent results report that in rats orally administered sesamin can improve oxidative stress induced by kainic acid-induced status epilepticus and middle cerebral artery occlusion [60, 61]. In this study, we have used quercetin at 10^{-7} M which is a relative high concentration compared to that reported *in vivo* in the pmol and nmol/g/tissue range [50]. However, for sesamin, we used 10^{-12} M concentration that should be physiologically attainable *in vivo*.

Currently, more and more *in vivo* studies point out the importance of the biotransformation of natural molecules and the magnitude of bioavailability of the parental molecule and/or its metabolites for the prevention of human diseases [59]. With respect to these *in vivo* studies, our *in vitro* experiments document for an anti-inflammatory response with both quercetin and sesamin in a glial-neuron coculture system. Although far from an *in vivo* trial, it remains one of the best *in vitro* paradigms to study the possible relationship between microglial production of proinflammatory cytokines and neuronal cellular death.

Nevertheless, the information obtained with this study is also valuable providing new insights into the cellular mechanisms of natural compounds as preventive and/or complementary therapies for human diseases.

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Review Article

The Role of Dietary Polyphenols on Adult Hippocampal Neurogenesis: Molecular Mechanisms and Behavioural Effects on Depression and Anxiety

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Although it has been long believed that new neurons were only generated during development, there is now growing evidence indicating that at least two regions in the brain are capable of continuously generating functional neurons: the subventricular zone and the dentate gyrus of the hippocampus. Adult hippocampal neurogenesis (AHN) is a widely observed phenomenon verified in different adult mammalian species including humans. Factors such as environmental enrichment, voluntary exercise, and diet have been linked to increased levels of AHN. Conversely, aging, stress, anxiety and depression have been suggested to hinder it. However, the mechanisms underlying these effects are still unclear and yet to be determined. In this paper, we discuss some recent findings addressing the effects of different dietary polyphenols on hippocampal cell proliferation and differentiation, models of anxiety, and depression as well as some proposed molecular mechanisms underlying those effects with particular focus on those related to AHN. As a whole, dietary polyphenols seem to exert positive effects on anxiety and depression, possibly in part via regulation of AHN. Studies on the effects of dietary polyphenols on behaviour and AHN may play an important role in the approach to use diet as part of the therapeutic interventions for mental-health-related conditions.

1. Introduction

A long-standing dogma in the brain sciences stated that new neurons were only generated during development. However, in the mid-1900s new evidence indicated the need for a change in this doctrine, as an unknown capacity in the adult mammalian brain started to be unraveled: adult neurogenesis.

Two regions in the adult mammalian brain, including human [1], can be pointed as neurogenic sites [2]: the subventricular zone (SVZ), located along the sides of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus.

The new neurons generated in the SVZ migrate through a precise route, the rostral migratory stream (RMS), and integrate into the olfactory bulb, where they continuously replace local neurons [3]. In the DG, new neurons are generated from two types of progenitors or precursors located in the SGZ [4]: type 1 hippocampal progenitors, which extend a radial process across the granular layer, ramifying in the inner molecular layer, and type 2 cells, which are hippocampal progenitors with short processes (Figure 1, also showing the 3 types of progenitor cells lying adjacent to the ependymal cell layer in the SVZ: type B cells, which are GFAP positive; type C transit amplifying cells; type A, which are migrating neuroblasts) [4].

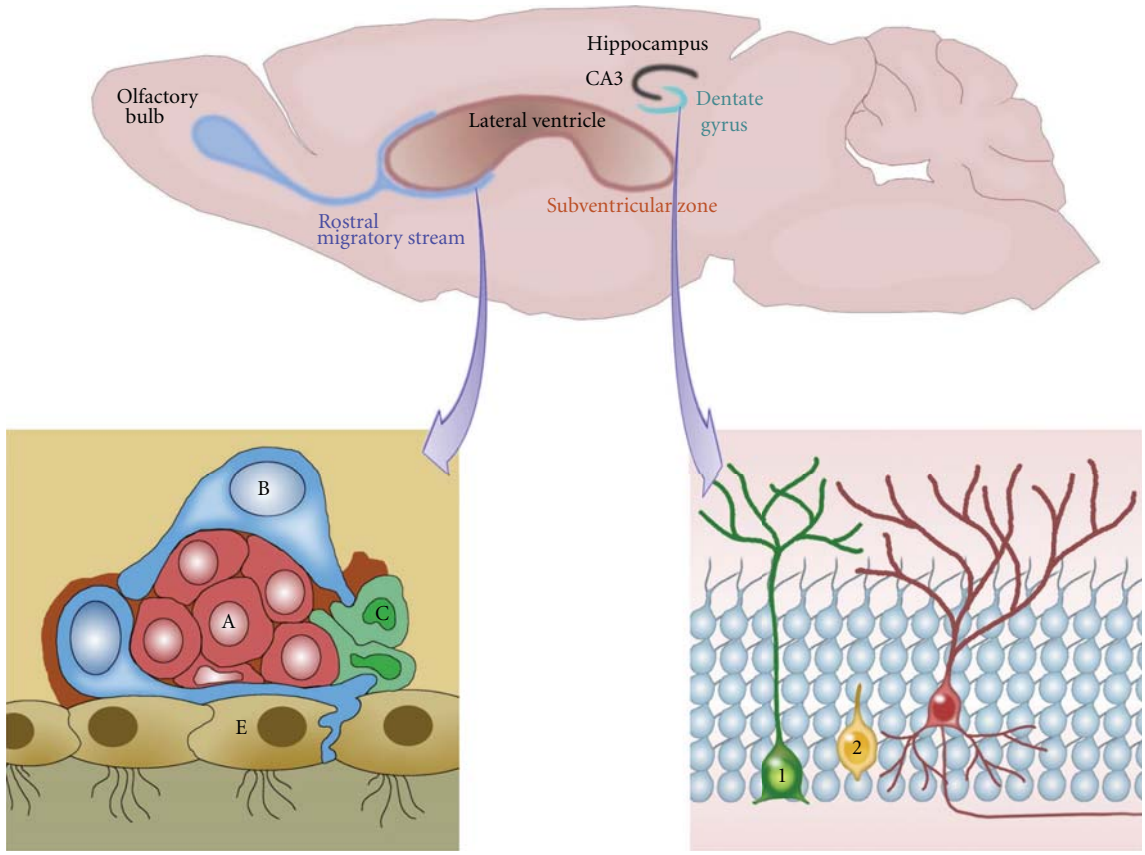

Figure 1: *Adult neurogenesis in the mammalian brain*. The figure illustrates the two well-known neurogenic sites in the brain: the subventricular zone (SVZ) and the dentate gyrus of the hippocampus (DG). Three types of progenitor cells are found lying adjacent to the ependymal cell layer (E) in the SVZ: A subset of relatively quiescent GFAP+ radial cells (type B cells) in the SVZ has the potential to serve as adult NSCs and generate rapidly dividing, transit-amplifying nonradial NSCs (type C cells), which in turn give rise to neuroblasts (type A cells) that migrate through the rostral migratory stream toward the olfactory bulb. In the adult SGZ, a population of GFAP+ Sox2+ radial cells corresponds to quiescent NSCs (type 1 cells). They coexist with actively proliferating, GFAP+ Sox2+ nonradial NSCs (type 2 cells) that generate both astrocytes and neuroblasts. Neuroblasts then migrate into the granule cell layer and mature into neurons (red cell).

The new neurons generated in the adult hippocampus, specifically in the SGZ of the DG, migrate to the inner layer of the granular zone, where they send/receive synaptic contacts [5].

Adult neurogenesis is a widely preserved phenomenon, being verified in different mammalian species such as mice, rats, guinea-pigs, monkeys, and humans [1, 6–12]. In both mentioned neurogenic sites, adult neurogenesis follows defined stages, identified as proliferation, cell fate determination, migration, and synaptic integration [5]. Many factors can impact the regulation of these different steps, possibly via the influence of factors released from vasculature [13, 14] and complex cell-cell interactions, between progenitor cells and other progenitors, astrocytes, and local/distal neurons [4].

Some environmental factors have been shown to influence adult hippocampal neurogenesis (AHN), such as voluntary exercise, enriched environment, and caloric restriction that increase the rates of AHN [15–17], whereas stressful conditions like aging or stress itself have a decreasing influence [8, 18].

Stress is one of the most potent negative modulators of hippocampal neurogenesis, as demonstrated in several species of mammals [19]. This effect has been demonstrated by different paradigms, such as exposure to predator odour [20], resident-intruder model of stress [8], psychosocial stress [21], inescapable shock [22], and others (reviewed in [23]). However, the mechanisms involved in this deleterious effect have not yet been totally clarified. There is evidence of a role for hormones and cytokines released during stress in modulating AHN. Indeed, AHN is very sensitive to the increase in corticoid levels [19], with these hormones negatively regulating it. It is also known that the hippocampus plays a role on the regulation of endocrine functions, possibly promoting a negative regulation of the hypothalamus-pituitary-adrenal (HPA) axis [24]. This involvement of the hippocampus is evidenced by the high concentration of glucocorticoid and mineralocorticoid receptors in this brain structure (reviewed in [25]). However, there is also evidence of a role for the newly generated neurons buffering the stress response. Indeed, a recent study has shown that the ablation of AHN led to a modulation of glucocorticoid levels after

moderate stress, with a sustained increase in the levels of these hormones even after a significant period following the stressful event [26]. This reinforces the modulating role of the hippocampus on the HPA axis through both AHN and the more established negative feedback via glucocorticoid receptors. This same study has suggested a relation between neurogenesis and the aetiology of depression. As stated, it has been proposed that depressed individuals have reduced hippocampal volume [27, 28]. In addition, there is increasing evidence from animal research that antidepressants might exert a proneurogenic effect in the hippocampus [29], and the ablation of neurogenesis has led to depressive behaviour in animals tested in the forced swimming test (FST) and in the sucrose preference test (SPT) [26, 30].

The role of AHN is still intensely debated. While several contradictory findings emerge when analyzing the literature, evidence in favour of a relevant role of adult-born neurons in hippocampus-dependent learning is compelling; spatial and associative memory is impaired in rodents under conditions that decrease AHN, whereas hippocampus-dependent learning tasks are solved better under conditions that increase AHN (reviewed in [31]).

AHN is also said to play a role in different neurodegenerative, neurological, and psychiatric disorders such as Alzheimer's and Parkinson's disease, anxiety, and depression. With regard to the latter, a small reduction of hippocampal volume has been found in human patients suffering from depression, suggesting an involvement of the hippocampus in mood disorders [27, 28]. Reduced AHN in different animal models of depression supports this observation as well as the ability of antidepressants to restore it [22, 28]. However, data from different studies are still controversial and the specific features that characterise the involvement of AHN in depression and anxiety are not absolutely clear. On the one hand, decreased AHN does not necessarily induce depressive behaviour in laboratory rodents [30, 32] and on the other hand, minimally normal levels of AHN have been shown to be necessary for the successful effect of some antidepressants [30, 33], and for buffering depressive behaviour [26].

Several studies during the last 5 years suggest that changes in diet can have a positive influence on neurogenesis, learning, and memory as well as cognition and mood (reviewed in [34]). As mentioned, laboratory rodents that were fed under calorically restricted conditions had a higher amount of AHN than *ad libitum* fed fellows, a process most likely regulated via the brain-derived neurotrophic factor (BDNF) [17]. Other positive effects on brain function were achieved by omega-3 fatty acids and vitamins, as well as by polyphenolic components of grapes, blueberries, cocoa, or teas (reviewed in [35, 36]). Polyphenolic compounds are phytochemicals known for their biological antioxidative, neuroprotective, and cognitive properties. For instance, it has been shown that different polyphenols can increase synaptic plasticity in the context of AHN [37–40] and also promote hippocampal long-term potentiation [41]. In addition, it has been verified that polyphenols can enhance learning and memory [42, 43] and reduce the risk of developing age-related neurodegenerative diseases [44, 45], possibly via a decrease in reactive oxygen species (ROS) production and inflammation in models of aging [46, 47].

Besides antidepressant drugs, different polyphenolic compounds such as catechins (flavanols/flavonoid from green tea), curcumin (nonflavonoid from tumeric/*Curcuma longa*), and resveratrol (stilbenoid produced naturally by several plants when under attack/found high in red grape skin) have been observed to have antidepresssant-like effects in rodents and human [48–52]. This suggests that polyphenols could be key compounds for the improvement of psychiatric disorders like depression or anxiety.

The facts that polyphenols have been shown to be helpful compounds against depression and that they can increase AHN suggest that these molecules might affect mood, and not only cognition, via AHN. In this way, this paper aims to address this hypothesis, discussing the effects of dietary polyphenols on mental health, particularly on depression and anxiety, and the possible molecular mechanisms underlying these effects via AHN regulation.

2. Polyphenols and AHN

2.1. Effects of Polyphenols on AHN. Many of the studies about the impact of polyphenols on the hippocampus have relied on the antioxidant properties of these molecules and their neuroprotective effect in different models of brain injury [53–61] (see Table 1 for details), with the majority of them not having addressed AHN. However, the emerging evidence of hippocampal plasticity offered by the mechanisms underlying AHN has made this phenomenon a promising target for pharmacological, environmental, and nutraceutical interventions, such as polyphenolic diet, raising the need for studies focusing on how these factors could impact the ability of the adult hippocampus to generate new functional neurons.

A positive role of a diet enriched in polyphenols and polyunsaturated fatty acids (LMN diet) on adult mice neurogenesis has been shown [37]. Following 40 days of LMN diet, different markers of AHN have been found to be increased in comparison to mice under control diet, such as the number of newly generated cells in the SGZ (as well as in the SVZ), with significantly more cells expressing the neuroblast marker doublecortin, suggesting that the LMN diet had an effect on neuronal populations. Indeed, the rise in neuronal differentiation was confirmed by the increased colocalization of the cell proliferation marker 5-bromo-2′-deoxyuridine (BrdU) and NeuN—expressed in mature neurons—in neurons of the granule layer of animals fed with the polyphenolic/fatty acid-enriched diet. Although more is yet to be specifically clarified, one of the possible mechanisms suggested for the increase in AHN by this special diet could be the induction of hippocampal plasticity factors such as insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R), as previously shown by short-term blueberry supplementation in rats [62]. In addition, the neurogenic potential of the LMN diet has also been suggested in a recent study that showed it to be capable of increasing to 70% the rate of cell proliferation in the SVZ of a mouse model of Alzheimer's disease [54]. However, since

Table 1: Effects of polyphenols, particularly green tea/epigallocatechin and curcumin, on different neurochemical and morphological aspects of the hippocampus. BB: blueberry; EGC: epigallocatechin; GT: green tea.

Reference	Polyphenol	Treatment	Model	Effect on hippocampus	Proposed molecular mechanism(s)
Xu et al. 2009 [53]	Curcumin	Curcumin (5, 10 and 20 mg/kg, p.o.) or imipramine (10 mg/kg, i.p.)	Sprague-Dawley rats	Curcumin prevented hippocampal dendritic remodeling under conditions of chronic stress	Normalizing corticosterone levels and downregulating pCaMKII and glutamate receptor levels
Fernández-Fernández et al. 2011 [54]	LMN diet (polyphenols and fatty acids)	Control or LMN diet for 5 months	Tg2576 male mice as a model of AD crossing them with 129S1/SvImJ females	Reduction of Aβ plaques in the hippocampus when administration of diet occurred before plaque formation	—
Joseph et al. 2010 [55]	Blueberry	HNCs treated with BB extract, BB fractions (e.g., proanthocyanidin, PAC) or control medium were exposed to dopamine (DA, 0.1 mM), amyloid beta (Aβ42, 25 μM) or lipopolysaccharide (LPS, 1 μg/mL)	NeuroPureTM E18 primary rat hippocampal cells	Results indicated that the degree of protection against deficits in Ca^{2+} recovery varied as a function of the stressor and was generally greater against Aβ42 and LPS than DA	Prevention of deficit in Ca^{2+} buffering, normalization of cyclic CREB, protein kinase Cγ (PKCγ) and increased expression of ERK
Narita et al. 2011 [56]	Grape seed extract (GSE: Koshu and Muscat Bailey)	50 mM glutamate for 30 min, in the presence or absence of various concentrations of GSEs	Hippocampal tissue isolated from newborn mice (C57/B6; P1)	Koshu: alleviated the acute inactivation of Erk1/2 and dendrite retraction in cultured hippocampal neurons exposed to a toxic concentration of glutamate (1.0 ng/ml) Muscat Bailey: no neuroprotective effect	High-affinity molecular targets
Fujishita et al. 2009 [57]	Grape seed extract (GSE, Koshu)	0, 1, 10, 30, 100 μg/mL for 24 h or 0, 1, 10, 30, 100 μg/mL for 2, 6, or 12 h.	Culture of hippocampal neurons and astrocytes	GSE upregulated (concentration (100 μg/mL) and time-dependently); various mRNAs for cytokines, particularly for interleukin-6 (IL-6)	GSE could protect neuronal cells from death by oxidative stress via upregulated IL-6 production in astrocytes
Assunção et al. 2011 [58]	Green tea/epigallocatechin	19 month-old rats were fed with GT since age of 12 Months	Wistar rats	GT treatment protected proteins and lipids against oxidation and prevented the increase of lipofuscin deposition	—
Assunção et al. 2010 [59]	Green tea	Rats fed with GT from 12 to 19 months of age versus controls aged 19 months	Wistar rats	GT increased CREB activation and the levels of BDNF and Bcl-2, but had no effect on activation of NF-κB subunits	Long-term GT ingestion improves antioxidant systems and activates CREB in the aging rat hippocampal formation, leading to neuroprotection mediated by downstream upregulation of BDNF and Bcl-2

Table 1: Continued.

Reference	Polyphenol	Treatment	Model	Effect on hippocampus	Proposed molecular mechanism(s)
Yin et al. 2008 [60]	Green tea/Epigallocatechin-3-gallate	Treatment by EGCG (10–50 μM)	Hippocampal neuronal culture; lead exposure	Lead exposure significantly inhibited the viability of neurons; treatment with EGCG effectively increased cell viability and decreased ROS formation	Free radical scavenging and antioxidative properties of EGCG
Xu et al. 2010 [61]	Green tea/epigallocatechin	GT polyphenols were administered orally to rats from 4 to 8 weeks after experimentally induced cerebral hypoperfusion (400 mg/kg per day or 100 mg/kg)	Wistar rats	Reduced lipid peroxidation and oxidative DNA damage after chronic cerebral hypoperfusion	Free radical scavenging and antioxidative properties of GT polyphenols

a fatty-acid-(F-A) exclusive diet has not been used, there is no conclusive evidence that the effects found are due only to polyphenols. In this sense, the contribution of FA or a synergistic effect of polyphenols and FA should be considered.

This positive effect of polyphenols on hippocampal neurogenesis has also been demonstrated *in vitro*, with low concentrations of curcumin stimulating cell differentiation in cultures of multipotent mouse neural progenitor cells. A similar effect has been demonstrated *in vivo,* with increased AHN in mice administered with this polyphenol [38] (see Section 3.2.2 for further discussion on the effects of curcumin on mental health and possible molecular mechanisms underlying it).

In addition to curcumin, the interaction between polyphenol and chronic stress has also been found with the administration of flavonoids. In this sense, administration of flavonoids extracted from Chinese herb Xiaobuxin-Tang (XBXT-2) significantly increased hippocampal neurogenesis in chronically stressed rats [39]. Additionally, XBXT-2 treatment reversed the stress-induced decrease of hippocampal BDNF and phosphorylated cyclic AMP-response element DNA-binding protein (pCREB) (Ser133) expression, two important factors closely related to hippocampal neurogenesis. Interestingly, as occurred with curcumin, the positive effects of these flavonoids were comparable to those achieved with imipramine treatment.

Dietary polyphenols are thought to be the most abundant antioxidants in foods and beverages [63], with particular potential to inhibit neuroinflammation (reviewed in [64]). Thus, with special regard to inflammation, oxidative stress and microglial activation—all factors known to decrease cell proliferation and neuroplasticity—it has been shown that neural progenitor cell proliferation and spatial memory performance are increased, with decreased microglial activation, in aged rats submitted to 4 weeks of treatment with NT-020—a natural diet based on the combination of polyphenols from blueberry and green tea, as well as antioxidant and anti-inflammatory amino acids like carnosine [65]. It is well established that one of the physiological markers of aging is the increase in circulating factors such as cytokines and chemokines, known to increase proinflammatory factors that exert a negative effect on the progenitor cell pools. The authors then suggest that the positive influence of the NT-020 diet on health promotion could occur via the promotion of proliferation and survival of neurons, as well as by anti-inflammatory actions that influence the stem cell niche of the aged brain [65].

Table 2 summarises the studies discussed before, on the effects of polyphenols on hippocampal cell proliferation and differentiation.

3. Polyphenols and Mental Health

3.1. Effects of Dietary Polyphenols on Depression and Anxiety: Behavioural Aspects and Proposed Mechanisms of Action. There is a growing body of data from animal and human studies supporting the role of a variety of dietary polyphenols in affecting behaviour and mood through anxiolytic and antidepressant-like properties (see Table 3). The varied mechanisms proposed for the effects of polyphenols on mental health and the mounting evidence for the role of each are suggestive of the complexity of the diverse interactions influencing mood and behaviour. Thus the multiple cellular and molecular mechanisms resulting in antidepressant-like or anxiolytic effects of a particular polyphenol can reveal potential targets at the level of the individual pathways which may collectively contribute to a common behavioural phenotype in the context of the etiologies of anxiety and depressive disorders and expand our understanding of interactions of these pathways.

The proposed mechanisms for polyphenol effects on mental health are not limited to their well-established antioxidant effects and are as varied as the different polyphenols themselves and the sources in which they are found. Chlorogenic acid is a common dietary polyphenol found in fruits such as plums, apples, and cherries and beverages such as tea and coffee which has been shown to have anxiolytic effects in animal studies [67, 76]. Chlorogenic acid has been demonstrated to have a number of effects on a cellular level, leading to several proposed mechanisms for its overall anxiolytic effects. In one study, the anxiolytic effect of chlorogenic acid was blocked *in vivo* by the benzodiazepine receptor antagonist flumazenil, suggesting that anxiety is reduced by activation of the benzodiazepine receptor [67]. *In vitro*, chlorogenic acid protected granulocytes from oxidative stress, which is another proposed important contributor to anxiety [67]. It has also been found to have neurotrophic effects *in vitro* that stimulate neuronal differentiation and neurite growth, supporting neuroplasticity, which also may contribute to its anxiolytic effects [66].

Other polyphenols have been shown to exert anxiolytic effects with proposed mechanisms that overlap those of chlorogenic acid. Like chlorogenic acid, the green tea polyphenol epigallocatechin-3-gallate (EGCG) has also been shown to have anxiolytic properties in animal studies with comparable results to a benzodiazepine anxiolytic drug [68]. *In vitro* work with cultured hippocampal neurons confirmed the specific modulation of the GABA-A receptor benzodiazepine site by EGCG [68].

However, other studies suggest that different pathways might contribute to the anxiolytic effects of other polyphenols. Anthocyanin polyphenols from *Vaccinium* berries (a genus which includes highbush blueberries, rabbiteye blueberries, and bilberries) also show anxiolytic effects in animal studies but *in* vitro studies have elucidated several different mechanisms which may be responsible for the anxiolytic property of this class of polyphenols [69, 77, 78]. The antioxidant properties of anthocyanins from rabbiteye blueberries were shown to reduce oxidative damage to neural DNA and this antioxidant neural protection was proposed as a mechanism for the anxiolytic property of berries [69]. Berry anthocyanins also inhibit monoamine oxidases (MOAs), providing neuroprotective effects and counteracting the MOA activity of lowering neural levels of serotonin, noradrenaline, and dopamine whose low levels have been implicated in the etiology of anxiety disorders [77]. Blueberry polyphenols also have anti-inflammatory

Table 2: Effects of different polyphenolic-based diets or treatments on the expression of cell proliferation and differentiation markers, especially in the adult hippocampus.

Reference	Polyphenol	Treatment	Model	Effect on AHN	Proposed molecular mechanism(s)
Valente et al. 2009 [37]	LMN diet (polyphenols and fatty acids)	*In vivo:* animals fed for 40 days with either a control diet or with LMN diet	*In vivo:* 129S1/SvImJ mice	*In vivo:* increased proliferation and differentiation in DG and SVZ	—
		In vitro: LMN cream (0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60,0.70, 0.80, and 1.00 mg/mL) for 24 h	*In vitro:* neuron-glial cell cultures (hippocampal and cortical) pretreated with H_2O_2	*In vitro:* cortical cells: antioxidant effect (approximately 80%) Hippocampal cells: antioxidant LMN range concentration restricted to 0.50 and 0.60 mg/mL; antioxidant capacity of approximately 100%	
So et al. 2008 [38]	Curcumin	*In vitro:* (0.1, 0.5, 1, 10, 20, and 50 uM)/well	Multipotent NPC; C57BL/6 mice	*In vitro:* increased proliferation (0.1,0.5 uM) (BrdU)	Curcumin stimulates the proliferation of embryonic cortical neural stem cells via the MAP kinase pathway
		In vivo: 500 nmol/kg body weight, once daily for 4 days		*In vivo:* increased proliferation and differentiation (NeuN)	
An et al. 2008 [39]	Flavanoids (XBXT-2)	XBXT-2 (25 or 50 mg/kg), imipramine (10 mg/kg, p.o.)	Sprague Dawley rats submitted to chronic stress	Increased proliferation (BrdU) Increased hippocampal BDNF	Increased neurogenesis and expression of hippocampal BDNF and pCREB as one of the molecular and cellular mechanisms underlying the antidepressant action of XBXT-2
Xu et al. 2007 [40]	Curcumin	Curcumin (5, 10 and 20 mg/kg, p.o.) or imipramine (10 mg/kg, i.p.)	Sprague-Dawley rats submitted to chronic stress	Increased proliferation (BrdU) Increased BDNF levels	Upregulation of 5-HT1A receptor mRNA and of BDNF levels
Fernández-Fernández et al. 2011 [54]	LMN diet (polyphenols and fatty acids)	Control or LMN diet for 5 months	Tg2576 male mice as a model of AD crossing them with 129S1/SvImJ females	Increased proliferation in the subventricular zone (SVZ) (BrdU)	—
Acosta et al. 2010 [65]	NT-020 diet (polyphenols, vitamin D3, and carnosine)	Aged: 135 mg/kg per day of NT-020 diet orally administered for 4 weeks versus young and aged controls treated with water by oral gavage	Fischer 344 rats	Increased proliferation (Ki67) Increased differentiation (DCX) Decreased activated microglia	Reduction of proinflammatory compound counteracts age-related decrease of neurogenesis
Ito et al. 2008 [66]	Chlorogenic acid	Polyphenol added to the medium to final concentration of 10 μM	Fetal rat hippocampal neurons	Promoted neuronal differentiation	—

Table 3: Effects of different dietary polyphenols on mental-health-related states and behaviour, particularly anxiety and depression. EGC: epigallocatechin; GT: green tea.

Reference	Polyphenol	Treatment	Model	Effect on mental health/behavior	Proposed molecular mechanism(s)
An et al. 2008 [39]	Flavanoids	XBXT-2 (25 or 50 mg/kg), imipramine (10 mg/kg, p.o.)	Sprague Dawley rats submitted to chronic stress	Antidepressant-like effect	Increased neurogenesis and expression of hippocampal BDNF and pCREBas one of the molecular and cellular mechanisms underlying the antidepressant action of XBXT-2
Duffy et al. 2008 [42]	Blueberry	2% blueberry extract versus control diet over 8 weeks	Fischer-344 rats submitted to kainate-induced learning impairment	Blueberry diet reduced memory impairment Less loss of CA1 pyramidal neurons	Possible involvement of MAPK
van Praag et al. 2007 [43]	Flavanoids (−) epicatechin	(−) epicatechin diet ad libitum versus control ad libitum	C57/Bl6 mice (runner versus non-runner)	(−) epicatechin improved memory in Morris water maze especially in combination with exercise	Increase of angiogenesis and spine density
Messaoudi et al. 2008 [48]	Cocoa	Effective doses of cocoa: 24 mg/kg/14 days and 48 mg/kg/14 days	Wistar rats	Antidepressant- like effects measured in the Forced swimming test	Reversal of oxidative damage
Sathyapalan et al. 2010 [49]	Cocoa	High cocoa liquor/polyphenol rich chocolate diet or cocoa liquor free/low polyphenol diet for 8 weeks	Human fatigue syndrome patients	Chalder Fatigue Scale score improved significantly after 8 weeks of the HCL/PR diet	
Xu et al. 2005 [50]	Curcumin	Curcumin (1.25, 2.5, 5, 10 mg/kg), or moclobemide (20 mg/kg), imipramine (10 mg/kg)	ICR mice	Curcumin also significantly inhibited immobility in FST	Antidepressant-like effects of curcumin may involve the central monoaminergic neurotransmitter systems
Xu et al. 2010 [51]	Trans-Reservatrol	*t rans*-Resveratrol (20, 40 and 80 mg/kg, via gavage)moclobemide (20 mg/kg, i.g.), imipramine (10 mg/kg, i.p.), and fluoxetine (10 mg/kg, i.p.)	ICR mice	Decreased the immobility time in mouse models of despair	Antidepressant-like effect of *trans*-resveratrol might be related to serotonergic and noradrenergic activation
Zhu et al. 2011[52]	Green tea/epigallocatechin	Orally administered green tea polyphenols (GTP; 5, 10 and 20 mg/kg) for 7 days Forced swimming test (FST) and tail suspension test (TST) 60 min after the last GTP administration	ICR mice	Antidepressive- like effects	May involve inhibition of HPA axis; possibility that chronic GTP treatment can reduce HPA axis hyperactivity in response to stress
Xu et al. 2009 [53]	Curcumin	Curcumin (5, 10, and 20 mg/kg, p.o.) or imipramine (10 mg/kg, i.p.)	Sprague-Dawley rats submitted to chronic stress	Curcumin reversed impaired spatial memory under conditions of chronic stress	Normalizing corticosterone levels and downregulating pCaMKII and glutamate receptor levels
Assunção et al. 2011 [58]	Green tea/epigallocatechin	19-month-old rats were fed with GT since age of 12 months	Wistar rats	Spatial learning abilities of GT-treated rats were significantly improved	Scavenging of free radicals

Table 3: Continued.

Reference	Polyphenol	Treatment	Model	Effect on mental health/behavior	Proposed molecular mechanism(s)
Xu et al. 2010 [61]	Green tea/epigallocatechin	GT polyphenols were administered orally to rats from 4 to 8 weeks after experimentally induced cerebral hypoperfusion (400 mg/kg per day or 100 mg/kg)	Wistar rats	Inhibited cognitive impairment Improved spatial learning and memory deficits induced by chronic cerebral hypoperfusion	Free radical scavenging and antioxidative properties of GT polyphenols
Bouayed et al. 200 [67]	Chlorogenic acid (Mirabelle)	20 mg/kg	Mouse model of anxiety	Decreased in anxiety-related behaviours	Anxiety is reduced by activation of the benzodiazepine receptor
Vignes et al. 2006 [68]	Green tea/epigallocatechin	Acute administration—drugs injected intraperitoneally in a volume of 100 μL per 20 g of body weight	Swiss OF1 mice	Anxiolytic effect in the elevated plus maze and passive avoidance tests	Anxiolytic effect could result from an interaction of the given polyphenols with GABAA receptors
Barros et al. 2006 [69]	Vaccinium berries	Water with berry extract over 30 days (0.6–1.0 mg/kg/day or 2.6–3.2 mg/kg/day)	Swiss mice	Decreased DNA damage in hippocampal tissue Anxiolytic effect in open-field test	Protective effect against free radical-induced DNA damage in the brain
Hou et al. 2010 [70]	Flavanols (Gingko biloba extract)	*In vivo:* 50 mg/kg per day for 4 months *In vitro:* 24 h incubation	*In vivo:* APPswe/P1D9 transgenic mice and wildtype *In vitro:* primary neuronal cells	*In vivo:* increased levels of BDNF in hippocampus; anti-depressant effects in wt mice *In vitro:* flavonols dose-dependently restored BDNF expression compared to vehicle control; increase of pCREB through glutamate-evoked activation	Increase in BDNF and glutamate-evoked activation of pCREB
Chen et al. 2010 [71]	Green tea/epigallocatechin	GTEs and EGCG 0.1 g/d	Wistar rats	Improved the cognitive impairments induced by stress	Involved with changes in plasma levels of catecholamines, 5-hydroxytryptamine, cytokines and expression of metallothioneins
Singal et al. 2004 [72]	Green tea/epigallocatechin	1. 50 μg i.p. of endotoxin LPS in 250 μL of pyrogen-freesaline 2. Green Tea Extract (GTE - 10, 25, 50, and 100 mg/kg)	Albino laca mice	Reversal of LPS-induced immobility	COX-2 inhibition by GTE

Table 3: Continued.

Reference	Polyphenol	Treatment	Model	Effect on mental health/behavior	Proposed molecular mechanism(s)
Sanmukhani et al. 2011 [73]	Curcumin	Acute study: treatment 24, 5, and1 h before test: curcumin 50 mg/kg, 100 mg/kg, vehicle, fluoxetine, and imipramine control Chronic study: treatment for 14 days, same doses as in acute	Acute study: Swiss albino mice Chronic study: Wistar rats	Antidepressant-like effects in FST and TST at 100 mg/kg in acute and chronic study	Increases neural levels of serotonin, noradrenaline and dopamine possibly via inhibition of MOA activity
Xu et al. 2006 [74]	Curcumin	Curcumin (2.5, 5, and 10 mg/kg, p.o.) or imipramine (10 mg/kg, i.p.)	Sprague–Dawley rats submitted to chronic stress	Curcumin reversed the effects of chronic stress on behaviour	Effects of curcumin on the behavioural deficits induced by chronic stress may be related to modulating effects on the HPA axis; increased in BDNF and pCREB proteins in specific brain regions may also be related
Wu et al. 2006 [75]	Curcumin	Diet with and w/o curcumin (500 ppm) for 4 weeks	Sprague-Dawley rats submitted to fluid percussion injury	Curcumin-fed injured animals had lower levels of oxidized proteins and counteracted cognitive impairment	Modulation of BDNF-system which acts on CREB and synapsin

properties mediated through inhibition of the expression of inflammatory cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), inhibition of activities of the transcription factor nuclear factor-$\kappa\beta$ (NF-$\kappa\beta$), and increased expression of the neurotrophic factor IGF-1 which may increase neural plasticity and neurogenesis [78]. Increases of these inflammatory cytokines and the transcription factor NF-$\kappa\beta$ have been associated with the development of mental health disorders and inhibition of these cytokines and NF-$\kappa\beta$ has been suggested as a method of treatment [79–82].

A number of polyphenols have been shown to have antidepressant-like effects as well. As was the case with polyphenols mediating anxiolytic effects, a variety of different mechanisms have been implicated for the antidepressant-like effects of different polyphenols. *Ginkgobiloba* extract contains many polyphenols including the flavonols quercetin, kaempferol, and isorhamnetin and has been shown to have antidepressant-like effects that could be due to its properties of increasing BDNF which would increase neuronal survival and plasticity or due its increase of pCREB through glutamate-invoked activation which would enhance synaptic strength and neuronal plasticity [70]. The ginkgo biloba flavonols quercetin and kaempferol also share the property of inhibiting the action of MOA's on serotonin and catecholamines with other polyphenols such as berry anthocyanins**,** the flavone apigenin in celery and the stilbene *trans*-resveratrol found in red wine [76, 77]. This MOA inhibition may contribute to antidepressant effects as elevated MOA activity is also linked to the etiology of depressive disorders [77]. The polyphenols in cocoa have also been shown to have antidepressant effects and to reduce the symptoms of chronic fatigue syndrome, possibly due to their anti-inflammatory properties mediated by inhibition of proinflammatory cytokines [48, 49, 83].

Green tea contains many polyphenols belonging to several molecular subclasses, including chlorogenic acid, pyrogallol, caffeic acid, (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), epicatechin-3-gallate (ECG), and (–)-epicatechin [71, 76, 84]. Green tea extracts have been shown to have antidepressant-like effects with multiple proposed mechanisms including antioxidant activity, regulation of adrenocorticotrophic hormone (ACTH) serum levels, inhibition of prostaglandins and inflammatory cytokines, increase of anti-inflammatory cytokines, and inhibition of MAO activity [52, 58, 71, 72, 85].

As can be seen in the examples of polyphenols discussed, many mechanisms of action for the antidepressant-like and anxiolytic behavioural effects of polyphenols show overlap between polyphenols and suggest shared cellular pathways that may interact and modulate each other.

A more detailed examination of a case in which the mechanisms of action for the antidepressant effects of the polyphenol have been elucidated may provide a clearer illustration of such modulation and interaction. The mechanisms of action for the polyphenol curcumin have been investigated in detail and provide an illustrative example of how polyphenol molecular and cellular pathways may interact with those established for conditions affecting neurogenesis and mental health such as stress and depression.

3.2. Proposed Curcumin Mechanisms of Antidepressant Action. The antidepressant effects of curcumin, a polyphenol which is the active ingredient in the spice turmeric, were investigated using the standard testing paradigms used to screen antidepressant drugs. The forced swimming test (FST) and tail suspension test (TST) are two standard animal models of depressive behaviour measurement in which the degree to which the animal ceases to struggle and becomes relatively passively immobile is used to assess depressive behaviour and both have been used as accurate methods for screening antidepressant properties of drugs when used in conjunction with locomotion-screening tests to distinguish any central nervous system stimulant properties [86, 87]. Curcumin was shown to dose-dependently reduce despair immobility behaviour in the TST and FST to a degree comparable to commonly prescribed tricyclic and selective serotonin re-uptake antidepressants [50, 73]. Further in-depth behavioural comparisons combined with *in vitro* studies revealed that curcumin increased neural levels of serotonin, noradrenaline, and dopamine and inhibited MOA activity with molecular mechanisms that appear to differ from typical tricyclic and selective serotonin reuptake antidepressants [50, 73].

However, there were additional cellular mechanisms and antidepressant effects of curcumin revealed by a different animal model of depression which was based on prolonged stress and proved to be more sensitive to these diverse mechanisms. This stress model allowed a more detailed examination of curcumin's multiple mechanisms of action and reflected their complexity, illustrative of the diversity of mechanisms through which a single polyphenol can exert effect. Significantly, several of these pathways also act to upregulate neurogenesis, offering additional context for investigation of the cellular and molecular mechanisms by which other polyphenols exert antidepressant-like and anxiolytic effects.

Prolonged stress has been shown to have a role in the etiology of depression in humans and the chronic unpredictable stress in animal models of depression has been shown to parallel the anatomical, neuroendocrine, and behavioural aspects of depression in humans [74, 88, 89]. Thus the chronic unpredictable stress animal model has been productive in elucidating cellular and molecular mechanisms involved in the behavioural phenotype of depression and shown that antidepressant effects may be related in part to modulating the responses of the hypothalamus-pituitary-adrenal (HPA) axis [74, 89–92] (Figure 2).

Curcumin has been shown to have the ability to block or reverse the stress-induced changes typical of HPA axis dysfunction to a level comparable to a typical tricyclic antidepressant, including behavioural escape-response performance deficits, physiological changes in the adrenal gland, increases in corticosterone levels, reduced glucocorticoid receptor (GR) mRNA expression, decreased levels of BDNF, and reduced levels of phosphorylated CREB [74] (Figure 3).

Curcumin effects on restoring GR mRNA expression are particularly important when viewed in the context of HPA axis function. When functioning normally, activation of the HPA axis begins with the perception of physical or

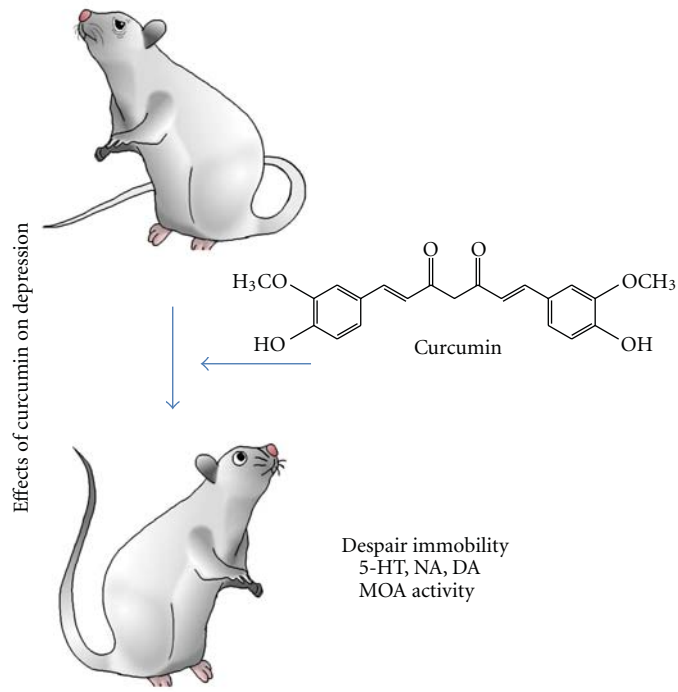

Figure 2: *Effects of curcumin on depression.* Administration of curcumin in rodent models of depression has been shown to ameliorate depressive-related behaviours, with decreased despair immobility, associated to increased levels of the neurotransmitters serotonin (5-HT), noradrenaline (NA), and dopamine (DA), and decreased activity of the enzyme monoamine oxidase (MOA).

psychological stress and results in stimulation of the adrenal cortex to release glucocorticoids (corticosterone in rodents and cortisol in humans). Glucocorticoids bind with the GR to regulate a number of systems in the body, including a self-limiting feedback mechanism acting to stop stimulation of glucocorticoid secretion and a GR feedback mechanism which lowers the production of inflammatory cytokines by inhibiting the activity of transcription factor NF-$\kappa\beta$ [93, 94].

Thus glucocorticoids normally operate their own negative-feedback mechanism to stop HPA axis activation and to inhibit inflammation response; yet this negative feedback effect on both systems has been shown ineffective in depression where high levels of cortisol co-exist with high levels of proinflammatory cytokines [82, 95, 96]. This apparent lack of responsiveness to the feedback mechanism is called glucocorticoid resistance and is attributed to dysfunction of GR signalling rather than to elevated glucocorticoid levels [82, 94, 97, 98].

Curcumin's antidepressant-like effects in increasing levels of stress-reduced GR mRNA expression may be one of the keys in restoring normal levels of GR signalling for GR-modulated feedback mechanisms and, coupled with curcumin's increase of phosphorylated CREB, may also have implications for restoration of neurogenesis. A recent study has shown that antidepressants increase GR expression and induce GR nuclear translocation and transcription activities through pathways involving cyclic AMP and protein kinase A (PKA) which cumulatively result in increased levels of neurogenesis [90]. This might elucidate the mechanism of antidepressant effects established by earlier works in which antidepressant drugs were shown to restore stress-reduced levels of neurogenesis and GR expression, offering further context for understanding Curcumin's effects on GR expression [19, 29, 98].

Curcumin has also been shown to inhibit NF-$\kappa\beta$ activation pathways [99, 100]. Activated NF-$\kappa\beta$ moves into the nucleus where it disrupts GR signalling, preventing proper functioning of GR feedback mechanisms [94, 97]. Activation of NF-$\kappa\beta$ also upregulates inflammatory cytokines which can have a number of effects also shown in the etiology of depression, including further dysregulation of the HPA axis, metabolism of monoamine neurotransmitters through elevated MOA activity, reduced neuronal plasticity, and reduced neurogenesis [77, 89, 94]. Therefore inhibition of NF-$\kappa\beta$ activation by curcumin has a number of antidepressant-like effects on the cellular level, preventing the reduced monoamine levels due to the elevated MOA activity, NF-$\kappa\beta$ disruption of GR signalling, and the upregulation of inflammatory cytokines whose elevated levels would otherwise inhibit neurogenesis and further disrupt GR signalling [89, 90, 101].

Curcumin administration in chronically stressed rats increased hippocampal neurogenesis via modulation of the HPA axis and upregulation of BDNF and 5-HT 1A

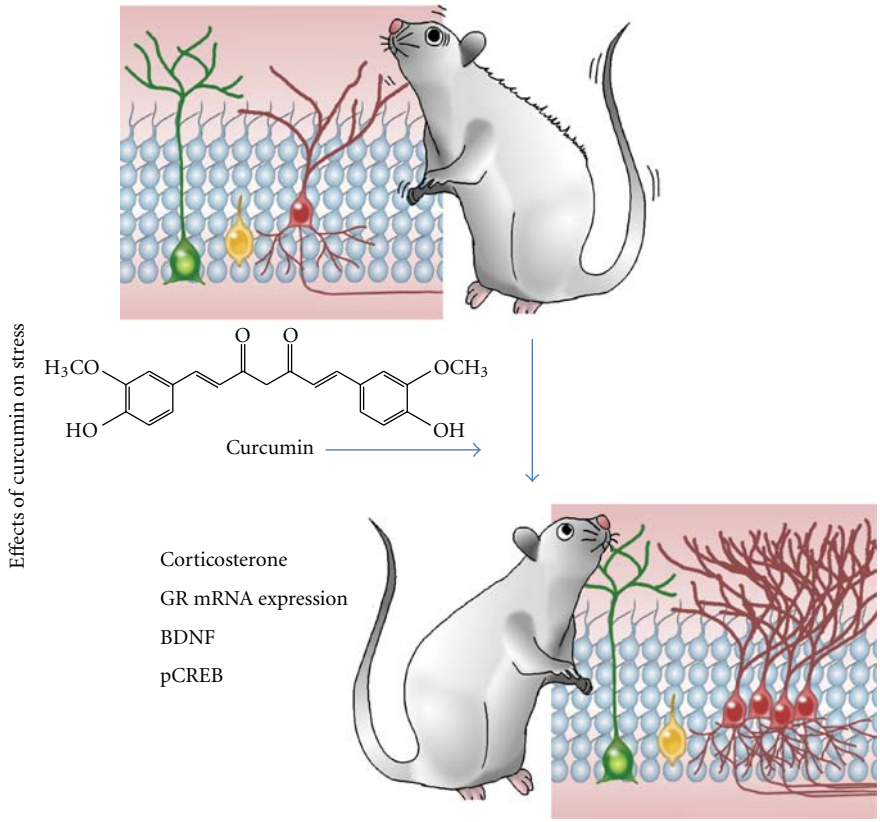

Figure 3: *Effects of curcumin on stress.* Administration of curcumin in rodent models of stress has been found to reduce stress-related behaviours, with decreased levels of the stress hormone corticosterone and increased levels of glucocorticoid receptor (GR) mRNA expression, brain-derived neurotrophic factor (BDNF), and phosphorilated cyclic AMP-response element DNA-binding protein (pCREB). Additionally, the decrease in AHN observed after exposure to stress has been found to be reversed following administration of curcumin.

receptors in the hippocampus [40]. Curcumin effects in restoring BDNF protein levels is important in the context of BDNF signalling in adult neurogenesis, shown to increase neuronal differentiation, survival, and dendritic arborisation [29, 102, 103].

However, modulation of serotoninergic signalling has proven fundamental to curcumin antidepressant and neurogenic effects. The antidepressant-like effects of curcumin are mediated in part through direct agonistic functions on 5-HT 1A and 5-HT 1B receptors and antagonistic effects on 5-HT 2C receptors [104]. The effects mediated by curcumin upregulation of 5-HT 1A receptors may be even more critical to neurogenesis as 5-HT 1A, 5-HT 1B, and 5-HT 2A receptors have been proven necessary for adult hippocampal neurogenesis and 5-HT 1A receptor antagonists significantly decrease cell proliferation in the hippocampus [105, 106]. The increased serotonin signalling resulting from upregulation of 5-HT 1A receptors and agonist effects on 5-HT 1A and 5-HT 1B receptors is also very pertinent to the antidepressant effects of curcumin as increased serotonin transmission has profound antidepressant effects as well as being associated with an increase in adult neurogenesis [105].

The effect of curcumin on 5-HT receptors also modulates GR signalling through serotonergic signalling activation of PKA. Activated PKA translocates to the nucleus where it can undergo protein-protein interactions with GR that optimise GR-DNA binding, stimulating GR signalling [94, 107]. In further interpathway modulation, activated PKA can also inhibit NF-$\kappa\beta$ from interfering with GR-DNA binding, thus increasing GR transcription activity through modulation of an additional pathway [94, 108].

Using the chronic unpredictable stress animal model of depression in combination with *in vitro* studies has provided details on many of the mechanisms through which curcumin exerted the antidepressant-like behavioural effects shown in FST and TST studies. As has been the case with many polyphenols, curcumin was shown to have multiple mechanisms of action that affect different cellular and molecular pathways, many of which have been shown to interact and modulate one another. Several of these pathways also act to upregulate neurogenesis both directly and indirectly, which is a further way in which curcumin may exert its antidepressant-like effects as some antidepressant drugs have been shown to exert neurogenesis-dependent behavioural effects [30, 109, 110].

4. Conclusion

Given the high prevalence of depression and anxiety in modern societies, unravelling the neurobiological basis of

these psychiatric conditions is one of the most challenging pursuits of science in the present days. The development of effective treatments for depression is likely to emerge from the identification of the mechanisms underlying its pathophysiological components [111]. In this context, studies on the effects of dietary polyphenols and elucidation of the mechanisms by which they exert these effects may play an important role as promising interventions in the field of mental health.

The present work discussed some recent findings addressing the effects of different dietary polyphenols on models of anxiety and depression and proposed mechanisms underlying these effects with particular focus on those related to AHN. As a whole, there is growing evidence for a positive effect of these plant- and food-derived components on behaviours related to depression and anxiety. The dietary polyphenols tested so far, however, appear to exert their effects through different and specific molecular pathways [112, 113], suggesting that the action of polyphenols is not uniform but highly specific. In addition, it should be noted that generalizing the effects of polyphenols as positive is still nonconsensual. For instance, on the one hand, the administration of 5,7,3′-trihydroxy-3,4-dimethoxyflavone (a flavonoid similar to kaempferol) led to significantly inhibited neurite outgrowth, and on the other hand, the polyphenol chlorogenic acid stimulated it [66].

Indeed, polyphenols are a very heterogenous group of natural chemical compounds that significantly differ with regard to their chemical properties. Studies have shown that the degree of polymerization and inherent properties such as molecular polarity can determine their potency in a range of actions, including antioxidation and anti-inflammation [83, 114]. These properties also affect the polyphenol level in different tissues, including the brain [36]. This diversity may explain why the same dietary source containing multiple polyphenols may show multiple effects as the effects of a single polyphenol may be either additive or altered when acting in the presence of other polyphenols [76, 78]. This synergistic effect should be considered in future investigations aiming to find effective interventions in the field of mental health.

Some polyphenols have been shown to influence anxiety- and depressive-like behaviours as well as AHN in different *in vivo* and *in vitro* models. However, further studies are necessary to clarify the potential of polyphenols in treating depression and anxiety. It is worth noting that a significant portion of the studies focusing on the effects of polyphenols on the hippocampus did not address AHN and used learning and memory paradigms as primarily models for studying neurodegenerative disorders like Alzheimer's disease. Given that AHN represents one of the most remarkable features of the central nervous system in terms of plasticity, more studies investigating the effects and mechanisms of polyphenols on hippocampal cell proliferation and differentiation may identify additional specific neurobiological targets for psychiatric-related behaviours. In addition, AHN has also been shown to correlate with anxiety [115–118] as well as emotional—and not only cognitive—learning and memory processes [119–121]. Also, it is clear that anxiety disorders present an important emotional mnemonic component [122]. Given the role of AHN in emotional learning and memory, future investigations that consider the potential effects of dietary polyphenols on depression and anxiety could expand our current understanding of these psychiatric conditions and provide evidence of their potential as tools for effective intervention.

Key Messages

(i) The adult hippocampus is capable of generating new neurons, but how these newly generated cells affect mental health and the different factors that can regulate AHN are still debated.

(ii) Polyphenol effects and bioavailability vary greatly due to their differing chemical, physical, and structural properties.

(iii) Plant-or food-derived polyphenols are widely known for their biological properties in enhancing cognition and neuroprotection in models of neurogenerative conditions, but some polyphenols can also increase cell proliferation and differentiation in models of anxiety and depression.

(iv) Different polyphenols exert their effects on AHN via different mechanisms of action, such as by activating the MAP kinase pathway or stimulating the expression and release of neurotrophic factors.

(v) Dietary polyphenols have been shown to affect mental health, having anxiolytic effects comparable to anxiolytic benzodiazepine drug chlordiazepoxide and antidepressant-like effects comparable to those of the SSRI antidepressant fluoxetine and TCA imipramine.

(vi) The anxiolytic and antidepressant-like effects of polyphenols are mediated through multiple molecular and cellular pathways, which interact and modulate one another.

Authors' Contribution

Nicole Cavegn and Alina Nix contributed equally to this work.

Conflict of Interests

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in, or financial conflict with, the subject matter or materials discussed in the paper, apart from those disclosed. No writing assistance was utilized in the production of this paper.

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Research Article

Investigation of Effects and Mechanisms of Total Flavonoids of *Astragalus* and Calycosin on Human Erythroleukemia Cells

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Flavonoids are found in most parts of plants and have been shown to have multiple biological activities such as anticancer, anti-inflammation, antibacteria, antivirus, and immune-stimulation. Existing data showed that the total flavonoids of *Astragalus* (TFA) can provide biological system with resistance to injury and can possess antimutagenic, atherosclerotic inhibition, and other biological effects. This study investigated the effects of TFA and calycosin (a compound isolated from TFA), on apoptosis induction, and cell cycle of human erythroleukemia cell line K562 by an array of techniques, including proliferation (MTT), PI staining, Annexin V/PI double staining, and RT-PCR. The experimental data showed that TFA and calycosin could inhibit the proliferation of K562 cells. The 50% inhibiting concentrations of TFA and calycosin were 98.63 μg/mL and 130.32 μg/mL, respectively. However, TFA and calycosin could not induce apoptosis in K562 cells, but could increase the number of the cells in the G_0/G_1 phase. The level of cyclin D1 mRNA in K562 cells decreased after the treatment with TFA and calycosin. This study provides new insights into the functional mechanism of total flavonoids of *Astragalus* and calycosin on human erythroleukemia cells.

1. Introduction

Leukemia is a malignant tumor causing serious harm to human health, especially because of its prevalence among children [1–3]. One of the treatments being explored involves the use of total flavonoids of *Astragalus* (TFA). TFA extracted from the Mongolia *Astragalus* are the main active antioxidant ingredients for scavenging free radicals [4]. Series of studies have shown that TFA can offer biological system resistance to injury, and have antimutagenic, antitumor, inhibition of atherosclerosis and other biological effects [4–9]. Furthermore, calycosin is one of the main chemical components isolated from the TFA, and it is not clear whether calycosin can exert inhibiting influence on tumor cells or not, since there have been no reports on the mechanism for the anticancer activity of calycosin. To provide some mechanistic understanding on how TFA and calycosin affect biological system, we carry out in vitro experiments to study the antitumor activity of TFA and calycosin. We use the K562 red leukemia cell lines as the target biological system to investigate the effects and mechanisms of TFA and calycosin on tumor cells. With MTT method, we can detect the effect of different concentrations of TFA and calycosin on K562 cells and study their influences on K562 cell apoptosis, cell cycle, and cyclin D1 mRNA level. This study provides new insights into the functional mechanism of TFA and calycosin on human erythroleukemia cells.

2. Materials and Methods

2.1. Reagents. TFA and calycosin were isolated and identified by the Department of Biochemistry at the Chinese People's Liberation Army Hospital. They were sterilized by the high-pressure sterilization process, dissolved with appropriate amount of DMSO, and diluted with culture medium to the required concentrations. MTT cell proliferation and cytotoxicity assay kit, Annexin V-FITC and cell cycle kit were obtained from the Keygen Biological Technology Development. RPMI1640 culture medium and fetal calf serum were purchased from Gibco. RT-PCR extraction kit and BioEasy SYBR Green I Real-Time PCR Kit Manual were

purchased from Beijing Shang Bai Company. Ribonuclease inhibitor was obtained from Biological Engineering, and M-MLV Reverse Transcriptase was purchased from Promega Biological Technology.

2.2. Apparatus. The following experimental apparatus were used in this study: CO_2 Cell constant temperature incubator (Sanyo), inverted microscope (Olympus), Multiskan MK3 Microplate reader (Thermo Lab), Clean bench (Beijing East Hall Instruments), Flow Cytometry (US Backman Kurt), Line-gene Fluorescence quantitative PCR detection system (Hangzhou Rike), HH.W21-Cr Thermostatic water tank (Beijing Changan Scientific Instruments), and MS1/MS2 Mini oscillator (Guangzhou Instrument and Dental Laboratory Technology).

2.3. Cell Lines and Cell Culture. Human erythroleukemia cells K562 (from the Chinese PLA General Hospital hematology Lab) were cultured in the 37°C, 5% CO_2 incubator with RPMI1640 culture solution, which contains 10% fetal calf serum. The culture solution was changed every 2-3 days. Experiments began when the cells showed a logarithmic growth.

2.4. Evaluation of the Effect of TFA and Calycosin on K562 Cell Proliferation. K562 cells are inoculated on a 96 orifice plate by 1×10^4 cells per well, and 10 μL of different concentrations of TFA and calycosin in the same medium were added to the wells. The four final concentrations of the TFA samples set are 20, 50, 100, and 200 μg/mL, respectively, and the final five concentrations of the calycosin sample set are 20, 50, 100, 200, and 400 μg/mL, respectively. Each concentration has six repeated wells and a blank control well with only 100 μL of the RPMI1640 medium. Testing samples in the plate were cultured in an incubator and after 24 h, 20 μL MTT was added to each well. After an additional 4-hour incubation at 37°C, 5% CO_2, the supernatant was aspirated, and 150 μL DMSO was added with proper mixing to terminate the reaction. The optical density (OD) value at 450 nm wavelength was measured, based on which the cancer cell growth inhibition rate was calculated. The calculation formula for the cell growth inhibition is as follows:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{A450 of Sample Group}}{\text{A450 of Control Group}}\right) \times 100\%. \quad (1)$$

The calculation method for median inhibitory concentration (IC50) is based on cell survival rate on the logarithm of dose map.

2.5. Evaluation of the Effect of TFA and Calycosin on Apoptosis of K562 Cells by Annexin V-FITC Double-Staining Method. K562 cells, which are in the logarithmic growth phase, were inoculated in 25 mL culture bottle with 1×10^7/L cell density. For both the TFA and calycosin groups and the control group (no drug), cells were collected and cultivated for 2, 4, 6, 12, and 24 h. Selection of the concentrations of TFA and calycosin is around the median inhibitory concentration. The cells were measured by flow cytometry and analysed for apoptosis population post marking the Annexin V and PI.

2.6. Evaluation of TFA and Calycosin Effects on K562 Cell Cycle by Flow Cytometry. Inoculate 5×10^6 cells in 5 cm^2 culture bottles; add TFA and calycosin at the concentrations of their IC50. After cultured for 24 hours, wash the cells twice with PBS, centrifuged at 2000 rpm/min for 5 min. After using PBS to blow cells into suspension completely, then fix the cells by 70% ethanol in volume at 4°C for 48 h, wash off ethanol before staining with PBS, centrifuge at 2000 rpm/min for 5 min and wash twice; add 100 μL RNase A and maintain in 37°C water bath for 30 min, and then add 400 μL PI staining and protect from light for 30 min at 4°C. Evaluate the cells by flow cytometric method and evaluation the changes of cell cycle. Calculate the cell proliferation index according to the formula of PI% = $(S + G_2/M)/(G_0/G_1 + S + G_2/M)$.

2.7. Evaluation of TFA and Calycosin on Cyclin D1 mRNA Levels by RT-PCR. The upstream and down primers are 5-CCTCG GTGTCCTA CTTCAAAT-3 and 5-TCCTCGCACT TCTGTTCCT-3, respectively. Inoculate 4×10^5/L cells in 25 cm^2 culture bottles. Add different concentrations of TFA and calycosin, with the TFA final concentrations at 50 and 100 μg/mL, and set a blank control well, incubated in the 37°C, 5% CO_2 incubator for 48 h. Attrite cells in liquid nitrogen add 1 mg cracking liquid RL for every 50–100 mg cell mass. Use a homogenizing instrument to homogenate the cells. Sample size should not exceed 1/10 of the lysate RL volume. Apply the RT-PCR technique to detect cyclin D1 mRNA levels in K562 cells.

2.8. Statistical Processing. The experimental data were represented by $\bar{x} \pm s$ and analyzed by single-factor analysis of variance t-test using SPSS13.0 statistical analysis software. Median inhibitory concentration (IC50) was determined using probability unit method (Probit), and the cell cycle using two-sample t-test method.

3. Results

3.1. The Inhibition of TFA and Calycosin on Proliferation of K562 Cells. The data showed that both TFA and calycosin have significant effects on the proliferation of K562 cells after treatment for 24, 48, and 72 h (Tables 1 and 2). TFA and calycosin showed dose-dependent inhibition of K562 cells proliferation. The median inhibitory concentrations (IC50) of TFA on K562 cells after treated for 24, 48, and 72 h are 98.63, 87.90 and 63.10 μg/mL, respectively. The median inhibitory concentrations (IC50) of calycosin on K562 cells after treated for 24, 48 and 72 h are 130.32, 123.03 and 122.18 μg/mL, respectively.

3.2. The Effect of TFA and Calycosin on Apoptosis of K562 Cells. On evaluation of the experimental results of apoptotic and necrotic cells in flow cytometry, no obvious changes in apoptosis of K562 cells after treated with TFA (100 μg/mL)

Table 1: The inhibition of TFA on proliferation of K562 cells.

TFA concentration (μg/mL)	24 h		48 h		72 h	
	OD value	Inhibition rate %	OD value	Inhibition rate %	OD value	Inhibition rate %
Control group	0.49 ± 0.02	0.5	0.59 ± 0.02	0.6	0.62 ± 0.04**	0.7
20	0.46 ± 0.03	5.8	0.46 ± 0.04**	22.7	0.48 ± 0.01**	23.5
50	0.42 ± 0.04**	14.9	0.40 ± 0.03**	31.4	0.32 ± 0.01**	48.9
100	0.28 ± 0.04**	44.1	0.34 ± 0.06**	42.8	0.24 ± 0.01**	62.4
200	0.06 ± 0.03**	88.5	0.16 ± 0.04**	72.9	0.09 ± 0.02**	85.0

Data expressed as mean $\bar{x} \pm s$, $^{**}P < 0.01$ compared with control group.

Table 2: The inhibition of calycosin on proliferation of K562 cells.

Calycosin concentration (μg/mL)	24 h		48 h		72 h	
	OD value	Inhibition rate %	OD value	Inhibition rate %	OD value	Inhibition rate %
Control group	0.48 ± 0.01	0.6	0.56 ± 0.02	0.1	0.70 ± 0.02**	0.01
20	0.41 ± 0.02	15.1	0.49 ± 0.03**	18.8	0.56 ± 0.02**	19.7
50	0.34 ± 0.02**	30.2	0.41 ± 0.05**	31.2	0.48 ± 0.01**	30.4
100	0.27 ± 0.01**	45.0	0.34 ± 0.02**	43.2	0.39 ± 0.03**	43.3
200	0.21 ± 0.02**	57.9	0.25 ± 0.01**	58.1	0.29 ± 0.01**	57.9
400	0.15 ± 0.01**	67.9	0.17 ± 0.01**	71.2	0.19 ± 0.01**	72.0

Data expressed as mean $\bar{x} \pm s$, $^{**}P < 0.01$ compared with control group.

for 2, 4, 6, 12, and 24 h were observed. Also no obvious changes in apoptosis of K562 cells after treated with calycosin (1300 μg/mL) for 2, 4, 6, 12, and 24 h were observed.

3.3. The Effect of TFA and Calycosin on Cell Cycle of K562 Cells. TFA (at 50 and 100 μg/mL) and calycosin (at 60 and 130 μg/mL) can significantly block the growth cycle of K562 cells in the stage of G_0/G_1 as compared with the control group. The cells in the phase of G_0/G_1 markedly increased and the cell in the stage of S markedly decreased (Tables 3 and 4).

3.4. The Effect of TFA and Calycosin on Cyclin D1 mRNA Level in K562 Cells. The cyclin D1 mRNA level was 3.58 ± 0.63 for the control sample. The cyclin D1 mRNA levels for the 50 and 100 μg/mL TFA treated samples were 2.23 ± 0.42 and 1.72 ± 0.21, respectively. Similarly, the cyclin D1 mRNA level was 3.31 ± 0.71 for the control sample, the cyclin D1 mRNA levels for the 50 and 100 μg/mL calycosin-treated samples were 2.27 ± 0.33 and 2.02 ± 0.25, respectively. Cyclin D1 mRNA level of K562 cells was significantly reduced after treated with either TFA or calycosin.

4. Discussions

Leukemia is one of the malignant tumors in hematopoietic system. It counts up to 5% in the total morbidity of cancers and with high mortality rate. K562 is the erythroleukemia cell line that is derived from human chronic myelogenous leukemia. Up to now, chemotherapy is still the most important and essential method in treating leukemia [10, 11]. The traditional chinese medicine (TCM) has made positive achievements in treating leukemia as well [12–14]. Many herbal medicines provide evident and curative effects [15–18].

Astragalus mongholicus Bunge is a kind of herbal medicine with a long history and extensive clinical applications. TFA is the active component isolated from *Astragalus* with peroxyl radical scavenging capacity [4]. Studies showed that TFA have anti-injure and antimutation activities [4, 5, 7, 8]. Furthermore, TFA have a significant inhibitory effect on human hepatocellular carcinoma BEL-7402 cells in vitro [6].

Because of the difference in DNA content, the cell cycle is divided into discrete phases: G_0/G_1, S, and G_2/M. Based on the experimental results, we calculate the distribution percentage of the studied cells in each phase using a software. It is known that tumor cells in the S phase is higher than normal cells [19], and we also observed a large number of cells are in S phase with active DNA synthesis. The experimental results further show that TFA causes the cells in the G_0/G_1 phase to increase, particularly cells in the G_1 phase increased, and the cells in the S stage were significantly reduced, which indicated that the proliferative activity of K562 cells had weakened upon treatment.

Cyclin D is a sensor for extracellular growth signals. The existence of growth factors lead to continuous expression of cyclin D. Cyclin D1 begins to express between the G_0 and G_1 phases and participates in the control of the G_1 phase by binding to cyclin-dependent kinases 4 (CDK4) and 6 (CDK6), leading to the progression of the cells into the S phase to proliferate. Deregulation of the cell cycle will be induced by uncontrollable expression of cyclin D1 or CDK4/CDK6. Studies indicate that many tumors have overexpression of cyclin D1, such as mantle cell lymphoma (CML), nonsmall cell lung cancer (NSCLC), breast cancer, head and neck cancer, and esophageal cancer [20, 21].

Table 3: The effect of TFA on cell cycle of K562 cells.

Groups	G_0/G_1 stage (%)	S stage (%)
Control group	31.79 ± 2.98	63.59 ± 2.47
TFA (50 μg/mL)	36.32 ± 2.56	55.41 ± 1.65**
TFA (100 μg/mL)	47.52 ± 1.73**	52.82 ± 1.33**

Data expressed as mean $\bar{x} \pm s$, $^{**}P < 0.01$ compared with control group.

Table 4: The effect of calycosin on cell cycle of K562 cells.

Groups	G_0/G_1 Stage (%)	S Stage (%)
Control group	31.61 ± 2.67	70.09 ± 2.08
Calycosin (60 μg/mL)	40.89 ± 2.56	57.47 ± 1.89**
Calycosin (130 μg/mL)	46.33 ± 2.88**	54.79 ± 1.94**

Data expressed as mean $\bar{x} \pm s$, $^{**}P < 0.01$ compared with control group.

Cyclin D1 is also an important regulatory protein for cell cycle. The level and activity of cyclin D1 reach to the peak at different phases with regular wave. During G_0, the cell is subjected to stimulation by extracellular mitogens and subsequently expresses cyclin D1 in the early part of G_1. Cyclin D1 participates in the control of the G_1 phase by binding to CDK4/CDK6. Cyclin D1-CDK4 complexes phosphorylate retinoblastoma1 (RB) by the array of LXCXE at its N-terminal. Upon RB phosphorylation by CDK4/6, RB dissociates from E2F-DP1 heterodimer, leading to the inactivation of its suppressor effect on DNA synthesis and the subsequent progression of the cell into S phase from G_1 [22]. There are few studies on the effects of cyclin D1 on leukemia either domestically or abroad. In this study, we find that TFA and calycosin can notably reduce the expression of cyclin D1, which is likely related to the effects of TFA and calycosin in suppressing the propagation of K562 and retaining more cells in G_0/G_1 phase.

In conclusion, TFA and calycosin had effects on inhibition of the proliferation of K562 cells. They are also attributed to arrest them in the G_0/G_1 phase and induce decreased cyclin D1 mRNA.

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Review Article

Protective Mechanisms of Green Tea Polyphenols in Skin

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Skin is frequently exposed to a variety of environmental, chemical, and genotoxic agents that contribute to disease and carcinogenesis. Ultraviolet light (UVR) is the main external stress that leads to immunosuppresion, oxidative stress, premature aging, and tumor formation. Scientists and health professionals emphasize the importance of prevention strategies to circumvent such unfavorable outcomes. Plant polyphenols are a promising approach to disease prevention and treatment. Green tea is an abundant source of plant polyphenols that exhibit significant antioxidant, chemopreventive, and immunomodulatory effects in protecting the skin.

1. Introduction

Ultraviolet radiation (UVR) is a major environmental source of damage to the skin. Its effect on the skin's biology and immune system plays a major role in photoaging, inflammation and carcinogenesis [1]. Approximately 3.5 million skin cancers are diagnosed annually in the United States and the incidence of nonmelanoma (NMSC) increased dramatically from 1996 to 2006 [2]. The morbidity and economic burden of this malignancy is significant as the estimated total direct costs for treatment of NMSC and melanoma are $1.5 billion and $249 million, respectively [3, 4]. Sun avoidance, regular use of sunscreen, and protective clothing are the recommended methods of preventing UVR-induced damage but patient compliance is a major challenge. For example, a recent study by Buller and colleagues surveyed 4837 adult skiers and snowboarders about their sunscreen use and reapplication. Only 4.4% of adults were compliant with the recommended guidelines of applying sunscreen up to 30 minutes before sun exposure and reapplication every 2 hours [5]. Therefore there is a need to identify additional photoprotection strategies to engage the community at-large about the importance and benefits of sun protection.

In the last few decades there has been a dramatic increase in the use of plant and herbal supplements as people are seeking different methods of disease prevention [6]. Green tea consumption has become a popular trend in western cultures as its beneficial effects in human disease have shown promising results. Scientists searching for alternatives to preventing and treating disease have recognized its powerful beneficial effects in many organ systems. Green tea extracts were found to be effective at suppressing environmentally induced breast cancer [7], inhibiting T lymphocyte expansion in autoimmune diseases [8], and suppressing inflammatory responses in coronary vessels in rodent experiments [9]. Favorable results have also been demonstrated in skin disease and carcinogenesis. Recent *in vitro* and *in vivo* animal and human skin studies also showed its anti-inflammatory, antioxidant, photoprotective, and chemopreventative effects after topical application and oral consumption [10–14]. This review will discuss the chemical properties of green tea that make it effective in skin biology and immunology and how its mechanisms of action play a role in antioxidant, photoprotective and chemopreventative functions in the skin.

2. Background

Polyphenols are naturally occurring chemicals derived from plants, fruits, nuts, and vegetables. They have been proven to

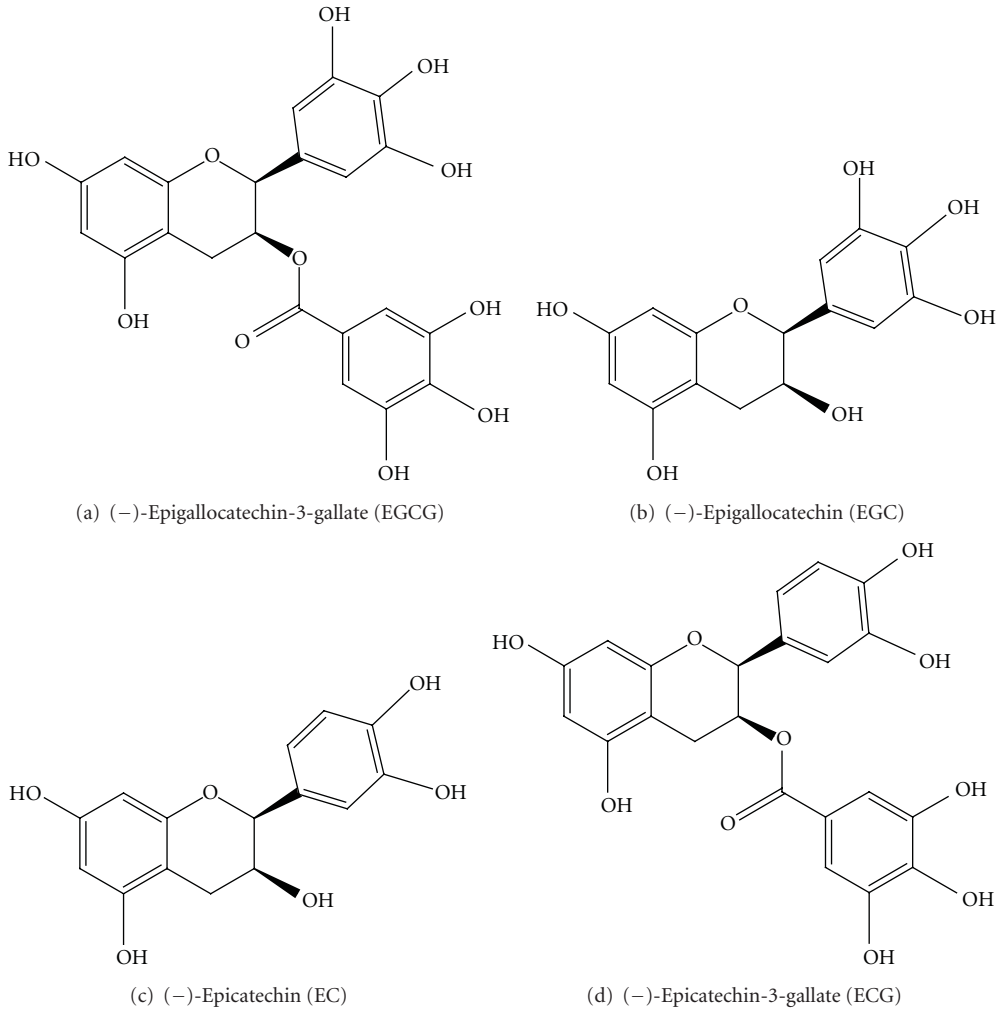

Figure 1: Structure of green tea polyphenols.

have many beneficial health benefits. Being widely abundant and relatively inexpensive, the use of polyphenols is highly attractive to researchers as a strategy for a cost-effective alternative to current pharmacologic therapeutics [15]. Tea is an important dietary source of plant polyphenols and next to water it is the second most commonly consumed beverage in the world. It is produced mainly from a single plant species *Camellia sinensis*. The tea plant originated in Southeast Asia over 4,000 years ago and is currently produced in over 35 countries with China, India, Sri Lanka, and Kenya generating three-quarters of the world's production. The six different types of tea (white, yellow, green, oolong, black, and post fermented teas) are categorized based on the wilting and enzymatic oxidization that takes place during processing [16].

There are three main types of polyphenols (flavonoids, stilbenes, and lignans) that are classified by the number of phenol rings they contain and the binding properties of the ring structures. The phenol rings are comprised of phenyl and hydroxyl group structures that possess anti-inflammatory, immunomodulatory and antioxidant properties [17]. Each class of polyphenols can be further subclassified by the interactions of their respective phenyl rings to carbon, oxygen, and organic acid molecules [18]. This creates the huge diversity of polyphenol compounds that can be found in many naturally occurring food products. Flavonoids are divided into 6 subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols. Majority of the green tea polyphenols (GTPPs) are monomeric flavanols called catechins. The four main catechin compounds are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC) (Figure 1). EGCG is the most abundant and extensively studied catechin with potent therapeutic effects in skin.

3. Skin Damaging Effects of Ultraviolet Light

Sunlight is an important source of energy to sustain life. However, except for vitamin D synthesis, it has several harmful effects to the skin. Solar UVR can be divided into three categories based on wavelength: UVA (320–400 nm),

UVB (280–320 nm), and UVC (<280 nm). High energy short wavelength UVC (<280 nm) and a portion of UVB (280–295 nm) are absorbed by the ozone layer and atmosphere; therefore it does not reach the Earth's surface where it is capable of causing extreme damage to DNA and biomolecular molecules [19]. Longer wavelength UVB (295–320 nm) makes up only 5–10% of atmospheric UVR but it has been implicated in a variety of skin diseases, nonmelanoma and melanoma skin cancers. DNA is a chromophore for UVB and this direct interaction produces cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6–4) mutagenic photoproducts that lead to tumor initiation and tumor promotion [20–22]. UVB also has indirect detrimental effects on the skin's immune system, oxidative stress responses, and photoaging [22, 23].

UVA radiation is far more abundant (90%) and penetrates much deeper into the epidermis and dermis of the skin. It is weakly absorbed by DNA but reacts with other nonDNA chromophores that lead to the formation of ROS which damage DNA, proteins, and lipids in the skin [24–26]. Singlet molecular oxygen produced by UVA targets DNA base guanine producing 8-oxo-7,8-dihydroguanine (8-odHG) which is an important marker of oxidative stress [27, 28]. With the increased use of high-intensity UVA tanning booths and UVB-absorbing sunscreens, human exposure to UVA has become a public health concern [29]. UVA-induced mutagenesis is still an area of debate; however, it is clear it plays a significant role in producing bipyrimidine photoproducts that have genotoxic effects [30]. *In vivo* human studies have also demonstrated the immunosuppressive effects of UVA and its increasing role in carcinogenesis [31]. In order to avoid potential mutations, UVR-induced DNA lesions are repaired by nucleotide excision repair (NER) and base excision repair (BER) mechanisms before DNA replication occurs. Additionally, stress signals created by UVR trigger protective signaling responses in the cell membrane, nucleus, and mitochondria that lead to cell cycle arrest or apoptosis [32–34]. Chronic and excessive UVR exposure overwhelms and depletes these cutaneous defense mechanisms. Therefore, compounds with antioxidant and cell repair potential are promising additions to our sun protection armamentarium.

4. Oral Consumption and Topical Application of GTPP

Human and animal studies using both topical and oral preparations of GTPP have shown significant protective effects against UV-induced skin damage and immunosuppression. As an external organ system, skin allows for direct pharmacological intervention with topical products. This mode of delivery also minimizes the potential for systemic toxicity. Topical application of EGCG in a hydrophilic ointment demonstrated better photoprotective properties versus oral consumption in mice [35]. In this *in vivo* study, topical application provided significantly greater benefit against UVB irradiation-induced depletion of antioxidant enzymes and signaling protein phosphorylation. These photoprotective functions of GTPP may be mediated through interactions with inflammatory signaling molecules. Upon UVR exposure Interleukin-12 (IL-12) is known to enhance NER enzyme activity in keratinocytes [36]. Meeran et al. proposed an IL-12-dependent mechanism of DNA repair by topically applied EGCG [37]. In this study, UV-induced suppression of CHS responses was maintained in IL-12 knockout mice in comparison to wild type mice. Subcutaneous injection with IL-12 three hours prior to UV exposure in the IL-12 knockout mice diminished the amount of CPD positive cells produced in contrast to the untreated group. Earlier studies using topical and orally consumed GTPP in mice decreased UVR-induced carcinogenesis, by inhibiting the activity of chemical tumor initiators and promoters [38–40].

Recent studies by Katiyar et al. demonstrated a dose-dependent decrease in UVR-induced immunosuppression via contact hypersensitivity response (CHS) to 2, 4-dinitrofluorobenzene in mice that were fed purified GTE [41]. This decrease in immunosuppression was persistent 4 weeks after resumption of a normal liquid diet in the animals. The authors further demonstrated that GTPs in drinking water of UV-irradiated mice reduced the migration of CPD positive cells to lymph nodes and improved the NER mechanisms. Similar results were seen in adult human subjects that ingested 7.5 mg of pure (commercially available) green tea brewed in 540 mL of boiling water. There was a significant decrease in UVR-induced DNA damage of peripheral white blood cells [42, 43]. Human studies using topically applied GTPP prior to exposure with 2-MED of SSR demonstrated a decrease in SSR-induced erythema, DNA damage, Langerhans cell damage, and production of 8-OHdG in healthy human subjects [44, 45].

5. Toxicity of GTPP

In general, GTPP has been shown to be well tolerated in animal and human trials. Topical preparations have the least harmful effects with minor irritation being the most significant finding [46]; however, adverse effects of oral consumption have been demonstrated. In a 9-month chronic study in fasting Beagle dogs, oral ingestion of green tea extract capsules caused unexpected morbidity and mortality (16 deaths out of 24 treated animals) in the treated versus control group resulting in early termination of the study group [47]. Clinical signs of toxicity, weight loss were observed as early by day 9 of this chronic study group with fasting animals. Follow-up studies by the authors demonstrated more favorable results when the green tea capsules were administered in a fed state. Although the exact mechanism of the toxicity was not determined, the authors suggested gastrointestinal irritation, organ, and hematologic evidence of immune-mediated hemolysis may have played a role in the toxicity of ingesting a capsular form of the green tea extracts. Studies by Isbrucker et al., where mice were fed liquid and powered purified green tea extracts, did not show any genotoxic effects [48].

In vitro culture experiments by Navarro-Peran et al. showed that EGCG inhibits the activity of dihydrofolate reductase (DHFR) which is an important enzyme

in nucleotide biosynthesis [49]. The authors suggested that this effective inhibition provides evidence for EGCG's chemotherapeutic mechanism of action. Their results provide an interesting insight into the reported association of maternal tea consumption and neural tube defects [50]. There are a limited number of published studies showing this teratogenic effect especially given the large amount of tea consumed globally. Experiments evaluating reproductive and developmental toxicity of EGCG in rats did not show teratogenic effects [51].

6. Antioxidant Activity of GTPP in Skin

The skin has a complex defense system to deal with harmful environmental and chemical substances but excessive or chronic exposure can overwhelm the system leading to oxidative stress and oxidative damage. In cells, reactive oxygen species (ROS) are formed during the energy-producing process of reducing molecular oxygen to water. These are superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$). An overproduction of ROS depletes physiologic ROS-scavenging enzymes (superoxide dismutase, and catalase) which cause damage to proteins, lipids, and DNA [52] that contribute to skin diseases, immunosuppression, and development of skin cancer. GTPPs have been shown to be effective in curbing these harmful effects because their chemical structures can chelate metal ions and decrease free radical damage to cellular structures [53, 54].

Photoaging is caused by chronic UV exposure. *In vitro* studies using cultured human skin fibroblasts pretreated with GTPP showed a decrease in hydrogen peroxide (H_2O_2)-induced ROS. In this study, the authors demonstrated the ability of GTPP to improve fibroblast cell shape and absolute cell numbers when compared to control groups [10]. To assess the effect of GTE on lipid peroxidation (LPO), Jorge et al. conducted an *in vitro* assay using liposomal phophatidylcholine structure. They demonstrated a significant decrease in the concentration of hydroperoxides after a 3-hour reaction with an oxidative compound [55].

The inhibitory effect of GTPP on hydrogen peroxide formation and cell signaling is paramount to its antioxidant properties. There are few *in vivo* human studies demonstrating this protective event. In 2001, Katiyar and colleagues demonstrated this protective effect in adult human volunteers exposed to a single dose of (4xMED) UV irradiation prior to topical EGCG administration [56]. These authors confirmed what is already known about EGCG inhibition of UV-induced H_2O_2, NO and LPO production. They also demonstrated blockage of UV-induced infiltration of ROS-producing $CD11b^+$ cells and restoration of epidermal antioxidant enzymes reduced glutathione, catalase, and glutathione peroxidase.

7. Mechanism of Action of GTPP

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine proteins that are involved in cellular functions in the skin including cell growth, differentiation, proliferation, and apoptosis [57]. These proteins include extracellular signal-regulated kinases (ERK), c-Jun NH_2-terminal kinases (JNK), and p38. MAPK signaling cascades and downstream effectors are triggered in response to UVR-induced oxidative and genotoxic stress. The activity of GTPP is likely due to free radical scavenging activity that prevents MAPK activation. Topical application of GTPP in SKH-1 hairless mice showed inhibition of UVB-induced phosphorylation of ERK1/2, JNK, and p38 expression [58]. These results were also seen in human dermal fibroblasts where EGCG inhibited the UVB-induced activation of these downstream effector pathways [59]. Bae et al. also demonstrated the attenuation of nuclear transcription factors c-Jun, p53, and c-fos within 30 mins of UVB irradiation of the cultured cells. UVB-generated hydrogen peroxide stimulates membrane epidermal growth factor receptors (EGFRs) that activate ERK proteins which contribute to cell proliferation and differentiation that may be involved in tumor promotion [60]. Pretreatment of normal human epidermal keratinocytes (NHEKs) with EGCG prior to UVB exposure inhibited H_2O_2 production and phosphorylation of ERK1/2, JNK, and p38 proteins [61]. Green tea extracts have been implicated in immunoregulatory signaling functions. Rodent experiments by Kim et al. demonstrated a dose-dependent decrease in histamine production by peritoneal mast cells incubated with GTPP [62]. Further experiments by these authors proposed that the altered histamine release was due to a GTE-mediated decrease in cAMP and calcium levels which led to a NFκB and p38 MAPK-dependent inhibition of proinflammatory cytokines, TNF-α and IL-6. Table 1 lists the major cellular and molecular targets of GTPP on normal skin.

8. Role of GTPP in Chemoprevention and Carcinogenesis

Cancer remains the second leading cause of death in the United States [15]. Studies have shown that 30–40% of all cases of cancers can be prevented by combining a healthy diet, exercise, and maintaining a healthy body weight, and more than 20% of all cases of cancer can be prevented just by consuming an ample and varied amount of fruits and vegetables [15, 63]. EGCG has been shown to inhibit tumor invasion and angiogenesis thereby preventing tumor growth and metastasis [64]. Dermatologists are considerably interested in green tea polyphenols (GTPP) as preventative products for skin cancer, as their use have shown promising results.

Skin cancer is the most common of all cancers; however it is very preventable and curable if diagnosed early. Chronic exposure to UVR is the key factor in initiating skin cancer. UVB radiation induces both direct and indirect biologic effects, including multiple effects on the immune system, inducing oxidative stress, and damaging DNA, all in which play an important role in the generation and maintenance of neoplasms [65]. *In vitro* and *in vivo* systems have both shown the protective effects from polyphenols on the biochemical processes that are induced or mediated by UV radiation, suggesting that routine use of polyphenols both topically and

Table 1: Summary of effects of green tea polyphenols on skin.

GTPP protective effect	Cellular and/molecular response	References
UV protection	Inhibits UVB-induced MAPK activation and phosphorylation of ERK1/2, JNK and p38.	[51–54, 57]
	Attenuates nuclear transcription factors, c-Jun p53, and c-fos	
Antioxidant	Free radical scavenging activity	[35, 48, 53, 54, 56, 57]
	Inhibits NOS, H_2O_2 production	
	Prevents UVB-induced depletion of antioxidant enzymes: catalase, glutathione peroxidase, superoxide dismutase, and glutathione	
	Inhibits UVB-induced LPO and protein oxidation	
Anti-inflammation	Prevents UV-induced depletion of CD1a + LC and APC Inhibits UV-induced infiltration of monocytes, macrophages, neutrophils	[37, 45, 55, 57, 60]
	Protects UVB-induced immunosuppression via IL-12 production	
	↓ in histamine release by mast cells	
Anticarcinogenesis	Inhibits DNA damage.	[45, 48, 59, 60]
	Inhibits UV-induced CPD, 8-OHdG formation	
	DNA repair enzyme activation	
	Modulates transcriptions factors AP1, NFKB	
	Inhibits tumor growth, progression and angiogenesis	

LC: langerhans cells; LPO: lipid peroxidase; MAPK: mitogen-activated protein kinase; NOS: nitric oxide synthase.

orally may provide effective protection against UV radiation and ultimately skin cancer [65].

Polyphenols are shown to possess anti-inflammatory, immunomodulatory, and antioxidant properties [65]. EGCG and green tea extract are non-toxic for humans and have a wide range of target organs making it significantly different than the standard form of preventative cancer drugs. Not only is EGCG widely distributed throughout the body, but studies also show that multiple oral administrations causes a synergistic effect leading to higher concentrations of EGCG in the cells. This effect was first seen in a study involving mice, where 3H-EGCG was administered and measured in the excretions. 24 hours after the intubation, radioactivity was still found in multiple organs including the skin. After multiple administrations of 3H-EGCG, radioactivity increased 4–9 times in most organs, suggesting that routine consumption or topical treatment may provide efficient protection against UV radiation in humans. These results eventually led to a study in humans, where researchers looked at green tea consumption and the average age of cancer onset. Cancer onset of male patients consuming more than 10 cups of green tea was approximately 3.2 years later than male patients who consumed less than 10 cups of green tea per day, and cancer onset for women drinking more than 10 cups of green tea per day was 7.3 years later. These results allowed researchers to determine the effective cancer preventative amount to be approximately 10 Japanese-size cups (120 mL/cup) of green tea per day, which is equivalent to about 2.5 g of green tea extract [66]. Another study observed that regular intake of EGCG increased the minimal dose of radiation required to induce erythema, suggesting that the EGCG is able to strengthen the skin's tolerance by inhibiting the UV-induced skin damage from the radiation [65]. From these findings it can be seen that orally consumed EGCG has two different mechanisms of action and can act as both a chemopreventive and photochemopreventive drug; it can protect the body by suppressing, slowing down, and reversing the process of carcinogenesis, as well as protecting the skin from damaging radiation caused by harmful UVB rays.

As mentioned before, UVB radiation induces oxidative stress and DNA damage, and also affects the immune system. In separate experiments, it has been shown that topical treatments containing EGCG significantly inhibits acute or chronic UV irradiation-induced protein oxidation in the skin of mice, suggesting that GTTP's may be able to reduce photo damage in the skin and prevent premature aging [45, 65, 67]. Another study showed that the pretreatment of mouse skin with EGCG inhibited UVB-induced infiltration of leukocytes, specifically $CD11b^+$ cells in the skin which mediate UV-induced immunosuppression. These infiltrating leukocytes can be a potential source of H_2O_2 and NO which play important roles in initiating and promoting tumor cells. Less damage to the epidermal structure of the mouse skin was also observed with the topical application of EGCG before being exposed to UVB light. The data collected in this study demonstrated the potent preventative effects of topical EGCG in mice against UV radiation-induced infiltration of leukocytes, suggesting that GTTP's may have preventative effects against the development of skin cancer in humans [68]. Inflammatory responses are implicated in skin disease, tumorigenesis, and tumor metastasis. EGCG effectively inhibited human melanoma cell culture growth by decreasing IL-1β secretion and NFκB activity [69].

9. Concluding Remarks

As discussed in this paper, GTPPs have important antioxidant, immunomodulatory, and photoprotective functions.

Their ability to modulate critical biochemical functions through topical and oral formulations makes GTPPs a promising candidate for chemoprevention and treatment of disease. Future collaborative studies are needed to clarify optimum dosing amounts that will provide therapeutic benefits.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Dietary Phenolic Acids Act as Effective Antioxidants in Membrane Models and in Cultured Cells, Exhibiting Proapoptotic Effects in Leukaemia Cells

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Caffeic, syringic, and protocatechuic acids are phenolic acids derived directly from food intake or come from the gut metabolism of polyphenols. In this study, the antioxidant activity of these compounds was at first evaluated in membrane models, where caffeic acid behaved as a very effective chain-breaking antioxidant, whereas syringic and protocatechuic acids were only retardants of lipid peroxidation. However, all three compounds acted as good scavengers of reactive species in cultured cells subjected to exogenous oxidative stress produced by low level of H_2O_2. Many tumour cells are characterised by increased ROS levels compared with their noncancerous counterparts. Therefore, we investigated whether phenolic acids, at low concentrations, comparable to those present in human plasma, were able to decrease basal reactive species. Results show that phenolic acids reduced ROS in a leukaemia cell line (HEL), whereas no effect was observed in normal cells, such as HUVEC. The compounds exhibited no toxicity to normal cells while they decreased proliferation in leukaemia cells, inducing apoptosis. In the debate on optimal ROS-manipulating strategies in cancer therapy, our work in leukaemia cells supports the antioxidant ROS-depleting approach.

1. Introduction

In the scientific literature, the term polyphenols refers also to phenolic acids, although from the strict structural point of view of purely chemically based definition they are monophenolic compounds, as widely stated by Quideau et al. [1]. The reason for including phenolic acids in the family of plant polyphenols lies in the fact that they are bioprecursors of polyphenols and, more importantly, they are metabolites of polyphenols. Many papers and reviews describe studies on bioavailability of phenolic acids, emphasizing both the direct intake through food consumption and the indirect bioavailability derived by gastric, intestinal, and hepatic metabolism of "true" polyphenols [2–8].

Two aspects have to be taken into account when dietary phenolic compounds are tested in cell cultures as models: seldom the free form, that is, aglycone, is the actual molecule reaching blood and tissues; and the concentration used should be similar to that found in the circulatory system, that is, nmol/L to low mmol/L [6, 9].

More than ten years ago, we began studying the antioxidant activity of phenolic acids deriving from the intestinal metabolism of polyphenols or coming directly from food, representative of the two major classes of phenolic acids, that is, hydroxycinnamic and hydroxybenzoic acids. We have focused on antioxidant properties of these phenolic acids, tested at low micromolar concentration, starting from experiments conducted in homogeneous solution and in

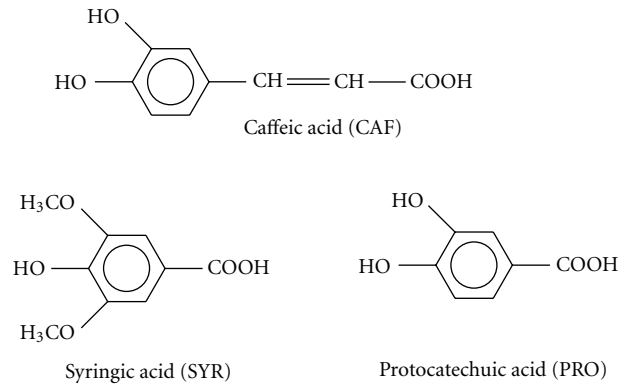

Figure 1: Chemical structures of studied phenolic acids.

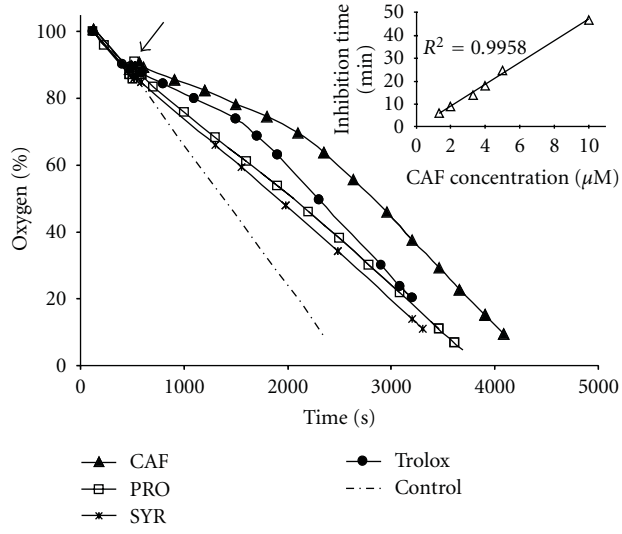

Figure 2: Oxygen uptake traces during AAPH (17 mM) induced peroxidation of PC (15 mM) unilamellar vesicles at 37°C and pH 7.2 in the absence of inhibitor (control) and in the presence of one of the various phenolic antioxidants, each at the same concentration (5 μM). The arrow shows antioxidant injection. Inset: relationship between CAF concentrations (1.3 to 10 μM) and inhibition periods measured.

membrane models. In particular, we have studied caffeic acid (CAF), a derivate of cinnamic acid carrying two hydroxyl groups in *ortho* position, syringic (SYR), and protocatechuic (PRO) acids, with a hydroxybenzoic structure: SYR with methoxyl groups in positions 3 and 5 and hydroxyl group in 4, and PRO with hydroxyl groups in positions 3 and 4.

Direct sources of CAF are dates, berries, fruit such as apricot, apple and kiwi, spices, herbs, vegetables, beverages such as coffee, wine, and, at a lesser extent, beer [10–13]. The main sources of SYR are swiss chard, olives, walnuts, dates, spices, and pumpkin [10, 14]. PRO, besides being one of the main anthocyanin metabolite [15], is present in cocoa powder, dates, chicory, olives, and onions [10].

These compounds have been studied mainly for their properties against oxidative damage leading to various degenerative diseases, such as cardiovascular diseases, inflammation, and cancer. Indeed, tumour cells, including leukaemia cells, typically have higher levels of reactive oxygen species (ROS) than normal cells so that they are particularly sensitive to oxidative stress. Differences in ROS levels between normal and cancer cells are due to the dysregulation of redox balance in neoplastic cells that develop when, for instance, intracellular production of ROS increases, or when antioxidant defences are depleted [16–22]. The importance of ROS in cancer is unequivocal, but scientists still not exactly understand how reactive species act [23]. Many reviews extensively describe sources, mechanisms, and involvement of ROS in cancer, and illustrate the role of cellular redox regulation in cancer therapy development [24–30]. In particular, recently Wang and Yi [31] well reviewed "two paradoxical ROS-manipulation strategies in cancer treatment," that is the opposite proantioxidant and antioxidant approaches. They propose the development of "redox signaling signature," a combinational set of parameters such as redox status, antioxidant enzymes expression, cell signalling, and transcription factor activation profiles in a given type of cancer cells, to be used as an index for choosing one of the two faces of the coin: ROS-elevating or ROS-depleting specific therapy against certain type of cancer cells. A way to kill cells is the induction of apoptosis, the programmed cell death. Whether this goal should be reached by diminishing or increasing ROS is controversial, likely depending on the "redox signaling signature" of each cell type. The advantage of such a strategy, that is, the induction of apoptosis *via* death signalling pathways, is that normal cells are not significantly affected since their basal ROS levels are lower and, therefore, they are less susceptible to redox changes. Recently, Halliwell observed that one of the various contribution to cancer played by reactive species is their ability to suppress apoptosis [23]. Akt is the prototypic kinase which promotes cellular survival: Akt enhances survival by directly phosphorylating key regulatory proteins of the apoptotic cascades [32]. In fact, the phosphoinositide 3-kinase (PI3K)/Akt pathway is constitutively active in many tumours. The phosphorylation of PI3K activates Akt and phosphorylation by p-Akt activates key survival proteins and inactivates proapoptotic substrates. Phosphorylation of these proteins decreases tumour susceptibility to apoptotic stresses. In other words, activation of the PI3K/Akt pathway is one of the mechanisms by which ROS modulate cell survival during carcinogenesis.

The inhibition of ROS through the use of antioxidants decreases the antiapoptotic pathways that are activated by ROS in cancer cells [29, 33]. Although some studies report that high levels of ROS turn on cell death signalling [25] and that Akt dephosphorylation leading to apoptosis is induced by ROS [34], many papers demonstrate the link between the increased redox stress in tumours with the PI3K/Akt pathway [35, 36].

Our previous studies investigating the effect of IL-3 on the M07e human acute myeloid leukaemia (AML) cell line noted that the prosurvival effect of this cytokine was mediated by NAD(P)H oxidase isoform Nox2-derived ROS production, and was suppressed by antioxidants, NAD(P)H oxidase (Nox) inhibitors or specific knockdown of Nox2

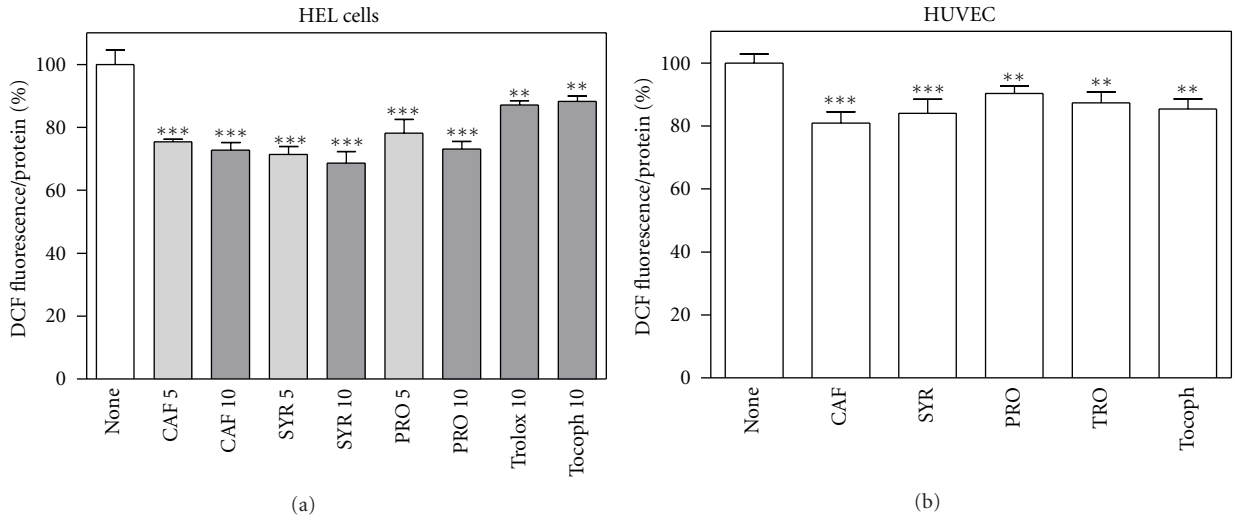

Figure 3: Antioxidant effect of phenolic compounds in HEL cells and HUVEC exposed to oxidative stress generated by 50 μM H_2O_2. Cells were preincubated with different compounds (5 or 10 μM for HEL cells, (a); 10 μM for HUVEC, (b)) for 20 hours, treated with H_2O_2 for 30 min, then ROS levels were measured by means of H_2DCFDA assay as described in Section 2. Results are expressed as means ± SD of three independent experiments, each performed in octuplicate. $^{**}P < 0.005$, significantly different from control cells; $^{***}P < 0.0005$, significantly different from control cells.

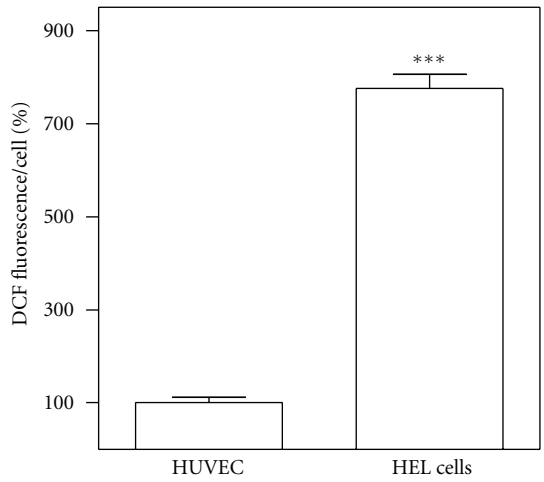

Figure 4: Comparison between basal ROS levels in HEL cells and in HUVEC. ROS levels were measured by means of H_2DCFDA assay as described in Section 2. Results are expressed as means ± SD of three independent experiments, each performed in octuplicate. Significant difference $^{***}P < 0.0001$.

expression [37, 38]. In a different AML cell line, B1647, expressing Nox2 and Nox4 [39], we showed that VEGF signalling and Nox activity are coupled, and that inhibitors of both Nox and VEGF receptor 2 are able to induce apoptosis in these leukaemia cell line [40].

Starting from our experience on antioxidant properties studied in model systems and on the role of ROS and their inhibitors in leukaemia cell proliferation, the aim of this work was to explore the antioxidant activity of three dietary phenolic acids in membrane models and in the leukaemia cell line, HEL, as well as to investigate the relationship among reactive species, cell proliferation, and apoptosis.

2. Materials and Methods

2.1. Materials. Egg yolk lecithin (phosphatidylcholine, PC) was purchased from Lipid Products (Redhill, UK). The thermolabile azo compound 2,2′-azobis(2-methyl-propionamidine) dihydrochloride (AAPH), caffeic acid (CAF), syringic acid (SYR), protocatechuic acid (PRO), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, TRO), α-Tocopherol (Tocoph), hydrogen peroxide, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, dichlorofluorescin diacetate), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Trypan Blue, Igepal CA-630, the fluorogenic substrates N-acetyl Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) for Caspase 3, N-Acetyl-Ile-Glu-Thr-Asp-7-amido-4-methyl-coumarin (Ac-IETD-AMC) for Caspase 8, and N-Acetyl-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin (Ac-LEHD-AFC) for Caspase 9, sodium orthovanadate, phenylmethanesulfonyl fluoride (PMSF), *N*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), *N*-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), protease inhibitor cocktail, Laemmli sample buffer containing 2-mercaptoethanol, mouse monoclonal antitubulin antibody were obtained from Sigma-Aldrich. HEL (Human erythroleukaemia) cell culture was from DSMZ (Braunschweig, Germany); HUVEC (Human Umbilical Vein Endothelial Cells) were kindly donated by Professor Claudio Muscari, Department of Biochemistry, University of Bologna; RPMI 1640 (with Hepes, with L-glutamine), foetal calf serum, Penicillin/Streptomycin, were purchased from PAA. ATPlite 1step luminescence kit was from PerkinElmer. Nitrocellulose

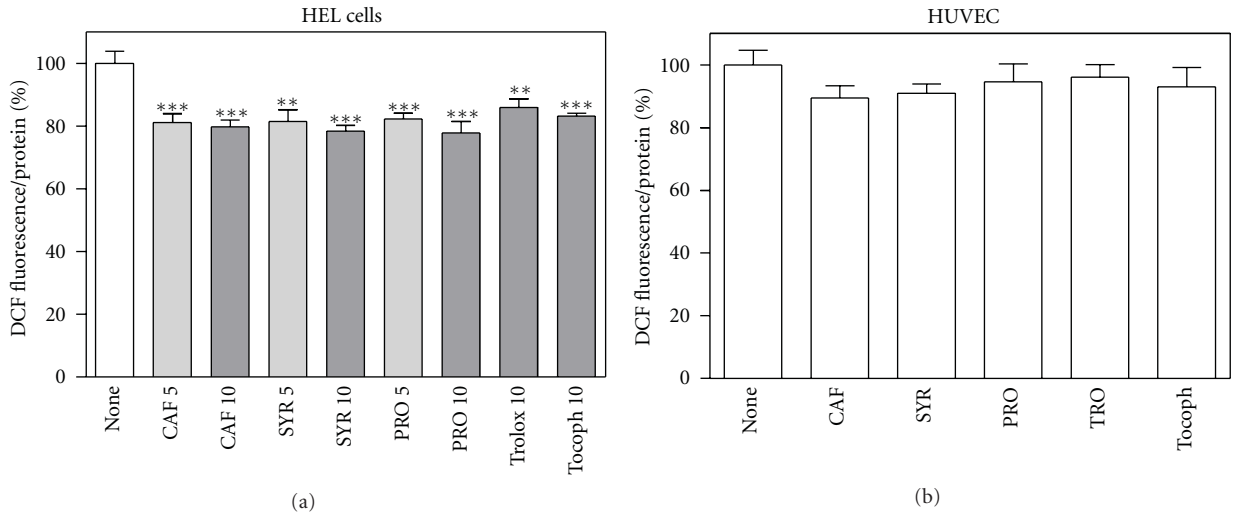

Figure 5: Effect of phenolic compounds on basal ROS levels in HEL cells and HUVEC. Cells were treated with different compounds (5 or 10 μM for HEL cells, (a); 20 μM for HUVEC, (b)) for 20 hours, then ROS levels were measured by means of H_2DCFDA assay as described in Section 2. Results are expressed as means ± SD of four independent experiments, each performed in octuplicate. $^{**}P < 0.005$, significantly different from control cells; $^{***}P < 0.0005$, significantly different from control cells.

membranes and Amersham ECL Plus Western Blotting Detection Reagents were from GE-Healthcare. Anti-caspase 3, anti-Bax, anti-Bcl-2, and anti-p-Akt antibodies were purchased from Cell Signaling Technology. Anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology. PageRuler Prestained protein ladder was from Fermentas, Thermo Fisher Scientific. All the other chemicals and solvents were of the highest analytical grade.

2.2. Preparation of Large Unilamellar Vesicles. Vesicles were prepared by adding in a round-bottom tube the appropriate amount of phosphatidylcholine. The solvent was carefully removed with a stream of nitrogen to obtain a thin film, then 0.6 mL of phosphate-buffered saline (PBS), pH 7.2, containing 1 mM Na_2EDTA were added. The film was vortex-stirred for 7 min and the milky suspension obtained was transferred into LiposoFast (produced by Avestin, Ottawa, Canada) and extruded 21 times back and forth through two polycarbonate filters (100 nm pore size, Nucleopore Corp., Pleasenton, CA) to obtain large unilamellar vesicles (LUVET). The total volume was then adjusted to give a final concentration of 15 mM PC. Ethanol solutions of phenolic acids were added to LUVET to obtain a final concentration between 1.3 and 10 μM of the acid.

2.3. Vesicle Autoxidation. Autoxidation experiments in the presence or absence of antioxidants were carried out by monitoring the oxygen concentration with a Clark-type electrode (Yellow Springs Instruments Co., OH). After thermal equilibration of LUVET at 37°C, the appropriate amount of AAPH was added to the suspension in order to obtain a final AAPH concentration of 17 mM, suitable for LUVET peroxidation initiated by water soluble azo compounds [41]. The reaction cell was always protected from room light to avoid initiator photodecomposition.

2.4. Cell Culture. Human erythromegakaryocytic leukaemia cell line (HEL) established from peripheral blood obtained from a patient with acute myelogenous leukaemia was grown in RPMI supplemented with 10% (v/v) heat-inactivate foetal calf serum supplemented with 100 U/mL penicillin and 100 g/mL streptomycin sulphate at 37°C in a humidified atmosphere maintained at 5% CO_2. The cells were treated with different concentrations (5, 10, 20, 50 or 100 μM) of CAF, SYR, PRO, Trolox, and α-Tocopherol, dissolved in ethanol or in ultrapure water, for 20 hours.

2.5. Measurement of Intracellular ROS. Cells (1×10^6/mL) were washed twice in PBS and incubated with 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 20 min at 37°C. H_2DCFDA is a small nonpolar, nonfluorescent molecule that diffuses into the cells, where it is enzymatically deacetylated by intracellular esterases to a polar nonfluorescent compound, that is oxidised to the highly green fluorescent 2′,7′-dichlorofluorescein (DCF). The fluorescence of oxidized probe was measured using a multiwell plate reader (Wallac Victor^2, PerkinElmer). Excitation wavelength was 485 nm and emission wavelength was 535 nm [42, 43].

2.6. Cell Viability and Proliferation. Viable cells were evaluated by the Trypan Blue exclusion test. Cell viability was also assayed by the MTT assay, since the reduction of tetrazolium salts is widely accepted as a reliable way to examine cell viability/proliferation [44]. Cells (2×10^4) were incubated in 96-well flat-bottomed plates with 0.5 mg/mL MTT for 4 h at 37°C. At the end of the incubation, blue-violet

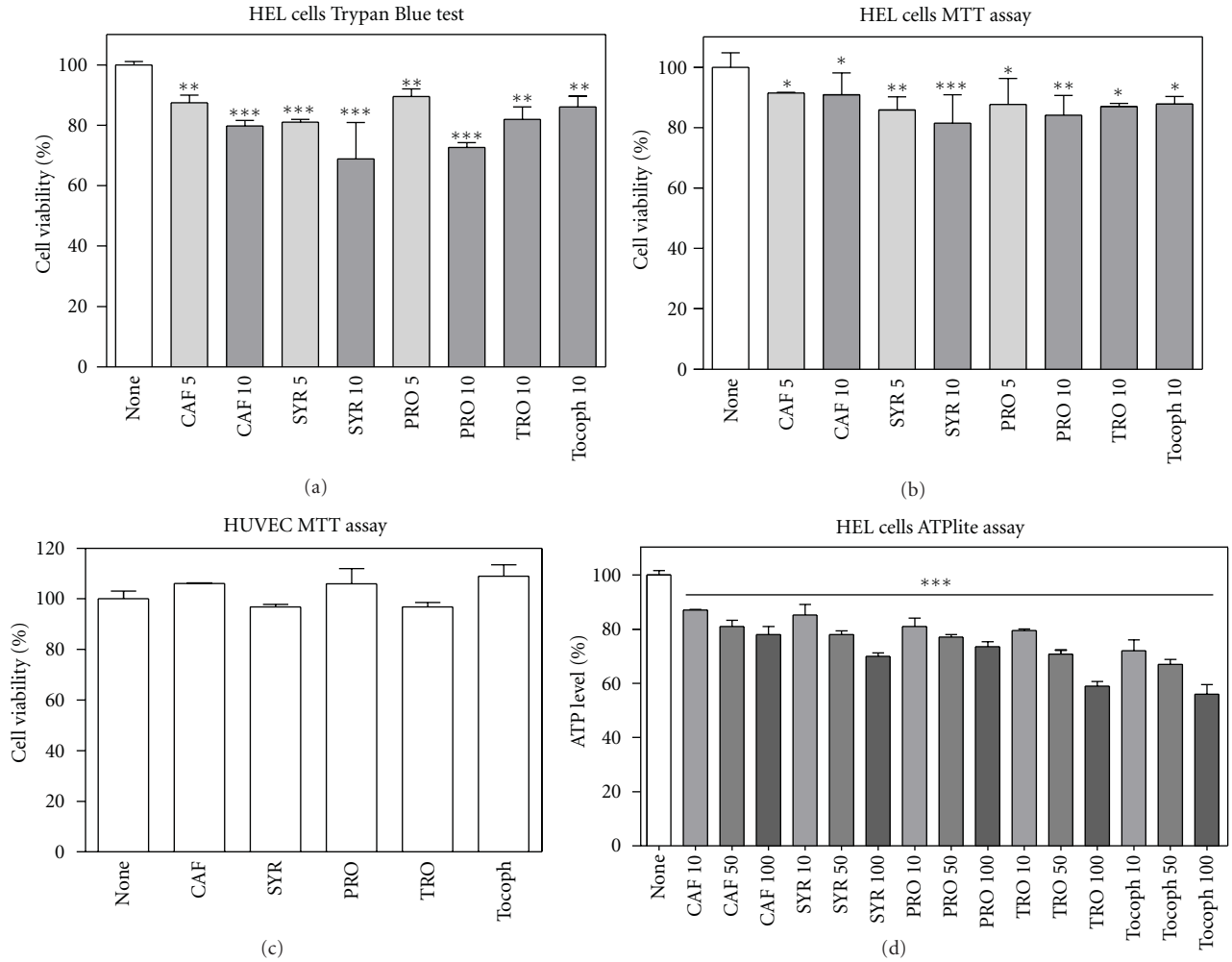

Figure 6: Effect of phenolic compounds on cell viability/proliferation. Cells were treated with different compounds (5 to 100 μM for HEL cells, (a), (b) and (d); 20 μM for HUVEC, (c)) for 20 hours. (a): Viability was estimated by Trypan Blue exclusion test. (b) and (c): Viability/proliferation was evaluated by MTT assay as described in Section 2. Results are expressed as means ± SD of three independent experiments, each performed in quadruplicate. $^{*}P < 0.05$, significantly different from control cells; $^{**}P < 0.01$, significantly different from control cells; $^{***}P < 0.001$, significantly different from control cells. (d): HEL cells viability, after 20-h treatment with 10, 50, or 100 μM compounds, was determined by the ATPlite 1step luminescence kit as described in Section 2. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. All values are significantly different from control ($^{***}P < 0.001$).

formazan salt crystals were formed and dissolved by adding the solubilization solution (10% SDS, 0.01 M HCl), then the plates were incubated overnight in humidified atmosphere (37°C, 5% CO_2) to ensure complete lysis. The absorbance at 570 nm was measured using a multiwell plate reader (Wallac Victor^2, PerkinElmer). In addition, cell viability was assessed by using the ATPlite 1step luminescence kit, an ATP monitoring system based on firefly (Photinus pyralis) luciferase. The emitted light, produced by the reaction of ATP with added luciferase and D-luciferin, is proportional to the concentration of ATP, present in all metabolically active cells. In a 96-well black clear-bottom plate, according to the supplier's instructions, 100 μL of the reconstituted reagent were added to each well containing 2×10^4 cells (100 μL), equilibrated at room temperature. The plate was shaken for 2 minutes at 700 rpm using an orbital microplate shaker, then the luminescence was measured using a multiwell plate reader (Wallac Victor^2, PerkinElmer).

2.7. Caspase Activity Assay. Ac-DEVD-AMC was used as fluorogenic substrate for caspase 3, Ac-IETD-AMC for caspase 8, and Ac-LEHD-AFC for caspase 9. After different treatments, cell lysates were incubated with specific substrates at 37°C for 15 min. The activity of caspase 3 and caspase 8 was measured following the hydrolysis of fluorogenic substrates resulting in the release of the fluorescent AMC; excitation wavelength was 370 nm, emission wavelength was 455 nm. The caspase 9 activity was determined by measuring the fluorescence of AFC; the excitation and emission wavelengths of AFC were 400 and 505 nm, respectively. Measurements were performed by a Jasco FP-777 spectrofluorometer.

2.8. Immunoblotting and Densitometric Analysis. Western blot analysis was done to detect procaspase 3, cleaved caspase 3, Bax, Bcl-2, p-Akt, and tubulin, by using the corresponding antibodies. Cells were lysed with a lysis buffer (1% Igepal, 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 0.1 mM PMSF, 0.1 mM TLCK, 0.1 mM TPCK, 1 mM sodium orthovanadate and protease inhibitor cocktail, pH 8.0) in ice for 15 min. Samples were added to Laemmli sample buffer containing 2-mercaptoethanol and kept at 95°C for 5 min to solubilise the proteins. Then they were separated on 10% SDS-polyacrylamide gel using a Mini-Protean III apparatus (Bio-Rad Laboratories). Proteins were transferred electrophoretically to nitrocellulose membrane at 100 V for 60 min. Nonspecific binding was blocked by incubating with Tris-buffered saline (TBS)/Tween, pH 8.0, containing 5% nonfat, dry milk for 1 h at room temperature. Nitrocellulose membranes were incubated overnight at 4°C with primary antibodies, washed 3 times with TBS/Tween, and incubated for 60 min at room temperature with secondary antibodies in TBS/Tween containing 5% nonfat dry milk. Membranes were washed and developed using Amersham ECL Plus Western Blotting Detection Reagents. Images of the blots were obtained using a CCD imager (Fluor-S Max MultiImager System, Bio-Rad Laboratories) and bands were acquired and analyzed by using Bio-Rad Quantity One analysis software. Cleaved caspase 3 immunoreactive bands were quantitated and expressed as the ratio of each band density to control band density. Bax and Bcl-2 immunoreactive bands were quantitated and expressed as the ratio of Bax band density to Bcl-2 band density for each sample.

2.9. Statistical Analysis. Results are expressed as means of at least three independent experiments with standard deviation. Differences between the means were determined by two-tailed Student's t test or by Newman-Keuls multiple comparison test following one-way ANOVA.

3. Results and Discussion

At first, the antioxidant activity of CAF, SYR, and PRO (see Figure 1) was investigated in large unilamellar vesicles of phosphatidylcholine, model membranes in which interfacial interactions, molecular packing, and dynamics of the lipid phase can be envisaged similar to those of natural membranes.

Autoxidation experiments were performed by monitoring the oxygen consumption with a miniaturized Clark-type electrode and by using the water soluble azo compound AAPH as initiator of radical peroxidation and Trolox as reference antioxidant. AAPH, owing to its hydrophilicity, gives rise to radical chain initiation at a constant rate in the aqueous phase according to a well-defined pathway [45], being a useful tool for understanding the effectiveness of hydrophilic and lipophilic peroxidation inhibitors against the attack of oxygen radicals to biomembranes from the external water environment.

Antioxidants were tested at several concentrations in the range 1–10 μM, and Figure 2 shows representative oxygen uptake traces acquired at 5 μM, optimum concentration to identify the inhibition period of good antioxidants in our experimental conditions, that is, 15 mM PC unilamellar vesicles and 17 mM AAPH at 37°C [46, 47]. The oxygen consumption traces reported in Figure 2, obtained at 37°C and pH 7.2, indicated that CAF behaves as a very effective antioxidant, showing both a longer inhibition time and a higher rate constant for the reaction with peroxyl radicals than those measured with the same amount of Trolox. Moreover, a linear relationship (r^2 = 0.996) was found between CAF concentrations and the inhibition periods in the concentration range here reported (see inset in Figure 2).

In the presence of similar amounts of SYR and PRO, the rate of oxygen consumption was somewhat reduced with respect to control experiments, but no evident inhibition time occurred. This reduction was much smaller than that observed when the same amounts of CAF or Trolox were added to the assay (Figure 2). Thus, SYR and PRO did not behave as effective antioxidants, since in their presence no evident inhibition time occurred. According to Pryor et al. [48] "compounds of this type are generally classed as retardants rather than as antioxidants," since they react with peroxyl radicals slowing the initiation and propagation steps of lipid oxidation, but do not completely stop it.

Many factors are responsible for the free radical scavenging activity of compounds *in vitro*. Structural, thermodynamic, and kinetic aspects have to be taken into account [47, 49–54]. The presence of a catechol moiety in the molecule is one of the most deeply studied features. A second aspect is the effect of ring substituents. For example, the acrylic group CH=CH−COOH in *para* position with respect to a phenolic OH is of some relevance in determining the good antioxidant properties of cinnamic acids such as caffeic acid; instead, the lower reactivity of protocatechuic acid is due to the presence of the electron withdrawing COOH ring substituent; electron donating OH groups have stronger effects then OCH_3 ones on antioxidant ability. A less frequently considered characteristic is the pK_a value of the hydroxyl group(s), that indeed lead to a very strong dependence of antioxidant activity on the pH of the buffer solution where compounds are tested. Finally, but not exhaustively, it is essential to contemplate the different hydrophilicity of molecules, resulting in a different partition of the inhibitors between the water and the lipid phase.

Consequently, we evaluated the ability of CAF, SYR, and PRO to reduce intracellular ROS content after oxidative stress generated by low nonlethal level of exogenous H_2O_2 in the human hematopoietic cell line HEL (human erythroleukemia) and in primary-cultured HUVEC (Human Umbilical Vein Endothelial Cells).

HUVEC and hematopoietic cells, such as HEL, have a common progenitor, known as hemangioblast, multipotent cell that is able to differentiate to both endothelial and hematopoietic cells. Although many cellular pathways are still unclear, the nearly centenarian hemangioblast hypothesis has now been confirmed [55], since several cellular events and pathways have been detected. Moreover, a strict relationship between angiogenesis and development of cancer is widely documented: it is now well established that VEGF and its receptors are involved in promoting both solid and liquid

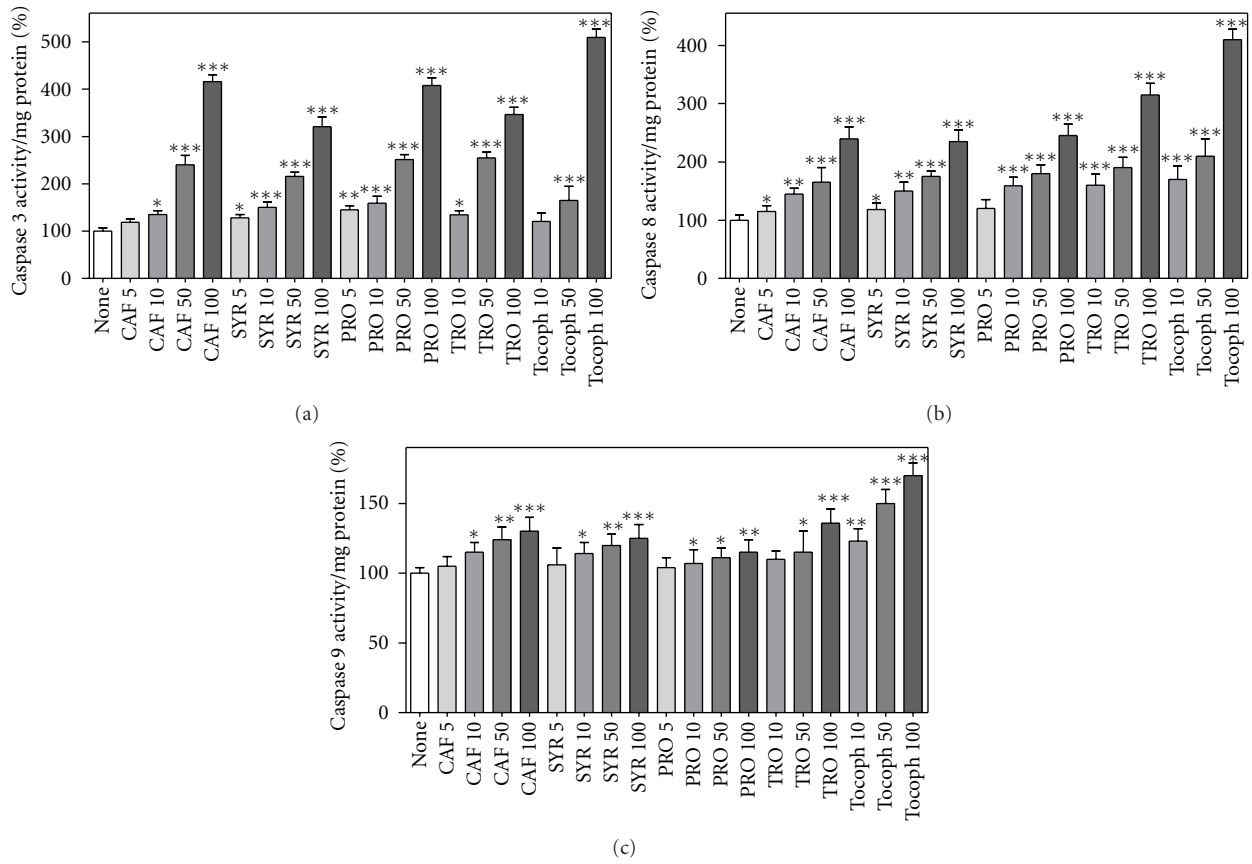

Figure 7: Caspase activity in HEL cells after phenolic compound treatment. HEL cells were incubated with different compounds (5, 10, 50, or 100 μM) for 20 hours, then cell lysates were incubated with three different substrates at 37°C for 15 min, that is Ac-DEVD-AMC as specific fluorogenic substrate for caspase 3 (a), Ac-IETD-AMC for caspase 8 (b), and Ac-LEHD-AFC for caspase 9 (c), as described in Section 2. Results are expressed as means ± SD of four independent experiments, each performed in triplicate. $^{*}P < 0.05$, significantly different from control cells; $^{**}P < 0.01$, significantly different from control cells; $^{***}P < 0.001$, significantly different from control cells.

tumour growth and proliferation [56–61]. Thus, HUVEC are "normal" cells frequently chosen to be compared with tumour cells [62, 63], in particular with HEL cell line in this study.

ROS levels were measured with the cell-permeant probe H_2DCFDA, commonly used to detect free radical/ROS production in cells, owing to the intracellular conversion to the highly green fluorescent DCF [64]. In general, dihydrofluorescein does not discriminate between the various reactive oxygen/nitrogen species, but it remains the most straightforward and versatile indicator of cellular oxidative stress.

As shown in Figure 3, the phenolic acids, preincubated for 20 hours, behaved as good antioxidant since they were able to decrease ROS levels after 30-min exposition to 50 μM H_2O_2 in both cellular systems; this reduction was comparable to that provoked by α-tocopherol, the most effective lipid-soluble antioxidant nutrient, and by its water soluble analogue, Trolox.

Many tumour cells are characterised by increased ROS generation compared with their noncancerous counterparts. Basal intracellular ROS levels were measured in HEL cells and compared with those exhibited by HUVEC. Figure 4 confirms the notion that cancer cells exhibit constitutively high levels of ROS, showing that ROS content in HEL cells is about eight times as high as in normal cells, HUVEC.

Subsequently, we investigated whether phenolic acids, at low concentrations, likely comparable to those present in human plasma [12, 65–67], were able to decrease basal reactive species. Graphs reported in Figure 5 show that in HEL cells the reduction was of about 20% after 20-h treatment, whereas no effect was observed in HUVEC even at higher concentrations.

A large body of literature reports that ROS may promote cellular proliferation and contribute to cancer development. In the last decade, we demonstrated that ROS are essential for cell survival in two different (erythro)megakaryocytic leukaemia cell lines, similar to HEL; Nox family is a major source of ROS (Nox4 and/or Nox2); ROS production can act as prosurvival factor that protects leukaemia cells from apoptosis, effect counteracted by antioxidants [37–40, 68–72].

In order to verify the role of ROS in tumour cell proliferation, we examined the effect of CAF, SYR, and PRO on cell viability, evaluated by Trypan blue exclusion test and by MTT assay (Figure 6), and compared it with Trolox and

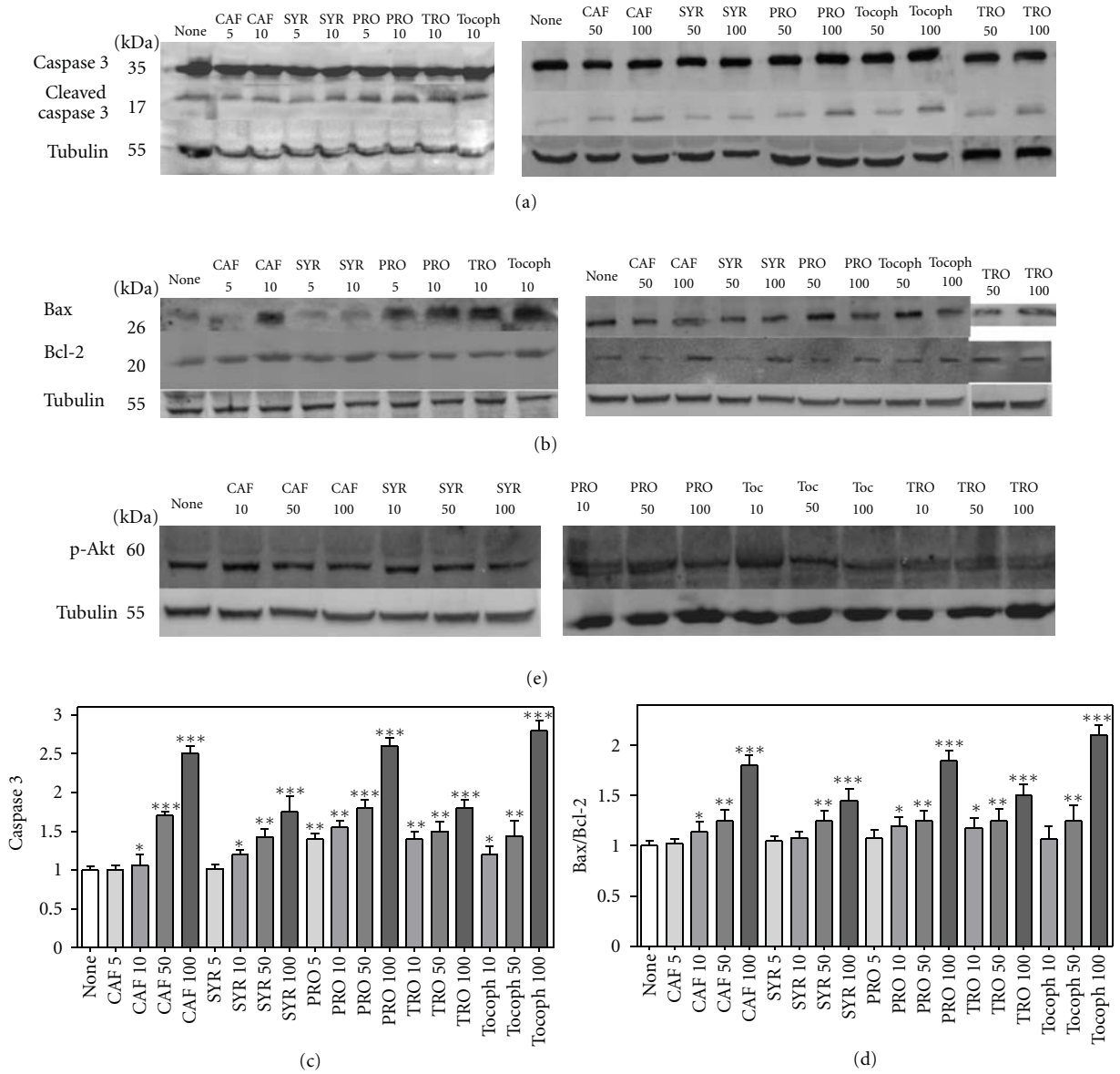

Figure 8: Effect of phenolic compounds on apoptosis. HEL cells were incubated with different compounds (5, 10, 50, or 100 μM) for 20 hours, then cell lysates were subjected to SDS-PAGE and Western blotting with the indicated antibodies as described in Section 2. Tubulin detection was used as a control. Representative immunoblots are shown. (a): anti-caspase 3; (b): anti-Bax and anti-Bcl-2; (e): anti-p-Akt. (c): Densitometric analysis of three independent Western blot assays for cleaved caspase 3 (17 kDa fragment). (d): Bax/Bcl-2 ratio from densitometric analysis of three independent experiments. $^{*}P < 0.05$, significantly different from control cells; $^{**}P < 0.01$, significantly different from control cells; $^{***}P < 0.001$, significantly different from control cells.

α-tocopherol effect. All the antioxidants slightly decreased leukaemia cell viability/proliferation (Figures 6(a) and 6(b)), whereas they did not affect HUVEC viability even at higher concentration (Figure 6(c)).

To exclude artifacts or interferences in MTT assay [73–76], although no direct reactivity toward the tetrazolium salt was detected *in vitro*, we performed complementary viability experiments by measuring intracellular ATP levels in HEL cells, also at higher phenolic acid concentrations. Measurements reported in Figure 6(d) confirmed the results obtained with the other methods.

Considering the lack of effect of tested compounds on HUVEC viability, further studies were conducted to characterise cell death induced by phenolic acids in leukaemia HEL cells, focusing on typical apoptotic features. Caspases play a central role in mediating various apoptotic responses and are activated in a sequential cascade of cleavages. The activation of an effector caspase, such as caspase 3, is executed by initiator caspases, such as caspases 8 and 9, through proteolytic cleavage after a specific internal aspartate residue, to separate the large and small subunits of the mature caspase. To detect the enzymatic activity of caspases,

three fluorogenic substrates were used: Ac-DEVD-AMC was employed as substrate for caspase 3; Ac-IETD-AMC for caspase 8; Ac-LEHD-AFC for caspase 9. Treatments of HEL cells with antioxidants stimulated the activity of all the tested caspases in a dose-dependent manner, as shown in Figure 7, where the fluorescence of AMC or AFC is reported.

Subsequently, we investigated the proapoptotic effect of phenolic compounds in HEL cells, performing SDS-PAGE followed by Western blot on cell lysates for detection of caspase 3, Bax, and Bcl-2 proteins (Figure 8). Western blot results on cleaved caspase 3 were in good agreement with outcomes obtained by fluorimetric assay, as shown in densitometric analysis of Figure 8(c). Moreover, phenolic acids were able to raise the expression of the proapoptotic Bax; in concert, a decrease of prosurvival Bcl-2 was produced. Bax and Bcl-2 are proteins involved in apoptosis acting in opposite way; thus, the Bax/Bcl-2 ratio is a useful index of apoptosis. Antioxidant compounds, after 20-hour incubation, caused a dose-dependent increase of this ratio (Figure 8(d)).

In mammals, one of the most efficient antiapoptotic survival pathway is represented by PI3K/Akt [77, 78]. Activated Akt is the principal mediator of prosurvival signalling regulated by PI3K [79]. The active phopshorylated form of Akt (p-Akt) promotes cell proliferation and survival by phosphorylating downstream molecules that regulate cell cycle and apoptosis [80]. Figure 8(e) shows that the treatment with phenolic molecules under study slightly decreased phosphorylation levels of Akt, confirming once again their proapoptotic role.

4. Conclusions

In this study, we evaluated the antioxidant activity of some phenolic acids, deriving both by direct absorption from food consumption and as a result of the cleavage of flavonoids by gut microflora. These compounds acted as chain-breaking antioxidants, with different effectiveness, in membrane models and were able to contrast intracellular ROS raise due to exogenous oxidative stress in both leukaemia and normal cells. Moreover, we observed that phenolic acids were able to scavenge reactive oxygen species in HEL cells, characterised by very high intracellular ROS levels. They exhibited no toxicity to normal cells, whereas they decreased proliferation in leukaemia cells, inducing apoptosis. Indeed, they rose caspase 3, 8, and 9 activity, increased the ratio of the apoptotic-related protein Bax/Bcl-2, and reduced Akt activation.

In the debate on optimal ROS-manipulating strategies in cancer therapy, our work in leukaemia cells supports the antioxidant ROS-depleting approach.

Abbreviations

CAF: Caffeic acid
SYR: Syringic acid
PRO: Protocatechuic acid
TRO: Trolox
Tocoph: α-Tocopherol
ROS: Reactive oxygen species
AML: Acute myeloid leukaemia
Nox: NAD(P)H oxidase
VEGF: Vascular endothelial cell growth factor
AAPH: 2,2′-azobis(2-methylpropionamidine) dihydrochloride
PC: Phosphatidylcholine
HEL: Human erythromegakaryocytic leukaemia cell line
H_2DCFDA: 2′,7′-dichlorodihydrofluorescein diacetate (also known as dichlorofluorescin diacetate)
DCF: Dichlorofluorescein
MTT: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
HUVEC: Human Umbilical Vein Endothelial Cells
Ac-DEVD-AMC: N-acetyl Asp-Glu-Val-Asp-7-amido-4-methylcoumarin
Ac-IETD-AMC: N-Acetyl-Ile-Glu-Thr-Asp-7-amido-4-methylcoumarin
Ac-LEHD-AFC: N-Acetyl-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin
AMC: 7-amido-4-methylcoumarin
AFC: 7-amido-4-trifluoromethylcoumarin.

Authors' Contribution

L. Zambonin and C. Caliceti: contributed equally to this work.

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Research Article

Protective Role of Malvidin-3-Glucoside on Peroxynitrite-Induced Damage in Endothelial Cells by Counteracting Reactive Species Formation and Apoptotic Mitochondrial Pathway

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The health-promoted benefits of anthocyanins, including vascular protective effects and antiatherogenic properties, have now been recognized, but the involved molecular mechanisms have not been well elucidated. Following our previous work on cytoprotective mechanisms of some anthocyanins against apoptosis triggered by peroxynitrite in endothelial cells, here we investigated the protective role of malvidin-3-glucoside, a major dietary anthocyanin, on such deleterious process, by exploring the interference on cellular reactive species formation and on apoptotic mitochondrial pathway. Preincubation of cells with 25 μM malvidin-3-glucoside protected efficiently endothelial cells from peroxynitrite-promoted apoptotic death, an effect which may be partially mediated by its ability to decrease the formation of reactive species after cell aggression, as assessed by the dichlorodihydrofluorescein diacetate assay and by carbonyl groups formation. Moreover, malvidin-3-glucoside inhibited mitochondrial apoptotic signaling pathways induced by peroxynitrite, by counteracting mitochondrial membrane depolarization, the activation of caspase-3 and -9, and the increase in the expression of the proapoptotic Bax protein. Altogether, our data expands our knowledge about the molecular mechanisms underlying the vascular protection afforded by malvidin-3-glucoside, and anthocyanins in general, in the context of prevention of endothelial dysfunction and atherosclerosis.

1. Introduction

In the last decade, there has been a remarkable increment in scientific knowledge concerning the beneficial role of anthocyanins, a large group of flavonoids widely distributed in human diet, in several oxidative-stress-related diseases, including atherosclerosis [1, 2]. Atherosclerosis, the main cause of cardiovascular disease, is a multifactorial disorder where endothelial dysfunction and inflammation plays a critical role [3, 4]. Peroxynitrite has been recognized as one relevant intervening factor in atherogenesis, as a strong endogenous oxidizing and nitrating species of several biomolecules. It is formed from the diffusion-limited reaction between nitric oxide (NO) and superoxide anion ($O_2^{\bullet -}$), both produced by immune system cells and vascular cells [5–7]. This reaction has one of the highest rate constants known for reactions with NO, justifying the toxicity of peroxynitrite at certain concentrations [8]. This reactive species can indeed reach high concentrations in vascular endothelium under shear stress and in atherosclerotic vessels, where it has been suggested to promote, in particular, LDL oxidation and extensive protein tyrosine residues nitration [9, 10], contributing to the production of the fatty streaks and subsequent atherosclerotic plaque formation. However, peroxynitrite may be involved in atherogenesis by other pathways, mainly by promoting the impairment of vascular reactivity and the disruption of critical cellular processes leading cells to apoptosis or necrosis [11, 12]. Therefore, counteracting peroxynitrite damaging effects is vital to endothelial integrity, and anthocyanins have already demonstrated their high peroxynitrite-scavenging activity, in several studies [13–15].

Epidemiological studies have evidenced that diets rich in vegetables and fruits are associated with a decreased risk in cardiovascular diseases [16, 17] and some of the major contributors are anthocyanins, whose intake has been

estimated to be up to 9-fold higher than that of other dietary flavonoids [16, 17]. Among dietary anthocyanins, malvidin-3-glucoside (Mv3glc) is one of the major constituents, in particular, in red wine [18]. Despite the controversy about physiologically available concentrations of anthocyanins, there is unquestionable data revealing that they are absorbed in their intact glycoside forms, including Mv3glc, appearing rapidly in the blood plasma and tissues after ingestion [19, 20]. Furthermore, the potential *in vivo* cumulative effects should also be taken into consideration. Therefore, recently, anthocyanins have been considered as promising bioactive molecules [21] in the search for potential functional foods and nutraceuticals, mainly in the context of atherosclerosis prevention [1, 22].

Although in earlier studies the biological activities of anthocyanins were closely related to their antioxidant properties, mainly ascribed to the B-ring hydroxyl groups and to the conjugated double bond system [23], their anti-inflammatory and antiatherogenic effects, among several others, cannot be explained solely on basis of these properties. In this context, there is a plethora of works, which has indicated other action mechanisms beyond such properties, namely, by interfering with crucial signaling pathways and gene regulation [24, 25].

Recently, we have shown that anthocyanins possessing either catecholic or monophenolic structures are able to counteract peroxynitrite-induced endothelial cells apoptosis through the inhibition of crucial signaling cascades, upstream and downstream of mitochondria [14]. Following this work, here we clarified the cytoprotective mechanisms for Mv3glc, an anthocyanin with 3′,5′-dimethoxyl substituents in the B-ring (Figure 1), providing new insights about the potential role of this feature on modulation of apoptotic mitochondrial signaling pathway. In fact, different patterns of hydroxylation and methoxylation, mainly on the B-ring, are known to modulate the antioxidant properties of polyphenols [26, 27] and thus could also account for their protective effects against endothelial cells under peroxynitrite injury, a process that involves the production of reactive species, which may be either directly or indirectly mediators of cellular signaling cascades. Thus, besides the antioxidant activity of Mv3glc, we assessed its capacity to counteract peroxynitrite-induced apoptotic effects by interfering in mitochondrial apoptotic signaling cascades, in primary cultures of bovine arterial endothelial cells (BAECs) as a typical endothelial cell model. This work indicates that preincubation of cells with 25 μM Mv3glc prevented several apoptotic events, such as the loss of mitochondrial membrane potential, the caspases-9 and -3 activation, and the increase in cytoplasmatic Bax levels, inhibiting peroxynitrite cell injury, an effect partially mediated by its ability to scavenge reactive species resulting from cellular oxidative aggression.

2. Materials and Methods

2.1. Reagents. Malvidin 3-*o*-β-glucoside chloride, purified from natural sources, was obtained from Extrasynthése (Genay, France). Mv3glc had purity above 97% as measured by HPLC and was used as solutions (5 mM) in DMSO and stored in the dark, under nitrogen atmosphere, at −80°C; the final solvent concentration did not exceed 1% by volume. Quercetin was from Sigma-Aldrich Co.

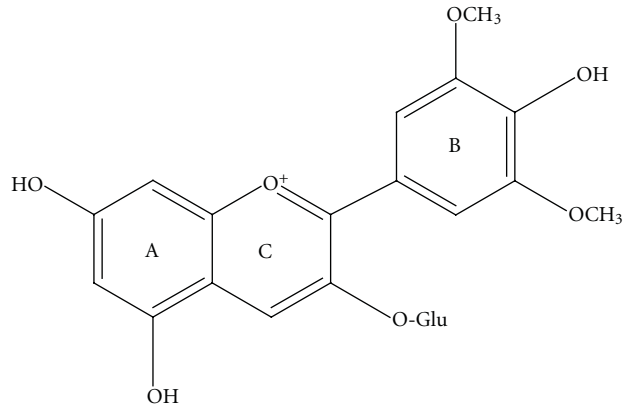

Figure 1: Malvidin-3-glucoside chemical structure.

Laboratory chemicals, namely, dihydrorhodamine 123 (DHR 123), dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT), collagenase, gelatin, Hoescht 33258, substrates for caspase-3 (Ac-DEVD-AMC), caspase-9 (Ac-LEDH-AFC), 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA), and streptomycin/penicillin were purchased from Sigma-Aldrich Co as well as other chemical reagents used.

For cell culture, Dulbecco's modified Eagle's medium (DMEM), trypsin 0.25%, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) pH 7.4 were obtained from Gibco-Invitrogen.

Primary specific mouse monoclonal antibody to Bax and anti-mouse IgG secondary antibody were obtained from Abcam (Cambridge, UK); mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich Co.

2.2. Primary Cultures of Bovine Aortic Endothelial Cells. Bovine aortic endothelial cells (BAECs) were obtained from thoracic aorta by treatment with collagenase (2 mg/mL). Cells were cultivated on gelatin-coated (0.2%) tissue culture plastic (bovine origin, Sigma-Aldrich Co), in DMEM supplemented with 10% heat inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5%CO_2. Endothelial cells, identified by their cobblestone morphology, were subcultured at confluence and used between the fourth and the tenth passages. Before each experiment, cells at 80% confluence were starved in serum-free medium for at least 6 h.

2.3. Peroxynitrite Synthesis and Peroxynitrite Treatment of Cells. Peroxynitrite was synthesized using a quenched flow reactor, as previously described [9]. The obtained peroxynitrite was then stored at −80°C under N_2 atmosphere.

Immediately before use, it was always quantified from its absorbance at 302 nm in 1 M NaOH ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$).

Peroxynitrite treatment of BAECs was performed as we have previously described [14, 28]. Briefly, $ONOO^-$, diluted in recently prepared NaOH 10 mM, was delivered (to give a final concentration of 500 μM) as a single bolus, against one side of the culture plate containing cells previously washed and equilibrated with PBS with calcium and magnesium, pH 7.4, while rapidly swirling the medium to ensure uniform exposure to $ONOO^-$ before decomposition. After 10 min, cells were gently washed with PBS, which was replaced with DMEM without serum for the required times. PBS was used to avoid interfering reactions of $ONOO^-$ with the medium constituents. No pH shift was observed during this treatment. The same volumes of either 10 mM NaOH (vehicle control) or decomposed peroxynitrite ($ONOO^-$ was decomposed in PBS or in 10 mM NaOH overnight) were used as controls. In experiments with Mv3glc, cells were preincubated with this compound for 14 h, and after this time the incubation medium was removed and the cells were submitted to $ONOO^-$ aggression, as described above. Thus, in these assays, Mv3glc was not present in the culture medium during the experiments with $ONOO^-$.

2.4. Cell Viability Test. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT) to formazan, as reported [29]. After incubation with the indicated concentrations of Mv3glc, for 14 h, cells in 6-well plates (0.5×10^6 cells/well) were washed with PBS and incubated with MTT (0.5 mg/mL) for 1 h, at 37°C, in the dark, in new serum-free culture medium. Then, after removing the medium, the formazan crystals were dissolved in DMSO (300 μL), and the extent of MTT reduction was quantified by the measurement of absorbance at 550 nm in a Synergy HT plate reader. Results were expressed as a percentage of control cells.

2.5. Morphology of Cells Nuclei. The morphology of cells nuclei was observed by fluorescence microscopy using the cell-permeable DNA-binding fluorescent dye Hoechst 33258. Briefly, peroxynitrite-treated cells were fixed with 4% (w/v) paraformaldehyde for 10 min, at room temperature, and subsequently stained with Hoechst 33258 (5 μg/mL) for 10 min, at same temperature, washed with PBS, and mounted using PBS : glycerol (3 : 1, v/v). Nuclear morphological changes were visualized in an inverted fluorescence microscope (Zeiss Axiovert 40) with a Zeiss DAPI filter. Cells with chromatin condensation as well as nuclear fragmentation were considered apoptotic, in contrast with the normal cells presenting homogeneously stained nuclei. Cells from at least nine randomly chosen microscope fields (400x) per sample were counted and the number of apoptotic cells was expressed as a percentage of the total number of cells.

2.6. Dihydrorhodamine Fluorescence Assay. The *in vitro* specific ability of Mv3glc to scavenge peroxynitrite was evaluated by the inhibition of peroxynitrite-mediated oxidation of dihydrorhodamine 123 (DHR123) to rhodamine 123, followed by the decrease in fluorescence, as previously described by Kooy et al. [30] with minor modifications. Mv3glc (5 mM stock solution) and DHR123 (5.78 mM stock solution) were dissolved in DMSO and stored at −80°C under nitrogen atmosphere. The working solutions were prepared daily in saline phosphate buffer pH 7.4 (50 mM Na_2HPO_4, 5 mM KCl, 90 mM NaCl, and 100 μM DTPA). Briefly, 25 μM DHR123 were added to saline phosphate buffer with 100 μM DTPA, at 37°C, containing Mv3glc (2–10 μM), and after adding peroxynitrite (1.2 μM in NaOH 0.1 M), the fluorescence intensity of oxidized DHR123 was measured in a Perkin-Elmer LS 50B spectrofluorometer, at 37°C in a thermostatized cuvette with magnetic stirring, at the excitation and emission wavelengths of 500 and 536 nm (slit widths of 2.5 and 3.0), respectively. The results were expressed in terms of % of control (DHR123 plus $ONOO^-$). Decomposed peroxynitrite in buffer did not oxidize DHR123. Quercetin was used as a reference compound.

2.7. Evaluation of Intracellular Reactive Species and of Carbonyl Group Formation. Intracellular reactive species were assessed by using the nonfluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA), which permeates cell membranes and may be oxidized by reactive species, yielding the fluorescent 2′,7′-dichlorofluorescein (DCF), essentially as previously described [31, 32]. Briefly, cells in 24-well plates (1×10^5 cells/well) were previously incubated with or without 25 μM Mv3glc and further submitted to above-referred treatment with peroxynitrite and incubated up to 6 h. Then, 20 min before the end of each hour, cells were incubated with 2 μM DCFHDA prepared in serum-free DMEM, at 37°C, in the dark; after washing of cells with PBS, they were maintained in serum-free DMEM and then observed by fluorescence microscopy (Zeiss Axiovert 40). Also, the fluorescence intensity measurements were obtained in a Synergy HT plate reader (Bio-Tek Instruments) (excitation and emission wavelengths at 485 and 530 nm, resp.). Results were expressed as fluorescence intensities (arbitrary units).

The peroxynitrite-promoted cellular oxidations were also followed in terms of the carbonyl group formation. The treated cells were lysed in HBS buffer supplemented with 1% protease inhibitor cocktail, pH 7.5 on ice. After 5 cycles of liquid N_2/37°C water bath, lysates were centrifuged at 15000 g for 20 min at 4°C, and the supernatants (total extracts) were collected and stored at −20°C. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, USA), based on the Bradford reaction.

The carbonyl content was determined in duplicates using an already established colorimetric assay [33]. Briefly, 800 μg of protein were incubated with 500 μL of 0.2% 2,4-dinitrophenylhydrazine (DNPH) (in 2 N HCl) for 1 h in the dark, and blanks were prepared using 2 N HCl alone. Samples and blanks were precipitated by the addition of an equal volume of 20% trichloroacetic acid and centrifugation at 10000 g for 5 min, 4°C. The supernatants were discarded and the pellets were submitted three times to 10 minutes

wash with 1 mL of ethanol : ethyl acetate (1 : 1 v/v), vortexing each 3 minutes during the process. The samples were then centrifuged at 10000 g for 3 min, 4°C. The final pellets were resuspended in 0.5 mL of 6 M guanidine-HCl, and the carbonyl contents were determined by measuring the absorbance of the hydrazones at 370 nm. Results were calculated as μmol carbonyl/mg protein using an extinction coefficient of 22000 M^{-1} cm^{-1}.

2.8. Measurement of Mitochondrial Membrane Potential. Measurement of the mitochondrial membrane potential ($\Delta\Psi m$) was performed by loading cells with the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), a membrane-permeable cationic fluorophore that accumulates inside mitochondria in a potential-dependent manner, which is visualized by a shift in its fluorescence emission from green (525 nm) to red (590 nm) [34]. Cells were preincubated with Mv3glc and treated with peroxynitrite as described above; at the indicated times, the supernatants were removed and cells were labeled with JC-1 (2 μg/mL) at 37°C, for 20 minutes. Further, after washing with PBS, cells were visualized in an inverted fluorescence microscope (Zeiss Axiovert 40) with an FITC filter set. In the different experiments, the ratios between the fluorescence intensities of the J aggregates, in mitochondria (excitation 550 nm, emission 600 nm), and of the monomers remaining in cytoplasm (excitation 485 nm, emission 535 nm) were used to obtain the red/green fluorescence ratios. Fluorescence intensities were measured in a Synergy HT microplate reader (BioTek Instruments, Inc, USA).

2.9. Measurement of Caspases Activities. Caspase activation was assessed, essentially, as previously described by Brito et al. [12], in cytosolic protein extracts. After cells incubation with peroxynitrite, with or without the anthocyanin, as above described, cells were washed with PBS and then scrapped on ice in a lysis buffer containing 1 mM sodium ethylenediaminetetracetic acid (Na-EDTA), 1 mM Na-EGTA, 2 mM $MgCl_2$-$6H_2O$, 25 mM HEPES (pH 7.5) supplemented with 0.1% CHAPS, 100 mM PMSF, 2 mM DTT, and 1% (v/v) protease inhibitor cocktail. After 5 cycles of freezing and thawing with liquid N_2 and 37°C water bath, lysated cells were centrifuged at 14000 g for 10 min at 4°C and the collected supernatants were stored at −80°C.

The enzymatic activities were determined in 25 μg of lysates protein in a reaction buffer solution (25 mM HEPES, pH 7.5 supplemented with 0.1% CHAPS, 5 mM DTT, and 100 mM PMSF). The reactions were initiated by addition of adequate fluorogenic substrates (100 μM) containing specific cleavage sites, linked to a fluorochrome: Ac-DEVD-AMC for caspase-3-like activity and Ac-LEDH-AFC for caspase-9-like activity. After incubation for 150 min at 37°C, the release of AMC or AFC was determined by fluorescence measurements at 380/460 nm or 400/505 nm (excitation/emission), respectively, in a Synergy HT microplate reader. Caspases activities were expressed as a percentage of the control, that is, cells incubated without neither peroxynitrite nor anthocyanin.

2.10. Western-Blot Analysis. Protein expression was evaluated by Western-blot, as essentially described [14]. Cytoplasmic protein extracts were obtained from lysed cells prepared in a solution containing 300 μL of 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and 1% protease inhibitor cocktail, pH 7.5, for 10 min on ice. After centrifugation at 5000 rpm for 5 min at 4°C, the supernatants were collected and stored at −20°C. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, USA), based on the Bradford reaction.

Thirty micrograms of reduced and denaturated total protein from each extract were separated by SDS/PAGE [10% (v/v)] and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, UK) by electroblotting. The membranes were blocked with skimmed milk in TBS buffer supplemented with 0.1% (v/v) Tween 20 (TBS-T: 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween) and then probed with antibodies against Bax for 2 h, at room temperature. After extensive washings with TBS-T, the blots were incubated with an alkaline phosphatase-conjugated secondary antibody for 1 h, at room temperature. Immunoreactive complexes were detected by chemifluorescence after blots exposition to enhanced chemifluorescent reagent (Amersham Biosciences) in a Typhoon 9000 scanner (Amersham Biosciences). β-Actin was used as a control for protein loading in the cytoplasmic extracts. Bands were analyzed using the ImageQuant TM software from Amersham Biosciences.

2.11. Statistical Analysis. All data were expressed as means ± SEM of at least 3 independent assays, each one in duplicate. Differences between groups were analyzed by one-way analysis of variance (ANOVA), Bonferroni's, Tukey's, or Student tests were used as appropriate. A value of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Protection of Endothelial Cells against Peroxynitrite-Mediated Apoptosis. In agreement with our previous reports [12, 14], peroxynitrite under this experimental conditions, induced apoptotic death in bovine aortic endothelial cells (Figure 2(a)), a process significantly reduced in cells previously incubated with Mv3glc. In fact, in that figure, it is evidenced that peroxynitrite cell treatment, after 6 h of cell incubation, as described in Section 2, led to about 30% of apoptotic cells, as assessed by nuclear condensation or fragmentation visualized by Hoechst staining, whereas in untreated cells these features were not detected (less than 2%). A reduction in about 70% of apoptotic cells was observed in preincubated cells with 25 μM Mv3glc, before $ONOO^-$ aggression, a result similar to those obtained with other anthocyanins. At this concentration, and during all the experience time, Mv3glc *per se* did not interfere with endothelial cells viability and prevented the peroxynitrite-induced decrease in cell viability, as evaluated by the MTT test (Figure 2(b)). Of note is that the anthocyanin was not present in the medium during peroxynitrite treatment and

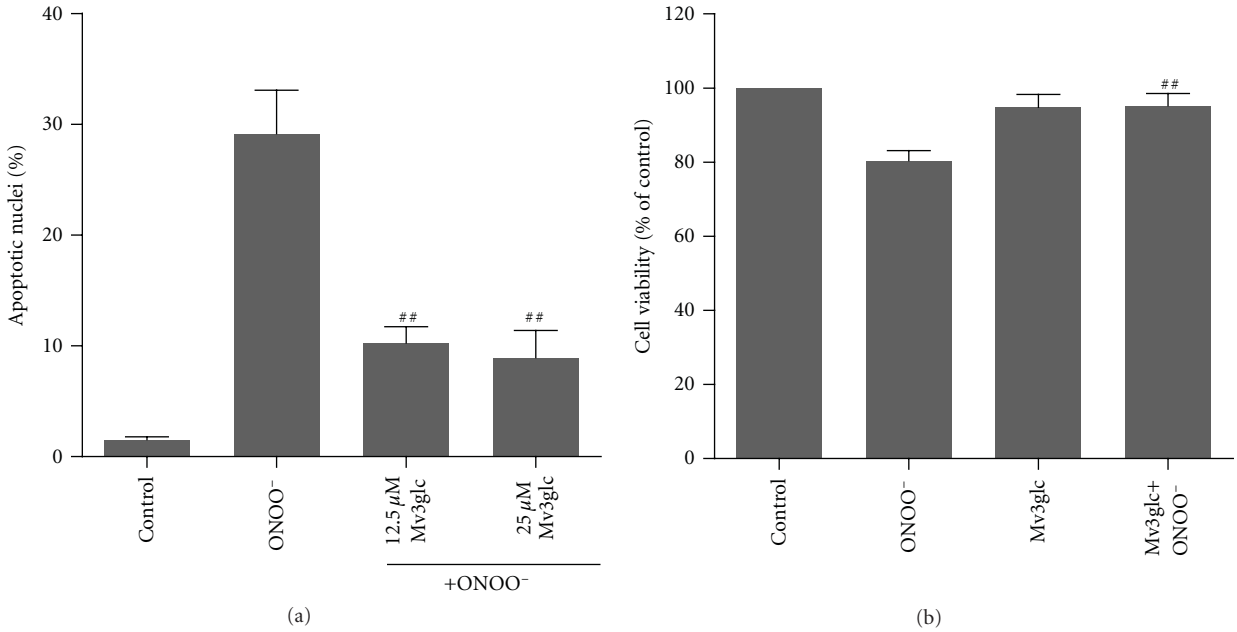

Figure 2: Mv3glc prevented peroxynitrite-mediated apoptotic and viability changes in endothelial cells. (a) Confluent BAECs were preincubated with either 12.5 or 25 μM Mv3glc for 14 h. Subsequently, cells were washed with PBS and treated with peroxynitrite, as described in Section 2. Morphological apoptotic changes were assessed after nuclei staining with Hoechst 33258. (b) Cellular viability of BAEC incubated for 14 h with 25 μM Mv3glc without further treatment or treated with peroxynitrite, as assessed by MTT reduction, and expressed as percentage of control cells. Control refers to the experiment in similar conditions without peroxynitrite and anthocyanin; $ONOO^-$ refers to the experiment with 500 μM peroxynitrite without anthocyanin. Values are mean ± SEM of at least five different experiments, each one assayed in duplicate. $^{***}P < 0.001$ versus control; $^{\#\#}P < 0.01$ versus $ONOO^-$.

afterwards, and after the incubation period, intact Mv3glc was detected in endothelial cells (3 nmoles/mg protein), as evaluated by HPLC (results not shown).

3.2. Peroxynitrite Scavenging Activity of Malvidin-3-Glucoside. It is known that the methoxy and hydroxyl substituents in the B-ring of malvidin-3-glucoside make this anthocyanin highly reactive toward radical species, although its role as physiological antioxidant has been questionable due to a probable instability under neutral conditions and to unknown reachable cellular concentrations [22]. The capacity of this compound to scavenge peroxynitrite, as compared with a reference antioxidant, quercetin, was previously assessed in a cell-free model system, in terms of inhibition of dihydrorhodamine oxidation promoted by this reactive species.

As shown in Figure 3, Mv3glc at very low concentrations (2–10 μM) strongly decreases the percentage of peroxynitrite-mediated DHR123 oxidation, revealing a high capacity of this flavonoid to scavenge peroxynitrite in a concentration-dependent way. It presented a relative low IC_{50} value (2.9 μM), as compared to quercetin (IC_{50} value of 1.12 μM), a well-known reference compound with a high peroxynitrite scavenging activity. The use of bicarbonate in the assay buffer did not alter significantly the IC_{50} values (results not shown).

3.3. Inhibition of Intracellular Formation of Reactive Species and of Carbonyl Groups during Peroxynitrite Aggression. The

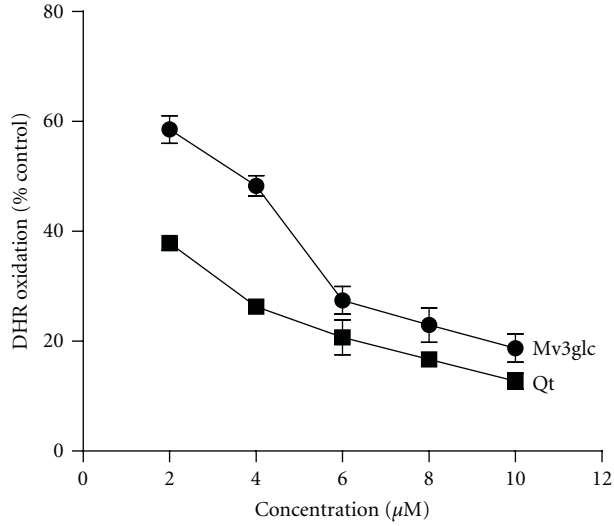

Figure 3: Mv3glc, at low concentrations, efficiently inhibit peroxynitrite-induced dihydrorhodamine 123 oxidation. Briefly, 25 μM DHR 123 was incubated with either Mv3glc or quercetin (Qt), used as a reference compound, at 37°C, and after addition of 1.2 μM peroxynitrite the fluorescence was immediately assessed, as described in Section 2. The measured fluorescence intensities were expressed in percentage relative to control (DHR123 plus $ONOO^-$ without Mv3glc or quercetin). Values are results of five independent experiments. $P < 0.001$ for all concentrations of Mv3glc or of quercetin versus control.

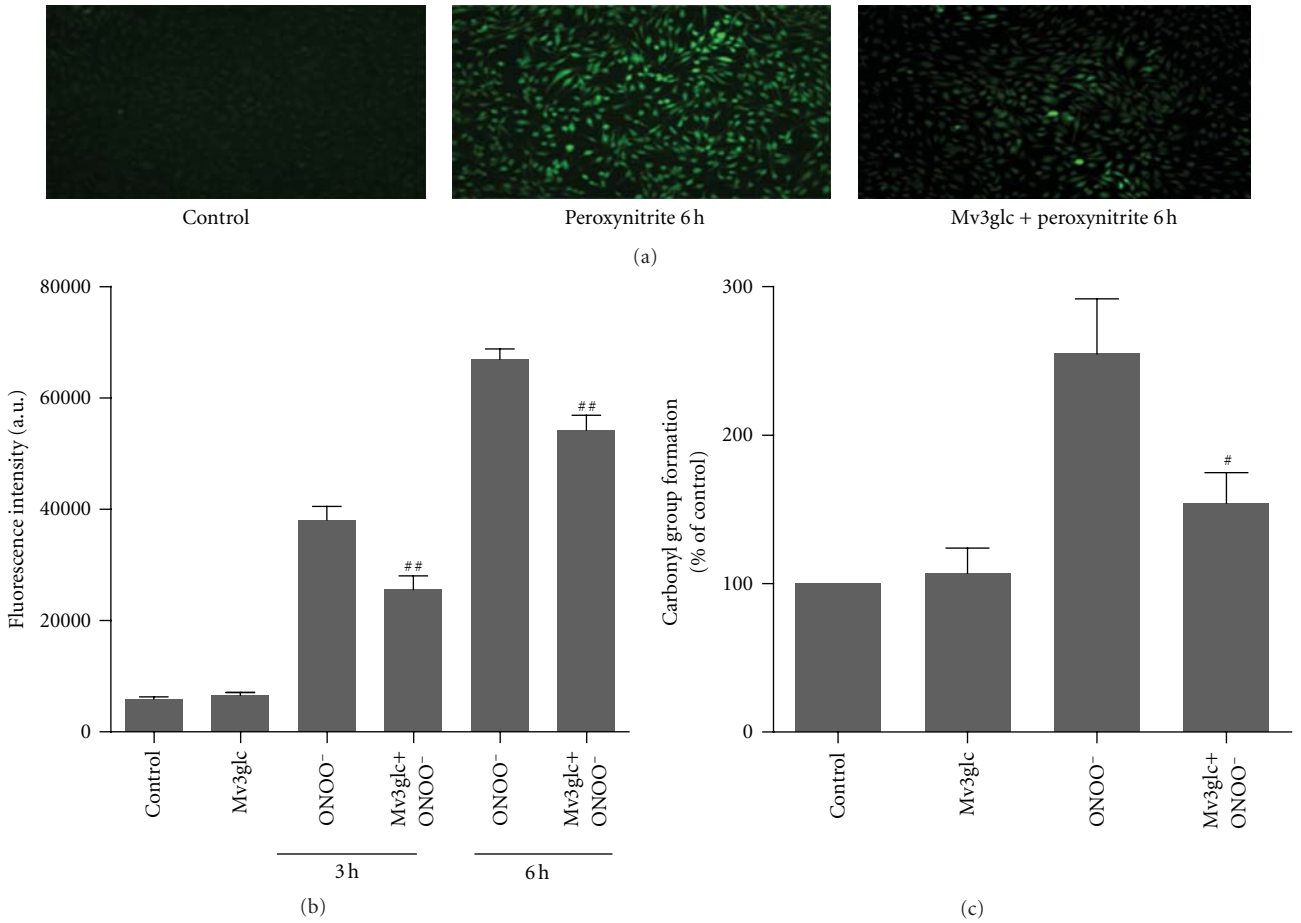

Figure 4: Mv3glc decreases peroxynitrite-mediated reactive species production (a, b) and carbonyl groups formation (c) in endothelial cells. BAECs were preincubated with 25 μM Mv3glc for 14 h and then submitted to peroxynitrite aggression, which was carefully removed after 10 min of incubation and maintained during the indicated times, in a serum-free medium, at 37°C, as described in Section 2. (a) Representative images of endothelial cells, at 6 h of incubation after $ONOO^-$ treatment, in the absence or presence of 25 μM Mv3glc, obtained by fluorescence microscopy (400 x). (b) Reactive species production was measured, at 3 h and 6 h of incubation, in terms of fluorescence intensity of DCF. (c) Carbonyl groups formation was assessed in total protein cell extracts obtained, as indicated in Section 2. Results are expressed as % of control, that is, cells incubated in similar conditions without either peroxynitrite or Mv3glc. Values are mean ± SEM of five experiments. $^{***}P < 0.001$ versus Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus $ONOO^-$.

effect of Mv3glc on peroxynitrite-induced oxidative stress on endothelial cells was also assessed by the detection of the dichlorofluorescein (DCF) form by fluorescence microscopy. This assay has been commonly used to assess cellular production of reactive oxidant species as a result of the oxidation of the nonfluorescent polar molecule dichlorodihydrofluorescein diacetate (DCFH-DA). This compound is passively loaded into whole cells, and once inside them, it is cleaved by esterases to yield DCFH, which may be oxidized to DCF by a variety of reactive species [35]; thus, an increase in DCF fluorescence is an index of cellular reactive oxidant species formation, as shown typically in Figure 4(a) at 6 h of incubation after peroxynitrite treatment. As expected, such aggressive treatment induced an increase in intracellular oxidative stress, as indicated by the increase in cell mean fluorescence, as early as 3 h of incubation, which continues to increase up to 6 h, according to the kinetic analysis previously reported [14]. This fluorescence was significantly reduced in cells preincubated with 25 μM Mv3glc before peroxynitrite addition, as shown typically in Figure 4(a) and presented in Figure 4(b) at 3 and 6 h of incubation after peroxynitrite treatment; at these times, the decreases in fluorescence were about 37 and 24%, respectively, relative to the assay with peroxynitrite stimulus without the anthocyanin.

On the other hand, the protein carbonyl formation in endothelial cells treated with peroxynitrite was also evaluated, as a marker for protein oxidation and recognized to be useful to estimate the extent of peroxynitrite oxidative damage to proteins [36]. In peroxynitrite-mediated endothelial cells injury, an increase in about 150% of carbonyl groups formation was observed, as evaluated by the general assay with DNPH [37] (Figure 4(c)). When preincubated with

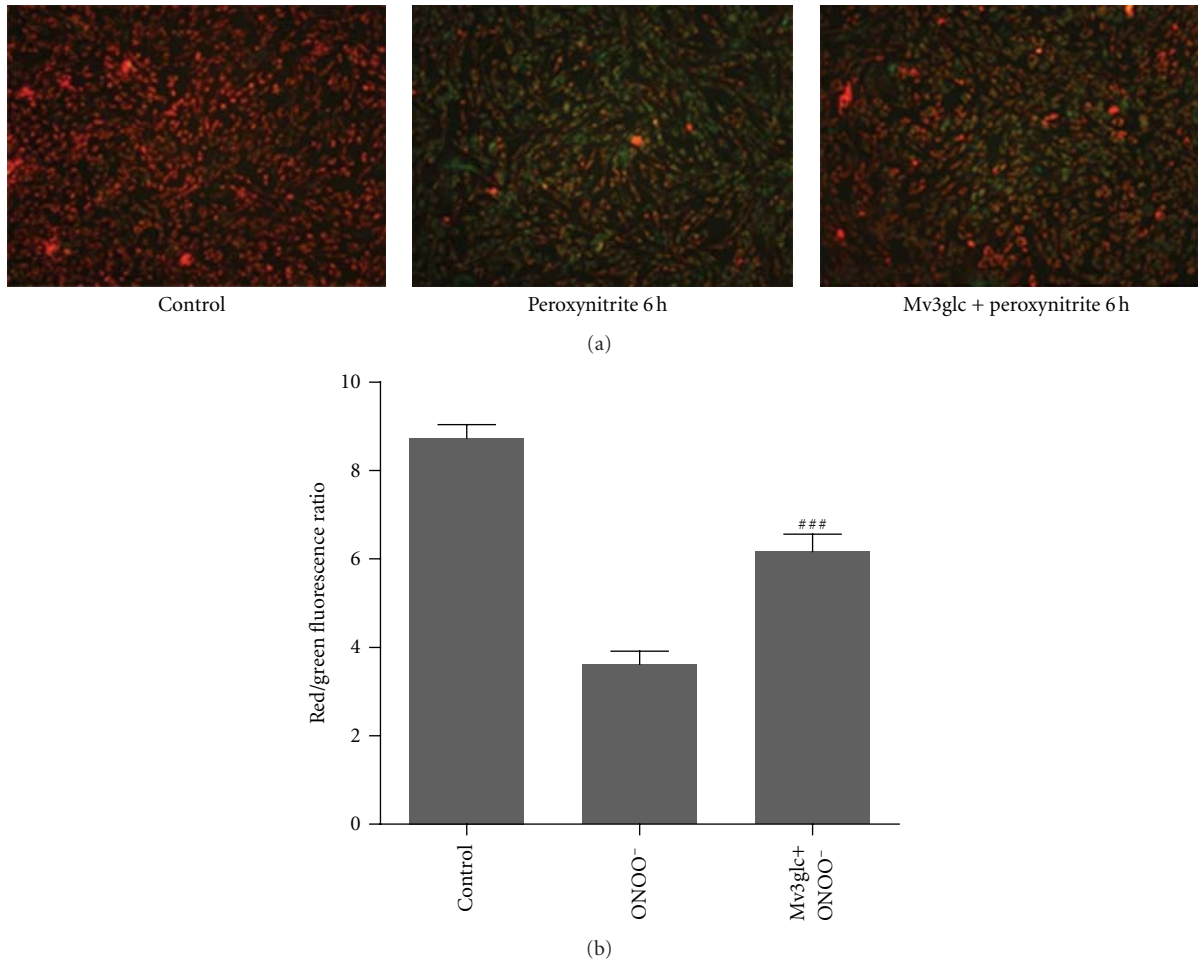

Figure 5: Mv3glc prevents peroxynitrite-induced depolarization of mitochondria. Endothelial cells, preincubated or not with 25 μM Mv3glc, were loaded with JC-1 fluorophore 20 min prior to the end of the 6 h of incubation with peroxynitrite and incubated for 20 min at 37°C, in the dark, as described in Section 2. Further, cells were visualized in a fluorescence microscope (a) and the fluorescence intensities of either the monomers (green) or J-aggregates (red) were assessed in a microplate reader (b). In (a), representative images of endothelial cells labeled with JC-1, treated with peroxynitrite in the absence or presence of Mv3glc, obtained by fluorescence microscopy (400x). In (b), quantification of mitochondrial membrane potential in terms of red/green fluorescence ratio, as measured in the same cell cultures. A decrease in red/green fluorescence intensity ratio indicates mitochondria depolarization. Values are mean ± SEM of five experiments, each one assayed in duplicate. $^{***}P < 0.001$ versus Control; $^{###}P < 0.001$ versus $ONOO^-$.

Mv3glc, a reduction in the carbonyl groups formation by about 70% occurred, pointing to a significant decrease in oxidative damage to cell proteins.

3.4. Inhibition of Peroxynitrite-Induced Depolarization of Mitochondria. It is well known that the loss of the mitochondrial membrane potential is a hallmark of cellular apoptosis. In order to evaluate whether inhibition of this event accounts for the antiapoptotic effects of Mv3glc, changes in the mitochondrial membrane potential were assessed after loading the cells with the fluorescent probe JC-1 previously submitted to peroxynitrite challenge. In cells with high mitochondrial membrane potential, the monomeric JC-1 dye accumulates in mitochondria and aggregates, emitting red fluorescence, but in cells undergoing apoptosis, the mitochondria depolarization prevents the entrance of JC-1 within the mitochondria. Thus, in these cells, JC-1 remains in the cytoplasm in a green fluorescent monomeric form (Figure 5(a)). Results in this figure show that in endothelial cells submitted to peroxynitrite injury, a strong decrease occurred in the cell population emitting red fluorescence accompanied by an increase in the population with green fluorescence, reflected in a decrease in the red/green fluorescence ratio of about 60% relative to the control, without peroxynitrite (Figure 5(b)).

The preincubation of cells with 25 μM Mv3glc before peroxynitrite aggression led to an increase in this ratio by 30% of the control with peroxynitrite, indicating that this anthocyanin prevented significantly the loss of peroxynitrite-mediated mitochondrial membrane potential.

3.5. Inhibition of Peroxynitrite-Induced Activation of Caspases-9 and -3. The mitochondrial depolarization is commonly related with the sequential activation of caspases-9 and -3

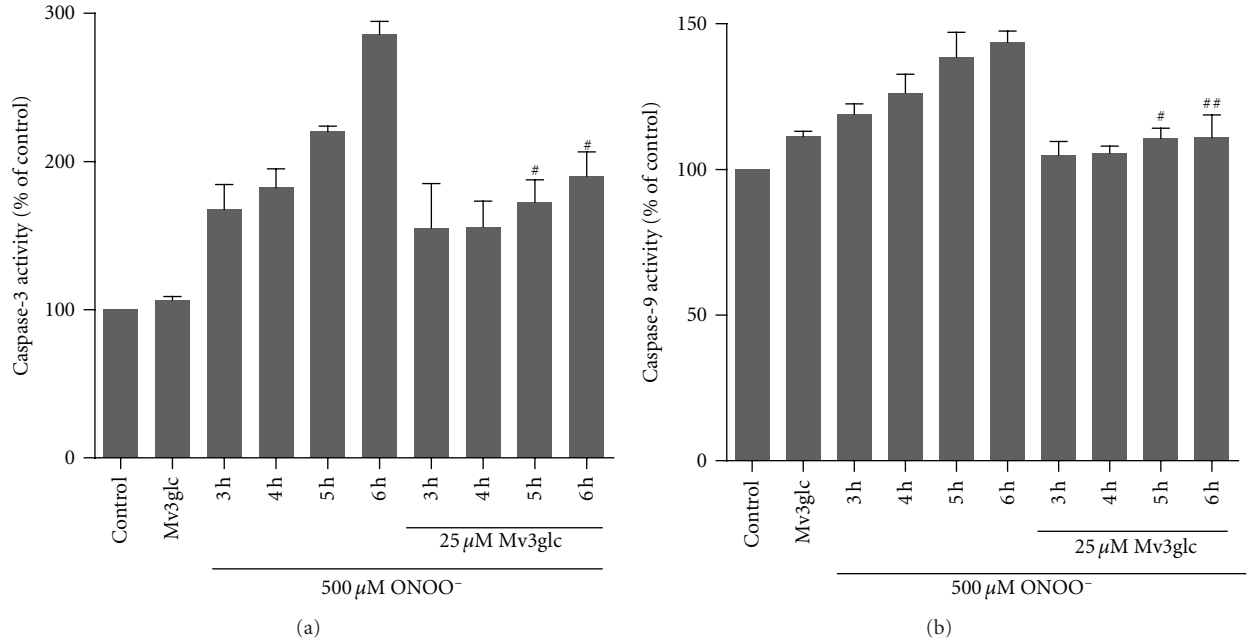

Figure 6: The activation of caspases-3 and -9 in endothelial cells submitted to peroxynitrite injury is significantly inhibited by the preincubation of cells with 25 μM Mv3glc, for 14 h. BAECs were treated with 500 μM peroxynitrite after this preincubation step, as described in Section 2, and graphs show the kinetic activities of cellular caspase-3 (a) and caspase-9 (b) induced by peroxynitrite treatment ($ONOO^-$) in the absence and presence of anthocyanin. Values are mean ± SEM of five different experiments, each one assayed in duplicate. For caspase-3, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Control; $^{\#}P < 0.05$ versus $ONOO^-$. For caspase-9: $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus $ONOO^-$.

[38], and previous data from our laboratory have already reported that peroxynitrite-induced endothelial cells apoptosis is mediated by activation of both caspases [12]. Figure 6 shows that preincubation of cells with 25 μM Mv3glc prevented almost completely the caspases-9 activity and led to a decrease in caspase-3 activity by about 60% at 6 h of incubation after peroxynitrite aggression.

Therefore, these results are in accordance with others previously reported indicating that in our experimental conditions, peroxynitrite triggers apoptosis through the mitochondrial or intrinsic pathway, as caspases-3 and -9 are the effector and the initiator caspases, respectively, of this cell death cascade and indicate that Mv3glc was able to disrupt this pathway, inhibiting cell apoptosis.

3.6. Decrease in Peroxynitrite-Induced Bax Levels. The Bcl-2 family of proteins has been established to play a crucial role in mitochondrial-mediated apoptosis [39, 40]. Data from our laboratory have already shown that peroxynitrite *per se* disrupts the intracellular balance between proapoptotic and antiapoptotic proteins by increasing Bax intracellular levels without affecting those of Bcl-2. We confirmed that peroxynitrite did not modify significantly the level of Bcl-2, even in the presence of Mv3glc (results not shown).

On the contrary, Bax expression level suffered a strong increase during the time course after peroxynitrite treatment, reaching about 2-fold the control at the 4th hour of incubation (Figure 7). Such increase was significantly reduced by about 70% in cells preincubated with Mv3glc, at the end of the third and fourth hours, after peroxynitrite injury.

4. Discussion

During the last years, there has been an increasing interest in dietary anthocyanins mainly due to the increasing knowledge of their broad pharmacological activities, in particular, in the context of prevention of atherosclerosis [41], and among these compounds, Mv3glc is one of the major constituents. Despite that the exact mechanisms, by which anthocyanins exert health beneficial effects, are still not well understood, it has been shown that they inhibit different proatherogenic pathways, namely, increasing resistance of LDL to peroxidation, modulating platelet aggregation, and promoting nitric oxide-mediated vasorelaxation [16, 42]. Concerned with the atherogenic process, peroxynitrite is a potent physiological oxidant and a crucial intervenient in its development, as above referred. The ability of anthocyanins to scavenge peroxynitrite *in vitro* is well known, mainly due to their electrodonating properties and as alternative substrates for nitration [43]. However, although research over the last decades has been focused on the correlation between biological activities and antioxidant properties, their role as critical cellular mediators with interference in signaling pathways and gene regulation is an important research field [44, 45]. Thus, following our previous work [14], here

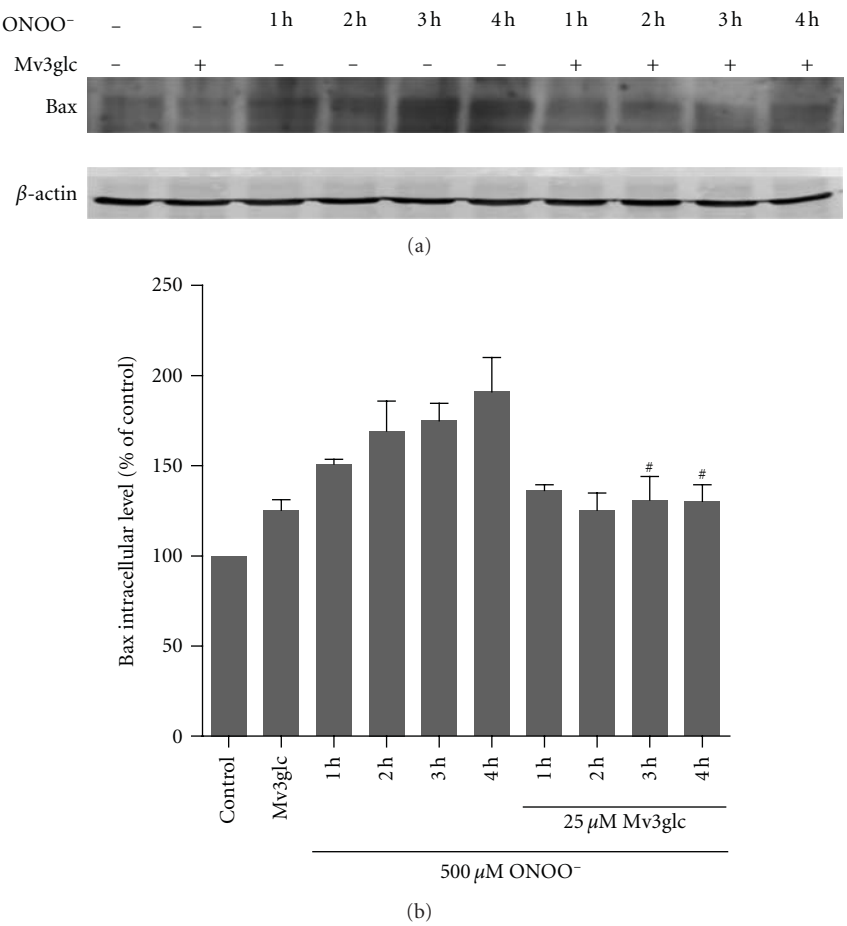

Figure 7: Mv3glc reduced the increase in Bax cytoplasmatic levels induced by peroxynitrite. BAEC were preincubated with 25 μM Mv3glc with no further treatment or post-treated with peroxynitrite for 10 min, as described in Section 2 and maintained during 6 h in a serum-free medium, at 37°C. Cytoplasmatic total protein extracts were then analyzed by immunoblot with specific antibody against Bax. Representative blots and densitometric quantifications of three independent experiments are shown. Results were normalized to endogenous β-actin and expressed as mean ± SEM of percentage of control. Values are mean ± SEM of four experiments, each one assayed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Control; $^{\#}P < 0.05$ versus $ONOO^{-}$.

we explored the cytoprotective mechanisms for Mv3glc in endothelial cells under peroxynitrite aggression.

Specifically, we demonstrated that Mv3glc, when previously incubated with endothelial cells, was able to protect them from peroxynitrite-mediated apoptotic cell death (Figure 2(a)) revealing that the potential steric hindrance due to its hydroxylation pattern and methoxy groups is not an impediment for its cellular uptake, allowing degrees of protection similar to other studied anthocyanins [14]. In fact, Mv3glc incorporates into endothelial cells under our experimental conditions, as verified by HPLC analysis (results not shown), in agreement with results previously reported [20].

In endothelial cells, Mv3glc counteracted the intracellular formation of reactive species after peroxynitrite-mediated insult, as evaluated by the DCF assay (Figure 4), in accordance with its high ability to scavenge peroxynitrite, as observed in the dihydrorhodamine oxidation assay (Figure 3). The observed reactive species were formed as a consequence of the primary insult to the cells, considering that the added peroxynitrite was removed after the incubation period, by washing cells carefully. Moreover, the ability of Mv3glc to neutralize the oxidative injury to cells triggered by such species is evidenced by a reduction in peroxynitrite-mediated carbonyl groups formation, the most commonly measured products of protein oxidation in biological samples [46], by about 70% relative to the assay without the anthocyanin (Figure 4). Proteins are major targets for damage due to their abundance and rapid rates of reaction with a wide range of reactive species, which are well recognized as relevant intervenients in atherogenic process, and the protein carbonyls have been reported as useful markers of protein oxidative events either *in vitro* or *in vivo* assays.

Therefore, the protection afforded by Mv3glc against peroxynitrite-mediated injury in endothelial cells may be partially mediated by its ability to scavenge reactive species formed as a consequence of cellular oxidative aggression.

Such cellular reactive species are thought to be responsible for triggering several deleterious events including mitochondrial-associated apoptosis. In accordance with previous studies from our laboratory and others, peroxynitrite, at lower concentrations, induces apoptosis by triggering the mitochondrial pathway, while at higher concentrations it leads mainly to cellular necrosis [12]. Although the used concentration of peroxynitrite (500 μM) is apparently high, the net exposure of cells to this species is much lower considering its short half life (less than 1 s, 37°C, pH 7.4), reaching levels more closed to those which have been estimated to be produced *in vivo* [47].

Actually, in our experimental conditions, peroxynitrite insult into endothelial cells induced apoptotic cell death, and Mv3glc, in a similar way to other anthocyanins with a different B-ring structure, was able to counteract this deleterious effect through the inhibition of crucial mitochondrial signaling cascades. In fact, the loss of mitochondrial potential due to peroxynitrite, as indicated by the green fluorescence resulting from the monomeric form of JC-1 outside the mitochondria, was prevented efficiently by cellular pretreatment with Mv3glc, as evidenced by the increase in JC-1 aggregated red fluorescence inside the mitochondria (Figure 5). This result is relevant, given the importance of mitochondria in regulating cell death pathways. Moreover, Mv3glc proved its effectiveness in preventing the activation of both caspases-3 and -9 (Figure 6), an event involved in mitochondrial-mediated apoptotic death pathway. Thus, Mv3glc ability to interfere with the apoptotic intracellular signaling cascade may be closely related to cell protection from mitochondrial disruption.

Recently, we have shown that peroxynitrite induces mitochondrial membrane permeabilization by interfering in the balance between pro- and antiapoptotic levels of the Bcl-2 family members, by increasing Bax levels without changes in Bcl-2, leading to a raise in Bax/Bcl-2 ratio [12, 14]. Bax is a proapoptotic protein that forms cytoplasmatic heterodimers with antiapoptotic Bcl-2 family members but when cells are submitted to an apoptotic insult it may suffer mitochondrial translocation, promoting outer membrane permeabilization and the subsequent mitochondrial-mediated apoptotic pathway. Similarly to other anthocyanins previously studied, namely, cyanidin-, delphinidin- and pelargonidin-3-glucoside [14], Mv3glc was capable of decreasing Bax intracellular levels induced by peroxynitrite stimulus (Figure 7), indicating that its protective effect may precede mitochondria depolarization and caspases activation. Besides, as Mv3glc counteracted the raise in Bax levels without affecting Bcl-2 levels, a decrease in the Bax/Bcl-2 ratio occurred, restoring the balance between pro- and antiapoptotic proteins.

In conclusion, our results support the potential benefits of Mv3glc, one of the most prevalent anthocyanins in diet, as a vascular protectant. Clearly, it protects endothelial cells against peroxynitrite-induced damage by counteracting reactive species formation and interfering in the regulation of apoptotic intracellular signaling mechanisms mediated by mitochondria. The potential steric hindrance due to its B-ring substitution pattern is not an impediment to its cellular action, allowing a protection profile similar to that reported for structurally different anthocyanins. Indeed, although the o-dihydroxy (catechol) structure of the B-ring, associated to a hydroxyl group in the 3 position, seems to confer to anthocyanins significant higher general antioxidant and peroxynitrite-scavenging capacities [14], the protection afforded by all the studied compounds, including the monophenolic pelargonidin and Mv3glc, against peroxynitrite-induced mitochondrial apoptotic effects in endothelial cells is not significantly different. All the tested anthocyanins showed ability to counteract such effects in a similar way, through the inhibition of crucial signaling cascades, suggesting that their cellular actions are much beyond their antioxidant activities. Despite the need for further *in vivo* studies to confirm these findings, our data together with other previously reported by us support mechanistically the health benefits of this class of dietary nutrients. Moreover, it reinforces the view that anthocyanins, including Mv3glc, can be promising molecules for development of functional foods and nutraceuticals to improve endothelial function, in the context of prevention of atherosclerosis.

Acknowledgments

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Research Article

The Effects of New Alibernet Red Wine Extract on Nitric Oxide and Reactive Oxygen Species Production in Spontaneously Hypertensive Rats

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We aimed to perform a chemical analysis of both Alibernet red wine and an alcohol-free Alibernet red wine extract (AWE) and to investigate the effects of AWE on nitric oxide and reactive oxygen species production as well as blood pressure development in normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHRs). Total antioxidant capacity together with total phenolic and selected mineral content was measured in wine and AWE. Young 6-week-old male WKY and SHR were treated with AWE (24,2 mg/kg/day) for 3 weeks. Total NOS and SOD activities, eNOS and SOD1 protein expressions, and superoxide production were determined in the tissues. Both antioxidant capacity and phenolic content were significantly higher in AWE compared to wine. The AWE increased NOS activity in the left ventricle, aorta, and kidney of SHR, while it did not change NOS activity in WKY rats. Similarly, increased SOD activity in the plasma and left ventricle was observed in SHR only. There were no changes in eNOS and SOD1 expressions. In conclusion, phenolics and minerals included in AWE may contribute directly to increased NOS and SOD activities of SHR. Nevertheless, 3 weeks of AWE treatment failed to affect blood pressure of SHR.

1. Introduction

Recent findings have supported the idea that natural bioactive compounds could be beneficial for the prevention and treatment of cardiovascular disease, although the benefits for individuals may depend on their genetic profile [1–7]. Among foodstuffs widely distributed in the human diet, red wine has attracted considerable interest as a potential source of bioactive compounds such as polyphenols and minerals [8–10].

To date several studies have proposed the mechanisms by which red wine could exert its beneficial effects toward cardiovascular disorders and hypertension in particular. These mechanisms mainly involve increased vasorelaxation and blood pressure reduction by enhancing nitric oxide synthase (NOS) activity and nitric oxide production [11–15]. Moreover, red wine could modulate activities of endogenous antioxidant enzymes, thereby enhancing the functions of body defense against the oxidative stress [16, 17].

Polyphenols and minerals are among the principal bioactive compounds present in red wines. While red wine polyphenolic compounds are well known for their direct free radical scavenging, antioxidant, anti-inflammatory, antiplatelet aggregation, and lipid metabolism improving activities [18, 19], the effects of minerals present in red wines have been studied much less. Red wine contains a number of trace elements such as magnesium, zinc, potassium, and manganese essential for the proper function of endogenous

antioxidant system [8, 20]. Mounting evidence suggests that minerals are able to counteract the development of cardiovascular, metabolic and other diseases [21, 22]. The positive effects of minerals in humans are related to their ability to enhance the activity of the body's antioxidant defense system by catalyzing antioxidant enzymes. For instance, several minerals found in foodstuffs, such as copper, zinc, and manganese, are essential for activity of superoxide dismutase (SOD), a key antioxidant enzyme [23, 24].

The modulation of nitric oxide synthase activity seems to be the most promising tool by which different polyphenolic compounds could regulate the blood pressure. It has been documented that oral administration of red wine polyphenolic compound can reduce myocardial fibrosis, prevent aortic thickening, attenuate the increase of aortic reactivity to norepinephrine, prevent the decrease in acetylcholine-induced endothelium-dependent relaxation, and, in rats, reduce blood pressure during NO deficiency. These alterations were associated with increased NOS activity, a moderate increase in endothelial NOS expression, and reduction of oxidative stress, the factors which may be responsible for the beneficial effect of the red wine polyphenolic compounds [25, 26]. Moreover, the study of Bernátová et al. demonstrated that red wine polyphenols (Provinols) even accelerate the decrease in blood pressure and improved structural and functional cardiovascular changes produced in developed form of N^G-nitro-L-arginine-methyl-ester (L-NAME)-induced hypertension [27].

Despite considerable attention paid toward cardiovascular disorders, experimental studies are still lacking. This study provides comprehensive chemical analysis of both red wine and red wine extract in order to evaluate the effect of red wine compounds on NOS and SOD activities, eNOS and SOD1 expressions, and blood pressure development in normotensive and spontaneously hypertensive rats.

2. Materials and Methods

2.1. Chemicals. Gallic acid, Folin-Ciocalteau reagent, potassium peroxodisulfate, potassium superoxide, Trolox, and SOD assay kit were obtained from Sigma-Aldrich. The ABTS assay used a commercial kit Randox TAS was purchased from Randox Laboratories, Antrim, United Kingdom. Lucigenin purchased from Fluka. All the other reagents were of analytical grade.

The wine examined was obtained from the Slovak State Institute of Viniculture (Modra, Slovakia). Samples of Alibernet red wine were subjected to the process of dealcoholization, and concentration, producing an alcohol-free ten times concentrated Alibernet red wine extract (AWE).

2.2. Alibernet Red Wine and AWE Characterization

2.2.1. Measurement of Total Antioxidant Capacity. In the Alibernet wine and AWE samples, TAC was measured using spectrophotometric assays on a Thermo Spectronic Genesys 20 UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). TAC was determined using a commercial kit Randox TAS (radical ABTS; Randox Laboratories, Antrim, United Kingdom) according to the manufacture instructions. Amounts of antioxidant power are expressed in Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents (mmol/L).

2.2.2. Measurement of Total Phenolic Content. Total phenolic content was determined using Folin-Ciocalteau reaction [28] by spectrophotometric assay on a Thermo Spectronic Genesys 20 UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Absorbance was measured at 760 nm using gallic acid as the standard. TPC is expressed in gallic acid equivalents (GAEs).

2.2.3. Determination of Minerals. The red wine and AWE elemental composition was determined by AAS method using acetylene/argon flame in Atomic Absorption Spectrometer (Varian Spectra AA 220 FS, Australia) for copper, zinc, selenium, and lead, ISE (Ion Selective Electrode) for potassium determination, and Photometry (Roche equipment, Modular E 170, Switzerland) for calcium, magnesium, phosphorus, and iron determination.

2.3. Animal Studies

2.3.1. Animals and Treatment. All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use.

Male 6-week-old normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) were divided into the control groups and groups treated with AWE (24,2 mg/kg/day) for 3 weeks ($n = 6$ in each group). The extract was given in tap water. To ensure that each animal received the complete amount of AWE, the calculated amount of liquid extract was given to each cage in the appropriate volume of water and adjusted to the animal water consumption. Daily water consumption was estimated individually for every animal 1 week before the experiment. During the experiment, water consumption was controlled. All animals were housed at a temperature of 22–24°C and fed with a regular pellet diet ad libitum. Systolic blood pressure (SBP) was measured by a noninvasive method of tail-cuff-plethysmography every week.

After 3 weeks of treatment, the animals were sacrificed and the body weight (BW) and heart weight (HW) were determined. Samples of the left ventricle, aorta, and kidney were used to determine NOS and SOD activities and eNOS and SOD1 protein expressions. Superoxide levels were measured in the left ventricle and aorta. Total antioxidant capacity and SOD activity were measured also in the plasma.

2.3.2. Total NOS Activity and eNOS Protein Expression. Total NOS activity was determined in crude homogenates of the LV, aorta, and kidney by measuring the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine as previously described by

Bredt and Snyder, with minor modifications by Pecháňová et al. [29, 30]. [^{3}H]-L-citrulline was measured with the Quanta Smart TriCarb Liquid Scintillation Analyzer (Packard Instrument company, Meriden, CT). Total NOS activity is expressed as pkat/g of proteins.

For eNOS protein expression, samples were prepared according to Pecháňová et al. [31]. Following the electrophoresis, proteins were transferred to nitrocellulose membranes and were probed with a polyclonal rabbit anti-eNOS antibody (Santa Cruz Biotechnology, Inc., USA). Bound antibody was detected using a secondary peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Inc., USA). The bands were visualized using the enhanced chemiluminescence system (ECL, Amersham, UK) and analyzed densitometrically using Photo-Capt V.99 software.

2.3.3. Total SOD Activity and SOD1 Protein Expression. The SOD activity was analyzed using the SOD assay kit (Fluka). The absorbance was read at 450 nm using a microplate reader (Thermo Scientific Multiscan FC, Finland). SOD activity was calculated using activity of SOD standards, and results were expressed in U/mL in plasma or U/mg of protein in tissues.

For SOD1 protein expression, following the electrophoresis, proteins were transferred to nitrocellulose membranes and were probed with a polyclonal rabbit anti-SOD1 antibody (Santa Cruz Biotechnology, Inc., USA). Bound antibody was detected using a secondary peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Inc., USA). The bands were visualized using the enhanced chemiluminescence system (ECL, Amersham, UK) and analyzed densitometrically using Photo-Capt V.99 software.

2.3.4. Total Antioxidant Capacity of Plasma. TAC in plasma was determined using decolorisation assay and expressed as trolox equivalent antioxidant capacity (TEAC). Antioxidant capacity of samples affect changes in decolorisation of ABTS radical (3 min, 37°C), measured spectrophotometrically at 720 nm [32]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard and expressed in mmol/L.

2.3.5. Measurement of Superoxide Level. The superoxide (O_2^-) was evaluated using lucigenin-enhanced chemiluminscence [33]. The aorta and left ventricle were cut into small pieces of up to 15 mg wet weight. After equilibration and adaptation to dark conditions, samples were added to lucigenin solution and measured every 30 s for 5 min in a Turner Designs TD-20/20 luminometer. Results are provided in relative luminescence unit/mg tissue (RLU/mg tissue).

2.4. Statistics. For Alibernet red wine and AWE characterization, data are presented as mean values ± standard deviation (SD; $n = 3$). The statistical significance was evaluated by Pearson's test, which is suitable for small numbers of samples, using GraphPad Prism version 4 software (GraphPad Software, San Diego, CA, USA).

For animal studies, one-way analysis of variance and the Duncan test were used for statistical analysis. Values were considered to differ significantly if the probability value was less than 0.05 ($P < 0.05$). Results are given as mean ± SD.

Table 1: Total antioxidant capacity and total phenolic content of Alibernet red wine and Alibernet wine extract.

Sample	TAC mmol/L	Total phenols GAE mg/L
Alibernet red wine	35,82	2039 ± 0,55
AWE	376,38	24172 ± 1,26

GAE: gallic acid equivalents, AWE: alibernet red wine extract.

3. Results

3.1. Alibernet Red Wine and AWE Characteristics

3.1.1. Total Antioxidant Capacity and Phenolic Content. Antioxidant capacity of Alibernet red wine was slightly above 35 mmol/L, whereas, in Alibernet wine extract, it reached 376 mmol/L. This means that approximately 10 times higher TAC was determined in AWE in comparison to Alibernet red wine. The results are visualized in Table 1. This also proves that the process of extraction had not affected the antioxidant properties of the red wine extract.

The total phenolic content of the Alibernet wine samples determined by the Folin-Ciocalteau colorimetric method was 2039 mg/L in Alibernet wine and above 24172 mg/L of gallic acid equivalents in Alibernet red wine extract.

Our research here establishes that dealcoholised Alibernet red wine extract has more than 11-fold higher total antioxidant capacity and total phenolic content compared to Alibernet red wine. This confirms that AWE has preserved all the essential properties of wine. This also reveals a significant positive relationship between total antioxidant capacity and the total phenolic content.

3.1.2. Mineral Content. The most predominant elements detected in Alibernet red wine and AWE were potassium (K), zinc (Zn), magnesium (Mg), and calcium (Ca). As with TAC and TPC, wine extract contains up to 10-fold higher values of these minerals. The results are visualized in Table 2.

3.2. Animal Studies

3.2.1. Animal Characteristics. SHR had significantly lower body weight and higher blood pressure compared to normotensive WKY. Alibernet wine extract treatment failed to change both body weight and blood pressure of WKY and SHR. Heart weight did not differ between groups. Results are shown in Table 3.

3.2.2. Total NOS Activity and eNOS Protein Expression. The Alibernet wine extract did not change total NOS activities in the tissues of WKY rats. However, it was able to increase significantly NOS activity in the left ventricle (Figure 1(a)), aorta (Figure 2(a)), and kidney (Figure 3(a)) of SHR compared to untreated control rats. Endothelial NOS protein expression was not changed significantly after the

Table 2: Elemental profile of Alibernet red wine and Alibernet wine extract (AWE).

Sample	K mg/L	Mg mg/L	P mg/L	Ca mg/L	Fe mg/L	Cu mg/L	Se μg/L	Pb μg/L	Zn mg/L
Alibernet red wine	1387,01	131,14	108,97	69,54	1,54	ND	ND	ND	354,30
AWE	9069,51	1334,07	ND	657,31	24,60	0,26	ND	7,83	5463,92

Data are expressed as mean values ($n = 3$). ND: not detected.

Table 3: Animal characteristics: body weight, heart weight, and blood pressure of WKY and SHR.

	WKY-C ($n = 6$)	WKY + AWE ($n = 6$)	SHR-C ($n = 6$)	SHR+AWE ($n = 6$)
Body weight (g)	249 ± 13	253 ± 11	211 ± 12*	216 ± 11*
Heart weight (g)	0,809 ± 0,044	0,841 ± 0,070	0,791 ± 0,046	0,812 ± 0,050
Blood pressure (mmHg)	107 ± 8	108 ± 6	167 ± 12*	176 ± 14*

WKY-C: control Wistar Kyoto rats; WKY + AWE: Wistar Kyoto rats treated with the Alibernet wine extract; SHR-CL: control spontaneously hypertensive rats; SHR + AWE: spontaneously hypertensive rats treated with the Alibernet wine extract. *$P < 0.05$ SHR versus WKY.

AWE treatment in both WKY and SHR (Figures 1(b), 2(b), and 3(b)).

3.2.3. SOD Activity and SOD1 Protein Expression. The Alibernet wine extract treatment failed to affect SOD activities as well as SOD1 protein expressions in the tissues of WKY rats. On the other hand, AWE treatment significantly increased SOD activity in the LV of SHR (by 54%; Figure 4(a)). However, it had no effect on left ventricular SOD1 protein expression (Figure 4(b)). Similarly, AWE treatment failed to affect SOD activity and SOD1 protein expression in the aorta, kidney, and plasma of SHR (data not shown).

3.2.4. Total Antioxidant Capacity of Plasma. Consistent with unchanged plasma SOD activity, there were no changes in the total antioxidant capacity of plasma in both WKY and SHR groups after AWE treatment (data not shown).

3.2.5. Measurement of Superoxide Level. Despite an increased tendency of superoxide level in the left ventricle and aorta of SHR, there were no significant changes between the strains. Similarly, AWE treatment did not reveal any significant changes in both LV and aorta of Wistar Kyoto and spontaneously hypertensive rats (Table 4).

4. Discussion

Emerging evidence suggests that minerals as well as polyphenolic compounds could play an important role in the proper functions of the endogenous antioxidant system as well as nitric oxide synthase activity.

Here, for the first time, an approach of simultaneous analysis of total phenolic content, minerals, and antioxidative capacities was employed to provide complex assessment of the red wine extract used in the experimental animal model. It has been confirmed that the Alibernet wine extract has up to ten times higher amounts of polyphenols and minerals compared to the Alibernet red wine. These results allowed us to conclude that processing the wine to obtain the extract did not have the negative impact on the content of minerals and phenolics of interest to present study. Moreover, we confirmed a strong positive relationship between the total antioxidant capacity and the content of total phenolic and minerals in both wine and the wine extract. These results are consistent with the findings published for red wines by other investigators [34, 35]. Those authors have experimentally verified correlation between polyphenolic content and the total antioxidant activity. In addition to analysis of polyphenolic content, we have also studied mineral content, which to a certain extent could affect the total antioxidant capacity in the red wine extract as it was shown in Table 1.

Minerals contained in foodstuffs play an important role in the body homeostasis. A growing amount of clinical evidence indicates relationships between levels of minerals and progression of certain diseases. Many recent studies documented the ability of selected minerals to counteract the development of cardiovascular, metabolic, and other diseases [36–40]. Positive effects of minerals in humans are related to their ability to enhance the activity of antioxidant defense system by catalyzing antioxidant enzymes. Several minerals found in foodstuffs, such as copper, zinc, and manganese, are essential for proper activity of superoxide dismutase, a key antioxidant enzyme [41, 42]. Experimental studies with zinc supplementation have revealed an augmentation of catalase, glutathione-s-transferase, and SOD activity after zinc supplementation to rats for 4 months [43].

Since results of chemical analysis of Alibernet wine extract showed relatively high concentrations of minerals, this may possibly contribute to changes in the activity of different antioxidant enzymes and/or nitric oxide synthase. Thus, SOD activity and SOD1 protein expression, NOS activity, and eNOS protein expression were analyzed together in experimental animals after Alibernet wine extract treatment. Western Blot analysis did not shown any significant changes in SOD1 and eNOS protein expressions after AWE treatment in the tissues investigated. On the other hand, AWE treatment increased significantly SOD activity in left ventricle and NOS activity in the LV, aorta, and kidney in the spontaneously hypertensive rats.

Three forms of SOD are present in humans and in all other mammals: cytoplasmic SOD1, mitochondrial SOD2, and extracellular SOD3 [44]. SOD1 is a Cu-Zn-dependent

Table 4: Level of superoxide in the aorta and left ventricle after treatment with AWE.

	WKY-C	WKY + AWE	SHR-C	SHR + AWE
Superoxide level in aorta RLU/mg	3,52 ± 1,25	5,66 ± 1,55	6,36 ± 2,47	7,11 ± 1,82
Superoxide level in LV RLU/mg	6,65 ± 1,60	8,12 ± 1,93	7,68 ± 1,67	8,01 ± 2,81

WKY-C: control group; WKY + AWE: animals treated with the Alibernet wine extract;
SHR-C: control group; SHR + AWE: animals treated with the Alibernet wine extract.

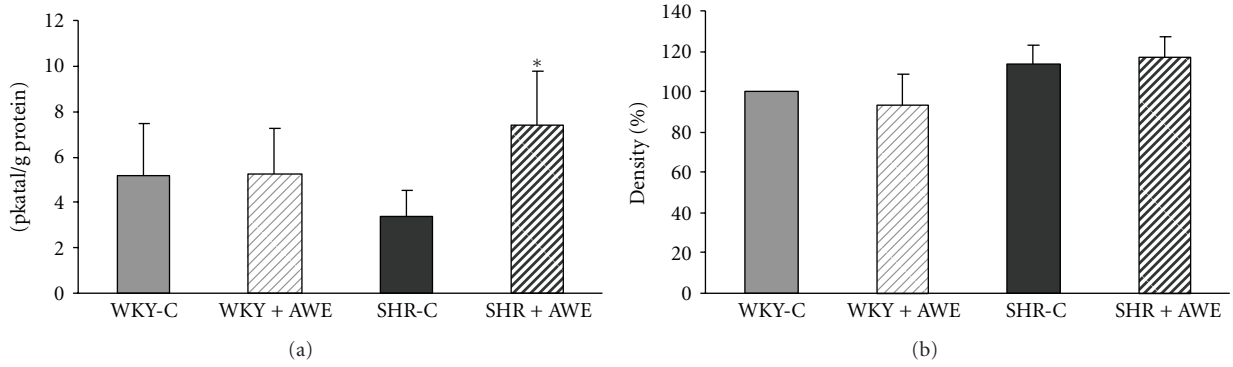

Figure 1: The effect of Alibernet red wine extract (AWE) treatment on total nitric oxid synthase (NOS) activity (a) and eNOS protein expression (b) in the left ventricle (LV) of Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). $P < 0.05$ as compared to respective control. WKY-C: control group; WKY + AWE: animals treated with the Alibernet wine extract; SHR-C: control group; SHR + AWE: animals treated with the Alibernet wine extract.

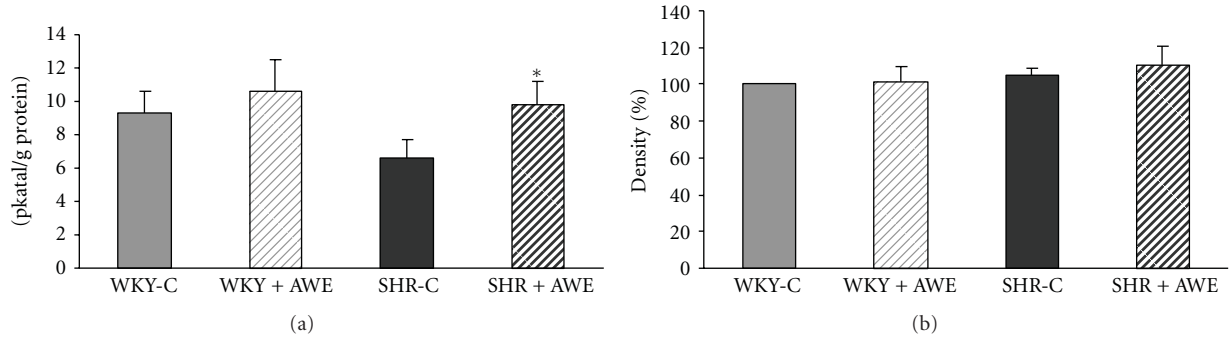

Figure 2: The effect of Alibernet red wine extract (AWE) treatment on total nitric oxid synthase (NOS) activity (a) and eNOS protein expression (b) in the aorta of Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). $P < 0.05$ as compared to respective control. WKY-C: control group; WKY + AWE: animals treated with the Alibernet wine extract; SHR-C: control group; SHR+AWE: animals treated with the Alibernet wine extract.

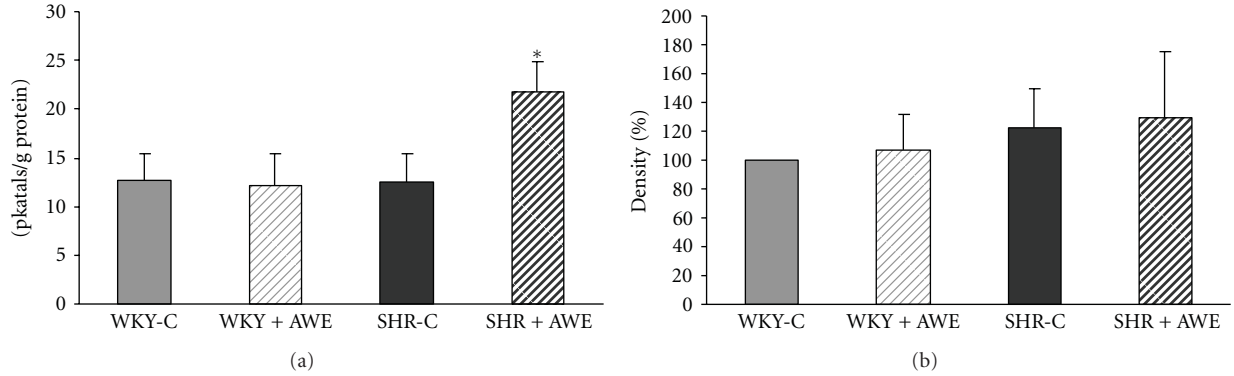

Figure 3: The effect of Alibernet red wine extract (AWE) treatment on total nitric oxid synthase (NOS) activity (a) and eNOS protein expression (b) in the kidney of Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). $P < 0.05$ as compared to respective control. WKY-C: control group; WKY + AWE: animals treated with the Alibernet wine extract; SHR-C: control group; SHR + AWE: animals treated with the Alibernet wine extract.

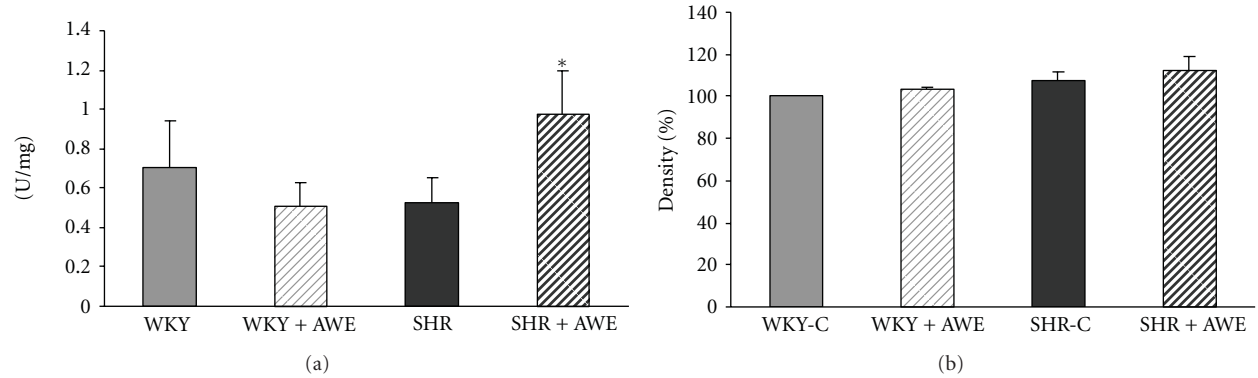

Figure 4: The effect of Alibernet wine extract (AWE) on superoxide dismutase (SOD) activity (a) and SOD1 protein expression (b) in the left ventricle (LV) of Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). $P < 0.05$ as compared to untreated animals. WKY-C: control group; WKY + AWE: animals treated with the Alibernet wine extract; SHR-C: control group; SHR + AWE: animals treated with the Alibernet wine extract.

isoform and is highly upregulated in the endothelial cells [45]. For this reason, SOD1 protein expression was analyzed. Since AWE treatment did not affect the level of SOD1 protein expression, we suppose that elevated level of Zn in AWE may directly increase SOD activity. We assume there is no increase in expression of SOD2 or SOD3 proteins after wine extract treatment. Of course, the polyphenol compounds involved in the extract may also contribute to increased SOD activity. These results are consistent with the recent study by Fernández-Pachón et al. [17] where an increased activity of SOD in human erythrocytes was observed after seven days of red wine consumption.

Zinc is one of the most important and ubiquitous trace elements in the body with significant antioxidant activity. Zinc is an important mineral in preventing free radical formation, protecting biological structures from damage and boosting the immune response [42, 46]. Moreover, Zhou et al. [47] have proven the key role of zinc in the proper function of nitric oxide synthase. In fact, NOS is catalytically active only as a dimer consisting of two subunits, the association of which is stabilized by tetrahedral coordination of Zn by thiol ligands at the dimer interface. That is why we also suggest that increased NOS activity after AWE treatment may be attributed to zinc. Besides zinc, AWE treatment could also increase the level of calcium—another important element necessary for endothelial NOS activation [48]. Our suggestion is confirmed by the fact that Western Blot analysis did not reveal any significant changes in endothelial NOS protein expression after AWE treatment of SHR. Thus Zn, Ca, and polyphenol compounds of AWE most probably directly increased NOS activity. We assume there is no increase in expression of the neuronal or inducible NOS proteins in the tissues investigated. López-Sepúlveda et al. [49] also did not see increased eNOS expression in the aorta of SHR after red wine polyphenol treatment. However, several other papers using different models documented increased eNOS expression after red wine polyphenol administration [15, 25]. We assume that differences in polyphenolic consumption, different models as well as different treatment duration may be responsible for differences in eNOS protein expression.

Our results demonstrating increase in NOS activity in all samples investigated are consistent with the recent publications by Auger et al. [13] and Madeira et al. [15] where the authors found elevated NOS activity after using different red wine extracts. A recent study of Khoo et al. [50] focused on vascular responses to the polyphenolic compound-quercetin using a combination of biochemical and vasoactive criteria has also confirmed a positive correlation between quercetin concentration and increased NO bioavailability in endothelial cells. This study has confirmed the ability of polyphenolic compounds to improve endothelial cell function by stimulation of endothelial NOS in nitric oxide production.

Alibernet red wine extract increased both NOS and SOD activities only in rats with spontaneous hypertension, where the abovementioned harmful effects are present. This means that polyphenols and minerals may affect only pathological mechanisms while they have no effect under normal conditions. Similar effects of red wine polyphenols were described in L-NAME-induced hypertension [25]. Nevertheless, in our study, increased SOD and NOS activities after 3 weeks of AWE treatment failed to affect blood pressure of SHR.

The authors of several papers on this topic showed reduction of blood pressure after administration of red wine polyphenols to SHR. However, duration of the treatment was generally longer than three weeks. López-Sepúlveda et al. [49] documented reduction of hypertension and vascular dysfunction in SHR after red wine polyphenols treatment (40 mg/kg by gavage) for 5 weeks. Chan et al. [51] also found improved endothelium-dependent dilation after 10-week oral administration of red wine polyphenols (100 mg/kg/day) to SHR. On the other hand, Botden et al. [52] in a study on humans did not see decreased peripheral or central blood pressure in subjects with high-normal blood pressure or grade 1 hypertension after red wine polyphenol intake at two different dosages for 4 weeks. Concerning our study, increased SOD and NOS activities after 3 weeks of AWE treatment failed to affect blood pressure of SHR. We assume that prolongation of AWE treatment could lead to the reduction of blood pressure as well. This, however, requires further investigation.

In conclusion, our study documented that phenolics and minerals presented in Alibernet wine extract, and zinc especially, may contribute directly to increased NOS and SOD activities in the tissues of spontaneously hypertensive rats. This finding enables us to suggest that the positive effects of fruits and vegetables are related to the increase in activity of the endogenous antioxidant enzymes and enhancement of antioxidant effects. This occurs together with an increase in NO production, important for vasodilation and blood flow improvement. This is particularly important in the case of hypertension, where elevated free radical and cytokine production, chronic inflammation, and the suppression of NO production are present.

Abbreviations

AWE: Alibernet red wine extract
NOS: Nitric oxide synthase
SOD: Superoxide dismutase
WKY: Wistar Kyoto
SHR: Spontaneously hypertensive rats
TAC: Total antioxidant capacity
GAE: Gallic acid equivalents
TPC: Total phenolic content
TEAC: Trolox equivalent antioxidant capacity.

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Review Article

Dietary Polyphenols as Modulators of Brain Functions: Biological Actions and Molecular Mechanisms Underpinning Their Beneficial Effects

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Accumulating evidence suggests that diet and lifestyle can play an important role in delaying the onset or halting the progression of age-related health disorders and to improve cognitive function. In particular, polyphenols have been reported to exert their neuroprotective actions through the potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuroinflammation, and the potential to promote memory, learning, and cognitive function. Despite significant advances in our understanding of the biology of polyphenols, they are still mistakenly regarded as simply acting as antioxidants. However, recent evidence suggests that their beneficial effects involve decreases in oxidative/inflammatory stress signaling, increases in protective signaling and neurohormetic effects leading to the expression of genes that encode antioxidant enzymes, phase-2 enzymes, neurotrophic factors, and cytoprotective proteins. Specific examples of such pathways include the sirtuin-FoxO pathway, the NF-κB pathway, and the Nrf-2/ARE pathway. Together, these processes act to maintain brain homeostasis and play important roles in neuronal stress adaptation and thus polyphenols have the potential to prevent the progression of neurodegenerative pathologies.

1. Introduction

A gradual increase in human life span, with people over the age of 60, is expected to double between 2000 and 2050 [1]. As the elderly population expands, the prevalence of both Alzheimer's disease (AD) and Parkinson's disease (PD) is likely to augment, therefore having profound economical and social implications. Although the exact cause is not yet finally known, it has been postulated that the behavioural and neuronal declines associated with these age-related neurodegenerative disorders are triggered by multifactorial events including neuroinflammation, glutamatergic excitotoxicity, increases in iron, and/or depletion of endogenous antioxidants [2–4]. Therefore, it becomes imperative to develop drugs that possibly exert neuroprotective actions in order to prevent or even reverse age-related health disorders. One such possibility is the use of nutritional substances such as polyphenols [5, 6]. For example, a large number of dietary interventions using polyphenol rich foods or beverages, in particular those using tea [7–9], Gingko Biloba [10, 11], cocoa [12, 13] and blueberry [14–16], have demonstrated beneficial effects on memory and learning in both animals and humans. Furthermore, individual flavonoids such as the citrus flavanone tangeretin, have been observed to maintain nigrostriatal integrity and functionality following lesioning with 6-hydroxydopamine, suggesting that it may serve as a potential neuroprotective agent against the underlying pathology associated with PD [17]. While historically research focused on their antioxidant properties [18], recent data support the view that polyphenols, and their *in vivo* metabolites, do not act as conventional hydrogen-donating antioxidants but may exert modulatory actions in cells through actions at protein kinase and lipid kinase signalling pathways [19] and may even involve hormetic effects to protect neurons against the oxidative and inflammatory stressors [20]. This paper will describe the potential of polyphenols to modulate neuroinflammation, to counteract neurotoxins induced neurodegenerative disorders, and to enhance memory, learning, and cognitive performances. Neuroprotective mechanisms through the ability of polyphenols to interact

with neuronal signaling pathways and to mediate endogenous cellular defense systems including sirtuin, NF-κB, Nrfs, and related pathways will be also presented.

2. Sources and Structures of Polyphenols

Polyphenols are a group of naturally occurring phytochemicals which are present in high amounts in fruits, vegetables, and natural products and are characterised by the presence of multiple hydroxyl groups on aromatic rings. These compounds are divided into two main categories: the flavonoids and nonflavonoids, based on the number of phenol rings and the way in which these rings interact.

2.1. Flavonoids. Flavonoids are polyphenolic compounds comprising 15 carbons, with two aromatic rings connected by a three-carbon bridge (C_6–C_3–C_6). Hydroxylation in position 3 of C-ring allows the differentiation of flavanonols from flavanones since they share a similar structure based on the 2,3-dihydro-2-phenylchromen-4-one skeleton. From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. Flavonoids share a common feature which consists of two aromatic carbon rings, benzopyran (A and C rings) and benzene (B ring) and may be divided in various subgroups based on the degree of the oxidation of the C-ring, the hydroxylation pattern of the ring structure, and the substitution of the 3-position. The main dietary groups of flavonoids are (1) flavones (e.g., apigenin, luteolin), which are found in parsley and celery. Hydroxylation on position 3 of the flavone structure gives rise to the 3-hydroxyflavones also known as the (2) flavonols (e.g., kaempferol, quercetin), which are found in onions, leeks, and broccoli; (3) isoflavones (e.g., daidzein, genistein), which are mainly found in soy and soy products. These compounds have a large structural variability, and more than 600 isoflavones have been identified to date and are classified according to oxidation level of the central pyran ring; (4) flavanones/flavanonols (e.g., hesperetin, naringenin/astilbin, engeletin), which are mainly found in citrus fruit, herbs (oregano), and wine; (5) flavanols (e.g., (+)-catechin, (−)-epicatechin, epigallocatechin, and epigallocatechin gallate (EGCG), which are abundant in green tea, red wine, and chocolate. Flavanols are found both as monomers and oligomers referred to as condensed tannins or proanthocyanidins. Variations in their structures lie in the hydroxylation pattern of the B ring and the presence of gallic acid in position 3. The lack of a double bond at the 2-3 position and the presence of a 3-hydroxyl group on the C ring create two centres of asymmetry; (6) anthocyanidins (e.g., pelargonidin, cyanidin, and malvidin), whose sources include red wine and berry fruits. These compounds exist as glycosides in plants, are water-soluble, and appear red or blue according to pH. Individual anthocyanins arise from the variation in number and arrangement of the hydroxyl and methoxy groups around the 3 rings (Figure 1).

2.2. Nonflavonoids. The nonflavonoid group may be separated into two different classes: (1) the phenolic acids, including the hydroxybenzoic acids (HBAs; C_1–C_3 skeleton) and hydroxycinnamic acids (HCAs; C_3–C_6 skeleton) and (2) the stilbenes (C_6–C_2–C_6 skeleton).

The most common phenolic acids are not present in plants in a free state but occur as simple esters of glucose, tartaric acid, and quinic acid [21], and variations in the structure mainly lie in the hydroxylation and methoxylation pattern of the aromatic cycle [22]. HBAs are derivatives of the hydroxybenzoic acids such as *p*-hydroxybenzoic, protocatechuic, and gallic acids and are mostly present in the form of glucosides and some esters with glucose. However, gallic acid is mainly esterified to quinic acid or catechins and usually present in polymeric forms as soluble tannins [21]. HCAs are found in a variety of foods, the most common being caffeic and ferulic acids and their derivatives. They are mostly present in ester forms bound to quinic, shikimic, or tartaric acids. Caffeic acid is generally the most abundant phenolic acid and is mainly found as the quinic ester, chlorogenic acid, in blueberries, kiwis, plums, and apples [23]. However, very high intake of chlorogenic acid is common among coffee drinkers because of very high concentrations (50–150 mg of chlorogenic acids in one cup (200 mL) of instant coffee [24] (Figure 1).

Stilbenes possess a 1,2-diarylethenes structure based on the C_6–C_2–C_6 backbone and are usually synthesized in plants in response to infection or injury [25]. Resveratrol, the main stilbene, can be found in the *cis* or *trans* configurations, either glucosylated (piceid) or in lower concentrations as the parent molecule of a family of polymers such as viniferins, pallidol, or ampelopsin A [26]. Major dietary sources of resveratrol include grapes, wine, and peanuts. Resveratrol is found in low concentrations (0.3–7 mg aglycones/L and 15 mg glycosides/L) in red wine, and thus it seems unlikely to produce protective effects at normal nutritional intakes (Figure 1).

3. Brain Localisation of Polyphenols

Despite the increasing amount of evidence for the bioavailability of polyphenols in the systemic circulation [22, 27–29] only little information is available regarding their ability to reach the central nervous system (CNS). In order for polyphenols to access the brain, they must first cross a tightly regulated, selectively permeable endothelial cell layer which isolates the CNS tissue from the vasculature, the blood-brain barrier (BBB). The BBB is permeable to nutrients and actively excludes many substances from the central nervous system [30]. Using *in vitro* models, initial studies have demonstrated that polyphenols permeation through the BBB is dependent on the degree of lipophilicity of each compound with less polar polyphenols or metabolites (i.e., *O*-methylated derivatives) capable of greater brain uptake than the more polar ones (i.e., sulfated and glucuronidated derivatives) [31]. Their brain entry will also depend on their interactions with efflux transporters, such as P-glycoprotein (Pgp) [32] and their stereochemistry. For example, both catechin and epicatechin could cross a cellular model of BBB in a time-dependent and stereoselectivity manner with epicatechin $\gg$ catechin [33]. The amount of nutrient or drug that

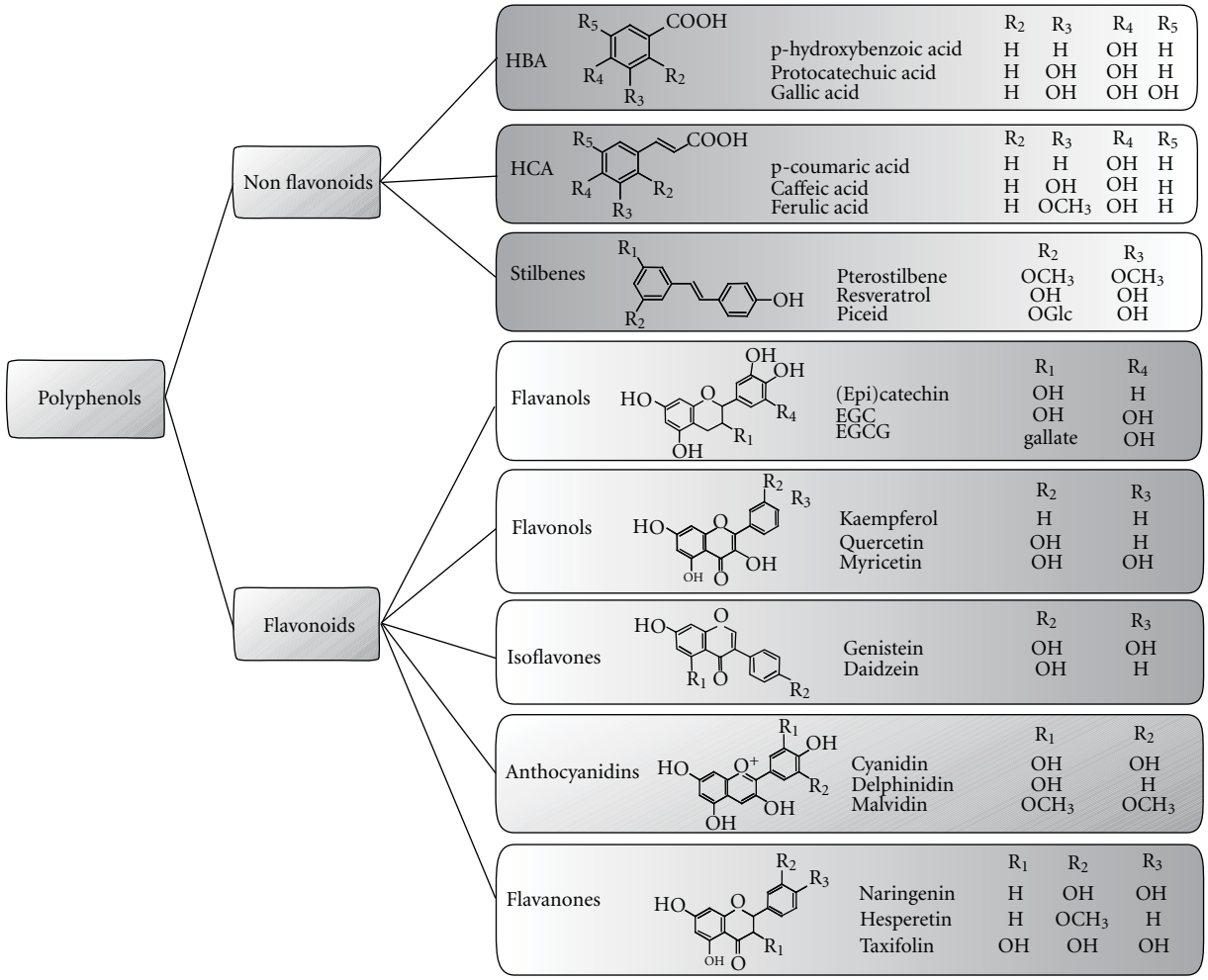

Figure 1: Structures of polyphenols. Polyphenols are a group of naturally occurring phytochemicals which are present in high amounts in fruits, vegetables, and natural products and are characterised by the presence of multiple hydroxyl groups on aromatic rings. These compounds are divided into two main categories, the flavonoids and non flavonoids, based on the number of phenol rings and the way in which these rings interact. For the flavonoid group, the major differences between the individual groups arise from the hydroxylation pattern of the ring-structure, the degree of saturation of the C-ring, and the substitution of the 3-position. HBAs, hydroxybennzoic acids; HCAs, hydroxycinammic acids.

penetrate into the brain was also investigated *in vivo*, with animal studies indicating that polyphenols are able to cross the BBB and to colocalise within the brain tissues independently of their route of administration. For example, naringenin was found in the brain following its intravenous administration [34], whilst epigallocatechin gallate [35], epicatechin [36], and anthocyanins [37, 38] were observed after oral administration. Although the uptake and distribution of dietary polyphenols within the brain are well documented, the question of the dose reaching the target tissues remains uncertain. Discrepancies in the findings mainly stem in the fact that studies reporting polyphenol brain uptake and concentrations often disregard residual blood as a potential confounder. Studies using exsanguinated, perfused animals or applying the recently published mathematical correction model [39] may therefore be more suitable for assessing polyphenol uptake and metabolism in the brain. Data deriving from such studies suggest that polyphenols usually localise in the brain at levels below 1 nmol/g tissue (see review by Schaffer and Halliwell [40]). Furthermore, several polyphenols have been identified in different regions of the rat [38, 41] and pig brains [42, 43] and usually accumulates in a nonregion-specificic manner [16, 44]. For example, recently, Janle et al. demonstrated that ^{14}C-labelled grape polyphenols did not show any regional differences in ^{14}C accumulation from anterior to posterior slices of the brain [44]. Collectively, these results indicate that polyphenols transverse the BBB and localise within the brain tissue, suggesting that they are candidates for direct neuroprotective and neuromodulatory actions. Nonetheless, our knowledge regarding polyphenol absorption, metabolism, tissue distribution, and intracellular accumulation and excretion remains insufficient, and future work is needed to better understand their biological effects.

4. Effect of Polyphenols on Memory, Learning, and Neurocognitive Performance

Accumulating evidence suggests that diet and lifestyle can play an important role in delaying the onset or halting the progression of neurodegenerative diseases and improving cognitive function [45–48]. With regards to diet, polyphenols have been associated with a reduced risk of developing dementia [45, 49], an improved cognitive performance in normal ageing [48] and an improved cognitive evolution [5]. More recently, high total polyphenol intake was also associated with better language and verbal memory but not with executive functioning. In particular, intake of catechins, theaflavins, flavonols, and hydroxybenzoic acids was positively associated with language and verbal memory, especially with episodic memory as assessed by the RI-48 test [50]. Although a positive correlation between dietary polyphenol consumption and cognitive decline has been mostly reported, a limited body of evidence is, however, suggestive that carrier of the *APOε4* genotype may influence the beneficial effect of polyphenols in relation to dementia and AD. For example, the frequent consumption of fruits and vegetables was associated with a decreased risk of all cause dementia (hazard ratio [HR] 0.72, 95% CI 0.53 to 0.97) especially amongst the *APOε4* noncarriers [51]. The relationship between polyphenols intake and *APOε* genotype is intriguing, and further work is required to gain a better understanding of the physiological and molecular mechanisms underlying such disparity.

Over the last years, there has been much interest in the neurocognitive effects of berries, in reversing age-related deficits in motor function and spatial working memory [14, 16, 52]. While the consumption of cranberry juice over a 6 weeks period in older adults has failed to report any cognitive benefits [53], consumption of both grape or blueberry juices in older adults with or without mild cognitive impairment (MCI) reported significant improvement in memory function after 12 weeks intervention [54, 55]. In addition to spatial memory, blueberry supplementation in aged animals has also been shown to improve "object recognition memory" [56] and "inhibitory fear conditioning learning" [57, 58]. Blueberry appears to have a pronounced effect on short-term memory [58] and has also been shown to improve long-term reference memory following 8 weeks of supplementation. [14]. Tests using a radial arm maze have supported these findings and have provided further evidence for the efficacy of blueberries [16]. Indeed, these have shown that improvements in spatial memory may emerge within 3 weeks, the equivalent of about 3 years in humans. Although not fully understood, evidence suggest that blueberry-derived polyphenols may enhance the efficiency of spatial memory by indirectly acting on the dentate gyrus (DG), an hippocampal subregion particularly sensitive to the effects of aging [59]. In particular, blueberry supplementation has been shown to significantly increase the precursor cells in the DG of aged rats [14]. Such link between hippocampal neurogenesis, cognitive performance, and aging may represent a potential mechanism by which polyphenol-derived foods may improve memory [60].

In addition to those with berries, human and animal studies with cocoa and tea flavanols have also provided further evidence that dietary polyphenols are beneficial in reversing the course of neuronal and behavioural aging [7, 61]. For example, two recent acute human studies have shown that cocoa flavanol consumption was able to improve working memory and attention [12, 13]. In addition, pure (−)-epicatechin (500 μg/g) was also observed to enhance the retention of mice spatial memory, especially when combined with exercise [62], suggesting that polyphenols may be causal agents in inducing the behavioural effects. Although the exact mechanisms underlying such behavioural changes remain to be elucidated, evidence suggests that flavanol-rich foods improve peripheral blood flow and surrogate markers of cardiovascular function [63–65]. In addition, CNS imaging studies in humans have demonstrated that the consumption of flavanol-rich cocoa may improve cerebral blood flow (CBF) in healthy older adults [66] and in young adults in response to a cognitive task [67]. These effects are particularly significant, as increased cerebrovascular function is known to facilitate adult neurogenesis [68] and to enhance vascularisation [69, 70], two events important in the maintenance of cognitive performances.

The flavonoid-rich plant extract, *Ginkgo biloba,* has also been shown to induce positive effects on memory, learning, and concentration [71, 72]. *Ginkgo biloba* has a prominent effect on brain activity and short-term memory in animals and humans suffering from cognitive impairment [11, 73] and promotes spatial learning in aged rodents [74, 75]. However, the pharmacological mechanisms by which *Ginkgo biloba* promotes cognitive effects remain unclear, although its ability to elicit a reduction in levels of reactive oxygen species (ROS) [76], to increase cerebral blood flow [77], to modulate membrane fluidity [78], to interact with muscarinic cholinergic receptors [79], to protect the striatal dopaminergic system [80], and to upregulate AMPA, calcium and chloride channels, and growth hormones [81] have been suggested as possible mechanisms underlying its actions in the CNS. Together, these data provide a strong indication that regular polyphenol consumption may have a positive effect on neurocognitive performance as we age (Figure 2).

5. Polyphenol Protection against Neuronal Injury Induced by Neurotoxins

There are a number of epidemiological studies which suggest that plant-derived polyphenol-rich foods or supplements might delay the initiation and progression of AD, PD, and related neurodegenerative disorders [5, 82]. With regard to AD, most of the preclinical studies of the effects of polyphenols have focused on models where there is increased production of beta-amyloid (Aβ), a small protein produced by the enzymatic cleavage of amyloid precursor protein (APP) [83]. For example, the chronic consumption of ferulic acid with the drinking water protected mice from the deleterious effects of an intracerebral injection of β-amyloid peptide [84]. More recently, using transgenic mouse models, studies have started to address the potential effect of polyphenol-rich diets on AD. Oral administration of

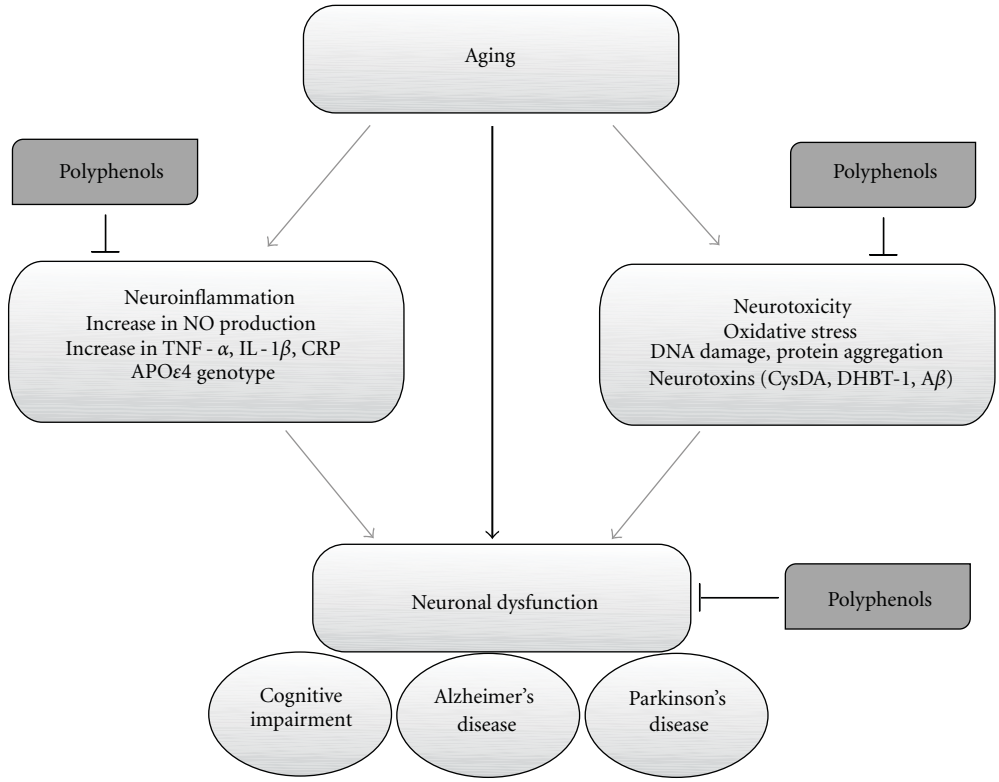

Figure 2: Modulation of neuronal dysfunction by dietary polyphenols. In ageing and neurodegenerative diseases, neuronal death can be triggered by specific genetic mutations, neurotoxins, and/or neuroinflammation. Initiating factors promote cellular alterations, including increases in oxidative stress, protein aggregation, DNA damage, and activation of apoptotic cascades. Dietary polyphenols have been observed to protect the brain against such cellular alteration through the modulation of neuronal function against endogenous neurotoxins and inhibition of glial-induced neuroinflammation. Aβ, amyloid beta; CysDA, 5-S-cysteinyldopamine; DHBT1, dihydrobenzothiazine 1; TNF-α, tumor necrosis factor alpha; IL-1β, interleukine-1 beta; CRP, C reactive protein; NO, nitric oxide.

epigallocatechin-3-gallate (EGCG) for 6 months in mice which overexpress the Swedish mutation of APP (APPsw), reduced Aβ pathology and improved cognition [85]. Similarly long-term green tea catechin administration also improved spatial learning and memory in senescence prone mice, by decreasing $A\beta_{1-42}$ oligomers and upregulating synaptic plasticity-related proteins in the hippocampus [86]. The antiamyloidogenic activity is not unique to EGCG and a number of other polyphenols bind to Aβ fibrils and prevent further fibrillization [87–89]. For example, gallic acid and catechin-rich grape seed polyphenolic extract (GSPE) inhibited cognitive deterioration coincident with reduced levels of soluble high molecular weight oligomers of Aβ [88]. Repeated intraperitoneal injection of nobiletin has similar effects [90]. The mechanisms underlying these changes are not clear but might be linked to increased nonamyloidogenic processing of APP, through stimulating the activity of α-secretase, which cleaves APP at a site which prevents the formation Aβ species [91, 92]. Alternatively, it is conceivable that polyphenols reduce Aβ plaque pathology by inhibiting amyloid aggregation and fibrillization either as a result of metal chelation activity [93–95] or by favouring the formation of nontoxic oligomers [96]. Additional mechanisms have been also suggested for the ability of polyphenols to delay the initiation of and/or slow the progression of AD-like pathology, including a potential to inhibit neuronal apoptosis triggered by neurotoxic species (e.g., oxidative stress and neuroinflammation) or disrupt amyloid β aggregation and effects on amyloid precursor protein processing through the inhibition of β-secretase (BACE-1) [97] and/or activation of α-secretase (ADAM10) (See review by Williams and Spencer [98]).

The potential utility of polyphenols in neurodegeneration extends beyond AD, and there is also considerable interest in their therapeutic potential in PD [47, 99]. There is good evidence to suggest that the consumption of green tea may have a beneficial effect in reducing the risk of PD [82], as has been extensively reviewed elsewhere [100, 101]. The efficacy of green tea is likely to be mediated by the effects of EGCG, which has been shown to attenuate the selective degeneration of dopamine neurons in animal models of PD induced by toxins including 6-hydroxydopamine [102] and MPTP [103]. In addition, the citrus flavonoid tangeretin has also been observed to be neuroprotective against 6-hydroxydopamine lesioning in a rat model of PD [17]. *In vitro* studies have also indicated that polyphenols might act to prevent PD pathology via their ability to prevent the formation of the endogenous neurotoxin, 5-*S*-cysteinyl-dopamine

(CysDA) [104, 105]. Such adducts may be generated by reactive species [105] and have been observed to be elevated in the human substantia nigra of patients who died of PD [104], suggesting that such species may be potential endogenous nigral toxins. However, CysDA-induced neuronal injury is counteracted by nanomolar concentrations of various polyphenols including pelargonidin, quercetin, hesperetin, caffeic acid, tyrosol, *p*-coumaric acid, and the 4′-*O*-Me derivatives of catechin and epicatechin [105, 106]. Furthermore, in presence of the flavanol, (+)-catechin, tyrosinase-induced formation of CysDA was inhibited by a mechanism linked to the capacity of catechin to undergo tyrosinase-induced oxidation to yield cysteinyl-catechin adducts [107]. In contrast, the inhibition afforded by flavanones, such as hesperetin, was not accompanied with the formation of cysteinyl-hesperetin adducts, indicating that it may be inhibited via direct interaction with tyrosinase [107]. Furthermore, the stilbene resveratrol also had a small inhibitory effect; however, its reaction with tyrosinase in the presence of L-cysteine led to the formation of dihydrobenzothiazine (DHBT-1) [107], a strong neurotoxin known to selectively inhibit the respiratory chain complex I, the alpha-ketoglutarate dehydrogenase (alpha-KGDH), and the pyruvate dehydrogenase complexes (PDHC) [108] (Figure 2). Collectively, these studies suggest that polyphenols have the potential to confer benefit in diverse neurodegenerative disorders. Some of the major neuroprotective mechanisms are discussed in more detail below.

6. Role of Polyphenols in Preventing Neuroinflammation

Although neuroinflammation plays a critical role in brain host defence, it also contributes to the underlying neuronal loss in neurodegenerative disorders, such as PD, AD [109–111] and to damages associated with cerebral ischemia [112]. Neuroinflammation is "driven" by activated resident glial cells (astrocytes and microglia) which result in invasion of circulating immune cells and the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6), nitric oxide ($NO^{\bullet}$), prostaglandin E2, chemokines, and reactive oxygen species (ROS). Amongst the numerous factors released by activated glial cells, excessive $NO^{\bullet}$ production has been reported to induce neuronal cell death by damaging the mitochondrial electron transport chain function in neurons [113] therefore resulting in neuronal ATP synthesis disruption and in increased generation of ROS [114]. Furthermore, NADPH oxidase activation, an important event in activated microglia-induced neurotoxicity, has also been suggested to mediate both superoxide ($O_2^{\bullet -}$) production and to release proinflammatory molecules such as TNF-α [115]. $NO^{\bullet}$ produced in microglia or astrocytes may react with $O_2^{\bullet -}$, produced by NADPH oxidase [116, 117], to generate the neurotoxic peroxynitrite radical ($ONOO^-$) [116]. $ONOO^-$ has been observed to inhibit mitochondrial respiration, induce caspase-dependent neuronal apoptosis, and to induce glutamate release resulting in excitotoxicity and neuronal death [116, 118]. Additionally, glial cytokine production may also play a deleterious role in neurodegenerative diseases by binding to specific cell surface receptors expressed in neurons and activating apoptotic pathways. For example, TNF-α binds to the tumour necrosis factor receptor-1 (TNFR1) which may lead to neuronal apoptosis [119, 120].

Since long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to lower the risk of AD in later life [121], there has been much interest in the development of new drugs capable of preventing neuroinflammatory-mediated brain injury. Emerging evidence suggests that dietary polyphenols may exert neuroprotective effects by suppressing the activation of microglia, which mediates inflammatory processes in the CNS. Although rather complex, the main anti-inflammatory properties of polyphenols include: (1) an inhibitory role on the release of cytokines, such as IL-1β and TNF-α, from activated glia; (2) an inhibitory action against iNOS induction and subsequent nitric oxide production in response to glial activation; (3) an ability to inhibit the activation of NADPH oxidase and subsequent ROS generation in activated glia; (4) a capacity to downregulate the activity of proinflammatory transcription factors such as NF-κB through their influences of a number of glial and neuronal signaling pathways, such as MAPK cascade (discussed in details below) [122, 123].

For example, the commonly consumed flavonol quercetin has been reported to inhibit neuroinflammation by attenuating nitric oxide production and iNOS gene expression in microglia [117, 124] and by preventing inflammatory cytokine production, thus preventing neuronal injury [125, 126]. However, one of the major physiological metabolites of quercetin, quercetin-3′-sulfate, failed to demonstrate any anti-inflammatory action [117]. Nevertheless, these studies have employed quercetin concentrations (10–50 μM) much higher than of those found in plasma after ingestion [28]. In contrast to this, epicatechin and catechin (10–300 nM) were observed to inhibit TNF-α release but not iNOS expression or nitric oxide production in primary glial cells [127] suggesting that flavanols at physiologically relevant concentrations may hold the potential to exert anti-inflammatory effects in the central nervous system. Polyphenols present in blueberry have also been reported to inhibit $NO^{\bullet}$, IL-1β and TNF-α production in activated microglia cells [128], and the flavanone naringenin was observed to be highly effective in reducing LPS/IFN-γ-induced glial cell activation [127]. Dietary polyphenols are also potent inhibitors of NADPH oxidase activity *in vitro*. A study comparing 45 polyphenolic compounds indicated that whilst both the flavanols (+)-catechin and (−)-epicatechin failed to inhibit NADPH oxidase, their relevant methylated metabolites exhibited strong NADPH oxidase inhibition through an apocynin-like mechanism [129]. Interestingly, other apocynin-like phenolic compounds, such as, ferulic acid, homovanillin alcohol, caffeic acid, tyrosol, and vanillic acid were also observed to inhibit NADPH oxidase activity, therefore indicating that smaller polyphenols, more structurally related to some colonic metabolites, may also serve as novel therapeutic agents in neuroinflammation (Figure 2).

There is also data which shows encouraging positive effects of polyphenols in animal and *in vitro* models relevant to multiple sclerosis (MS), a chronic debilitating disease which is characterised by demyelination, progressive

irreversible axonal damage and inflammation [130]. For example, EGCG delivered orally reduces symptom severity in the autoimmune encephalomyelitis model of relapsing-remitting MS by reducing inflammation and increasing neuroprotection [131]. Quercetin has also been reported to be effective in the Experimental Autoimmune Encephalomyelitis (EAE) mouse model, and reduces T-cell proliferation *in vitro* at concentrations exceeding 10 μM [132]. Micromolar concentrations of luteolin, apigenin, fisetin, and quercetin (but not morin or hesperetin) were reported to suppress the production of the cytokine interferon-gamma (IFNγ) from lymph-node-derived T cells but, paradoxically, worsen clinical severity in the EAE model. More recently, resveratrol protection against EAE was associated with rises in IL-17/IL-10 and with repressed macrophage IL-6 and IL-12/23 p40 expression [133]. Thus, the studies to date show promising proof of concept of beneficial effects of polyphenols in suppressing immune and inflammatory responses in models of MS.

7. Mechanisms Underpinning the Beneficial Effects of Polyphenols

It has generally been assumed that the health benefits of polyphenols were linked to their capacity to directly scavenge free radicals and other nitrogen species *in vitro* [134–137]. However, the concentrations at which they exert such antioxidant activity are unlikely to be easily achieved *in vivo* as many polyphenols have very limited bioavailability and are extensively metabolised therefore reducing their antioxidant potential [19]. During the last years, a new realisation of how nutritional antioxidants may function has been envisaged, and recent findings have suggested that in lower amounts, typical of those attained in the diet, polyphenols may activate one or more adaptive cellular stress responses pathways [93, 138–140]. Activation of such hormetic pathways in neurons results in the production of several types of cytoprotective proteins including neurotrophic factors, protein chaperones, antioxidant and phase II enzymes, and antiapoptotic proteins [141, 142]. One particular protective pathway which is receiving considerable attention in regard to hormesis in the nervous system involves the transcription factor NF-E2-related factor-2 (Nrf2). Nrf2 binds to the antioxidant-responsive element (ARE) with high affinity and plays a central role in the upregulation of genes implicated in the regulation of the cellular redox status and the protection of the cell from oxidative insult [143, 144]. Under basal conditions, Nrf2 interacts with a cytosolic repressor protein Keap1 (Kelch ECH associating protein) limiting Nrf2-mediated gene expression [145]. In cells exposed to oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus, where it activates ARE-dependent transcription of phase II and antioxidant defence enzymes, such as glutathione-S-transferase (GST), glutathione peroxidase (GPx), and heme oxygenase-1 (HO-1) [146].

Most polyphenols have been reported to respond in a bell-shaped dose-response manner, presenting cellular toxicity at high concentrations while inducing light chemical stress at lower doses with activation of physiological hormesis in cells [142], resulting in overexpression of defensive genes such as those activated by Nrf2. For example, resveratrol was observed to protect PC12 cells against H_2O_2-mediated oxidative stress [147] and to attenuate cerebral ischemic injury in rat [148] via the activation of Nfr2 and the upregulation of HO-1. The caffeic acid phenethyl ester (CAPE), the active component of propolis, protected nigral dopaminergic neurons in an experimental mouse model of dopaminergic neurodegeneration through the modulation of heme oxygenase-1 and brain-derived neurotrophic factor (BDNF) [149]. The ethyl ferulate (EFE), a lipophilic polyphenol also found in propolis, was observed to protect rat neurons against oxidative stress via the induction of Nrf2/HO-1 [150]. The flavanol (−)-epicatechin prevented stroke damage through the Nrf2/HO1 pathway [151], and increased glutathione levels in primary astrocytes through an upregulation of ARE-mediated gene expression [152]. Although a positive correlation between dietary polyphenol consumption and brain function has been mostly reported, evidence is also suggestive that *APOε4* carriers may not benefit from the frequent consumption of fruits and vegetables rich in such phytochemicals. Indeed, previous findings suggest that *APOε4* carriers are less responsive towards the anti-inflammatory, paraoxanase-1 inducing, and blood pressure lowering activity of quercetin [153–155]. Such diminished responsiveness of the *APOε4* versus *APOε3* genotype (approximately 55–60% of the Caucasians population are homozygotes for the *ε3* allele) may be attributed to an impaired Nrf2 signalling and to a lower activity of Nrf2 target genes including glutathione-S-transferase, heme oxygenase-1, and NAD(P)H dehydrogenase, quinone 1 [156].

Several upstream signaling cascades may either individually, or in a combined manner, activate Nrf2. These include selective actions on a number of protein kinase and lipid kinase signalling cascades, most notably the PI3K/Akt and MAP kinase pathways which regulate prosurvival transcription factors and gene expression [19]. In general, *in vitro* studies have reported that polyphenols, at submicromolar concentrations, activate ERK, as determined by measuring increased phosphorylation of this enzyme. For example, both the flavanol (−)-epicatechin (0.1 and 0.3 μM) [139] and the citrus flavanone hesperetin at nanomolar concentrations [140] were observed to activate ERK1/in cortical neurons. Furthermore, EGCC was reported to restore ERK1/2 activities in 6-hydroxydopamine-treated or serum-deprived neurons [102]. ERK activation often leads to the activation of CREB, a transcription factor considered to be critical in the induction of long-lasting changes in synaptic plasticity and memory [157, 158]. CREB activation regulates the expression of a number of important genes, including BDNF, thus playing a pivotal role in controlling neuronal survival and synaptic function in the adult central nervous system [159, 160]. Regulation of BDNF is of particular interest as it is linked with the control of synaptic plasticity and long-term memory [161], and recent studies have shown that spatial memory performance in rats supplemented with blueberry correlates well with the activation of CREB and with increases of BDNF in the hippocampus [58]. Fisetin,

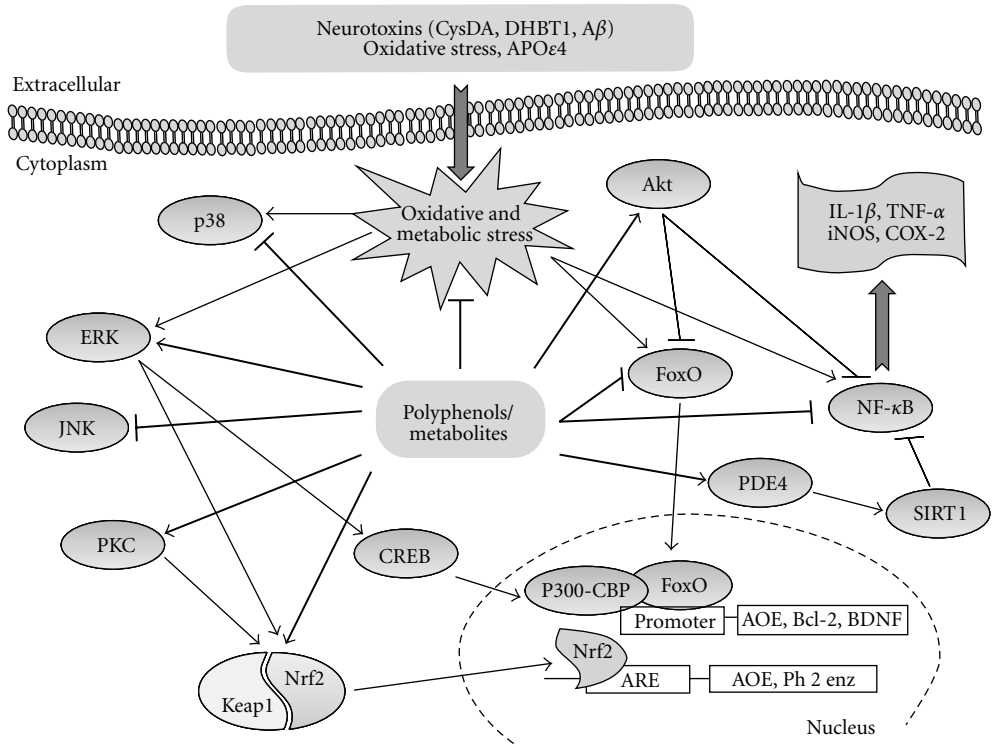

Figure 3: Mechanisms underlying the biological effects of polyphenols. Polyphenols and their *in vivo* metabolites activate cellular stress-response pathways resulting in the upregulation of neuroprotective genes. For example, both PKC and ERK can activate the nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 then translocates to the nucleus and binds to the antioxidant response element (ARE) in genes that encode cytoprotective proteins such as antioxidant enzymes (AOE) and phase 2 (Ph2) enzymes. The transcription factor cAMP-response-element-binding protein (CREB) is also activated by ERK, which induces the expression of brain-derived neurotrophic factor (BDNF), a mediator of neurohormesis. In addition, polyphenols can also regulate the transcription factor NF-κB, which can mediate adaptive cellular stress responses by reducing the expression of inflammatory cytokines. Activated SIRT1 may also inhibit NF-κB and so can reduce the cellular stress response. Another important pathway activated by metabolic and oxidative stress involves transcription factors of the forkhead (FoxO) family, which modulate genes that encode antioxidant enzymes and other stress-response proteins.

a polyphenol found in strawberries, has also been shown to improve long-term potentiation and to enhance object recognition in mice by a mechanism dependent on the activation of ERK and CREB [162].

As well as effects on the ERK/CREB/BDNF axis, polyphenols are also known to modulate the activity of an enzyme system associated with neuroprotection, Akt (also known as PKB). One of the major enzymes which controls Akt/PKB activity is the lipid kinase, PI3K. In cortical neurons, polyphenols such as the citrus flavanone hesperetin (0.1 and 0.3 μM) cause the activation of Akt/PKB and the consequent inhibition of proteins associated with cell death such as apoptosis signal-regulating kinase 1 (ASK1), Bad, caspase-9 and caspase-3 [140]. The activation of Akt by flavonoids in hippocampal neurons has been shown to trigger the increased translation of specific mRNA subpopulations [163], including the activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) [58]. Arc is also under the regulatory of control of both BDNF [164] and ERK signalling [165]. Increased Arc expression may facilitate changes in synaptic strength, and the induction of morphological changes in dendritic spines [166]. In support of this, studies have indicated that changes in neuronal morphology occur in response to flavonoid supplementation [8], and that certain polyphenols can influence neuronal dendrite outgrowth *in vitro* [167–169].

In addition to the previously described signalling systems, two additional pathways that are known to play important roles in neuronal stress adaptation are those involving the transcription factor NF-κB and the protein sirtuin-1 (SIRT1) [170]. In neurons activation of NF-κB can prevent cell death induced by a range of insults including exposure to excitotoxins and oxidative stress [171]. Numerous polyphenols have been shown ascribe to inhibit NF-κB in different cell types. For example, quercetin (50 μM) suppresses NF-κB in a microglial cell line [117]. Apigenin (5–15 μM) blocks LPS stimulation of the NF-κB pathway in RAW 246.7 macrophages and reduces κB-transcriptional activity [172]. Catechin (0.13–2 mM) has been reported to increase mouse microglial cell survival following exposure to the oxidative agent tert-butylhydroperoxide (tBHP) by suppressing NF-κB activation [173]. The flavone wogonin (50 μM) was shown to reduce NF-κB activation in C6 glioma cells and prevent microglial activation [174], and baicalein is reported to inhibit NO. Production in and NF-κB activity in microglia [175, 176]. Although these data give proof of principle that NF-κB is a potential target of polyphenols, the concentrations

required for positive effects of those particular compounds *in vitro* are supraphysiological and difficult to be achieved through the diet. While it is likely that the antioxidant effects of the polyphenols used in those studies account for the positive effects on suppressing NF-κB activation, at dietary relevant concentrations (0.1–1 μM), different classes of polyphenol were unable to suppress NF-κB-signaling pathways in primary astrocytes [177]. Despite the fact that polyphenols may be effective compounds at suppressing neuroinflammation *in vitro*, the NF-κB signalling system is unlikely to be regarded as the primary signalling system responsible for their effects *in vivo*.

The protein SIRT1 can also be activated by polyphenols resulting in cell proliferation and cell survival. Cellular substrates of SIRT1 include the tumor suppressor p53, the transcription factor NF-κB, the forkhead box class O (FoxO) family of transcription factors, the peroxisome proliferator-activated receptor (PPAR)-γ, the PPAR-γ coactivator 1α (PGC-1α), and endothelial nitric oxide synthase (eNOS) [178]. In the realm of polyphenols, resveratrol has been the most extensively studied for its ability to modulate SIRT1 both *in vivo* and *in vitro* [179, 180]. However, the observed activation of SIRT1 by resveratrol *in vitro* now appears to be an artefact of the assay used, therefore raising doubt on the direct resveratrol-SIRT1 connection [181]. Recently, further insight into the mechanisms by which resveratrol interact with sirtuins has been proposed. Using a model of aged-related metabolic phenotype, Park et al. identified phosphodiesterase (PDE) enzymes as direct targets and proposed that resveratrol indirectly activates SIRT1 through a signaling cascade involving cAMP, Epac1, and AMPK [182]. Although these results provide important new mechanisms by which resveratrol interacts with sirtuins, the supraphysiological dose used in these experiments must be taken with caution when translating these results to *in vivo* dietary intervention. SIRT1 also plays an important role in the regulation of neurodegenerative disorders [183], and several findings have now converged on the notion that activation of sirtuins by polyphenols could be extended to degenerating neurons. For example, resveratrol, was observed to protect both *C. elegans* and mouse neurons against the cytotoxicity of the mutant polyglutamine protein huntingtin through a mechanism involving Sir-2.1 and SIRT1 activation, respectively [184]. Furthermore, resveratrol decreased cell death associated with neurons cultured from a mutant huntingtin (109Q) knock-in mice, in a manner that is reversible by two SIRT1 inhibitors, sirtinol and nicotinamide [183]. Finally, overexpression of SIRT1 and resveratrol treatment markedly reduced NF-κB signaling stimulated by Aβ and had strong neuroprotective effects, therefore linking SIRT1-NF-κB activity to AD [185] (Figure 3).

8. Conclusion

The neuroprotective actions of dietary polyphenols involve a number of effects within the brain, including a potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuroinflammation, and the potential to promote memory, learning, and cognitive function. While many of the mechanisms underpinning their beneficial effects remain to be elucidated, it has become clear that they in part involve decreases in oxidative/inflammatory stress signaling increases in protective signaling, and may also involve hormetic effects to protect neurons against oxidative and inflammatory stressors. Most of the dietary polyphenols that have been shown to be protective against age-related disease are all chemically reactive and nearly all are electrophilic. Such chemical features renders these molecules capable of influencing the redox potential of their target cells and to modulate series of transcriptions factors that result in the activation of phase I and phase II metabolism genes. Nonetheless, much of the data obtained on their bioactivity derived from short-term basis *in vitro* or *in vivo* studies where the dose used was not of nutritional relevance. Although at the moment, the balance of evidence that does suggest that polyphenol effects contribute to the benefits of a high intake of fruits and vegetables, the extent of their contribution *in vivo*, and at physiological relevant concentrations remains uncertain. More work needs to be done to prove whether this class of compounds is most likely to result in health benefits and to determine their beneficial effects in slowly developing neurodegenerative disorders. In view of their multiple biological activities, the consumption of polyphenol-rich foods throughout life holds a potential to limit neurodegeneration and to prevent or reverse age-dependent deteriorations in cognitive performance. However, the therapeutic and pharmacological potential of these natural compounds still remains to be translated in humans in clinical conditions. Moreover, efficacy in RCT is also needed to support the relatively consistent epidemiological and mechanistic evidence. Despite this lack of efficacy data and the uncertainty of their effects *in vivo*, investigations into the absorption and metabolism of various polyphenols in humans indicate that there are common pathways for the metabolism of the majority of polyphenols, notably via their bacterial metabolism in the large intestine [186, 187]. Consequently, research on developing dietary polyphenols for applications in neurodegenerative disorders should prioritise investigations of smaller polar polyphenols for brain bioavailability and bioactivity. The challenge ahead therefore is to proceed cautiously until rigorous randomized controlled clinical trials have been undertaken to determine empirically whether polyphenols and/or their metabolites have efficacy in individuals affected by dementia and other neurodegenerative conditions.

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Research Article

Curcumin and Other Polyphenolic Compounds in Head and Neck Cancer Chemoprevention

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Despite clear results of observational studies linking a diet rich in fruits and vegetables to a decreased cancer risk, large interventional trials evaluating the impact of dietary micronutrient supplementation, mostly vitamins, could not show any beneficial effects. Today it has become clear that a single micronutrient, given in supernutritional doses, cannot match cancer preventive effects of whole fruits and vegetables. In this regard polyphenols came into focus, not only because of their antioxidant potential but also because of their ability to interact with molecular targets within the cells. Because polyphenols occur in many foods and beverages in high concentration and evidence for their anticancer activity is best for tissues they can come into direct contact with, field cancerization predestines upper aerodigestive tract epithelium for cancer chemoprevention by polyphenols. In this paper, we summarize cancer chemopreventive attempts with emphasis on head and neck carcinogenesis and discuss some methodological issues. We present data regarding antimutagenic effects of curcumin and epigallocatechin-3-gallate in human oropharyngeal mucosa cultures exposed to cigarette smoke condensate.

1. Introduction

About two-thirds of head and neck squamous cell carcinomas (HNSCC) can be attributed to heavy tobacco and alcohol consumption. These cancers develop predominantly in men in their sixties [1]. Tobacco carcinogens exert their harmful effects in large fields of upper aerodigestive tract mucosa during decades of smoking. Thus, mucosa cells stepwise accumulate genetic alterations that drive cells towards malignancy. Slaughter's concept of field cancerization best explains head and neck carcinogenesis, that HNSCC patients often suffer from syn- or metachronous cancers and have a high risk for local recurrences or second primary tumors. According to this concept, head and neck squamous cell carcinomas arise from multifocal precancerous lesions within large areas/fields of *condemned* mucosa in the oral cavity, pharynx, and larynx [2]. It seems obvious that in the same manner chemical carcinogens harm the epithelium, preventive agents might protect it. Cancer chemoprevention was initially supposed by Sporn in 1976. The term describes the use of specific natural, biologic, or synthetic agents to reverse, suppress, or prevent the development of epithelial cancer. Sporn reasoned that it usually takes several years until the final, invasive stage of epithelial malignancies is reached. Therefore, the progression of precancerous lesions could be stabilized, arrested, or reversed [3]. 10 years later, Hong and colleagues showed in a landmark study that following treatment with 13-cis-retinoic acid, the size of oral leukoplakia, a known precancerous lesion of the oral cavity, decreased in 67% of patients given the drug, compared with 10% in the placebo arm. Moreover, the vitamin A derivate significantly reversed dysplasia [4].

2. Chemoprevention of (Head and Neck) Cancer

Cancer is a largely preventable disease. Thirty years ago, Doll and Peto estimated that about 35% of all cancer deaths in the United States of America might be attributable to dietary factors [5]. During the 1980s and 1990s, researchers intensively investigated the role of dietary factors in upper aerodigestive

tract cancers. Multiple case-control studies showed an inverse correlation between fruit and vegetable intake and the risk for cancer of the oral cavity and pharynx [6–9]. Peto et al. also hypothesized in 1981 that β-carotene might reduce the incidence of all cancers, especially lung cancer [10]. In 1994, evidence had accumulated that β-carotene and the synthetic retinoid isotretinoin have chemopreventive effects in multistep field carcinogenesis of the upper aerodigestive tract and lung [11].

In 1988 a large interventional trial was launched including more than 18 000 men and women at high risk for lung cancer. The Beta-Carotene and Retinol Efficacy Trial (CARET) tested the combination of 30 mg β-carotene and 25 000 IU vitamin A/per day against placebo. Surprisingly, the trial needed to be stopped 21 month, before completion because of clear evidence of no benefit and substantial evidence of potential harmful effects. There were 28% more lung cancers and 17% more deaths in the active intervention group [12].

Another large-scale interventional trial evaluated the effects of α-tocopherol (vitamin E) and β-carotene on cancer incidences. The ATBC trial included 29 133 male cigarette smokers randomly assigned to receive α-tocopherol, β-carotene, a combination of both or placebo. Median followup was 6.1 years. In the β-carotene group, no decrease in cancer incidences was seen, but rather an increase at several sites, including the lung and stomach. The vitamin E group had fewer incidences in prostate and colorectal, but more stomach cancers [13]. Subgroup analyses for the upper aerodigestive tract showed no effect of either agent on overall incidence of oral, pharyngeal, and laryngeal carcinomas or mortality from these tumors. The results suggested a minor protective effect of β-carotene on incidences of early stage larynx cancers [13]. The EUROSCAN study looked for positive effects of retinyl palmitate (vitamin A) and N-acetylcysteine on overall survival, event-free survival and incidence of second primary tumors of head and neck and lung cancer patients. In 2592 patients, 60% suffering from head and neck, 40% from lung cancer, no benefit for the intervention group could be shown [14].

After decades of intensive research the scientific community ended up with little evidence for beneficial effects, but clear evidence for harmful effects of micronutrients. In 1999, Byers discussed the reasons why there is this large gap between the findings of observational studies and interventional trials. He found several factors that need to be considered. First, observational studies generally reveal weaker relationships between cancer and nutrients taken as dietary supplementation, most probably because of variability in reporting of doses taken and patterns of use. Second, the randomized controlled trials completed followed the investigational paradigm of pharmacology by testing micronutrients in supernutritional doses. The basic assumption that high doses of a single nutrient would reproduce the effects of the complex mixture of nutrients found in whole foods might be wrong. But whole foods or whole-diet interventions, he emphasized, cannot easily be incorporated in those trials, certainly not in a blinded way. Third, for reasons of feasibility, such trials evaluate only individuals at high risk for cancers within a short period of time. Since randomized controlled trials are designed to answer narrowly defined questions, it may be unwise to study only those at high risk [15].

3. Rationales for Head and Neck Cancer Chemoprevention

Chemoprevention of head and neck cancer is exceptionally interesting because many cancers develop from premalignant lesions, often readily accessible for clinical and histopathological examination. Moreover, as a result of field cancerization, HNSCC patients are at high risk for local recurrences or second primary tumors, an excellent endpoint for clinical trials. Due to life style factors and health behaviors, HNSCC patients often exhibit poor dietary conditions before treatment [16]. Besides smoking, the strongest predictor of survival, low fruit intake, is negatively associated with survival [17].

Upper aerodigestive tract mucosa is the primary target of tobacco smoke, a complex mixture of about 7000 chemical compounds including a high concentration of oxidants [18]. One cigarette puff contains 10^{14}–10^{16} radicals, mainly reactive oxygen species (ROS) [19]. Thus, cellular antioxidant defense capacity is easily exceeded leading to oxidative damage of macromolecules such as proteins, lipids and DNA. While the former can be replaced during physiological turnover, genetic material needs to be repaired [20]. During carcinogenesis, chemicals and oxidative stress lead to DNA alterations and evoke an inflammatory response that causes even more oxidative stress [21]. In addition, besides their intrinsic DNA-damaging properties, elimination of tobacco carcinogens, causes oxidative stress, not only during metabolic activation but also by being conjugated with and thereby depleting glutathione, a major nonenzymic antioxidant of the cell [22]. One of the most frequent ROS-induced DNA alterations is 8-oxo-guanine, which leads to frequent misincorporations of adenine in the opposite DNA strand. The resulting G : C to A : T transversion represents the most predominant somatic mutations in a wide range of epithelial malignancies [23]. Hence, tobacco smoke derived oxidative stress is a major source of DNA damage and mutagenicity. Therefore, orally administered high doses of potent antioxidant micronutrients such as β-carotene, α-tocopherol, or ascorbic acid in order to compensate oxidative stress seemed to be the most promising strategy for upper aerodigestive tract cancer chemoprevention—but, as stated earlier, failed. On the molecular level, a possible explanation for this failure is that high doses of antioxidants may have prooxidant effects, as it was supposed by Halliwell for vitamin E [24]. We showed that vitamin C loses its DNA protective effects in oxidative stressed cells at high concentrations and increases DNA damage caused by hydrogen peroxide [25].

4. Polyphenols in Cancer Prevention

In the late 1990s epidemiologists became aware of a phenomenon called the *French Paradox*. Despite high levels of risk factors such as smoking, hypertension, diabetes, and dietary fat, France has a comparably low mortality rate from

cardiovascular diseases. This observation was at least partly explained by high consumption of wine. Pronounced in the southwest of France, high levels of red wine intake also seemed to decrease mortality rates from lung cancer [26]. The validity of this conclusion was later questioned because the protective effect was not seen in other countries with similar or even higher wine consumption than France, but much attention was drawn to the potential health benefits of grapes and wine. Phenolic acids and polyphenols, in total 1.2 g per litre red wine, came into focus [27].

5. Resveratrol and Tannins

Resveratrol is a natural phenol produced by several plants. The phytoalexin was detected in high concentrations in the skin of red grapes and is believed to protect them from environmental stress and infections. In red wines, its concentration is approximately 2.0–40.0 μM [28]. Regarding HNSCC, only experimental data is available so far. El Attar and Virji reported in 1999 that resveratrol was effective inhibiting growth and proliferation of an oral cancer cell line at concentrations found in red wines [29]. Other studies proved its effectiveness in preventing intestinal and colon cancer in rodents. Due to its poor systemic bioavailability, human interventional trials are difficult to conduct, but at the moment one study is ongoing regarding possible effects on colonic mucosa from colon cancer patients [30, 31]. The strongest evidence of anticancer action of resveratrol exists for tumors derived from tissues with which resveratrol can come into direct contact [32], thus making it a prime candidate for HNSCC chemoprevention.

Tannins are also found in large quantities in red wine and were shown to act as both anti-initiating and antipromoting agents in experimental animals [33]. In mouse epidermal JB6 cells, tannins blocked epidermal growth factor-induced tumor promotion [34].

6. Quercetin

Quercetin is another polyphenol found in fruits, nuts, herbs, and vegetables, as well as in wine. It is normally present in its glycosylated form, but digestive cleavage of the glycosides catalyzed by β-glycosidases already begins in the oral cavity [35]. It is a potent scavenger of (oxygen) radicals and chelator of metal ions involved in ROS production [36], and it induced the expression of human 8-oxo-guanine DNA glycosylase, an enzyme involved in the repair of oxidative damaged DNA, in Caco-2 cancer cells [37]. But, quercetin is a two-edged sword, because in concentration greater than 40 μM, it increased oxidative stress in Chinese hamster ovary cells [38]. Chemopreventive effects in mice were shown by De and colleagues. Orally administered quercetin restricted the progression of cervical dysplastic lesions [39]. In humans, it has a comparable poor systemic bioavailability as resveratrol [30].

7. Epigallocatechin-3-Gallate (EGCG)

Tea is one of the most widely consumed beverages in the world. Flavanols, commonly referred to as tea catechins, notably epicatechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3-gallate (EGCG), represent about 3–10% of the dry weight of black tea and 30–42% of green tea, respectively. Orally administered, bioavailability of EGCG was found to be about 16% in rats. There seems to be no considerable presystemic hepatic metabolism [40]. Even though antioxidant properties of tea catechins are well established *in vitro* [41], the rationale for chemoprevention trials is largely based on its molecular mechanisms. Dong and colleagues identified vimentin, insulin-like growth factor 1 receptor and Ras-GTPase-activating protein SH3 domain-binding protein 1 as high affinity binding targets for EGCG, all of which were shown to be involved in EGCG-mediated growth inhibition in various cancer cell lines [42–44]. Of particular interest regarding head and neck carcinogenesis, EGCG was demonstrated to have inhibitory effects on epidermal growth factor receptor (EGFR) signalling pathways as detected in esophageal cancer, epidermoid carcinomas and colon cancer cell lines [45–48]. Further targets of EGCG and molecular mechanisms of action are reviewed by Yang and colleagues [49]. Despite these laboratory findings, a meta-analysis by the Cochrane collaboration of 51 studies including more than 1.6 million participants could not find sufficient evidence for cancer chemopreventive effects of drinking (green) tea [50].

8. Curcumin

Curcumin, the yellow pigment in tumeric, is widely used as a spice and has various properties such as antioxidant, immunomodulation, antiangiogenesis, and induction of apoptosis [32]. It was shown to effectively inhibit growth of normal human oral epithelial cells and cell lines derived from both oral precancerous lesions and squamous cell carcinomas [51]. Moreover, curcumin decreased incidence and volume of chemically induced oral cancers in rats [52]. In a prospective trial of patients at high risk for the development of epithelial cancer in several organs, oral intake of curcumin up to 8 g/day had no toxic effects in humans and led to histologic improvement of oral leukoplakia in 2 of 7 patients during 3 months of administration [53]. In 25 patients with oral leukoplakia treated with 900 mg curcumin, 80 mg desmethoxycurcumin and 20 mg bisdesmethoxycurcumin per day, serum and salivary vitamin C and E levels were found to increase, while markers for oxidative stress in serum and saliva decreased [54]. In head and neck cancer cell lines, curcumin was shown to target various molecular pathways including caspase-3 dependent signalling, Notch-1 and NF-κB pathways. This resulted in the induction of apoptosis and general growth inhibition of the cell lines [55–59].

9. Methodological Considerations

Some theoretical points regarding chemopreventive research need to be considered. Growth inhibitory or proapoptotic effects of polyphenols in cancer cell lines do not indicate chemopreventive, but rather chemotherapeutic effects. The use of cancer derived cell lines to evaluate possible effects of polyphenols on various cellular signalling pathways and protein expression should at least be questioned.

In this respect, epidermal growth factor receptor (EGFR) biology is a good example. About 80–90% of HNSCC show high expression of EGFR. Since high EGFR levels have been detected in premalignant lesion and increasing levels were found during malignant progression, the receptor is a widely accepted biomarker for head and neck carcinogenesis [60]. This made the receptor an interesting target for chemoprevention on the basis of anticipated inhibition of malignant transformation.

We investigated the role of EGFR biology in the context of chemical carcinogenesis caused by benzo(a)pyrene (B(a)p) and found that EGFR stimulation significantly reduces B(a)p-induced DNA fragmentation in premalignant oropharyngeal mucosa cells exhibiting high EGFR expression. This effect was totally abrogated when the receptor was blocked in advance [61, 62]. Thus, in premalignant mucosa of head and neck cancer patients, EGFR stimulation protects the cell from B(a)p, most probably as a result of EGFR downstream activation of a multidrug resistance efflux pump [63] capable of extruding the carcinogen from the cell [64, 65]. Furthermore, evidence is mounting that EGFR levels correlate with tobacco consumption and might, hence, represent a physiological response to its carcinogenic impact.

On the other hand, in HNSCC EGFR serves as an independent prognostic marker associated with resistance to nonsurgical therapies and poor survival [66]. Particularly, increased EGFR expression was linked to poor response to platinum-based chemotherapy. Cetuximab, a monoclonal EGFR antibody, was shown to be effective in cisplatin-resistant cancers [67]. The very same efflux pumps involved in B(a)p-extrusion are implicated in the resistance to chemotherapeutic drugs like cisplatin [68]. Named according to their function in antimicrobial and anticancer chemotherapy, it has now become clear that these pumps rather confer a general defense against xenobiotics and are highly conserved in all living organisms [69].

Therefore, EGFR inhibition, for example, by EGCG, certainly leads to growth inhibition in cell line experiments and might render cancer cells more sensitive to cytotoxic drugs. But it might also diminish cellular defense against carcinogens in premalignant mucosa. This illustrates that the modification of cellular signalling pathways or protein expression might have a different impact in non-/premalignant cells and cancer cells, respectively.

For the above reasons the most appropriate *in vitro* model for cancer chemopreventive trials has not yet been identified. Animal models show their strength when potential chemopreventive agents suppress or even reverse artificially induced malignant transformation of epithelial cell despite the fact that it is obviously not the primary organism of interest. In our laboratory we use tissue cultures of fresh biopsied human upper aerodigestive tract mucosa since many years. First described by Steinsvåg and colleagues for nasopharyngeal adenoid tissue [70], we applied the model for nasal and oropharyngeal mucosa. Samples harvested during surgery on lower nasal turbinates, palatine tonsils and soft palate are kept in culture for several weeks until three-dimensional tissue cubes consisting of a connective tissued core and completely coated with ciliated or squamous epithelium have emerged. For the evaluation of carcinogenic impact of xenobiotics on upper aerodigestive tract mucosa, mostly isolated mucosa cells are applied. Compared to cells kept in their surrounding tissue, however, single cells may have only a limited metabolic competence, not only for xenobiotics but also for endogenous-derived compounds. Moreover, single cells are not best suited for repetitive tests because of considerable loss of cellular material. As mentioned above, head and neck carcinogens strike their targets in a chronic manner. Nasal or oropharyngeal mucosa cultures can easily be exposed to multiple incubations with xenobiotics without considerable cellular damage in terms of viability and can be transferred by careful aspiration between containers without cellular loss. Moreover, in these tissue cultures, primary mucosa cells survive for several weeks. Therefore, our tissue culture model represents the primary target tissue of inhaled or ingested xenobiotics and carcinogens. It allows repetitive incubations not only with chemical carcinogens but also with chemopreventive compounds [71].

10. DNA Protection by Polyphenols in Human Mucosa Tissue Cultures

A carcinogen is defined as a physical, chemical, or biological agent or a combination of agents that produces cancer in an organism. The *International Agency for Research on Cancer* (IARC) recently classified tobacco smoke as *carcinogenic to humans* (group 1) [72]. This classification also applies for benzo(a)pyrene (B(a)p), which was recently upgraded from group 2B (*possibly carcinogenic to humans*) to group 1 [73]. The majority of known chemical carcinogens are also mutagens, hence, agents that produce a genetic event resulting in a heritable genetic change [74]. B(a)p and its activated metabolite benzo(a)pyrene diol epoxide (BPDE) are well-known mutagens [75]. BPDE binds to DNA *in vitro* and *in vivo* and forms adducts, particularly within the p53 tumor suppressor gene [76]. As described above, oxidative stress also leads to DNA alterations. Although hydrogen peroxide, widely used to induce oxidative stress, causes DNA damage *in vivo* and *in vivo* [77], IARC found only inadequate evidence for its carcinogenicity in humans and limited evidence for carcinogenicity in experimental animals, therefore classifying it as group 3 (*not classifiable as to its carcinogenicity to humans*) [78].

Using nasal and oropharyngeal mucosa tissue cultures, we previously evaluated the ability of epigallocatechin-3-gallate (EGCG), tannins, and quercetin to prevent DNA damage. Cultures were incubated with the polyphenols before DNA was damaged by cigarette smoke condensate (CSC), BPDE, or hydrogen peroxide. Genotoxicity was quantified by the comet assay. To test EGCG, oropharyngeal mucosa cultures were incubated with EGCG before DNA fragmentation was introduced by BPDE, which was previously shown to induce dose-dependent DNA migration detectable by the comet assay in human cells [79]. The treatment with EGCG significantly decreased BPDE-induced DNA damage in a dose-dependent manner. In a further series of tests, tissue cultures were incubated with EGCG on only 1 or on 4 days for 30 minutes. DNA damage was induced

by CSC during an 18-hour-incubation period. Here, DNA damage was decreased by 28% after 1 and by 47% after 4 incubations [80]. Similar results were seen when cultures were exposed to tannins during 30 minutes on 3 days. DNA fragmentation caused by BPDE went down by more than 40% [81].

In cultures produced of nasal mucosa quercetin significantly prevented DNA damage caused by hydrogen peroxide, again in a dose-dependent manner [82].

For the study presented here we used oropharyngeal mucosa. Cultures were incubated with curcumin, EGCG or both. DNA damage was introduced by cigarette smoke condensate and quantified using the comet assay.

11. Materials and Methods

11.1. Tissue Material. The trial was approved by the ethics committee of Ludwig-Maximilians-University, Munich, Germany. After given informed consent, mucosa samples were harvested during surgical therapy of chronic tonsillitis and/or obstructive sleep apnoea syndrome. Tissue samples were obtained during tonsillectomy and uvulopalatopharyngoplasty. After excision, samples were covered with 0.9% NaCl solution.

11.2. Cell Culture Procedure. After immediate transport to the laboratory, specimens were dissected into mucosal cubes of 1 mm^3 excluding deeper layers. Specimens were then transferred into a tube containing 5 mL Phosphate Buffered Salina (PBS, Gibco invitrogen, Eggenstein, Germany), washed three times in PBS, and placed in 24-well plates, one fragment in each well. Bottoms of wells were coated with 30 mL 0.75% Agar Noble (DIFCO, Detroit, USA) dissolved in 30 mL Dulbecco's modified eagle medium (DMEM), 6 mL 10% fetal calf serum (Gibco), 75 μL nonessential amino acids (Gibco), 240 μL penicillin-streptomycin (Sigma Aldrich, Steinheim, Germany), and 120 μL amphotericin B (Gibco), thus preventing adhesion to the surface. Cultures were now covered with 250 μL Bronchial Epithelial Cell Growth Medium (BEGM, supplemented with Bovine Pituitary Extract, insulin, hydrocortisone, epinephrine, triodothyronine, transferring, and retinoic acid; Promocell; Heidelberg, Germany) per well. After about 21 days incubated at 37°C, 5% CO_2, and 100% relative humidity, mucosa cultures were completely coated with epithelium. Growth medium (250 μL BEGM) was renewed every second day; every seventh day multiwell plates were changed. When transferred, cultures were mildly aspirated with a pipette, thus preventing damage to the cells as far as possible.

11.3. Incubations. In a first experimental arm, 20 mucosa cultures were incubated with 1 μmol/L curcumin for 60 minutes on 1 or 4 consecutive days. The concentration was determined by dose-response experiments (data not shown). BEGM was replaced twice after all incubations. Directly after the last incubation, cultures were exposed to cigarette smoke condensate (CSC, 0.7 mg/mL; produced of Marlboro Flavor Mix; Analytisch-Biologisches Forschungslabor, Munich, Germany) for 18 hours. All reagents were solved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany), which was used as negative control. In a second experimental arm, 20 cultures were exposed to 1 μmol/L curcumin for 1 hour and 0.5 μmol/L EGCG for 30 minutes to evaluate possible synergistic effects. Again, CSC was incubated for 18 hours.

11.4. Comet Assay. To quantify resulting DNA damage the alkaline version of the single cell microgelelectrophoresis (comet assay) was applied. The assay is capable of detecting DNA double- and single-strand breaks as well as alkaline labile sites and transient repair sites [83].

Mucosa cultures underwent enzymic digestions for 1 hour after being covered with a solution of 50 mg protease (Biochrom, Heidelberg, Germany), 10 mg hyaluronidase (Roche, Mannheim, Germany), and 10 mg collagenase P (Roche) dissolved in 10 mL BEGM. Thereafter, connective tissue and extracellular matrix components were carefully removed. Histolytic enzymes were neutralized with fetal calf serum (Gibco), and the cell suspension was washed twice in cold PBS (Gibco). Cell viability was monitored by the trypan blue dye exclusion test.

Comet assay was carried out according to the standard protocol [84]. DNA migration was measured using the image analysis software Komet 3.1 (Kinetic Imaging, Liverpool, UK) and quantified by the percentage of DNA in the tail (% tail DNA) [85]. 80 cell nuclei per slide were randomly selected without knowledge of pretreatment.

11.5. Statistical Analysis. Significant differences in DNA damage were calculated using the Wilcoxon Signed-Rank test by the SPSS 18.0 software (SPSS GmbH, München, Germany). Alpha level was set at 0.05 prior statistical analyses and adjusted according to the Bonferoni correction because of multiple testing. Significant α-levels are indicated in figures.

12. Results

Cell viability verified using the trypan blue staining test was constantly >90%, thus excluding major cytotoxic effects of the substances tested.

In the first experimental arm, DMSO used as the solvent for all other chemicals, as well as curcumin added on 1 or 4 days, did not cause considerable DNA damage. CSC did induce DNA fragmentation mean % tail DNA was 19.1. Previous exposure to curcumin within 60 minutes led to a reduction of DNA damage by 31.4% to 13.1% tail DNA. When cultures were incubated with curcumin on 4 days, DNA fragmentation was reduced by 47% to 10.1% tail DNA, which reflects a significant further decrease of CSC-induced genotoxicity (see Figure 1).

These results were confirmed in the second experimental arm. Again, the solvent, curcumin, and EGCG did not cause DNA fragmentation (data not shown). Curcumin decreased CSC-induced DNA fragmentation by 25.8% (1 day) and 47.1% (4 days), respectively. EGCG added on 1 or 4 days did also significantly reduce CSC-caused genotoxicity. % tail DNA went down from 24.0 to 18.8 (21.7%) and to 13.8, respectively (42.5%; see Figure 2).

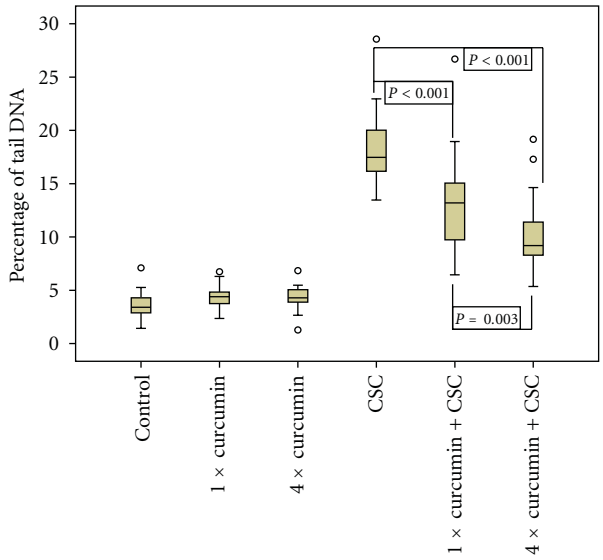

Figure 1: DNA damage (% tail DNA) caused by cigarette smoke condensate (CSC), after previous incubation with Curcumin on 1 day or on 4 days ($n = 20$; o = outlier value, * = extreme value; α-level = 0.02).

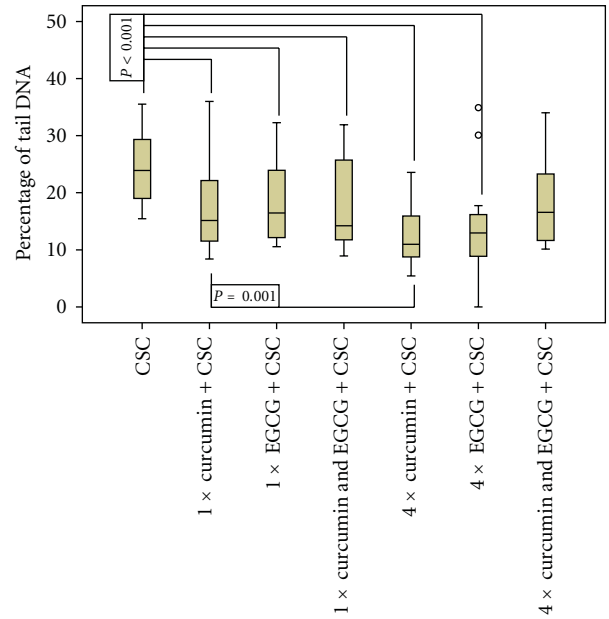

Figure 2: DNA damage (% tail DNA) caused by cigarette smoke condensate (CSC), after previous incubation with curcumin, epigallocatechin-3-gallate (EGCG), or both, on 1 day or on 4 days ($n = 20$; o = outlier value; α-level = 0.006).

Both polyphenols added together did not show synergistic effects. When incubated in 1 day, the combination of both substances did not further increase the effect of both curcumin and EGCG, but still significantly reduced genotoxicity of CSC. When added on 4 days, the combination of both substances did no longer significantly reduce CSC-induced genotoxicity after α-level was set to 0.006 according to the Bonferoni correction (see Figure 2).

13. Conclusion

In our study, mucosa cultures were incubated with curcumin, epigallocatechin-3-gallate (EGCG), or both on 1 day or on 4 consecutive days. After all incubations, the growth medium was replaced twice. Finally cultures were exposed to cigarette smoke condensate (CSC) for 18 hours. The result was a highly significant reduction of CSC-caused genotoxicity as evaluated by the comet assay in all experiments except for the combination of curcumin and EGCG incubated on 4 days. No additive effects were detected when curcumin and EGCG were added simultaneously. Compared to the other polyphenols tested in our laboratory, curcumin was most effective preventing tobacco-related DNA damage. All compounds reduced genotoxic effects of B(a)p or CSC in a dose-dependent manner [80, 81].

Bearing in mind that tobacco smoke is carcinogenic to humans and responsible for the vast majority of head and neck cancers, our results demonstrate promising chemopreventive potentials of curcumin and EGCG. Since we used oropharyngeal mucosa cultures as a primary human target tissue of tobacco smoke, the experimental setting represents a good compromise between *in vivo* studies in animals and human cell line experiments. Taken together, this study and previous results of our laboratory indicate that dietary polyphenols are capable of preventing tobacco-related genotoxicity in upper aerodigestive tract mucosa. A diet high in fruits and vegetables remains the major source of polyphenolic compounds and was repeatedly shown to lower the risk for HNSCC. Unfortunately, many heavy smokers do not exploit this source due to life-style habits.

Despite the disappointing findings of large interventional trials evaluating the impact of dietary micronutrient supplementation, mostly vitamins, on cancer incidences, the clear results of observational studies remain valid. Even if it could not be shown that one single agent or a combination of two can be as effective as whole fruits and vegetable, it is still in this diet and therefore a matter of time, until the network and interactions of micronutrients involved are better understood. Vitamin E, for example, is a complex mixture of 16 chemical compounds including 4 tocopherols. In the western diet, the most prevalent vitamin E compound is γ-tocopherol and high serum levels were shown to be inversely associated with cardiovascular diseases. Gamma Tocopherol is poorly retained after intestinal absorbtion, and α-tocopherol, widely used for dietary supplementation, reduces further serum concentration of γ-tocopherol [86–88]. Moreover, several vitamins in high supernutritional doses might be more harmful than protective. The old sentence of Paracelsus "dosis facit venenum" seems to be particularly true in this regard.

It becomes clear that micronutrient supplementation cannot mimic a diet rich in whole fruits and vegetables. Polyphenols recently came into focus, not only because of their powerful antioxidant effects but also because of their ability to interact with cellular signalling pathways. In this regard, as stated above, we need to carefully distinguish

chemopreventive and rather chemotherapeutic effects. Nevertheless, polyphenols showed promising anticancer and antimutagenic action. The fact that several phenolic compounds are present in beverages in high concentrations makes them good candidates for head and neck cancer chemoprevention. Since evidence for anticancer effects is best for tissues they can come into direct contact with, upper aerodigestive mucosa, target of field cancerization, seems to be one of the best-suited tissues.

Abbreviations

HNSCC: Head and neck squamous cell carcinoma
ROS: Reactive oxygen species
EGCG: Epigallocatechin-3-gallate
EGFR: Epidermal growth factor receptor
B(a)p: Benzo(a)pyrene
BPDE: Benzo(a)pyrene diol epoxide
CSC: Cigarette smoke condensate.

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Review Article

Polyphenols: Key Issues Involved in Chemoprevention of Prostate Cancer

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Prostate cancer is is the most common solid neoplasm and it is now recognized as one of the most important medical problems facing the male population. Due to its long latency and its identifiable preneoplastic lesions, prostate cancer is an ideal target tumor for chemoprevention. Different compounds are available and certainly polyphenols represent those with efficacy against prostate cancer. This review take a look at activity and properties of major polyphenolic substances, such as epigallocatechin-3-gallate, curcumin, resveratrol and the flavonoids quercetin and genistein. Although the current studies are limited, mechanisms of action of polyphenols added with the lack of side effects show a a start for future strategies in prostate chemoprevention.

1. Introduction

Chemoprevention is defined as the use of specific natural (dietary) or synthetic agents to prevent, delay, or slow the carcinogenic process. Prostate cancer is an ideal target disease for chemoprevention thanks to long latency, high incidence, tumor marker availability (prostate-specific antigen, PSA), identifiable preneoplastic lesions, and because it is a very heterogeneous disease with a large subgroup of patients with nonaggressive disease [1].

Up to now the two principal targets for prostate cancer chemoprevention have been inflammation and hormonal stimulation: between all prostate cancer risk reduction studies, the only ones who were successful are PCPT (finasteride) and REDUCE (dutasteride).

Although most prostate cancers behave indolently and are undiagnosed during life, it is still the second most common cause of cancer death in men and it will become a considerable health problem in the next millennium, as the adult population is increasing [1, 2].

The incidence and mortality of prostate cancer shows strong variations worldwide with the highest rates in North America, Australia, Western and Northern Europe and the lowest rates in Japan and other Asian countries [1].

These wide variations have been explored and nutritional factors were found to play a role together with genetic, behavioral, occupational, and environmental ones. Since prostate cancer typically develops later in life, identifying botanical compounds to prevent or delay disease progression may have a positive effect on quality of life and reduce healthcare costs of the aging population [3].

According to the National Cancer Institute, about 400 compounds have been listed as potential chemopreventive agents and about 40 of these are currently under clinical evaluation. This paper systematically examines the data on the prostate cancer chemopreventive action of polyphenols, a class of diet constituents that showed notable efficacy in preclinical models of carcinogenesis, analyzing in particular the mechanisms of action of some polyphenolic substances, such as epigallocatechin-3-gallate, curcumin, resveratrol and the flavonoids quercetin and genistein (Table 1) [4].

Table 1: Summary of studies on polyphenols and their respective mechanisms of action.

Compound	Type of study	Cell culture system or animal studies	Concentration used	Mechanisms of action
Epigallocatechin-3-gallate	In vitro [7, 8, 10]; in vivo [9]; in humans [12, 13]	LNCaP and PC-3 [8]; athymic nude mice [9]; LNCaP and DU-145 [7]	In vitro: 10–40 micromol/L [8]; in vivo: EGCG (62%) [9]; in humans: 600 mg/d [12]; 5 cups/day [13]	Activation of caspase-6 and -9 [8]; reduction of expression of androgen receptor [9]; inhibition of (MMP-2 and -2) and VEGF [10]
Curcumin	In vitro [16, 19, 22–25]; in humans [28]	LNCaP [16, 20], DU145 and LNCaP [19]; LNCaP [20]; L1210 mouse leukemia cell line [22]; PC-3 and DU145 [23]; PC-3 [24]; DU-145 [25]	In vitro: 30 μmol/L [16]; 10 μM [20]; 1–100 μM [19]; 0–45 μM [22]; 1–10 μM [23]; 2–5 μM [24]; 10 mg/mL [25]; in humans: 0.4 to 3.6 g [28]	Reduction of MDM2 protein and induction of gene NKX3.1 [16]; induction of apoptosis and the activation of procaspase-3 and -8 [19] and caspase-9 and -3 [24]; reduction of MMP-2 and -9 [25]; decrease of PGE-2 [28]
Resveratrol	In vitro [29, 30]	LNCaP, DU-145, PC-3 [29]; LNCaP and DU-145 [30]	In vitro: 2–40 μM [29]; 1 μmol [30]	Inhibition of the formation of free radicals [29] and induction of apoptosis [30].
Quercetin	In vitro [31–35]	PC-3 and LNCaP [31, 35]; LNCaP [32]; PC-3 [33, 34]	In vitro: 20–40 μM [31]; 100 microM [32–35]; in humans: 24 μg [36]	Activation of caspase-9 and caspase-3 [31]; induction of apoptosis [32, 33]; downregulation of the expression of MMP-2 and -9 [34]; suppression of androgen receptor [35]
Genistein	In vitro: [37–39]; in vivo: [40]	LNCaP and PC-3 [37]; LNCaP, DU-145 and PC-3 [38]; PC-3 [39].	In vitro: 10–20 μM [37]; 20 μM [38]; 0–250 μM [39] in humans: 30 mg [41]	Inhibition of angiogenesis and downregulation of TGF-β and EGF [37, 42]; increase of the glutathione peroxidase (GPx)-1 [38]; decrease of metastases by 96% [39]; serum prostate specific antigen (PSA) decreased by 7.8% [41]

2. Epigallocatechin-3-Gallate

Epigallocatechin-3-gallate (EGCG) represents the major polyphenolic constituent present in green tea, the most popular beverage next to water, that has potential to be developed as a chemopreventive agent for prostate cancer (Figure 1). The tea plant (*Camellia sinensis*) has been cultivated in Asia for thousands of years, and Green tea has been used for centuries in China, Japan, and Thailand as a traditional medicine with a variety of applications [5].

Over the last two decades many epidemiological studies have evaluated the chemopreventive properties of green tea, suggesting that increasing intake of green tea is correlated with significant decrease in the development of prostate cancer [6].

Due to its anticarcinogenic effects, such as inhibition of growth proliferation, induction of apoptosis, induction of phase II detoxifying enzymes, and reduction of oxidative damage to DNA, various in vitro and in vivo studies have more specifically shown that consumption of green tea polyphenols is associated with decreased risk and/or slower progression of prostate cancer [5].

According to cell-culture studies, EGCG induced apoptosis and cell cycle arrest in many cancer cells without affecting normal cells. Particularly in prostate cancer cells, EGCG activates growth arrest and apoptosis primarily via p53-dependent pathway that involves the function of both p21 and Bax such that downregulation of either molecule confers a growth advantage to the cells. In androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate carcinoma cells, EGCG inhibited COX-2, (inducible enzymatic isoform, rapidly induced by growth factors, tumor promoters, oncogenes, and carcinogens) without affecting COX-1 expression at both the mRNA and protein levels [7].

A study published in 2007 tested the effect of epigallocatechin-3-gallate alone and in combination with specific COX-2 inhibitors on the growth and apoptosis of human prostate cancer cells both in vitro and in vivo. This study

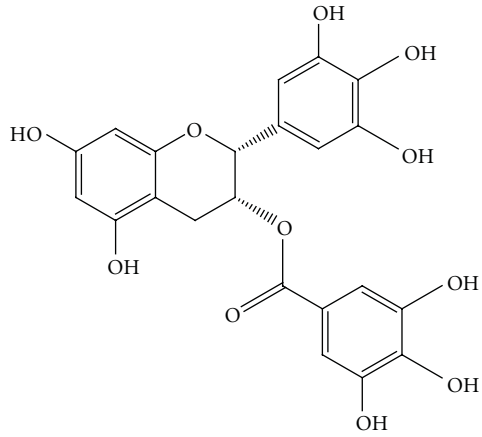

Figure 1: Epigallocatechin-3-gallate.

demonstrated a synergic action and an increased efficacy of selective COX-2 inhibitors in combination with polyphenols, from green tea, for inhibition of growth of human prostate cancer cells both in vitro and in vivo. It was observed that this effect was mainly due to increased apoptosis after increased activation of caspase-6 and caspase-9 [8].

It has been shown that ester bond-containing tea polyphenols, such as EGCG, potently and specifically inhibit the chymotrypsin-like activity of the proteasome in vitro and in vivo at the concentrations found in the serum of green tea drinkers, causing growth arrest in the G(1) phase of the cell cycle [7].

In a study published in 2006 the combination treatment with EGCG, green tea extract, water extract of black tea, and theaflavins was shown to reduce gene expression and protein expression of androgen receptor in the athymic nude mice implanted with androgen-sensitive human CaP CWR22Rv1 cells that resulted in induction of apoptosis, decrease in the levels of VEGF protein, reduction in the level of serum PSA, and a reduced tumor volume [7, 9].

Furthermore, this polyphenolic compound seems to inhibit tumor expression of matrix metalloproteases (MMP-2 and MMP-9) and vascular endothelial growth factor (VEGF), which are overexpressed in angiogenesis, and thereby prevent the invasion and the metastatic spread of cancer [10].

In a mouse model of orthotopic androgen-sensitive human PCa, the combination of soy phytochemical concentrate, black tea, and green tea significantly reduced tumorigenicity. This association synergistically inhibited tumor angiogenesis, final tumor weight, metastasis and significantly reduced serum concentrations of both testosterone and dihydrotestosterone in vivo [11].

A prospective, double-blind, placebo-controlled study, using a defined product of green tea in capsule form in men with HG-PIN, observed a 90% reduction in developing Pca. This was the first study that has shown the effectiveness of green tea polyphenols for the treatment of premalignant lesions of prostate cancer [12].

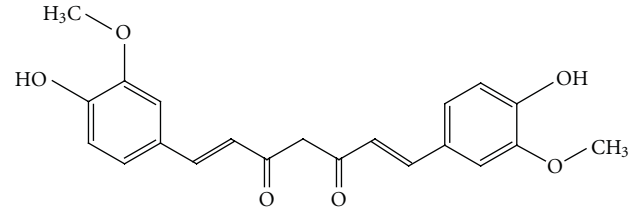

Figure 2: Curcumin.

In the Japan Public Health Center-based prospective study, 49,950 men aged 40 to 69 completed a questionnaire on the basis of their green tea consumption habit. Consumption was associated with a dose-dependent decrease in the risk of advanced PCa. The multivariate relative risk was 0.52 for men drinking 5 or more cups/day compared with less than 1 cup/day [13].

However, further studies are needed so that the EGCG can be safely considered as chemopreventive agents for prostate cancer.

3. Curcumin

Curcumin (diferuloylmethane) is a major chemical component of turmeric (*Curcuma longa* Linn.) and is used as a spice to give a specific flavor and yellow color to food in the Indian subcontinent (Figure 2) [14].

It has been used for centuries throughout Asia not only as a food additive but also as cosmetic and as a traditional herbal medicine to treat a variety of inflammatory conditions and chronic diseases. Over the past decade, several studies have substantiated the potential prophylactic or therapeutic value of curcumin and have unequivocally supported reports of its anticarcinogenic properties, such as its ability to influence a diverse range of molecular targets within cells. To date, no studies have reported any toxicity associated with the use of curcumin in either animals or humans [15].

The chemopreventive properties of curcumin are attributed to its effect on several targets including transcription factors, growth regulators, adhesion molecules, apoptotic genes, angiogenesis regulators, and cellular signaling molecules. It has been shown that curcumin has the ability to induce apoptosis in both androgen-dependent and androgen-independent prostate cancer cells acting through the downregulating apoptosis suppressor proteins and other crucial proteins such as the androgen receptor. In PC-3 (hormone-independent line possessing dysfunctional androgen receptors) and LNCaP (hormone-sensitive cells), curcumin significantly altered microfilament organization and cell motility. In PC-3, human prostate cancer cell line, curcumin reduced MDM2 protein and mRNA and enhanced the expression of the tumor suppressor p21/WAF1, a gene that encodes a potent cyclin-dependent kinase inhibitor of cyclin-CDK2 and -CDK4 complexes, inducing apoptosis and inhibiting proliferation.

Furthermore, curcumin inhibited androgen receptor-mediated induction of NKX3.1 expression and decreased the

expression of androgen receptors and the binding activity to antioxidant response element directly [16].

NKX3.1, a gene located nearly on 8p21.2, is involved in the initiation stage of prostatic tumorigenesis. There is considerable evidence that loss of NKX3.1 expression, along with PTEN heterozygosity, a gene that codes for a lipid phosphatase and functions as a negative regulator of phosphoinositol-3-kinase (PI3K) signaling, is found at high frequency in CaP [17].

NKX3.1 gene encodes a home-box-containing transcription factor that functions as a negative regulator of epithelial cell growth in prostate tissue. Thus, cellular NKX3.1 protein levels are critical for maintenance of the prostate epithelial phenotype. Experiments conducted on LNCaP and PC-3 cells demonstrated that inflammation and in particular overproduction of TNF-α and IL-β lead to rapid ubiquitination and proteasomal degradation of NKX3.1 protein through phosphorilation of serine-196 [18].

In a study, it was found that treatment of prostate cancer cells with curcumin (1–100 μM) suppresses both constitutive (DU145) and inducible (LNCaP) NF-κB activation and potentiates TNF-induced apoptosis. Curcumin treatment (50–100 μM) induced apoptosis in both cell types, which correlated with the downregulation of the expression of Bcl-2 and Bcl-xL and the activation of procaspase-3 and -8 [19].

A study also showed that curcumin blocks enhanced the effect of PSA expression by L-mimosine and dimethyloxalylglycine, prolyl hydroxylase inhibitors, which induce hypoxia condition [20].

In hormone refractory prostate cancer, it was found that curcumin, in addition to conventional treatment, may decrease prostate cancer aggressive proliferation and potentiate activity of taxane therapy increasing cytotoxicity and delaying prostate cancer cell resistance to these chemotherapeutic drugs [21–23].

In combination with radiation, curcumin (2–5 μM) showed significant enhancement of radiation-induced clonogenic inhibition and apoptosis in PC-3 cells and significant activation of cytochrome c and caspases-9 and -3. These mechanisms suggest that this natural compound acts by overcoming the effects of radiation-induced prosurvival gene expression in prostate cancer [24].

Others in vitro and in vivo studies have also demonstrated the inhibitory effects exerted by treatment with only curcumin against the growth and invasiveness of DU-145 prostate cancer cells. The inhibition of tumor cell invasion was due to reductions in MMP-2 and MMP-9. Curcumin was also shown to induce a marked reduction of tumor volume. This compound may therefore have a role as a chemopreventive agent and/or adjuvant therapy in the treatment of prostate cancer, probably as a nontoxic dietary supplement [25].

Until now, few clinical data about curcumin have been performed in humans, despite the large amount of study in vitro and in animals. For these reasons, it is not yet known the pharmacokinetics properties of curcumin and its efficacious doses.

In a pilot study of a standardized oral *Curcuma* extract, doses up to 180 mg of curcumin per day were administered to patients with advanced colorectal cancer for up to 4 months without overt toxicity or detectable systemic bioavailability [26]. A subsequent study has suggested that doses up to 8 g could be administered daily to patients with premalignant lesions for 3 months without overt toxicity [27].

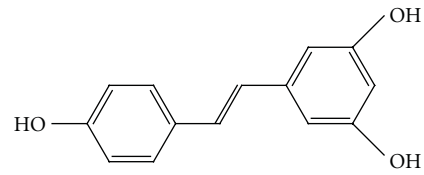

Figure 3: Resveratrol.

In a phase I clinical trial of oral curcumin, fifteen patients with colorectal cancer refractory to standard chemotherapy consumed a capsule with a dose escalation between 0.4 g and 3.6 g daily for up 4 months. A daily dose of 3.6 g curcumin engendered 62% and 57% decreases in inducible PGE-2, suggesting a possible use of this compound for prevention outside the gastrointestinal tract [28].

4. Resveratrol

Resveratrol (trans-3,4′,5-trihydroxystilbene, $C_{14}H_{12}O_3$) is a plant-derived polyphenolic phytoalexin produced by the enzyme stilbene synthase in response to infection by the pathogen Botrytis cinerea and to a variety of stress conditions, such as vicissitudes in climate, exposure to ozone, sunlight and heavy metals (Figure 3). It exists in two isoforms: trans-resveratrol and cis-resveratrol where the transisomer is the more stable form. Resveratrol is present in red grapes, peanuts, some common drinks, and dietary supplements [43].

It has broad-spectrum beneficial health effects including anti-infective, antioxidant, and cardioprotective functions, but it has gained considerable attention because of its potential cancer chemopreventive properties [44]. In this regard, resveratrol represents such an ideal molecule, due to its relatively low toxicity and capacity to target multiple signaling molecules that collectively promote cancer cell survival and tumor growth. It was demonstrated that this natural compound can modulate many intracellular cancer targets, which affect cell growth, inflammation, apoptosis, angiogenesis, invasion and metastasis. It is also able to potentiate the apoptotic effects of cytokines, such as TRAIL, chemotherapeutic agents, and gamma radiation [45].

Many in vitro studies have investigated the antiproliferative or proapoptotic effects of resveratrol in human prostate cancer cells, and its mechanism of action. Resveratrol was found to inhibit the growth of LNCaP cells (hormone-sensitive cells), DU-145 (androgen-independent cells), and PC-3 (hormone-independent line possessing dysfunctional androgen receptors) in a concentration-dependent manner. It has also been shown to exert a strong inhibitory effect on the formation of free radicals in human macrophages, reducing oxidative stress within premalignant cells, and to

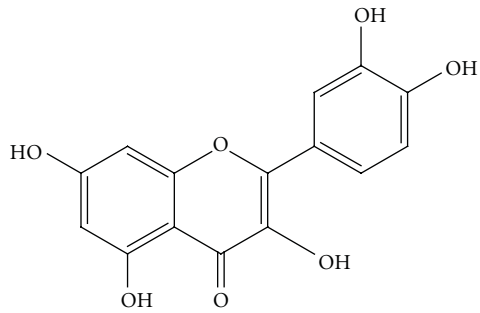

Figure 4: Quercetin.

decrease the production of NO in PC-3 and DU-145 cells, reducing growth and spread of prostate cancer [29].

About its proapoptotic effects, it has been shown to induce apoptosis in LNCaP and DU145 prostate cancer cell lines through different PKC-mediated and MAPK-dependent pathways [30].

Furthermore, resveratrol-mediated apoptosis has been associated with p53 activation and also occurs via the death receptor Fas/CD95/APO-1 in various human cancer cells [45].

It is also possible that resveratrol exerts its chemopreventive action in part by modulating the expression or function of androgen receptor [29].

Another interesting chemopreventive mechanism related with this compound is represented by sensitization effect. Research from in vitro and in vivo studies indicate that resveratrol can overcome chemoresistance in tumor cells by modulating apoptotic pathways, downregulating drug transporters, downmodulating proteins involved in tumor cell proliferation, and inhibiting NF-κB and STAT-3 pathway [44].

Goldberg et al. reported that, after an oral dose of resveratrol (25 mg/70 kg), catechin (25 mg/70 kg), and quercetin (10 mg/70 kg) to healthy human subjects, these compounds appeared in serum and urine predominantly as glucuronide and sulfate conjugates and free polyphenols accounted for 1.7–1.9% (resveratrol), 1.1 to 6.5% (catechin), and 17.2 to 26.9% (quercetin) of the peak serum concentrations and more than 80% is absorbed. The absorption of trans-resveratrol was the most efficient as judged by peak serum concentration (16-17% of dose consumed) [46].

5. Quercetin

Quercetin is the main representative of the flavonol class and a polyphenolic antioxidant found in a variety of fruits and vegetables, highly concentrated in onions, broccoli, apples, grapes (red wine), and in soybeans (Figure 4).

This flavonoid, besides having antioxidant and anti-inflammatory activities, has been shown to possess potent antiproliferative effects against various malignant cells, although its molecular mechanism involved in chemoprevention of prostate cancer remains unclear in many respects [47]. Quercetin treatment has been associated with selective antiproliferative effects and induction of cell death, predominantly through an apoptotic mechanism, in cancer cell lines. This compound seems to be able to induce apoptosis through multiple mechanisms: causing arrest in the G_1 phase of the cell cycle or through interaction with cell cycle-regulated proteins, like cyclin D1 and CDK4; releasing cytochrome c and activating caspase-9 and caspase-3; through inhibition of PI3K, an enzyme involved in the pivotal cell survival pathway, synergizing the effect of ECGC [31].

Epidemiological studies and preliminary data have shown that quercetin inhibits the onset/growth of prostate cancer. It was noted that there is a 27% risk reduction for prostate cancer for those who consume at least 24 μg of quercetin a day [36].

In human prostate carcinoma LNCaP cells, quercetin inhibited the PI3K/Akt pathway, suppressed the phosphorylation of Bad, proapoptotic Bcl-2 family member, and subsequently altered the interaction between Bcl-xL and Bax, leading to cytochrome c release, activation of caspases and consequently apoptotic death [32].

It was also found that quercetin inhibits the proliferation of PC-3 cells causing a significant decrease in Cdc2/Cdk-1 and cyclin B1 protein expressions and increasing hypophosphorylated level of pRb and this may be attributed to decreased expression of growth responsive genes and subsequent growth inhibition of PC-3 cells [33].

Another important chemopreventive activity of quercetin might be to reduce the risk of prostate cancer metastasis. Tumor invasion and metastasis represent a multistep process that depends on the activity of many proteins. Proteolytic degradation of the extracellular matrix components is a central event of this process, primarily due to the action of matrix metalloproteinases.

A study showed that this natural compound inhibits the expression of MMP 2 and 9 in prostate cancer cells (PC-3). As it has been detected that MMP-2 and 9 expressions were regulated by MAP kinase signaling pathways and quercetin is an inhibitor of several kinases including MAP kinases and tyrosine kinases, it is reasonable to speculate that quercetin might have downregulated the expression of MMP-2 and -9 through inhibition of protein kinases [34].

In addition, quercetin appears to have the ability to suppress the function of androgen receptor, pivotal molecule in normal development of the prostate and in the development and progression of prostate cancer. Quercetin-mediated inhibition of the androgen receptor transcription activity in prostate cancer cells may be caused, at least in part, by the formation of a protein complex containing c-Jun, Sp1, and androgen receptor, but further investigation will be necessary to examine whether other factors are also involved in this protein complex [35].

6. Genistein

Genistein (4′,5,7-trihydroxyisoflavone), the predominant isoflavone in human nutrition, is derived mainly from soybeans but also from other legumes, including peas, lentils, or beans (Figure 5) [38].

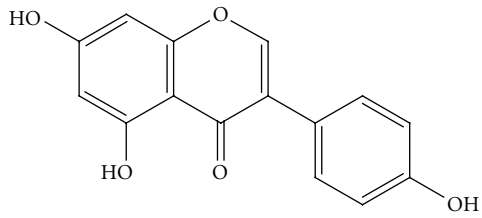

Figure 5: Genistein.

Genistein has many important health benefits, such as lowering the incidence of cardiovascular diseases, prevention of osteoporosis, attenuation of postmenopausal problems, reduction of body mass and fat tissue. It also has chemopreventive properties, and in particular genistein has been shown to inhibit growth of both androgen-dependent and -independent prostate cancer cells in vitro. Several mechanisms have been proposed for genistein anticarcinogenic activity: inhibition of protein-tyrosine kinase, with the result of alleviating the growth of cancer cells by inhibiting PTK-mediated signaling mechanisms; inhibition of topoisomerases I and II and protein histidine kinase with antiproliferative or proapoptotic effects; antioxidant effects, through inhibition of the expression of stress response related genes; inhibition of NF-κB and Akt signaling pathways, both of which are important for cell survival; the inhibition of angiogenesis; the downregulation of transforming growth factor-beta (TGF-β), and the inhibition of epidermal growth factor (EGF) [37, 42].

In vitro studies have also demonstrated that this natural compound downregulates the androgen receptor of PCa cells via the estrogen receptor β, resulting in a modified response to hormonal stimuli, inhibits several steroid-metabolizing enzymes such as 5-α-reductase or aromatase creating a more favorable hormonal milieu and a protective effect against prostate cancer, blocks the cell cycle progression at G_1, and inhibits PSA expression [38].

With regard to its antioxidant activity, a study examined the effect of genistein on human prostate cancer (LNCaP and PC-3) cells. To obtain the gene expression profile of genistein in LNCaP cells, it was performed cDNA microarray analysis. This survey has shown that while the expression of many genes, including apoptosis inhibitor (survivin), DNA topoisomerase II, cell division cycle 6 (CDC6), and mitogen-activated protein kinase 6 (MAPK 6), was downregulated, the glutathione peroxidase (GPx)-1 gene expression level was upregulated with a subsequent increase of GPx enzyme activities.

The tumor initiation and progression are often attributed to oxidative stress and the generation of ROS, which exceed cell ability of metabolize and detoxify them.

In addition to causing genetic changes, ROS may lead to epigenetic alterations that affect the genome and play a key role in the development of human carcinogenesis [40].

More specifically, ROS production is associated with alterations in DNA methylation patterns.

Furthermore, ROS-induced oxidative stress can contribute to gene silencing by mechanisms that involve aberrant hypermethylation of tumor suppressor gene promoter regions and thus lead towards progression to a malignant phenotype [48].

Oxygen radicals may cause damage to DNA and chromosomes, induce epigenetic alterations, interact with oncogenes or tumor suppressor genes, and impart changes in immunological mechanisms, like mutation of nuclear encoded genes such as *TP53*, promotes carcinogenesis [49, 50].

ROS are further determinant for the activation of inflammatory pathways that play a key role in cancer progression. Inflammation in cancer involves a close interplay between tumor-associated immune cells and the tumor cells themselves. Activation of NF-κB and AP-1 in immune cells, induced by ROS, determines production of inflammatory cytokines such as TNFα and IL-6 that have been demonstrated to be important in tumor progression [51].

Since ROS are considered key participants in the progression cancer, the antioxidant effect of genistein might prevent tumor invasion or metastasis in prostate cancer cells inhibiting production of matrix metalloproteinase, cell motility and degradation of the basement membrane. [52].

The antimetastatic potential of genistein was evaluated by a study through the development of an animal model, a murine model of human PCa metastasis. It has been demonstrated that genistein inhibits initial steps in the metastatic cascade, namely, cell detachment and cell invasion and for the first time induces flattening of cell nuclei in vivo, a measure of increased cell attachment. Furthermore, genistein, through inhibition of phosphorylation, has been shown to inhibit activation of p38 MAPK and FAK (promotility proteins) in vivo, blocking cell motility. Genistein decreased metastases by 96%, induced nuclear morphometric changes in PC3-M cells indicative of increased adhesion (i.e., decreased detachment) but did not alter tumor growth. This study showed for the first time that dietary concentrations of genistein can inhibit prostate cancer cell metastasis, but more specific analysis on the genistein effects upon human prostate cells needed [39].

Another interesting study has investigated the potential additive and synergistic effects of genistein and resveratrol for suppressing prostate cancer in the Simian Virus-40 T-antigen (SV-40 Tag) targeted probasin promoter rat model, a transgenic model of spontaneously developing prostate cancer. It has been shown that high dose genistein and resveratrol treatments, reducing cell proliferation and increasing apoptosis mainly through the modulation of sex steroid receptor and growth factor signaling, suppress the most severe grade of prostate cancer in these transgenic animals [53].

In a randomized, placebo-controlled, double-blind phase II clinical trial, fifty-four study subjects were recruited and randomized to treatment with genistein 30 mg ($n = 23$) or placebo ($n = 24$) for 3 to 6 wk prior to prostatectomy. Serum prostate specific antigen (PSA) decreased by 7.8% in the genistein arm and increased by 4.4% in the placebo arm, without adverse events and with beneficial effect on blood cholesterol [41].

7. Conclusions

Since prostate cancer is one of the most important medical problems afflicting male population, chemoprevention strategies represent a promising approach to reduce the incidence and mortality of this. Unfortunately, scientific evidences about polyphenols should be still demonstrated and well-conducted clinical studies are needed to clarify the efficacy of these molecules on prevention of PCa.

It should be better regulated the wide use of different dietary agents; in fact, being extractive products and not synthetic products, they may elicit a great variability of therapeutic results.

Among all mechanisms of action, these compounds have shown antioxidant effects by similar molecular pathways, indicating a possible use of the associations of these polyphenols, but few results have been developed.

Although a large amount of studies in vitro have been conducted until now, few clinical trials, using precise concentrations of these compounds, have been performed. For these reasons, we encourage to conduct further investigations and more extensive studies to obtain conclusive evidences. Despite all, different biomolecular mechanisms of action are promising, suggesting a real application in prostate cancer prevention. Inhibition of angiogenesis, induction of apoptosis, and reduction of tumor volume represent a start for future strategies in prostate chemoprevention, and understanding the underlying mechanisms of action, especially on humans, may change the natural history of this tumor.

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Review Article

Pleiotropic Protective Effects of Phytochemicals in Alzheimer's Disease

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Alzheimer's disease (AD) is a severe chronic neurodegenerative disorder of the brain characterised by progressive impairment in memory and cognition. In the past years an intense research has aimed at dissecting the molecular events of AD. However, there is not an exhaustive knowledge about AD pathogenesis and a limited number of therapeutic options are available to treat this neurodegenerative disease. Consequently, considering the heterogeneity of AD, therapeutic agents acting on multiple levels of the pathology are needed. Recent findings suggest that phytochemicals compounds with neuroprotective features may be an important resources in the discovery of drug candidates against AD. In this paper we will describe some polyphenols and we will discuss their potential role as neuroprotective agents. Specifically, curcumin, catechins, and resveratrol beyond their antioxidant activity are also involved in antiamyloidogenic and anti-inflammatory mechanisms. We will focus on specific molecular targets of these selected phytochemical compounds highlighting the correlations between their neuroprotective functions and their potential therapeutic value in AD.

1. Introduction

Alzheimer's disease (AD) is a decisive challenge to the health care system throughout the world and it is the result of a long chain of events leading to neuronal dysfunction and impairment in memory and cognitive abilities. The two core pathological hallmarks of AD are senile plaques (SPs) and neurofibrillary tangles (NFTs). To date, there are several experimental reports supporting the idea that oxidative stress is associated with the early development of AD [1]. In addition, recent studies suggest that inflammatory processes may significantly contribute to the progression of AD [2]. Specifically, the aggregation of beta-amyloid (Aβ) oligomers activate different signaling pathways through interactions with neuronal membranes causing oxidative stress and inflammatory responses. Furthermore, Aβ plaques can interfere with the neurotransmitter acetylcholine (ACh) affecting synaptic transmission and initiate inflammatory mechanisms that produce reactive oxygen species (ROS) [3]. Another cause that leads to cell death in AD is the hyperphosphorylation of tau protein that normally stabilizes the microtubules. When tau presents a high level of phosphorylation it becomes dysfunctional; therefore the microtubule collapse and the resulting NFTs block neurotransmitters and neuronal signaling. However, AD is a multifaceted neurodegenerative disorder and the researchers do not know enough about the biology of AD to identify the right targets. Since we do not have a comprehensive picture of the disease, the therapeutic landscape for AD is wide open. Moreover, it is necessary to emphasize that the new therapies must be based on molecular target and biomarkers. For instance, a good biomarker would be useful in the clinic but it could also help to design drugs to slow the decline [4]. Currently, a successful treatment is lacking and the medications available do not delay or modify

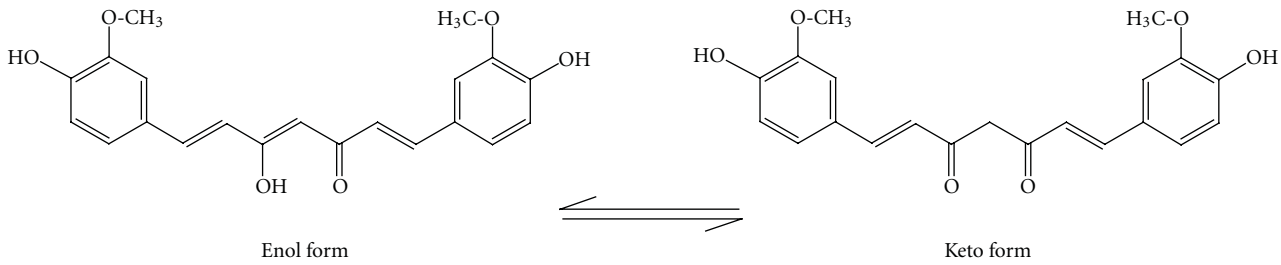


Figure 1: Chemical structures of Curcumin. Curcumin belongs to the class of curcuminoids and the presence of double bonds increases its potency and reactivity. The phytochemical curcumin undergoes keto-enol tautomerism.

the disease progression even though several potential drug targets have been identified. In this scenario, plant-derived compounds with multiple target mechanisms might play a role in drug development and discovery. A number of studies demonstrated potential health-promoting properties in the use of natural products as therapeutics for AD [5, 6]. Moreover several epidemiological reports have documented the influence of dietary habits on the incidence of neurodegenerative disorders. In particular, it was suggested a significant positive correlation between the consumption of polyphenolic phytochemical-rich foods and the prevention of certain neurological diseases, including AD [5]. Although these findings need to be interpreted with caution and it is still early to define such compounds as neuroprotective, several observations raise the possibility that they might have protective effects and might be able to slow the progression of AD. Among the numerous natural products of emerging interest with anti-AD properties, we will focus on some polyphenolic phytochemicals and on their potential role as antiamyloidogenic, anti-oxidative, and anti-inflammatory activities, highlighting specific molecular targets that might play a crucial function in the neuroprotection from AD.

2. Some Candidates Polyphenolic Phytochemicals for the Neuroprotection from AD

Polyphenols are a class of plant-derived substances characterized by the presence of more than one phenol structural unit. In the plants, they are involved in the defence from pathogens attacks or stress induced by chemical and physical damage. These compounds exert their protective action also in the animals by modulating several intracellular processes that preserve the neurons. In the following sections, we include some polyphenolic compounds, such as curcumin, (−)-epigallocatechin-3-gallate (EGCG) and resveratrol, that have received attention as alternative candidates for AD therapy.

2.1. Curcumin. Curcumin (1,7-bis [4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) or diferuloylmethane is extracted from the rhizome of *Curcuma longa* [7]. The structure is often shown in the keto form, but recent NMR studies demonstrated that curcumin exists in solution as keto-enol tautomers [8] (Figure 1). Numerous pieces of evidence suggest that curcumin may be a promising therapy for AD because it has different neuroprotective activities, including antioxidant [9], anti-inflammatory [10] and antiamyloidogenic properties [11]. Curcumin has been demonstrated to have a strong antioxidant neuroprotective effects, scavenging ROS [12] and neutralizing nitric-oxide-(NO-) based free radicals [13]. However, one of the issues of curcumin as a therapeutic agent in the treatment of AD is its poor water solubility [14], which is one reason for its low bioavailability following oral administration or through parenteral route [15]. The poor bioavailability is one of the causes of its failure in randomized control trials for AD. The structural features of curcumin that can contribute to the antioxidant activity are the phenolic and the methoxy group on the phenyl ring and the 1,3-diketone system. Moreover, the antioxidant activity of curcumin increases when the phenolic group with a methoxy group is at the ortho position [16, 17]. The orthomethoxy group can form an intramolecular hydrogen bond with the phenolic hydrogen, making the H-atom abstraction from the orthomethoxyphenols surprisingly easy [18]. The H abstraction from these groups is responsible for the remarkable antioxidant activity of curcumin. Moreover, the reactions of curcumin with free radicals produce a phenoxyl radicals and a carbon-centered radical at the methylene CH_2 group [19] (Figure 2). Additional experimental reports supporting the antioxidant property of curcumin were provided by Lim and coworkers using an AD transgenic mouse model which demonstrated that curcumin reduces brain levels of oxidized proteins containing carbonyl groups [20]. *In vivo*, the antioxidant activity of curcumin may be mediated through antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). Curcumin has been shown to serve as a Michael acceptor, reacting with glutathione (GSH) and thioredoxin [21]. Depletion in cellular GSH levels is an important measure of oxidative stress, which is implicated in the pathogenesis of AD. A study on postmortem brain of AD patients has revealed decreased levels of GSH in some area of the brain [22]. Also, the GSH levels were low in the red blood cells of male AD subjects, confirming an association between GSH and AD [23]. Noteworthy, there are some studies reporting the restorative effect of curcumin on

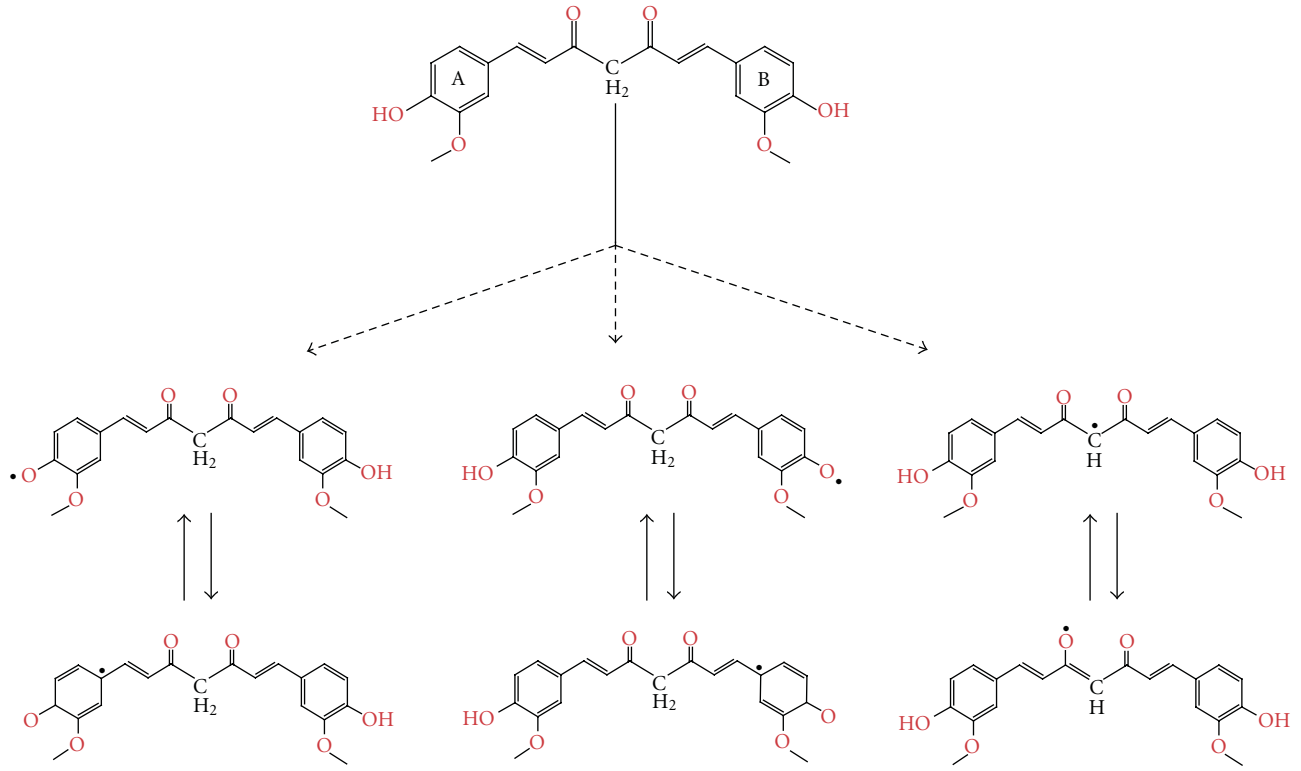
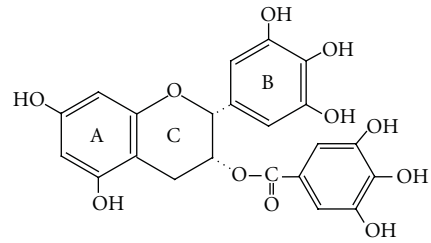


Figure 2: Reaction mechanism of curcumin with free radicals. The reactions produce phenoxyl radicals and carbon-centered radical at the methylene CH_2 group.

GSH depletion. For instance, it was demonstrated that curcumin is able to replenish the intracellular GSH pool by changing the nuclear content and/or activation of specific transcription factors such as 12-tetradecanoate 13-acetate (TPA-) responsive elements (TRE) and electrophilic response element (EpRE) [24]. Moreover, curcumin enhances the antioxidant enzyme activities of SOD and CAT in the striatum and mid-brain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) injected mice [25]. Taking into account that *in vivo* evidence showed that peroxynitrite induces Alzheimer-like tau hyperphosphorylation, nitration, and accumulation [26], it was reported that curcumin mediates the direct detoxification of reactive nitrogen species such as peroxynitrite, thus exerting an antioxidant activity [27]. Furthermore, the pieces of evidence to support a role of oxidative stress in AD brain with elevated levels of lipid peroxidation increasing [28]. Oxidative damage of lipids generates toxic aldehydes such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) leading to cell death. Important cytopathologies in AD brain include a decreased activity of all electron transport chain complexes [29]. In particular, complex IV decreases in AD, which causes release of oxidants during mitochondrial electron transport [30]. It was reported that excessive Aβ binds to regulatory heme, triggering functional heme deficiency and causing the key cytopathologies of AD. Additionally, Aβ-heme complex is a peroxidase and curcumin significantly inhibits the peroxidase activity of Aβ-heme [31]. The Tg2576 mouse model of AD exhibits impaired mitochondria metabolic activity in the spinal cord and curcumin partially suppressed the mitochondrial impairment reversing motor function deficits [32]. Interestingly, curcumin treatment abrogates lipid peroxidation protecting mitochondria from oxidative damage and apoptosis in cortical neurons [33]. Moreover, curcumin has been also shown in PC12 cells to provide protection against the deleterious effects of 4-HNE on mitochondrial redox metabolism, cytochrome c release, and DNA fragmentation [34]. The increased level of oxidative stress in AD is reflected by the increased brain content of iron (Fe^{2+}) and copper (Cu^{2+}) both capable of stimulating free radical formation. In addition to its properties of quencher, curcumin showed to be able to bind Cu^{2+} and Fe^{2+} ions [35]. Since these redox-active metals ions can intensify Aβ aggregation, curcumin may prevent this aspect of AD pathogenesis. Other reports suggested that curcumin regulates Fe^{2+} metabolism by modulation of Fe^{2+} regulatory proteins; therefore it may act as an iron chelator [36]. Significantly, *in vivo* studies reported that another divalent metal cation such as zinc (Zn^{2+}) is highly enriched in Aβ plaques [37, 38] but its role in the amyloid landscape is still poorly understood and under investigation. However, even though curcumin more readily binds to the redox-active metals such as Cu^{2+} and Fe^{2+}, it was also reported relatively weak affinity for the redox-inactive metal Zn^{2+} which might exert a small protective effect against Aβ by inducing metal chelation [35]. Recently, a systematic review

highlighted the importance of inflammatory processes in the pathogenesis of AD [39]. AD secretes increasing levels of multiple inflammatory mediators, and considering the anti-inflammatory characteristic of curcumin, it was reported that this polyphenol reduced the level of interleukin-1β (IL-1β), a proinflammatory cytokine that appears elevated in the brains of AD-like mice [20]. Findings on the anti-inflammatory effects of curcumin were also provided by Jin et al. demonstrating that this natural phenol reduces the release of proinflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) [40]. Indeed, curcumin abolished the proliferative effects of IL-6 because it inhibits the phosphorylation of signal transducer and activator of transcription 3 (STAT3) [41]. In a similar manner, curcumin downregulates the transcription factor activator protein 1 (AP1) through direct interaction with its DNA binding motif [42] and inducing the inhibition of IL-1α and TNF-α [43]. Several experimental lines suggest that the anti-inflammatory capacity of curcumin is associated to the reduction of the activity of nuclear transcription factors NF-kβ signaling pathway [44]. NF-kβ enhances the transcription of proinflammatory genes, such as inducible nitric oxide synthase (iNOS). In inflammatory cells, iNOS catalyzes the synthesis of NO, which can react with superoxide to form peroxynitrite which damages proteins and DNA. Curcumin has been found to inhibit NF-kβ-dependent gene transcription and the induction of iNOS in animal studies and macrophages cell culture [45, 46]. Probably, the inhibition of AP1 and NF-kβ occurs through the chromatin remodelling activity of curcumin that is able to modulate some histone deacetylases (HDAC) activity [47]. Moreover, curcumin attenuates the inflammatory responses through the inhibition of lipoxygenase and cyclooxygenase-2 (COX-2) enzymes, which are responsible of the synthesis of proinflammatory prostaglandins and leukotrienes [48]. Interestingly, the anti-inflammatory and neuroprotective effects of curcumin against dopamine induced neuronal death have also been demonstrated by Lee and coworkers which established that the inflammatory conditions induced by microglial activation are the main target for curcumin [49]. Noteworthy, curcumin exhibits protective effects on neuronal cells by inhibiting the aggregation of Aβ into oligomers and clearance effect on the exsting Aβ [50]. A very interesting *in vivo* approach with multiphoton microscopy showed the ability of curcumin to cross the blood-brain barrier (BBB) and disrupt amyloid plaques [51]. Additionally, in aged female rats with induced AD-like phenotype, curcumin prevented Aβ-induced spatial memory deficits in the Morris water maze assay, postsynaptic density loss, and reduced Aβ deposits [52]. As mentioned above, curcumin is able to clear amyloid plaques through several mechanisms and an additional activity that may be relevant is the induction of heat shock proteins (HSPs) molecular chaperones that are able to block protein aggregate formation [53]. However, even though several experimental research showed that curcumin exhibit high affinity binding to Aβ aggregates, one study reported the relationship between the tautomeric structures of curcumin, its derivatives, and their Aβ-binding activities. In particular, the results achieved by UV-visible spectroscopy revealed that the enolization is crucial for the binding and the enol forms of the curcumin derivatives are the predominant binding species for Aβ aggregates [54]. These important findings may represent a novel strategy for the design of therapeutic drugs or diagnostic tools in AD. Recently, Longvida, a curcumin formulation, has been evaluated in a Phase II Alzheimer's clinical trial (NCT01001637). Taking into account the low bioavailability of curcumin and its inability to reach required blood concentrations necessary to affect disease markers, Longvida is a solid lipid curcumin particle (SLCP) preparation and it was reported relatively higher bioavailability of SLCP compared to generic curcumin extract. Furthermore, this formulation is able to maintain plasma concentration of curcumin above the threshold required for the biological activity [55].

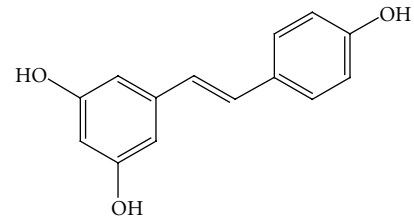


EGCG

Figure 3: Chemical structure of (−)-Epigallocatechin-3-gallate. EGCG contains three heterocyclic rings, A, B, and C, and the free radical scavenging property of EGCG is attributed to the presence of trihydroxyl group on the B ring and the gallate moiety at the 3′ position in the C ring.

2.2. (−)-Epigallocatechin-3-gallate (EGCG). EGCG ([(2*R*,3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl) chroman-3-yl] 3,4,5-trihydroxybenzoate) (Figure 3) is the most common phenolic constituent of green tea with several pharmacological activities associated with different beneficial health effects. It was well documented a powerful free radical scavenging activity for this catechin which might be attributed to the presence of the trihydroxyl group on the B ring and the gallate moiety esterified at the 3′ position in the C ring [56]. Furthermore, it was demonstrated in a human model of BBB the pharmacokinetics of catechin and epicatechin that could cross the BBB in a time-dependent manner [57]. EGCG penetrates the BBB at a low rate and the bioavailability after oral administration was approximately 5% [58]. It should be noted that high doses of EGCG were associated to death in rat hippocampal neuron through the mitochondrial-dependent pathway [59] and also that at high concentrations it has a prooxidant/proapoptotic activity [60]. However, considering that Aβ can induce mitochondrial dysfunction, it was also demonstrated that EGCG treatment is able to restore mitochondrial respiratory rates, altered mitochondrial membrane potential, and ROS production or ATP levels [61]. An increasing number of publications reports the ability of EGCG to modulate multiple biological pathways. Indeed, it has been shown to

regulate several biomedically important targets and to exert neuroprotection in many ways. In addition to the anti-inflammatory properties, EGCG exerts protection by regulating different survival genes and controlling numerous antioxidant protective enzymes [62]. Advanced glycation end-products are involved in the neuronal injury associated with several neurodegenerative disorders. EGCG increased SOD activity and protected against glycation end products-induced neurotoxicity by decreasing ROS and MDA [63]. Another demonstration that EGCG may have preventive and/or therapeutic potential in AD has been shown in BV2 microglia cell lines and in rat hippocampus where EGCG treatment increased cellular GSH pool through elevated mRNA expression of gamma-glutamylcysteine ligase (GCL) which provides neuroprotection from Aβ cytotoxicity [64]. On D-galactose-treated aged mice, EGCG treatment led to the increment of SOD and GSH-Px activities decreasing MDA contents in the hippocampus [65]. Moreover, it is interesting that the attenuation of monoamine oxidase (MAO) activity may provide protection against oxidative neurodegeneration. EGCG supplementation in adult rat brains was able to exert an inhibitory action on MAO-B preventing physiological peroxidation [66]. As mentioned above for the curcumin, EGCG acts as an antioxidant protecting rat hippocampal neurons against NO stress-induced neuronal damage by deoxidizing peroxynitrate/peroxynitrite produced after ischemia [67]. Recently, it was established the pivotal role of iron in neurodegeneration and recent studies examined the effect of EGCG in the Fe^{2+} chelating process demonstrating neurorestorative activity and Fe^{2+}-chelating properties [62]. Considering that the binding of EGCG to Fe^{2+} is essential for its antioxidant activity, among 12 phenolic compounds tested EGCG is the most potent inhibitor of the Fe^{2+}-mediated DNA break [68]. A considerable number of evidence have elucidated the importance of several cell signaling pathways in the neuroprotective action of EGCG. Several studies indicate that EGCG affects mitogen-activated protein kinases (MAPK), NF-kβ and protein kinase C (PKC) pathways [69]. In support of these observations, EGCG has been shown to mediate the phosphorylation of PKC promoting the survival of human neuroblastoma SH-SY5Y cells from Aβ and 6-hydroxydopamine (6-OHDA)-induced neurotoxicity [70]. Other evidence on the pharmacological actions of EGCG and its potential therapeutic applications to various neurodegenerative diseases such as AD were provided by Kim et al. EGCG in human astrocytoma U373MG cells suppressed NF-kβ activation and phosphorylation of MAPK p38 and the c-Jun N-terminal kinase [71]. Additional investigations have indicated that EGCG prevented the expression of COX-2, iNOS, the release of NO, and proinflammatory cytokines from astrocytes and microglia by inhibiting MAPK signaling cascades [72]. Moreover, administration of EGCG prevented lipopolysaccharide-(LPS-) mediated apoptotic cell death through the reduction of the levels of Aβ and inhibited the elevation of the expression of iNOS and COX-2 [73]. Considerably, EGCG is able to modulate enzymes that are involved in amyloid precursor protein (APP) processing and reduces the formation of β-amyloid plaques in cell culture and *in vivo* [74]. Intraperitoneal administration of EGCG attenuated brain Aβ neuropathology and improved cognitive function in a transgenic AD mouse model [75]. In particular, EGCG inhibits the fibrillogenesis of Aβ through the binding to the natively unfolded polypeptides and preventing their conversion into toxic aggregates intermediates [76]. Considering the inhibitory function of EGCG on the Aβ generation, it was previously shown that catechins are able to inhibit formation, extension, and destabilization of β-amyloid fibrils [77] and EGCG mediates the block of β-secretase activity [78]. Additionally, Obregon and coworkers studied the involvement of three candidate α-secretase enzymes in EGCG-induced nonamyloidogenic APP metabolism. The results showed that a-disintegrin and metalloprotease-10 (ADAM-10) is necessary for EGCG-mediated α-secretase cleavage activity in APP processing; thus potential stimulators of ADAM-10 such as EGCG could prevent the amyloidosis associated to AD [79]. A further study revealed that through the inhibition of extracellular signal-regulated protein kinase (ERK) and NF-kβ pathways, the treatment with EGCG in mutant AD mice improved memory function enhancing the α-secretase function and reducing the activities of β-and γ-secretases with subsequently decrease in the levels of Aβ [80]. It has also been reported synergistic effects between EGCG and fish oil on the decrease in AD-like pathology in Tg2576 mice [81] and in a recent study Li et al. showed that the administration of this or similar compound may improve spatial memory preventing the decrease in the proteins involved in the synaptic function and structure [82]. EGCG has a wide array of biological effects and it is a promising compound which has been proven efficacious in AD animal models. Lastly, EGCG has an excellent tolerability and has resulted in ongoing Phase II/III clinical trials (NCT00951834).



Resveratrol

Figure 4: Chemical structure of resveratrol. The 4′-OH in resveratrol provides its chemical and biological features.The transfer of protons or hydrogen atoms to reactive species appears to be crucial to its antioxidant mechanism.

2.3. Resveratrol. Resveratrol (5-[(E)-2-(4-hydroxyphenyl) ethenyl] benzene-1,3-diol) is a phytoalexin polyphenolic compound (Figure 4) found in grapes and other plants. In recent years many studies have reported interesting insights about the antiaging effects of resveratrol in different organisms including nematodes, yeast, rat, and mice. Indeed, resveratrol modulates various systems that protect cells

providing neuroprotective features both *in vitro* and *in vivo* in models of AD. Many studies reported that the central nervous system (CNS) is one of the resveratrol's targets. This compund is able to pass the BBB [83] but the bioavailability is low because it is quickly metabolized into glucuronide and sulfate conjugates. Several lines of evidence indicate a strong antioxidant functions together with other pharmacological activities, therapeutic and protective properties [84]. Regarding the radical-scavenging activity, structural studies and theoretical calculations demonstrate that in the antioxidant reaction of resveratrol the hydroxyl group at the 4′-position is much easier to subject to oxidation than other hydroxyl groups [85]. Intraperitoneally administration of resveratrol exerts neuroprotective properties upregulating several endogenous antioxidant enzymes such as SOD and CAT [86]. Prolonged administration of resveratrol improves colchicine-induced cognitive impairment, reduces MDA and nitrite levels, and restores depleted GSH [87]. However, it is important to emphasize that resveratrol can exhibit prooxidant activities in the presence of transition metal ions such as Cu^{2+}, leading to oxidative breakage of cellular DNA [88]. A substantial amount of research has attributed to this phytocompound the capacity to increase the activity of SIRT1 that are NAD^+-dependent class III histone deacetylases [89]. Consequently, resveratrol appears to possess the ability to activate sirtuins and to mimic caloric restriction [84]. In a mouse model of AD, a calorie-restricted diet attenuates AD pathogenesis through an increase in SIRT1 activity [90]. Additionally, it was reported that caloric restriction reduces Aβ deposition and Aβ-associated neuropathology in different animal models [91, 92]. In a meaningful way Kim et al. showed in transgenic AD mouse model that resveratrol reduced neurodegeneration through a decrease in the acetylation of known SIRT1 substrates, for example, peroxisome-proliferator-activated receptor gamma coactivator alpha (PGC-1α) and p53 [93]. SIRT1 activated by resveratrol protects cells against Aβ-induced ROS production and reduces amyloid neuropathology in the brains of Tg2576 mice [94]. Taking into account that resveratrol can be considered a neuroprotective compound in the context of AD, it is possible to speculate that the ability to counteract Aβ toxicity can occur through its antioxidant properties but also through SIRT1 activation. Definitely, resveratrol is reported to possess antiamyloidogenic activity in several studies, for example, the treatment with this stilbenoid resulted in the inhibition of β-amyloid peptide polymerization even though the antiamyloidogenic mechanism is still unknown [95]. As illustrated by Marambaud and colleagues, resveratrol promotes clearance of intracellular Aβ by activating its proteasomal degradation [96]. Moreover, SIRT1 overexpression reduces Aβ pathology in APP-expressing neuronal cultures by delaying Aβ synthesis [96, 97]. A recent work offers interesting insights into the effects of resveratrol on the polymerization, cell toxicity, and destabilization of Aβ fibril suggesting that resveratrol disrupts Aβ hydrogen binds thus preventing fibril formation, destabilizing preformed fibril without affecting oligomerization [98]. Furthermore, in a different study it was noticed that the protective effects of resveratrol on β-amyloid protein-induced toxicity in rat hippocampal cells are related to activation of PKC [99]. It is noteworthy to mention that resveratrol might be involved in the attenuation of neuroinflammatory responses because it is able to reduce the concentration of 8-iso-prostaglandin F2α, an indicator of free radical generation [100]. It has also been shown that resveratrol inhibits COX-1 but in contrast it does not affect the expression of COX-2 [100]. Since NF-kβ signaling activation plays an important role in the neurodegeneration, another link between AD and neuroprotective activity of resveratrol is its ability to reduce the expression of genes modulated by NF-kβ, such as iNOS, prostaglandin E2 (PGE2), as well as cathepsin and NO [101]. One of the main findings reported by Lu et al. was that resveratrol attenuates LPS-stimulated NF-κB activation in murine primary microglia and astrocytes and suggests that the inflammatory responses induced by LPS could be limited by resveratrol, with different potencies [102]. Studies performed in ischemia-reperfusion models have demonstrated that resveratrol inhibits peroxisome proliferator-activated receptors alpha (PPARα) [103] and reduces NF-kβ p65 expression [104]. Moreover, resveratrol was found to activate AMPK and reduce cerebral Aβ levels and deposition in the mice cortex [105]. Using electron microscopy and biochemical methods, it was reported that resveratrol prevents the abnormal expression of peroxiredoxins but also mitochondrial structural abnormalities in a mouse model of primary AD and Aβ-incubated mouse neuroblastoma cells [106].

Currently, resveratrol is under Phase III clinical trials (NCT00678431) studies to determine the effects in mild-to moderate AD in combination with glucose and malate.

3. Activation of the Keap1/Nrf2 System for Neuroprotection by Curcumin, EGCG, and Resveratrol

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a conserved master regulator of cellular antioxidant responses. As mentioned above, multiple pieces of evidence support the role of oxidative stress in the pathogenesis of AD [1]. Therefore, Nrf2 appears to be a good candidate to provide neuroprotection in AD. Nrf2 belongs to the CnC (Cap'n'Collar) family leucine zipper transcrption factors and regulates the expression of genes encoding antioxidant and detoxifying proteins such as glutathione S-transferase (GST), glutathione synthetase (GSS), heme oxygenase 1 (HO-1) and NAD(P)H:quinone oxidoreductase [12]. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1 (Kelch-like ECH-associating protein 1), which facilatites its polyubiquitylation and proteasome-mediated degradation. Keap1 functions as a sensor of stress signals and the exposure to electrophiles, oxidants, or xenobiotics disrupts Keap1-Nrf2 complex, thus stabilizing Nrf2 and allowing it to accumulate in the nucleus. Nrf2 activates the trascrption of its target genes via antioxidant response elements (AREs) in their promoter regions binding as a heterodimer with the members of Maf and Jun family [12]. To date, only few pieces of evidence show that the activation of Nrf2 and of its cytoprotective genes by curcumin, EGCG, and resveratrol

treatments is sufficient to protect against AD. However, Chen et al. reported that resveratrol is able to increase the expression of HO-1 and glutathione protecting PC12 cells from oxidative stress via activation of Nrf2-ARE signaling pathway [107] which indirectly suggests a potential role in AD treatment. Similarly, using primary neuronal cultures, resveratrol was able to significantly induce HO-1, presumably through the activation of Nrf2 [108]. Yet, curcumin induces HO-1 increasing tolerance of the brain to stresses and providing an important antidegenerative function in AD pathogenesis [109]. Moreover, curcumin activates GST [110] restoring GSH content in the brain and improving cognitive deficits [111]. Recently, consistent with the potential role of Nrf2 as therapeutic target in AD, it was observed that the incubation of $Nrf2^{+/+}$ astrocytes with curcumin led to a significant induction of phase II enzymes [112]. Additionally, data from our laboratory have shown the ability of low dose EGCG to stimulate HO-1 expression in rat cultured neurons. In this study, Nrf2 was found to be upregulated in neurons exposed to nontoxic concentrations of EGCG, suggesting that this compound may induce HO-1 via the activation of Nrf2 [113]. These results are in agreement with another study, where it was showed the ability of epicatechins to protect neurons and reduce brain infarct size of mice. Moreover, neuroprotection was abolished in neurons derived from knockout mice for HO-1 and Nrf2 [114]. In conclusion, Nrf2 is an attractive target for the discovery of natural neuroprotective agents against AD and these few examples can already be considered promising.

4. Cocktail of Drugs for Neuroprotection

Given the complexity and the multiple etiological nature of AD and other neurodegenerative disorders, a successful treatment may require a cocktail of compounds. Indeed, therapeutic approaches that are based on single biological mechanisms or targets may be inadequate. Also considering that certain regions of the brain respond differently to the treatments or are more affected than others, a cocktail of drugs may be more effective. Despite this, almost no studies have been done with a combination of neuroprotective drugs, especially with curcumin or resveratrol. However, new drug candidates for AD should be able to act on multiple brain targets for the treatment of cognition impairment, motor dysfunction, depression, and neurodegeneration. It is evident that the neurodegenerative disorders require multiple-target therapies to counteract the heterogeneous pathological aspects of the disease. For instance, a multifunctional neuroprotective-neurorescue compound might be endowed with properties that include (1) antifibrils formation and fibrils destabilizing action; (2) promotion of neurite outgrowth; (3) a direct neutralization of free-radicals-induced oxidative stress; (4) maintenance of mitochondrial integrity; (5) modulation of the activity of antioxidant detoxifying enzymes; (6) reduction in AβPP/α synuclein translation; (7) activation of transcription factors; (8) attenuation of reactive free-iron pool. To date, it is plausible that some of these actions may result only from a combination therapy of more compounds. In human neuroblastoma cells, EGCG causes a rapid decrease in proapoptotic factors whereas R-apomorphine upregulates anti-apoptotic proteins [115] but both compounds are also iron chelators; therefore they complement each other and induce a synergistic neuroprotective action. However, it should be underlined that plant polyphenols are recognized as multifunctional agents for neuroprotection, providing polypharmacological activities in addition to their established radical scavenging action. Therefore, multidrug medication therapy can be effective because single-target approach may be inadequate for heterogeneous disorders but at the same time one compound with two or more mechanisms of action, targeted at different pathological aspects of the disease may offer a good therapeutic efficacy.

5. Conclusions

The pathogenesis of AD is multilateral and its polyetiological origin requires new drug candidates capable to operate on multiple brain targets for the treatment of cognition and motor dysfunction, depression, and neurodegeneration. In this paper we present some phytochemical entities able to act on specific targets implicated in the pathogenesis of AD. The neuroprotective activity of curcumin, EGCG, and resveratrol has been demonstrated *in vitro* and in various models of neurodegenerative diseases *in vivo*. Consequently, it is reasonable to propose these substances as promising resources in the development of new medications for AD aimed to prevent and/or to treat this neurodegenerative disorder. Additionally, even though there are limits for their widespread use, such protective molecules appear to be innocuous, tolerate, inexpensive, and available. However, their efficacy and utility in the clinical pharmaceutical is still an open question because an exhaustive amount of experimental evidence is still missing. In addition, although the neuroprotective effects of the phytocompounds above described are attractive for their multiple biological activities, more long-term studies should be performed at least to determine their effects in slowing the development of AD. Furthermore, it is still unclear which is the ideal concentration for the compound to be in the active forms and exert its beneficial effects. In conclusion, polyphenols have revealed to be in the field of neurosciences promising neuroprotective compounds with great potential that continues to expand.

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Research Article

Quercetin Reduces Inflammatory Responses in LPS-Stimulated Cardiomyoblasts

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Flavonoids possess several biological and pharmacological activities. Quercetin (Q), a naturally occurring flavonoid, has been shown to downregulate inflammatory responses and provide cardioprotection. However, the mechanisms behind the anti-inflammatory properties of Q in cardiac cells are poorly understood. In inflammation, nitric oxide (NO) acts as a proinflammatory mediator and is synthesized by inducible nitric oxide synthase (iNOS) in response to pro-inflammatory agents such as lipopolysaccharide (LPS), a causative agent in myocardial depression during sepsis. In the present study, we evaluated the protective effect of Q on rat cardiac dysfunction during sepsis induced by LPS. Pretreatment of H9c2 cardiomyoblasts with Q inhibited LPS-induced iNOS expression and NO production and counteracted oxidative stress caused by the unregulated NO production that leads to the generation of peroxynitrite and other reactive nitrogen species. In addition, Q pretreatment significantly counteracted apoptosis cell death as measured by immunoblotting of the cleaved caspase 3 and caspase 3 activity. Q also inhibited the LPS-induced phosphorylation of the stress-activated protein kinases (JNK/SAPK) and p38 MAP kinase that are involved in the inhibition of cell growth as well as the induction of apoptosis. In conclusion, these results suggest that Q might serve as a valuable protective agent in cardiovascular inflammatory diseases.

1. Introduction

Systemic bacterial infection is associated with multiorgan dysfunctions including heart failure, which is the main cause of morbidity and mortality in septic patients [1, 2]. Lipopolysaccharides (LPSs), a major constituent of bacterial outer membrane, have been demonstrated to play a critical role in the initiation of the pathophysiological cascades [3–5]. Under septic conditions, excessive LPS activates numerous types of cells upon recognition by toll-like receptor 4 (TLR-4) resulting in enhanced production of proinflammatory cytokines that contribute to myocardial dysfunction [6–8]. Recent studies have shown that cardiomyoblasts express TLR-4 through which LPS has direct adverse effect on cardiomyocyte physiology [9, 10]. Activation of TLR-4 by LPS triggers NF-κB signaling and results in decreased cardiomyocyte contractility and substantial expressions of proinflammatory cytokines such as intercellular adhesion molecule-1 (ICAM-1) [11, 12], tumor necrosis factor-α (TNF-α) [13, 14], and inducible nitric oxide synthase (iNOS) [13]. The increased expression of iNOS, especially in non-inflammatory cells, may have deleterious effects [15–17]. Potential subcellular mechanisms involved in these harmful consequences include excessive direct reactions of NO with a wide variety of proteins and enzymes including reactions with amino, thiol (SH), diazo, and tyrosyl groups, and with heme and Fe^{2+} or sulfur centers [18]. Moreover, unregulated NO production, also associated with oxidative stress, can result in the generation of peroxynitrite and other reactive nitrogen species (RNS) that alter protein function via nitration and oxidation reactions [16, 19, 20]. It has been demonstrated that reducing systemic inflammatory response could improve myocardial function [21]. Activation of multiple stress signaling processes such as oxidative stress and mitogen-activated protein kinases (MAPKs) plays pivotal roles in the pathogenesis of septic cardiac dysfunction [22]. While activation of ERK1/2 MAP-kinase has been identified to enhance cell growth and migration, the stress-activated

protein kinases JNK/SAPK and p38 MAP-kinase are involved in the inhibition of cell growth as well as the induction of apoptosis [23].

Quercetin (Q) is a flavonoid and more specifically a flavonol that possesses a broad range of pharmacological properties, including anti-inflammatory effects [24], antiproliferative effects [25], and protective effects against oxidative stress [26]. Foods rich in quercetin include apples, black and green tea, onions, red wine, red grapes, citrus fruit, broccoli and other leafy green vegetables, cherries, and a number of berries including raspberries and cranberries [27]. Normally, human quercetin plasma concentrations are in the nanomolar range, but after quercetin intake, they may reach the micromolar range [28, 29]. A recent study demonstrated that low to moderate oral dose of quercetin for two weeks increased plasma quercetin concentrations dose-dependently in healthy individuals [30], confirming its bioavailability. In a previous study, we have demonstrated that Q is uptaken by the cardiomyoblast H9c2 cell line and counteracts cardiac oxidative stress via both its well-known antioxidant activity and through the modulation of two key fundamental protein kinases involved in prosurvival signaling pathways, Akt and ERK1/2 [31]. Moreover, we have shown that Q is able to strongly upregulate different antioxidant and phase 2 enzymes in neonatal rat cardiomyocytes demonstrating its indirect antioxidant activity [32].

In this paper, we evaluated the anti-inflammatory effects of Q in cardiac cells, focusing on the possible mechanisms by which this polyphenol counteracts LPS-induced inflammatory responses.

2. Materials and Methods

2.1. Materials. PhosSTOP was purchased from Roche Diagnostics (Mannheim, Germany). CelLytic M, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), mammalian protease inhibitor mixture, DMEM, fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), quercetin, and all other chemicals of the highest analytical grade were purchased from Sigma Chemical (St. Louis, MO).

2.2. Cell Cultures. Rat embryonic heart-derived myogenic cell line H9c2 (European Collection of Cell Cultures Salisbury, UK) was grown in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM) in a humidified incubator with 5% CO_2 at 37°C. Cells were split 1 to 4 at subconfluence (80%). H9c2 cells were seeded at a density of 5×10^4 cells/cm^2 72 hours prior to treatments.

2.3. Measurement of Nitric Oxide Production. Released nitrite, a stable product of NO in aqueous medium, was measured using the Griess Reagent System (Promega, Madison, WI, USA). Briefly, H9c2 cells were treated with 30 μM Q for 2 hours prior to stimulation with 10 μg/mL LPS for 24 hours. At the end of this time period, the culture medium was mixed with an equal volume of sulfanilamide solution (1% in 5% phosphoric acid) and of N-1-naphtylethylenediamine dihydrochloride solution (0.1% in water). The absorbance was measured at 540 nm. Nitrite concentrations were determined from a calibration curve of standard 0.1 M sodium nitrite concentrations 0.5–25 μM against absorbance.

2.4. Detection of Intracellular Reactive Oxygen Species. The formation of ROS was evaluated using a fluorescent probe, DCFH-DA, as described by Wang and Joseph [33]. Briefly, H9c2 cells were pretreated with 30 μM Q for 24 h and then incubated with 5 μM DCFH-DA in PBS for 30 min. After DCFH-DA removal, the cells were stimulated with 10 μg/mL LPS for different periods (0.5–24 hours). Cell fluorescence was measured using 485 nm excitation and 535 nm emission with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, Perkin-Elmer, Wellesley, MA). Intracellular antioxidant activity was expressed as percentage of control cells.

2.5. Caspase-3 Activity Assay. The activity of caspase-3 was measured by hydrolysis of Ac-DEVD-AMC by caspase-3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin moiety (AMC) [34]. H9c2 cells were treated with 30 μM Q for 2 hours prior to stimulation with 10 μg/mL LPS for different periods (0.5–24 hours). Cells were lysated in lysis buffer (50 mM Tris, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA/EDTA, 1 mM sodium pyrophosphate, 10 mg/mL phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, and 1 mg/mL aprotinin) and then centrifuged 5 min at 5,000 g and the supernatant was added to the assay buffer (100 mM HEPES pH 7.0, 5 mM dithiothreitol, 0.1% CHAPS, 10% sucrose, and 0.15 mM Ac-DEVD-AMC). The specific cleavage of the fluorogenic peptide Ac-DEVDA-MC was monitored following AMC cleavage at 370 nm excitation and 455 nm emission. One unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per minute at 25°C under the conditions described.

2.6. Western Immunoblotting. H9c2 cells were treated with 30 μM Q and after 2 hours were stimulated with 10 μg/mL LPS for different periods (0.5–24 hours). Cells were washed with ice-cold PBS and proteins extracted with CelLytic M cell lysis reagent with mammalian protease inhibitor mixture (1 : 100 dilution) and PhosSTOP. Proteins were boiled at 98°C for 3 min in loading buffer (62.5 mM Tris, pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.0025% bromophenol blue). Protein extracts were separated by SDS-PAGE (20 μg/lane) and then transferred onto nitrocellulose membrane (Hybond-C; GE Healthcare, Buckinghamshire, UK) at 110 V for 90 min using Tris-glycine buffer. Membranes were then incubated in a blocking buffer containing 5% (w/v) skimmed milk and incubated with anti-NOS2, anti-phospho SAPK/JNK (Thr^{183}/Tyr^{185}), anti-SAPK/JNK, anti-phospho p38 MAPK, anti-p38 MAPK (Cell Signaling Technology, Beverly, MA), and anti-β-actin (Sigma), overnight at 4°C on a three-dimensional rocking table. The blots were then incubated with goat anti-rabbit

IgG conjugated to horseradish peroxidase for 60 min at room temperature. The results were visualized by chemiluminescence using ECL advance reagent according to the manufacturer's protocol (GE Healthcare). Semiquantitative analysis of specific immunolabeled bands was performed using a Fluor S image analyzer (Bio-Rad, Hercules, CA, USA).

2.7. Protein Concentration. The protein concentration of the cell lysates was determined by the Bio-Rad Bradford protein assay (Bio-Rad Laboratories).

2.8. Statistics. Each experiment was performed at least three times, and all values are represented as means ± SD. One-way analysis of variance (ANOVA) was used to compare differences among groups followed by Bonferroni's test (Prism 5, GraphPad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Effect of Q on iNOS Expression and NO Production. The first aim of the study was to identify the effect of LPS treatment on iNOS expression (Figure 1). LPS treatment (10 μg/mL) of H9c2 cells for 0.5 hours did not influence iNOS protein expression. On the contrary, LPS stimulation for 2 and 24 hours was able to significantly induce iNOS. In particular, LPS increased the enzyme protein expression in a time-dependent manner ($r = 0.9888$, $P < 0.05$). Treament with 30 μM Q alone for 2 hours had no effect on iNOS protein expression, while pretreatment with Q, prior to LPS exposure, was able to significantly reduce iNOS protein expression at each LPS stimulation time.

We next evaluated total nitrite release as an indicator of NO production (Figure 2). As at 24 hours LPS stimulation had the strongest effect on iNOS protein expression, we used this time to stimulate H9c2. In agreement with the previous results, LPS significantly increased the release of NO in the culture medium, 30 μM Q alone did not influence NO production, and pretreatment with 30 μM Q before LPS stimulation significantly reduced NO production to level comparable to control cells.

3.2. Effect of Q on Intracellular ROS Production. To verify if the increase in RNS was accompanied by a concomitant increase in ROS production, we evaluated intracellular ROS accumulation in H9c2 cells stimulated with LPS for different periods in the presence or the absence of 30 μM Q (Figure 3). Intracellular ROS levels were significantly increased in cells stimulated with LPS for 24 hours compared to control cells, as indicated by the increase in DCF fluorescence. However, the increase in DCF fluorescence was significantly decreased by Q pretreatment suggesting that Q was capable of reducing intracellular ROS accumulation following LPS stimulation.

3.3. Effect of Q on LPS-Induced Apoptosis in H9c2 Cells. Q demonstrated antiapoptotic effects in different cell types,

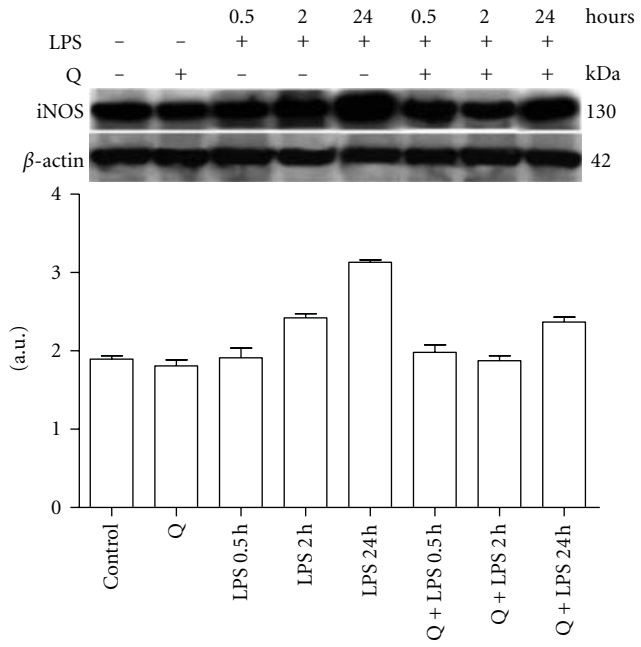

Figure 1: Effect of Q on iNOS expression in LPS-stimulated H9c2 cells. Cells were pretreated with 30 μM Q for 2 hours before stimulation with 10 μg/mL LPS. After the indicated time points, cells were harvested and lysed. Crude homogenates (20 μg) were immunoblotted with an antibody that detects endogenous levels of iNOS (NOS2). Equal loading was verified with an anti-β-actin antibody. Densitometric analysis of the protein bands was performed using Bio-Rad Quantity One 1-D Analysis software. Each column represents the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^*P < 0.05$ compared to Control; $^\circ P < 0.05$ compared to the corresponding LPS exposure times.

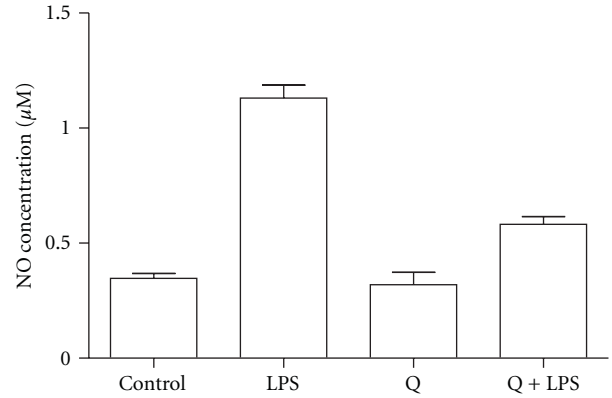

Figure 2: Effect of Q on NO production in LPS-stimulated H9c2 cells. Cells were pretreated with 30 μM Q for 2 hours before stimulation with 10 μg/mL LPS for 24 hours. After the treatment, the culture medium was collected for NO assay. The concentration of NO was determined by the Griess reagent using $NaNO_2$ as standard. Each column represents the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^*P < 0.05$ compared to Control; $^\circ P < 0.05$ compared to LPS.

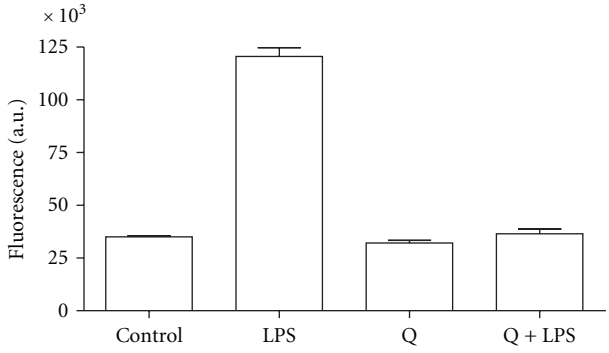

Figure 3: Effect of Q on intracellular ROS production in LPS-stimulated H9c2 cells. Cells were pretreated with 30 μM Q for 2 hours before stimulation with 10 μg/mL LPS. After 24 h intracellular ROS were measured using the peroxide-sensitive fluorescent probe DCHF-DA. Each column represents the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^*P < 0.05$ compared to Control; $^\circ P < 0.05$ compared to LPS.

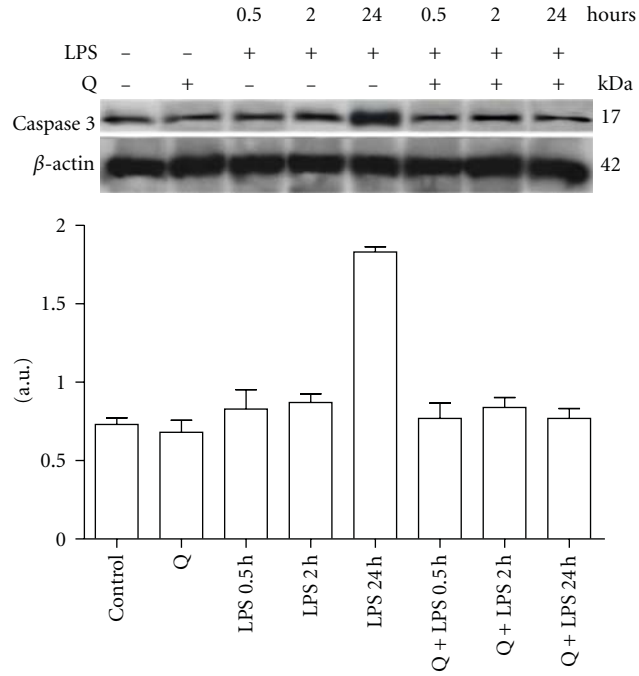

Figure 4: Effect of Q on cleaved caspase 3 protein expression in LPS-stimulated H9c2 cells. Cells were pretreated with 30 μM Q for 2 hours before stimulation with 10 μg/mL LPS. At the indicated time points, cells were harvested and lysed. Crude homogenates (20 μg) were immunoblotted with an antibody that detects endogenous levels of cleaved caspase 3. Equal loading was verified with an anti-β-actin antibody. Densitometric analysis of the protein bands was performed using Bio-Rad Quantity One 1-D Analysis software. Each column represents the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^*P < 0.05$ compared to Control; $^\circ P < 0.05$ compared to the corresponding LPS exposure times.

therefore, we next investigated whether Q protects cardiomyoblasts from LPS-induced apoptosis. H9c2 cells were pretreated with 30 μM Q before exposure to 10 μg/mL LPS for different exposure times and cleaved caspase 3 protein expression (Figure 4) and caspase 3 activity (Figure 5) were evaluated. Immunoblot analyses showed that stimulation with LPS for 24 hours induced a significant cleavage of caspase 3 in respect to control cells, while 0.5 and 2 hours LPS exposure times had no effect on cleaved caspase 3. Pretreatment with Q before LPS stimulation was able to strongly reduce cleavage of caspase 3 to level comparable to control cells. Q alone did not modify caspase 3 cleavage. These data were confirmed by caspase 3 activity assay. Only 24 hours LPS stimulation was able to significantly increase caspase 3 activity and pretreatment with Q significantly reduced caspase 3 activity to level comparable to control cells, demonstrating the antiapoptotic effect of Q against LPS-induced damage.

3.4. Effect of Q on LPS Activation of MAPK in H9c2 Cells. To better clarify the mechanisms behind Q protection against LPS induced apoptosis, we investigated the role of Q in the modulation of two fundamental proapoptotic signaling pathways in H9c2 cells: JNK and p38 MAPK. H9c2 cells were pretreated with 30 μM Q before exposure to 10 μg/mL LPS for different exposure times and cell lysates were analyzed by immunoblotting with anti-phospho-p38 and anti-p38 antibodies (Figure 6) or anti-phospho-JNK and anti-JNK antibodies (Figure 7). LPS treatment for 0.5 and 2 hours significantly activated p38 MAPK as measured by phospho/total p38 ratio, with the highest increase at 0.5 hours. At the longest exposure time p38, MAPK was no longer activated as phospho/total p38 ratio was comparable to control cells. Q was able to significantly inhibit p38 MAPK activation. LPS treatment for 0.5 hours significantly activated JNK as measured by phospho/total JNK ratio, while after 2 and 24 hours LPS stimulation did not influence JNK phosphorylation. Q was able to significantly reduce JNK activation after 0.5 hours LPS exposure time at level comparable to control cells.

4. Discussion

In this study, we have evaluated the anti-inflammatory role of Q in H9c2 cells demonstrating that this polyphenol attenuated LPS-induced inflammatory events inhibiting iNOS induction, reducing NO production and oxidative stress, and counteracting apoptosis through the modulation of two key protein kinases p38 MAPK and JNK.

A range of clinical conditions are associated with a dysregulation of inflammatory responses. Although the most common of these is sepsis, high concentrations of cytokines are also generated by ischemia-reperfusion, trauma, acute rejection, antigen-specific immune responses, and different acute inflammatory states [35]. Several studies suggest a beneficial effect for cardiac dysfunction through inhibition of cardiac inflammatory processes in sepsis [13].

LPS of Gram-negative bacteria has been recognized as a causative agent in myocardial depression during sepsis [36].

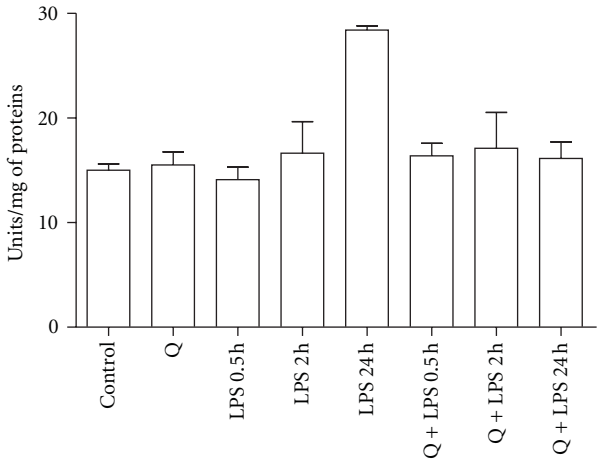

Figure 5: Effect of Q on caspase 3 activity in LPS-stimulated H9c2 cells. Cells were pretreated with 30 μM Q for 2 hours before stimulation with 10 μg/mL LPS. At the indicated time points, caspase 3 activity was measured spectrofluorimetrically in cell lysates as reported in Section 2. Each column represents the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^*P < 0.05$ compared to Control; $^\circ P < 0.05$ compared to the corresponding LPS exposure times.

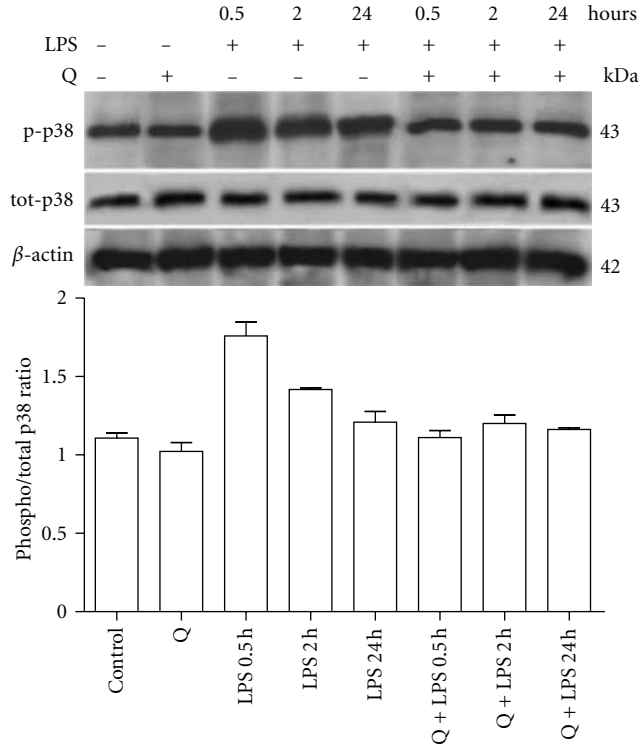

Figure 6: Effect of Q on p38 MAPK activation in LPS-stimulated H9c2 cells. Cells were pretreated with 30 μM Q for 2 hours before stimulation with 10 μg/mL LPS. At the indicated time points, cells were harvested and lysed. Crude homogenates (20 μg) were immunoblotted with anti-phospho-p38 and anti-p38 antibodies. Equal loading was verified with an anti-β-actin antibody. Densitometric analysis of the protein bands was performed using Bio-Rad Quantity One 1-D Analysis software. Each column represents the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^*P < 0.05$ compared to Control; $^\circ P < 0.05$ compared to the corresponding LPS exposure time.

It has been shown that the inflammatory responses induced by LPS in cardiomyocytes are characterized by an increased production of ROS which leads to the activation of transcription factors and intracellular signaling pathways and to the induction of inflammatory mediators, including TNF-α, ICAM-1, and iNOS. All of these mediators may be involved in the depression of cardiac function [13, 37, 38]. In the cardiovascular system, NO produced by iNOS is a major pathophysiologic mediator of septic shock [39] and has been shown to mediate the negative inotropic effects of cytokines [40]. In agreement with previous studies, performed using cardiomyocytes stimulated with LPS for at least 20 h [41, 42], we demonstrated that iNOS protein expression and NO production were significantly increased in LPS-stimulated H9c2. On the contrary, a study of Chen et al. [43] demonstrated an increase in iNOS mRNA level while NO production was not influenced by a 4 h LPS stimulation in rat cardiomyoblasts. This discrepancy could be ascribed to the lower LPS exposure time that is probably too short to significantly increase NO in the culture medium. In our study, Q treatment was able to significantly reduce both iNOS expression and NO production.

As NO may react with ROS such as the superoxide radical to yield the highly reactive oxidant species peroxynitrite, leading to more aggressive oxidative and nitrosative stress [44], we evaluated the intracellular ROS production in LPS-stimulated H9c2 cells. As expected, LPS significantly increased intracellular ROS level while Q pretreatment was able to reduce this production to level comparable to control cells. We previously demonstrated the ability of Q to reduce oxidative stress in H9c2 cells [31] and in rat cardiomyocytes [32] through both a direct and indirect antioxidant mechanisms.

The action of NO has been related to the induction of programmed cell death, or apoptosis, in various cells [45]. The capacity of NO to induce apoptosis was first appreciated by Albina et al. [46], who showed that NO caused apoptosis in macrophages. Many reports suggest that during the ischemia-reperfusion event, NO mediates tissue injury [47, 48]. Moreover, induction of iNOS by cytokines in primary rat cardiac myocytes was associated with an increased myocytes apoptosis, which was ameliorated by the administration of an inhibitor of NO synthase, which blocked the expression of iNOS in response to cytokines [45]. These results suggest that NO is markedly involved in cardiomyopathy, which leads to the contribution of impaired cardiac function. Our results demonstrated that Q was able to counteract LPS-induced apoptosis by inhibiting caspase-3 activation and significantly reducing caspase-3 activity. Different studies have demonstrated that the mechanism underlying the cytoprotection of Q may be mediated by

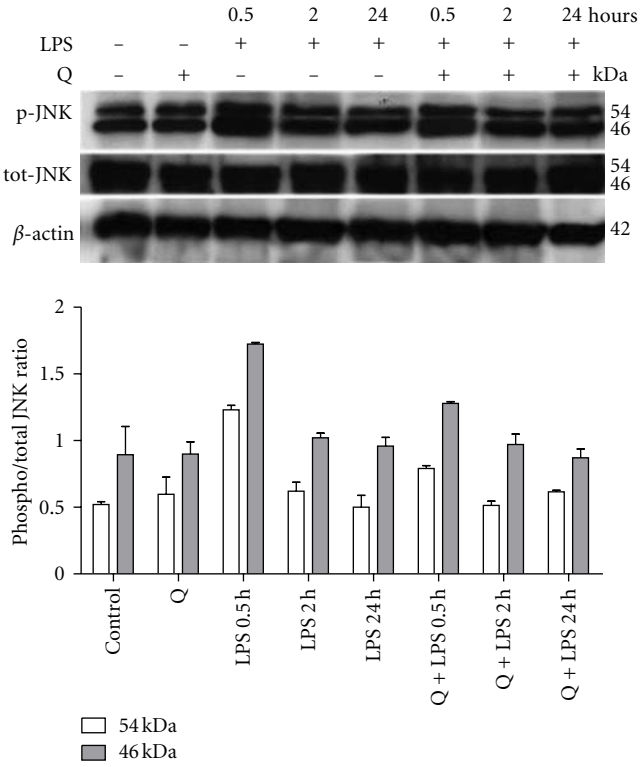

Figure 7: Effect of Q on JNK activation in LPS-stimulated H9c2 cells. Cells were pretreated with 30 μM Q for 2 hours before stimulation with 10 μg/mL LPS. At the indicated time points, cells were harvested and lysed. Crude homogenates (20 μg) were immunoblotted with anti-phospho-JNK and anti-JNK antibodies. Equal loading was verified with an anti-β-actin antibody. Densitometric analysis of the protein bands was performed using Bio-Rad Quantity One 1-D Analysis software. Each column represents the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^*P < 0.05$ compared to Control; $^\circ P < 0.05$ compared to the corresponding LPS exposure time.

inhibition of NF-κB activation [44, 49, 50], which controls the expression of inflammatory mediators [51]. A crucial step in the activation of NF-κB is the degradation of IκBa [52]. LPS stimulates the canonical NF-κB activation pathway through degradation of IκBa and phosphorylation of NFκB p65 subunit [53, 54], NF-κB p65 subsequently translocates from the cytoplasm to the nucleus and, in turn, triggers a large amount of genes encoded for inflammatory mediators [54].

The mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated c-Jun N-terminal kinases 1 and 2 (JNK) and p38 MAP kinases, plays an important role in cell fate decisions and has been implicated in death/survival signaling in cardiac myocytes [55]. Evidence also indicates that ROS contribute to LPS-stimulated MAPKs signaling pathways in myocardial cells [13]. These findings are in line with the crucial role of MAPKs signaling and oxidative stress in sepsis-induced myocardial contractile dysfunction [13, 37, 38]. Data reported in this paper demonstrated that LPS stimulation activates both p38 MAPK and JNK. In particular, JNK activation was quick and transient and after 2 h LPS stimulation JNK phosphorylation was comparable to control cells, while p38 MAPK activation lasted for at least 2 h. Our data are in agreement with the results of Peng et al. [56] that demonstrated an immediate and transient increase in p38 MAPK activation after LPS stimulation, which was followed by TNF-α production in the myocardium and the inhibition of p38 MAPK activation improved cardiac function and survival during endotoxemia in mice. Activation of JNK signal transduction cascades has been implicated in the regulation of hypertrophic and apoptotic responses in the myocardium [57]. Peng et al. [58] showed that LPS increases JNK activation in cultured cardiomyocytes. It has been demonstrated that the activation of JNK/SAPK is crucial for NO toxicity in H9c2 cardiac muscle cells [59], moreover, Liu et al. [60] observed that incubation of LPS-treated myocardial cells with the JNK1/2 inhibitor SP600125 resulted in marked inhibition of LPS-induced phosphorylation of IκB, degradation of IκB and upregulation of TNF-α, leading to myocardial apoptotic responses.

Q pretreatment was able to reduce the activation of the two kinases to values comparable to control cells, demonstrating that the antiapoptotic effect of Q could be also mediated by the modulation of p38 MAPK and JNK whose detrimental role in sepsis has been largely demonstrated.

In conclusion, Q inhibited iNOS induction in LPS-stimulated H9c2 cells in a time-dependent manner, reduced NO production, and counteracted LPS-induced apoptosis. This protective effect might be mediated by the suppression of the activation of p38 MAPK and JNK. Therefore, these results suggest that Q might serve as a valuable protective agent in cardiovascular and inflammatory diseases.

Acknowledgments

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Research Article

An Investigation of the Relationship between the Anti-Inflammatory Activity, Polyphenolic Content, and Antioxidant Activities of Cooked and *In Vitro* Digested Culinary Herbs

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There is little research on how cooking and digestion affect the anti-inflammatory activity of culinary herbs. Thus, the aim of this paper was to investigate this activity following cooking and *in vitro* digestion of the common culinary herbs, rosemary, sage, and thyme, and the relationship between their anti-inflammatory activity, polyphenol content, and antioxidant capacity. The anti-inflammatory activity of uncooked (U), cooked (C), cooked and *in vitro* digested (C&D), and standardised (STD, 30 mg/mL) culinary herbs was assessed by measuring their effect on interleukin 8 (IL-8) release from stimulated human peripheral blood lymphocytes (PBLs) and Caco-2 cells. The trolox equivalent capacity (TEAC) and estimated total phenolic content of the herbs were also determined. There was a significant decrease in IL-8 release from PBLs stimulated with H_2O_2 incubated with (U), (C), (C&D), and (STD) herbs and from Caco-2 cells stimulated with TNFα incubated with (C&D) and (STD) herbs. PBLs pre-incubated with (C&D) herbs prior to stimulation (H_2O_2 or TNFα) caused a significant inhibition in IL-8 release. The significant correlations between TEAC and estimated phenolic content and the anti-inflammatory activity suggest a possible contributory role of polyphenols to the anti-inflammatory activity of the culinary herbs investigated.

1. Introduction

There is growing evidence concerning the health-promoting potential of culinary herbs in a dietary context [1, 2], and the group of compounds that appear to be key contributors to this potential are the polyphenols. Polyphenols are major nonnutrient constituents of many common culinary herbs. Multiple studies, both epidemiological and experimental, suggest that polyphenols possess anti-inflammatory and antioxidant activity that may contribute, via the diet, to the prevention of chronic diseases such as cancer, cardiovascular disease, inflammatory bowel disease, and Alzheimer's [2–6]. Some polyphenols (caffeic acid, rosmarinic acid) known to be present in significant amounts in Labiatae culinary herbs, including rosemary, sage, and thyme [7], have been shown to affect the inflammatory response by inhibiting the action of proinflammatory cytokines such as IL-8 [8, 9]. However, their mechanism of action has yet to be fully elucidated [2, 6]. Culinary herbs are traditionally used in small amounts in the flavouring of food. Furthermore they are often cooked prior to consumption and, as with all foods, are affected by digestive processes following ingestion. Therefore, to gain a better understanding of the role that such herbs play in contributing to the dietary intake of polyphenols and their purported health-promoting properties, the impact of both cooking and digestion on the biological properties of these herbs needs to be established. Thus, the aims of this study were to determine the impact of cooking and *in vitro* digestion on the anti-inflammatory properties of the culinary herbs, rosemary, sage, and thyme and to determine whether this activity is associated with their polyphenol content and antioxidant capacity.

2. Material and Methods

2.1. Reagents. Human salivary α-amylase (100 units/mg; 1 unit corresponds to the amount of enzyme that liberates 1 μmol of maltose per minute at pH 6.9 at 25°C), porcine pepsin (800–2.500 units/mg of protein; 1 unit gives a ΔA280 of 0.001 per minute at pH 2 at 37°C), porcine pancreas pancreatin (4x U.S. Pharmacopeia), bile salts, hydrochloric acid (HCl), sodium bicarbonate ($NaHCO_3$), 1,2 ethyl acetate, formic acid, acetic acid, natural product reagent (NP), polyethylene glycol (PEG), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, methanol, rosmarinic acid, sodium hydroxide (NaOH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Folin Ciocalteu reagent (FC), sodium carbonate (Na_2CO_3), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%) (Trolox), phosphate buffered saline 5 mM at pH 7.4 (PBS), potassium persulphate ($K_2S_2O_8$), catalase (from bovine liver; 1 unit corresponds to the amount of enzyme which decomposes 1 μmol H_2O_2 per minute at pH 7.0 and 25°C), hydrogen peroxide (H_2O_2), tumor necrosis factor alfa (TNFα), 12 and 48 well plates (Costar), Millex disposable syringe filter units, (pore size 0.22 μm, Millipore), and grade 1 filter paper (Whatman) were all purchased from Sigma Aldrich, Poole, UK. Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L), 1% nonessential amino acids (NEAA), L-glutamine, RPMI 1640 medium, 100 U/mL penicillin/100 μg/mL streptomycin and heat inactivated fetal bovine serum, 24 well plates (Coning), 15 mL and 25 mL flasks with vented caps (Coning), and Ficoll-Paque Plus were purchased from Fisher Scientific, Loughborough, Leicestershire, UK. Caco-2 cells passage 44 were purchased from the European Collection of Cell Culture (ECACC), Health Protection Agency, Salisbury, UK. The Quantikine Elisa kit assay D8000C was purchased from R&D systems, Oxon, UK.

2.2. Culinary Herbs. Rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) dried and certified organic were purchased from Neal's Yard Remedies, Richmond, Surrey, UK and stored in air-tight containers in the dark at room temperature.

2.3. Preparation of Herb Samples. To mimic the cooking of these herbs as closely as possible, quantities of these herbs normally used in the preparation of food were used.

Uncooked herb samples (U) were prepared by adding herbs (1 g) to dark glass vials. These were then covered with water (25 mL, 37°C) infused for 10 minutes, filtered (grade 1 filter paper), and then filter sterilised (pore size 0.22 μm). The mean amounts of plant material in these samples were estimated by allowing the water in these extracts to evaporate under a fan overnight until no moisture was visible and weighing the dry material (rosemary (5.1 $\pm$ 0.4 mg/mL), sage (6.5 $\pm$ 0.7 mg/mL), and thyme (10.3 $\pm$ 0.1 mg/mL), $n = 3$).

Cooked herb samples (C) were prepared by heating herbs (1 g) in a Teflon stir frying pan for 10 minutes (without oil, as oils are known to possess antioxidant activity [10]); herbs were then prepared as described above.

Cooked and (*in vitro*) digested (C&D) herb samples were prepared by heating the herbs (1 g) in a Teflon stir frying pan for 10 minutes (as described above). The herbs were then passed through a model of *in vitro* digestion with a simulated buccal fluid [11] and a simulated gastric and intestinal fluid [12, 13]. Herbs (C) (1 g) were added to simulated buccal fluid (14 mL, pH 7.0, 37°C) containing α-amylase (1.4 units/mL) for 2 minutes in a preheated glass pestle and mortar. To simulate mastication, herbs were lightly crushed once. For the simulated gastric fluid, each buccal stage mixture was acidified to pH 2.0 with HCl (5 mL, 0.1 mol/L). A solution (1 mL) of pepsin (40 mg/mL) in HCl (0.1 mol/L) was then added, and the mixture was then poured into a glass bottle, with a screw cap, and placed in a water bath (Grant, SS40-2. Fisher, UK) shaking at a rate of 190 strokes per minute so as to mimic the contractions of the human stomach. The mixtures were left in the simulated gastric fluid for 1 hour at 37°C. For the simulated intestinal fluid, the pH of the mixture was raised to pH 5–5.5 by adding $NaHCO_3$(1 M, drop wise). A solution, containing $NaHCO_3$ (5 mL, 0.1 M), pancreatin (2 mg/mL), and bile salts (12 mg/mL), was added and the mixture incubated for 2 hours (37°C) in a water bath shaking at a rate of 190 strokes per minute so as to simulate the human small intestine during digestion. Sodium hydroxide (NaOH, 1 M) was added drop by drop to maintain the pH at 7.5. Control digests were also prepared (no herbs), and all samples were filtered (grade 1 filter paper) and filter sterilised (pore size 0.22 μm).

The pH of (U), (C), and (C&D) herb samples was adjusted to 6.0 $\pm$ 0.2 using HCl (0.1 M) (as pH via changes in charge can affect the solubility and chelating capacity of antioxidants [14]) and stored at −80°C.

Preliminary studies carried out using the trypan blue exclusion test showed that the control digest and (C&D) herb samples affected the viability of Caco-2 cells, possibly due to the digestive enzymes from the *in vitro* digestion process. To insure that these enzymes would not interfere with either of the cell assays, digestive enzymes were deactivated for all (C&D) herb samples by placing the entire preparation of each (C&D) herb samples and control digests in a glass test tube and placing this tube in boiled water for 5 minutes.

To gain a better understanding of the impact of the food matrix on the digestion of polyphenols in these culinary herbs, the effect of digestion on rosmarinic acid was carried out. Rosmarinic acid was chosen as has been determined from the literature to be a major polyphenol in the herbs selected for this study [7, 15, 16]. Rosmarinic acid (10 mg in 1 mL solution of 100 μL ethanol and 900 μL distilled water) was digested using the same *in vitro* process described above; however, it was not heated in a Teflon stir frying pan for 10 minutes as this was not practical. It was then compared to nondigested rosmarinic acid (10 mg/mL) to which the amount of distilled water added was equivalent to the amount of digestive fluids used throughout the *in vitro* digestion process.

Preliminary studies showed that the amount of plant material present in the extracts of the herb samples prepared

varied; therefore standardised amounts of plant material (STD) were prepared in order to assess the effect of the same amount of plant material for each herb investigated. This was achieved by adding herbs (1 g) to dark glass vials. The herbs were then covered with water (25 mL, boiled), infused for 10 minutes at room temperature, filtered (grade 1 filter paper), and filter sterilised (pore size 0.22 μm). The water in these extracts was left to evaporate under a fan overnight until no moisture was visible. The resulting dry material was weighed and resuspended with sterile distilled water at 30 mg of herb material/mL. The pH was then adjusted to pH 6 $\pm$ 0.2 and the samples stored at -80°C.

2.4. Determination of Antioxidant Capacity and Estimated Phenolic Content of Herb Samples. The antioxidant capacity of herb samples (U), (C), (C&D), and (STD) was determined using the ABTS radical scavenging capacity assay [17]. Herb samples (10 μL) were added to $ABTS^{*+}$ stock solution (990 μL) (with an absorbance of 0.700 $\pm$ 0.02 nM), and absorbance values were read, after 5 minutes at 730 nm at room temperature, on a Helios β spectrophotometer, (Unicam, UK). Trolox standards, prepared in ethanol, were diluted using PBS (0–20 μM) and assayed with the samples, and blanks, to determine the trolox equivalent capacity (TEAC). Gallic acid equivalents (GAEs) were determined for herb samples (U), (C), (C&D), and (STD) using the Folin Ciocalteu (FC) reagent as described previously [18]. However, bearing in mind that this reagent is reactive towards nonphenolic compounds, including ascorbic acid, it has been argued that this assay gives no more than a good "ballpark" estimation of total phenolic content [19]. Thus the values from this assay are expressed as estimated total phenolic content. Diluted herb samples and blanks (100 μL) and gallic acid standards (0.5–0.05 mg/mL) were added to 12 well plates (Costar); then FC reagent (200 μL) was added, followed by the addition of distilled water (2 mL) and Na_2CO_3 (15%) (1 mL). Solutions were incubated for 2 hours and measured at 25°C at 765 nm on a Cary 50 Microplate Reader (Varian Inc, UK).

2.5. Determination of Antioxidant Activity and Polyphenol Profile Using Thin-Layer Chromatography (TLC). Herb samples, pH 6.0 (5 μL), were spotted on to silica TLC plates (10 cm $\times$ 10 cm) and air-dried. The solvent system used was 1,2 ethyl acetate, formic acid, acetic acid, and water (100 : 11 : 11 : 26) [20]. TLC plates were then air dried and sprayed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.2% in methanol) to highlight antioxidant activity or natural product reagent (NP) and polyethylene glycol (PEG) to determine the presence of polyphenols. Yellow areas, against a purple background, corresponded to antioxidant activity. Areas of blue corresponded to phenolic acids, and green/yellow areas corresponded to other polyphenolic compounds. Reference standards (5 μL, 500 ppm) of rosmarinic acid were also run.

2.6. Cell Culture. Ethical approval was obtained from the Ethics Committee of the Faculty of Science, Kingston University, UK. Subjects were recruited once they had read the participant information sheet and had given informed consent. Blood collections and storage procedures were carried out in accordance with the UK Human Tissue Act [21]. Peripheral blood lymphocytes (PBLs) were isolated from heparinised venous whole blood (15 mL) using Ficoll-Paque Plus and then cultured in RPMI media supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/100 μg/mL streptomycin, placed in 15 mL flasks with vented caps (Coning) to incubate at 37°C and 5% CO_2 atmosphere for 24 hours and used promptly for cytotoxicity tests or ELISA assays. Caco-2 cell lines were established and maintained by serial passage in a standard sterile tissue culture environment [22]. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (500 mL) containing glucose (4.5 g/L), 1% nonessential amino acids (NEAA), supplemented with 1% L-glutamine, and heat inactivated fetal bovine serum (10%). Cells were placed in 25 mL culture flasks with vented caps (Coning), incubated at 37°C and 5% CO_2 and grown for 14 days prior to carrying out cytotoxicity tests or ELISA assays.

2.7. Cytotoxicity Tests on PBLs and Caco-2 Cells Exposed to Herb Samples. In order to ensure that herb samples were not toxic to the cell lines, cytotoxicity tests were performed. PBLs cells were seeded at a concentration of 1×10^5 cells/mL in 48 well plates (coning) and incubated with herb samples (diluted 1 : 10 in fresh FBS-free RPMI media supplemented with 100 U/mL penicillin/100 μg/mL streptomycin). Caco-2 cells seeded at 2×10^5 in 24 well plates (Coning) were grown for 14 days; the media were replaced every 3-4 days. Cells were washed 3 times in PBS and then incubated with herb samples (diluted 1 : 10 in FBS-free DMEM with glucose (4.5 g/L), 1% nonessential amino acids (NEAA), supplemented with 1% L-glutamine). Both cell lines were placed in an incubator (37°C) and 5% CO_2 for 24 hours with herb samples and controls and assessed for cytotoxicity using the trypan blue exclusion test.

2.8. Assay for Catalase-Like Activity. The potential catalase-like activity for each herb sample was investigated to rule out the potential inactivation of H_2O_2 by the samples through catalase-like activity. The assay was carried out using standard manometry; catalase was used as the positive control [23].

2.9. Investigation of the Anti-Inflammatory Activity of Herb Samples on PBLs and Caco-2 Cells. The anti-inflammatory activities of the herb samples were assessed in two ways so as to provide insight into how the herb samples affected the action of the proinflammatory factors H_2O_2 and TNF-α. First, PBLs and Caco-2 cells were coincubated with (U), (C), (C&D), and standardised (STD) herb samples, diluted 1 : 10 in respective medium, and H_2O_2 (2 mM) or TNFα (100 μg/mL) for 24 hours. Cells were also preincubated for 3 hours with (C&D) herb samples, diluted 1 : 10 in medium, to determine if the herbs following cooking and *in vitro* digestion were able to protect the cells from proinflammatory

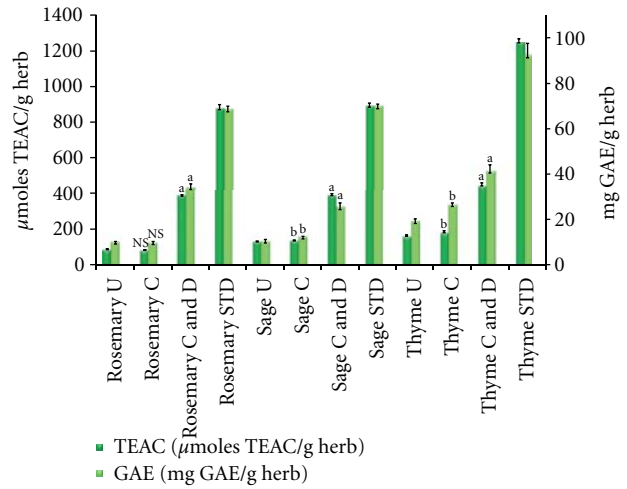


Figure 1: Antioxidant capacity (TEAC) and total phenolic content (GAE) of herb extracts. Data are presented as the mean ± SD ($n = 3$). NS: no significant differences between TEAC rosemary (U) and rosemary (C) ($P \geq 0.05$) and no significant differences between GAE rosemary (U) and rosemary (C) ($P \geq 0.05$). $^{a}P \leq 0.001$ (C&D) herb samples significantly different from their (U) & (C) counterparts. $^{b}P \leq 0.05$ (C) herb samples significantly different from their (U) counterparts. STD: standardised herb sample (30 mg/mL).

factors. These cells were then exposed to H_2O_2 or TNFα 24 hours at the concentrations stated above. PBL and Caco-2 controls were set up, in their respective media, with H_2O_2 or TNFα only at the concentrations stated above. Spontaneous release of IL-8 was assessed by incubating cells in media only (in the absence of herb samples, H_2O_2 or TNFα). Cells were also exposed to (U), (C), (C&D) blank digest, and (STD) samples (diluted 1 : 10 in their respective media) to determine they if they affected IL-8 release in the absence of H_2O_2 or TNFα.

Media were then collected and their IL-8 concentrations determined using the quantitative sandwich enzyme immunoassay ELISA for interleukin-8 kit (carried out as per manufacturer's instructions) to ascertain if its release was affected. Plate absorbance values were read at 450 nm with a correction at 540 nm using a Cary 50 Microplate Reader (Varian Inc, UK).

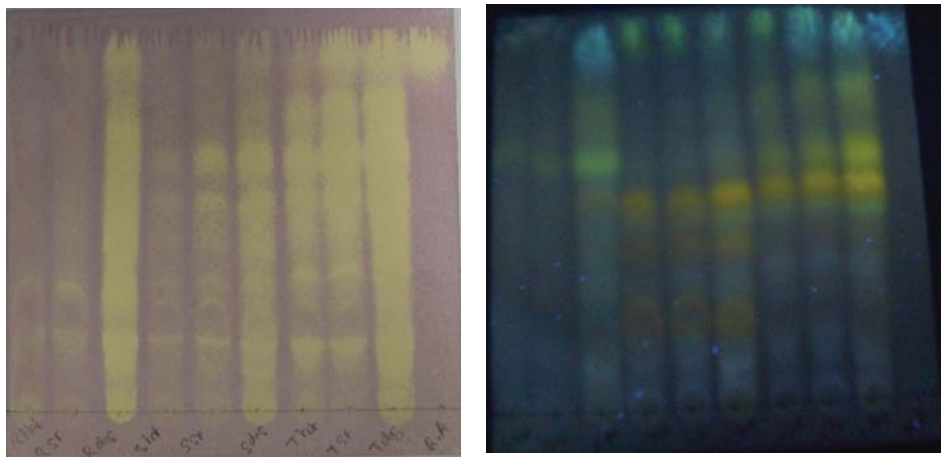
2.10. Expression of Data and Statistical Analysis. Data are expressed as means of triplicate analysis ± SEM, ($n = 3$) unless otherwise stated. Statistical analyses were performed using SPSS for windows (Version 17). The antioxidant capacity was expressed in μmoles TEAC/g herb and estimation of total phenolic content in mg GAE/g herb. Mean values for blank digest samples were subtracted from respective TEAC and GAE values for C&D herb samples. ANOVA with post hoc Tukey were used to compare TEAC and GAE of (U), (C), (C&D), and STD herb samples. The independent sample t-test was used to compare the TEAC of rosmarinic acid digested to rosmarinic acid nondigested. The percentage inhibition of IL-8 release from PBLs or Caco-2 cells was determined using the following equation: ((IL-8 release from respective control cells (stimulated by H_2O_2 or TNFα)-IL-8 release from cells coincubated or preincubated with herb samples and H_2O_2 or TNFα)/IL-8 release from respective control cells (stimulated by H_2O_2 or TNFα)) $\times 100$. As percentages are bound data, the percentages for IL-8 inhibition were checked for normality using the Shapiro-Wilk test. ANOVA with post hoc Tukey was then used to compare the percentage inhibition by herb samples (U), (C), (C&D), and (STD) preincubated with H_2O_2 or TNFα against their respective controls. Pearson's correlation coefficients (r) with level of significance ($P \leq 0.05$) (2-tailed) were used to compare all herb samples irrespective of treatments for TEAC and GAE assay results, and TEAC and GAE with percentage inhibition of TNFα or H_2O_2 stimulated IL-8 release by PBLs or Caco-2 cells.

3. Results

3.1. Antioxidant Capacity and Estimated Total Phenolic Content of Herb Samples. The antioxidant capacity (TEAC) and estimated total phenolic content (GAE) of the (C&D) herb samples were significantly higher than those of their (C) counterparts ($P \leq 0.001$) which were significantly higher than those of the (U) herb samples ($P \leq 0.05$) with the exception of rosemary ($P \geq 0.05$ for both TEAC and GAE assays) (Figure 1). The TEAC and GAE for the (STD) herb samples were significantly higher than (U), (C), and (C&D) samples ($P \leq 0.001$) (Figure 1). (STD) thyme was higher than (STD) sage and rosemary for both assays. For TEAC values for rosmarinic acid there was no statistical difference ($P = 0.692$) between nondigested rosmarinic acid (2901 ± 149.1 μmoles TEAC/g) and digested rosmarinic acid (2973.1 ± 140.6 μmoles TEAC/g), ($n = 3$).

3.2. Antioxidant Activity and Polyphenol Analysis Using TLC. For TLC plates sprayed with DPPH, all the (C&D) herb samples provided a more intense yellow colour compared to their undigested (U) and (C) counterparts. This difference in colour indicated a higher antioxidant activity (Figure 2(a)). For the TLC plates sprayed with NP and PEG, phenolic acids appeared in shades of blue towards the top of the plates, and other polyphenolic compounds appeared in shades of green and yellow in the centre of the plates (Figure 2(b)). The colours of phenolic acids and other polyphenolic compounds were more intense for the (C&D) herb samples, compared to their (U) and (C) counterparts, indicating higher levels of phenolic compounds. For (C) herbs, there was no difference in colour intensity for rosemary, a marginal increase in colour intensity for sage and an evident increase in colour intensity for thyme when compared with their (U) counterparts (Figures 2(a) and 2(b)).

3.3. Herb Samples Cell Cytotoxicity Tests and Catalase-Like Activity. The viability of both PBLs and Caco-2 cells exposed to (U), (C) (C&D), and (STD) herb samples diluted 1 in 10 in respective media for 24 hours was not significantly different from that of the controls ($P > 0.05$, data not shown).



(a) (b)

Figure 2: Percentage inhibition of IL-8 release by H_2O_2 or TNFα exposed PBLs pre-incubated or co-incubated with herb samples. (a) TLC plate spotted with herb samples (5 μL, pH 6) and rosmarinic acid (0.5 mg/mL) and sprayed with 2% DPPH. The yellow areas correspond to antioxidant activity. A: rosemary (U), B: rosemary (C), C: rosemary (C&D), D: sage (U), E: sage (C), F: sage (C&D) G: thyme (U), H: thyme (C), I: thyme (C&D), J: rosmarinic acid (U) uncooked, (C) heated, (C&D) heated, and digested. (b) TLC plate spotted with herb samples (5 μL, pH 6) and rosmarinic acid (0.5 mg/mL) and sprayed with natural product reagent (NP) and polyethylene glycol (PEG). Phenolic acids appear in shades of blue towards the upper part of the plate and other polyphenolic compounds appear in a range of shades of green and yellow in the centre of the plate. A: rosemary (U), B: rosemary (C), C: rosemary (C&D), D: sage (U), E: sage (C), F: sage (C&D) G: thyme (U), H: thyme (C), I: thyme (C&D), J: rosmarinic acid (U) uncooked, (C) heated, (C&D) heated and digested.

None of the herb samples exhibited catalase-like activity indicating that H_2O_2 was not inactivated by these samples.

3.4. Spontaneous Release of IL-8 from PBLs and Caco-2 Cells Compared to TNFα and H_2O_2 Stimulated PBL and Caco-2 Cell Controls. Spontaneous IL-8 release was detected for both PBLs (855.5 ± 10.7 pg/mL, $n = 8$) and Caco-2 cells (60.4 ± 1.1 pg/mL, $n = 6$). Exposure of cells to TNFα significantly increased IL-8 release compared to nonstimulated cells ($P \leq 0.05$) for both PBL (1011 ± 63.9 pg/mL, $n = 6$) and Caco-2 Cells (183 ± 14.5 pg/mL, $n = 6$). Exposure of cells to H_2O_2 significantly increased IL-8 release compared to nonstimulated cells ($P \leq 0.05$) for both PBLs (1144.6 ± 47.5 pg/mL, $n = 6$) and Caco-2 Cells (91.8 ± 8.2 pg/mL, $n = 6$). None of the herb samples had a significant effect on spontaneous IL-8 release from either cell lines.

3.5. Effect of Coincubation of Herb Samples with H_2O_2 or TNFα. For (U), (C), and (C&D) herb samples there were decreases in IL-8 release from PBLs but these decreases were only significant for PBLs, stimulated by H_2O_2 ($P \leq 0.05$, Figure 3) with the exception of (C) rosemary. (C&D) herb samples significantly decreased the release of IL-8 from Caco-2 cells stimulated by TNFα ($P \leq 0.001$, Figure 4). (STD) herb samples significantly inhibited the release of IL-8 from PBLs stimulated by H_2O_2 and PBLs stimulated by TNFα ($P \leq 0.001$, Figure 3). (STD) herb samples significantly decreased the release of IL-8 from Caco-2 cells stimulated by TNFα ($P \leq 0.001$, Figure 4). (U), (C), (C&D), and (STD)

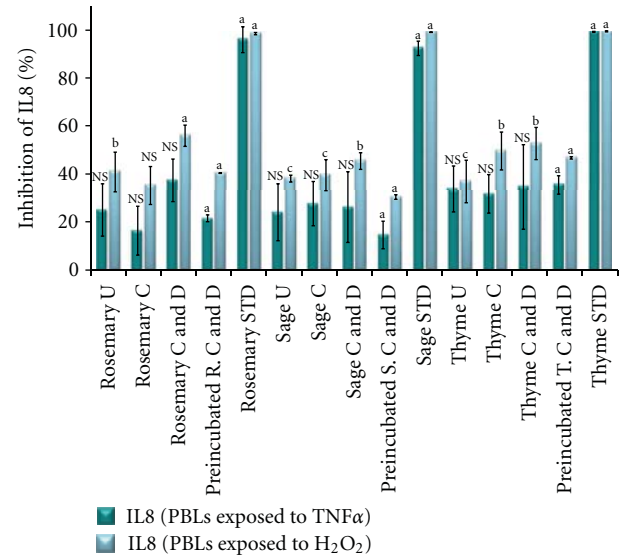


Figure 3: Percentage inhibition of IL-8 release by TNFα exposed Caco-2 cells pre-incubated or co-incubated with herb samples. (R.): Rosemary, (S.): Sage, (T.): Thyme. Data are presented as the means ± SEM ($n = 3$). %inhibition of IL-8 = [(IL-8 release from control PBLs (stimulated by H_2O_2 or TNFα)-IL-8 release from PBLS co-incubated/pre-incubated with herb samples)/IL-8 release from control PBLs (stimulated by H_2O_2 or TNFα)-] ×100. Significant differences between IL-8 released from control PBLs and PBLs co-incubated/pre-incubated with herb samples, $^aP \leq 0.001$, $^bP \leq 0.01$, $^cP \leq 0.05$, $n = 3$. NS: nonsignificant difference between IL-8 release for control PBLs and that of PBLs co-incubated with herb samples and TNFα or H_2O_2, or pre-incubated with herb samples.

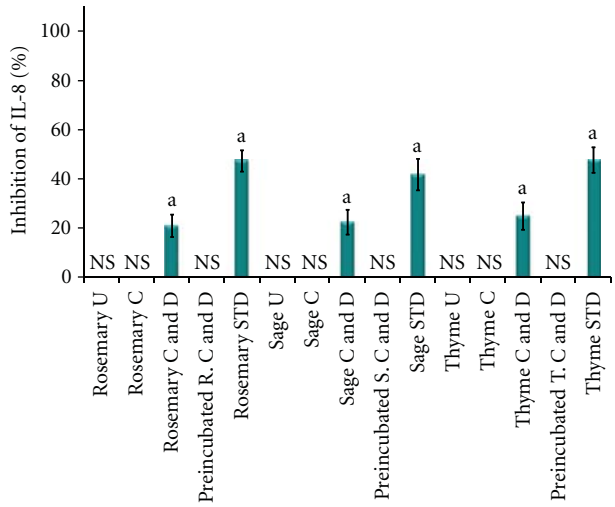


Figure 4: Percentage inhibition of IL-8 release by TNFα exposed Caco-2 cells pre-incubated or co-incubated with herb samples. (R.): Rosemary, (S.): Sage, (T.): Thyme. Data are presented as the Means ± SEM ($n = 3$). %inhibition of IL-8 = [(IL-8 release from control Caco-2 cells (stimulated by TNFα)-IL-8 release from Caco-2 co-incubated/pre-incubated with herb samples)/IL-8 release from control Caco-2 cells (stimulated by TNFα)] $\times 100$]. Significant differences between IL-8 released from control PBLs and PBLs co-incubated/pre-incubated with herb samples, $^{a}P \leq 0.001$, ($n = 3$). NS: nonsignificant difference between IL-8 release for Caco-2 cells control (TNFα) and that of Caco-2 cells co-incubated with herb samples and TNFα, or pre-incubated with herb samples.

herb samples had no effect on IL-8 release from Caco-2 cells stimulated by H_2O_2(data not shown).

3.6. Effect of Preincubation with Herb Samples prior to Adding H_2O_2 or TNFα. Preincubation with (C&D) herb samples caused a significant decrease in IL-8 release from PBLs stimulated by H_2O_2 or TNFα ($P \leq 0.001$, Figure 3). Preincubating the Caco-2 cells with (C&D) herb samples had no effect on IL-8 release when stimulated by H_2O_2 or TNFα (Figure 4).

3.7. Correlations between Antioxidant Capacities, Total Phenolic Contents, and Anti-Inflammatory Activities of Culinary Herbs, Irrespective of Treatments. There was a strong correlation between TEAC and GAE ($r = 0.989$, $P \leq 0.01$), (Table 1) and between the %decrease in IL-8 release caused by herb samples for H_2O_2 or TNF-α stimulated PBLs and their estimated total phenolic content and antioxidant capacity ($P \leq 0.01$, Table 1). There was a strong and significant correlation between the %decrease in IL-8 release from TNF-α stimulated Caco-2 cells and their estimated total phenolic content and antioxidant capacity ($r = 0.892$, $P \leq 0.01$, Table 1).

4. Discussion

The aims of this study were, first, to investigate what effect cooking and digestion *in vitro* have on the anti-inflammatory activity of the culinary herbs rosemary, sage, and thyme and, second, to investigate if there is an association between the anti-inflammatory activity and the antioxidant capacity and estimated total phenolic content of these herbs.

Based on the co- and preincubation experiments, this study indicates that rosemary, sage and thyme (U), (C), (C&D) and (STD) significantly decrease IL-8 release via inhibition of, and protection against, the action of H_2O_2 or TNFα. The anti-inflammatory action of the herbs was far greater for the PBLs than for the Caco-2 cells. In fact, with the exception of (C&D) and (STD) herbs in TNFα stimulated Caco-2 cells, none of the other herb samples showed any anti-inflammatory action based on the inhibition of IL-8 release for both H_2O_2 or TNFα stimulated Caco-2 cells. The Caco-2 cell line is a transformed human colonic adenocarcinoma; however, the PBLs are mature "healthy" lymphocytes, and these differences might explain the difference observed in IL-8 release [24].

Cooking the herbs did not consistently significantly increase their estimated total phenolic content or antioxidant capacity, compared to (U) herbs in the current study. Other studies that have looked at the impact of cooking on various plant foods concur that differences in polyphenol activity content are due to both the food matrix and the cooking techniques employed [25–27]. Cooking followed by *in vitro* digestion did elicit significant increases in TEAC and GAE compared to (U) and (C) herbs in the current study. In relation to the literature the impact of digestion on polyphenols using *in vitro* models resulted in decreases in activity in fruits juices [28] and either no changes or decreases in herbal teas (infusions from powdered herbs) [29]. These differences may be due to the nature of the food matrix (whole herbs were used in the current study) as well as the type of *in vitro* model of digestion used.

Analysis using TLC showed the presence of phenolic acids and other polyphenolic compounds in the (U) herb samples which agrees well with the literature [16, 20, 30]. However, to the authors' knowledge, no TLC work has been done to determine the polyphenolic profiles of rosemary, sage, and thyme following cooking and digestion *in vitro*. TLC results from the present study did not suggest that new compounds were formed or lost as a result of cooking as the profiles for (C) versus (U) herb samples were identical for rosemary and more intense in colour for sage and thyme but not dissimilar. The *in vitro* digestion process increased the levels of phenolic acids and other polyphenolic compounds. These observations are supported by the increases in the antioxidant capacity, activity (TLC), and estimated total phenolic content data. Rosmarinic acid, measured via the TEAC assay, was not affected by *in vitro* digestion, and although the effect of cooking on rosmarinic acid was not investigated, the lack of difference in TEAC and GAE in (C) compared to (U) herbs strongly indicates that polyphenols in these herbs, including rosmarinic which is a predominant polyphenol in these herbs, were not decreased as a consequence of the cooking method used.

A positive linear correlation has been established between polyphenols and antioxidant capacity of herbs [16]. Polyphenols are thought to be most concentrated in the vacuoles of

Table 1: Correlations between antioxidant capacities, estimated total phenolic content, and %decrease in IL-8 release irrespective of treatments.

Correlations	r	P
GAE versus TEAC	0.989	≤0.01
%decrease in IL-8 for PBLs exposed to TNFα versus GAE	0.914	≤0.01
%decrease in IL-8 for PBLs exposed to TNFα versus TEAC	0.901	≤0.01
%decrease in IL-8 for PBLs exposed to H_2O_2 versus GAE	0.922	≤0.01
%decrease in IL-8 for PBLs exposed to H_2O_2 versus TEAC	0.905	≤0.01
%decrease in IL-8 release for Caco-2 exposed to TNFα versus GAE	0.876	≤0.01
%decrease in IL-8 release Caco-2 exposed to TNFα versus TEAC	0.892	≤0.01

paraveinal and parenchymal cells below the epidermis [31]. Thus, the increase in antioxidant capacity and estimated phenolic content is likely to be due to the breakdown of the herbs' plant cell walls via the *in vitro* digestion process, facilitating the release of polyphenols. The antioxidant capacity, estimated total phenolic content, and anti-inflammatory effects of (STD) herb samples were by far the greatest (thyme>sage, rosemary). This is most likely to be due to a greater concentration of plant material (7.3 ± 2.4 mg/mL for (U) and 30 mg/mL for (STD) herb samples) and thus a greater concentration of polyphenols resulting in a dose-dependant response. This is further supported by the much greater decrease in IL-8 release that resulted when stimulated cells, (with the exception of H_2O_2 stimulated Caco-2 cells), were coincubated with (STD) herb samples, thus corroborating the role of polyphenols as significant contributors to the anti-inflammatory properties of these herbs.

The mechanism of action to explain the effect of the herbs on IL-8 release is unclear. However, evidence suggests that polyphenols may play a role in the anti-inflammatory activity reported in the current study. Rosmarinic acid is the predominant polyphenol in rosemary, sage, and thyme [7, 15, 16, 30, 32]. The effectiveness of rosmarinic acid in reducing inflammation by inhibiting the proinflammatory PKC/NF-κB pathway was demonstrated in mice [33]. Therefore it is possible to speculate that the rosmarinic acid present in the herbs studied may be responsible, at least in part, for the IL-8 inhibition observed. In addition, caffeic acid, another polyphenol shown to be present in the herbs investigated [7], was shown to inhibit both H_2O_2 and TNFα stimulated IL-8 release from Caco-2 cells [9]. This study indicated that inhibition occurred at the transcriptional and post-transcriptional level.

One clear difference observed in this study was that the inhibition of IL-8 release from PBLs stimulated by H_2O_2 was generally greater (and more statistically significant) than for those stimulated by TNFα. This difference may be because TNFα and H_2O_2 may affect different inflammatory pathways within the cells or may affect the same inflammatory pathways differently [24].

It remains unclear why none of the herb samples inhibited IL-8 release from Caco-2 cells exposed to H_2O_2. Differences in H_2O_2 and TNFα induced release of IL-8 were also reported [9] with caffeic acid, a metabolite of rosmarinic acid. The authors demonstrated that caffeic acid (2.00 mmol/L) was able to inhibit the H_2O_2 or TNFα induced release of IL-8 from Caco-2 cells; however, whilst it was shown that caffeic acid inhibition appeared to occur via the inhibition of their expression of IL-8 mRNA, it did not suppress the TNFα-induced increase in the IL-8 mRNA expression. That study suggests that the mechanisms by which polyphenols suppress the stimulation of IL-8 production and its subsequent release may differ based on the inflammatory stimulant. However, these results are in disagreement with the current study where only TNFα-stimulated IL-8 release was inhibited by the herb samples. As stated above, caffeic acid is a polyphenol found in all three culinary herbs investigated [7], although one must bear in mind that the concentration of polyphenols is affected by numerous factors including seasonal variations [34, 35] and the place of provenance [15, 36], as well as presale processing and storage [37] and also cooking [25, 26, 38].

The strong and significant correlations between percentage inhibition of IL-8 release from the stimulated PBLs with TEAC and GAE, irrespective of treatments, suggest that there is an association between the antioxidant capacity, polyphenol content, and the anti-inflammatory activity of the herbs investigated [5, 9, 39, 40]. In conjunction with literature concerning the anti-inflammatory activity of individual polyphenols, this association does provide compelling evidence that the polyphenols in these culinary herbs are major contributors to the inhibitory effects on IL-8 release reported. However, it is not clear if this action is in any way due to their antioxidant activity. Analytical methods would need to be employed to confirm this hypothesis and to clarify which polyphenols, including their metabolites, are responsible for the biological activities observed.

Whilst (STD) herb samples may not have a physiological relevance in terms of dietary intake, their use removed the impact that the amount of plant material has on antioxidant capacity, estimated total phenolic content, and anti-inflammatory activity when comparing the herbs. In addition, it also helped to demonstrate a dose-dependent relationship between the amount of herb and the anti-inflammatory activity. The dose-response relationship is of particular interest since the amount of herbs used in the preparation of food varies [41] and also varies between individuals. A recent study showed that the consumption of herbs and spices ranged from 0.0 to 10.0 g/person/day [42].

5. Conclusion

In conclusion, that study shows that the culinary herbs rosemary, sage, and thyme, in quantities used for cooking, possess significant anti-inflammatory activity that may be due to their polyphenol content. Further work is required to fully elucidate the polyphenols responsible for this action and their mechanisms of action.

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Review Article

Anticancer Activity of Green Tea Polyphenols in Prostate Gland

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Numerous evidences from prevention studies in humans, support the existence of an association between green tea polyphenols consumption and a reduced cancer risk. Prostate cancer is one of the most frequently diagnosed male neoplasia in the Western countries, which is in agreement with this gland being particularly vulnerable to oxidative stress processes, often associated with tumorigenesis. Tea polyphenols have been extensively studied in cell culture and animal models where they inhibited tumor onset and progression. Prostate cancer appears a suitable target for primary prevention care, since it grows slowly, before symptoms arise, thus offering a relatively long time period for therapeutic interventions. It is, in fact, usually diagnosed in men 50-year-old or older, when even a modest delay in progression of the disease could significantly improve the patients quality of life. Although epidemiological studies have not yet yielded conclusive results on the chemopreventive and anticancer effect of tea polyphenols, there is an increasing trend to employ these substances as conservative management for patients diagnosed with less advanced prostate cancer. Here, we intend to review the most recent observations relating tea polyphenols to human prostate cancer risk, in an attempt to outline better their potential employment for preventing prostate cancer.

1. Introduction

In the past decade, prostate cancer (PCa) has been one of the most frequently diagnosed male neoplasias in the Western countries, and despite recent important progress, it continues to represent a major cause of cancer-related mortality. The reasons of this high incidence are unknown. Racial and ethnic differences in PCa incidence and mortality are well known, with African-American men being at the greatest risk for diagnosis, followed by Caucasian and Hispanic men. Asian-Americans seem to be at the lowest risk for PCa [1]. Generally, PCa appears to be sporadically inherited (less than 10%). These observations highlight the hypothesis that interactions between multiple genetic and ambient factors are significative determinant in PCa development.

Diet is believed one of the most probable and determinant environmental risk factors. The hypothesis results were strengthened by ecological studies showing that the PCa incidence rapidly increases in Asian immigrants that have assimilated Western diet and way of living, and in Asian men that, although living in their original countries, are contaminated by Western lifestyle, tending to substitute soy, tea, fish,

fruits, and vegetables consumption with red meat and fatty food [2]. The molecular mechanisms, through which racial, genetic, environmental conditions affect PCa development, are still a matter of discussion.

Numerous experimental evidences suggest that both dietary and lifestyle factors act by promoting chronic inflammation and/or oxidative stress leading to DNA damage, epigenetic modifications, or other alterations associated with cancer initiation. Altogether, the experimental data so far produced suggest that antioxidant and anti-inflammatory agents may play a promising role for PCa prevention [3]. In fact, (1) the proliferative inflammatory atrophy (PIA) has been proposed as a precursor to prostatic intraepithelial neoplasia (PIN) that merges with high-grade PIN (HGPIN) in about 34% of cancerous lesions [4]. Chronic inflammation may damage epithelial cells and lead to proliferative lesions, likely PIN lesions, and prostatic carcinomas precursors [5]; (2) several evidences have suggested that oxidative stress, following from the imbalance of reactive oxygen species (ROS) production and cellular antioxidant defences, is one of the most critical aging-associated factor on prostate carcinogenesis. Cumulative ROS effect possibly results in lipids, proteins, and DNA damage [6]. Prostate gland is known to be particularly vulnerable to oxidative stress, probably because of inflammation and hormonal deregulation processes and epigenetic modifications, frequently occurring in the organ.

It is worth to underline that PCa is a suitable target for primary chemopreventive interventions, since it is a unique malignancy that generally grows very slowly, before symptoms arise. As a consequence, it offers a relatively long time period for therapeutic interventions and, because of its long latency, it is typically diagnosed in 50-year-old men or older, when even a modest delay in the disease progression could significantly improve the patient quality of life.

Considering that most of the known chemotherapeutic treatments against PCa carry side effects risk, there is an increasing trend to employ conservative management for patients diagnosed with less advanced PCa, that may not require treatment. These types of tumors, in fact, are relatively indolent, almost never relapse after local therapy, and probably require a simple watchful waiting.

In order to obtain new additional opportunities improving nontoxic chemopreventive strategies, dietary substances consumption, especially tea polyphenols, can represent an important clinical challenge.

Tea, the most popular worldwide consumed beverage after water, obtained from the dried leaves of the plant *Camellia sinensis*, has been studied extensively for its effects on cancer prevention. The major polyphenols in green tea, generally known as catechins, (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC), display meta-5,7-dihydroxyl groups on the A ring [7] and di- or trihydroxyl groups on the B ring, that represent the principal site of antioxidant reactions [8]. EGCG and ECG, harboring D ring (gallate), present maximal antioxidant activity (Figure 1). The above characteristics allow tea polyphenols to react with ROS (superoxide radical, singlet oxygen, hydroxyl/peroxyl radical, peroxynitrite) [9] and prevent, as strong metal ion chelators, ROS output following several compounds autooxidation. Due to the ability of acting as good donors for hydrogen bonding, an accurate prediction about tea polyphenols solubility and permeability remains, at the moment, an elusive target [10].

Tea polyphenols have been shown to inhibit tumorigenesis and tumor progression, at different organ sites, in different animal models for human cancer. Many evidences highlighted that these compounds affect enzyme activities and signal transduction pathways, resulting in cell proliferation suppression, apoptosis enhancement, as well as angiogenesis and cell invasion inhibition, finally inhibiting the development of the disease.

To date, an association between green tea polyphenols consumption and reduced cancer risk is also supported by human cancer prevention studies, although epidemiological evidences have not yet yielded conclusive results on their chemopreventive and anticancer effect against PCa, possibly owing to different confounding factors [11].

2. Mechanisms of GTCs Action in PCa Cell Lines

Several mechanisms involved in GTCs inhibition of cancer formation/progression are recently reviewed by numerous Authors [12–15]. Certainly, GTCs, through their antioxidant activity, are able to quench ROS and chelate transition metals, produced during all the carcinogenesis stage. However, it has been reported that also GTCs can be a source of ROS generation, inducing oxidative stress and consequently activating apoptotic pathways [16].

GTCs, and especially EGCG, are capable of modulating a plethora of cell signalling pathways crucial for cancer cells transformation and survival, including, but not limited to, the mitogen-activated protein kinase (MAP-kinase), the nuclear factor-kappa B (NFκB), and the insulin-like growth factor (IGF)/IGF-1 receptor pathways.

With regard to the prostate-specific processes GTCs are able to affect androgen receptor (AR) downregulation and prostate-specific antigen (PSA) expression [14, 17].

Here below, we report the most probable GTCs mechanisms of action in some PCa cell lines.

2.1. Inhibition of Cell Proliferation and Cell Cycle Arrest. GTCs exhibit ant-proliferative effects versus both androgen-sensitive and androgen-insensitive human PCa cells. The effect is mediated by cell cycle deregulation and cell death induction [18].

We showed that GTCs action is cancer specific, since GTCs is capable of inducing growth arrest both in SV-40 immortalized prostate epithelial cells (PNT1a) and in tumorigenic androgen-independent PCa cells (PC3), while normal human prostate epithelial cells were not significantly affected, even when EGCG was administered at higher doses [19]. The IC_{50} of EGCG ranges from about 40 to about 200 μM, depending on the cell line type (LNCap < PNT1a < DU145 < PC3), as well as the length of the experiment, ranging from 24 to 72 hours [18, 20]. Our results were confirmed by other authors in normal fibroblasts [20, 21].

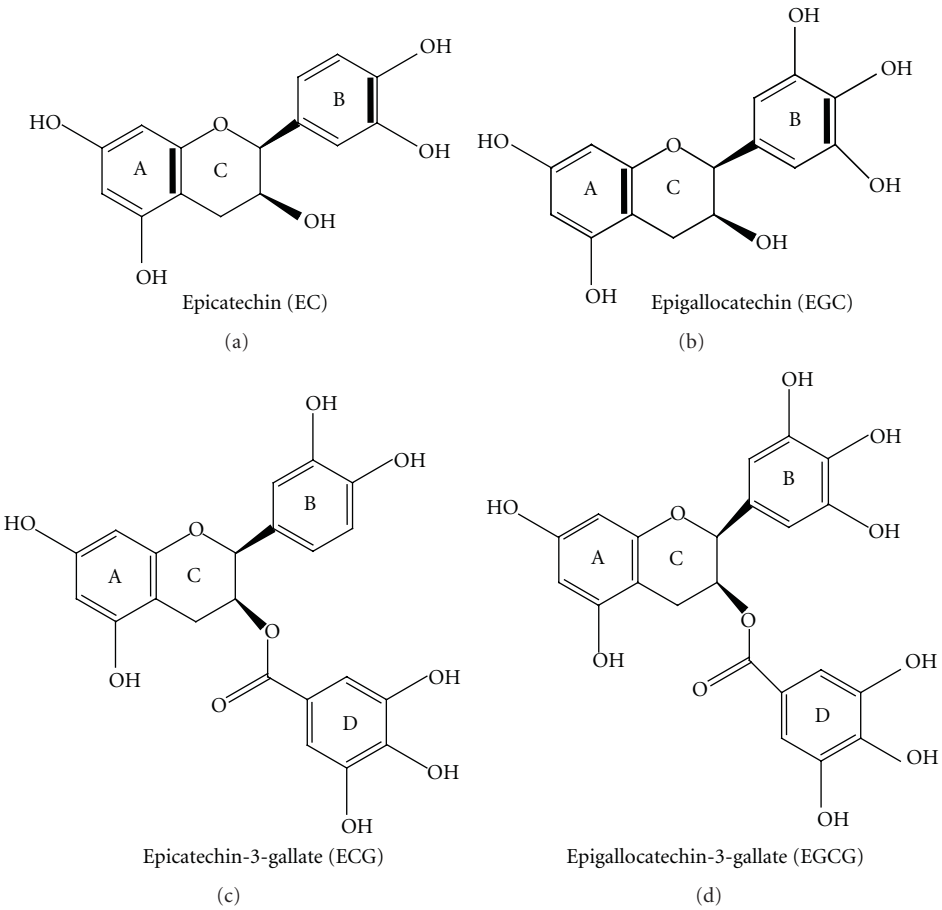

Figure 1: Chemical structure of the green tea catechins obtained from the dried leaves of the plant *Camellia sinensis*.

Both in LnCap and DU145 cells, EGCG treatment resulted in the cell cycle arrest at G_0/G_1 phase (dose dependent) and the cyclin kinase inhibitor WAF1/p21 and apoptosis induction, irrespective of the AR presence or p53 cell status. Cells harbouring an active p53 protein respond to lower EGCG doses than cells with a mutated p53 [18].

It was shown that EGCG treatment of LNCaP and DU145 cells causes an induction of G_1-phase cyclin kinase inhibitors, that, in turn, inhibit the cyclin-cycline-dependent kinase (CDK) complexes operative in the G_0/G_1 phase, thereby causing a cell cycle arrest, a possible irreversible process ultimately leading to apoptotic cell death [22].

2.2. Apoptosis. Hastak et al. [23] reported that EGCG induces apoptosis in LNCaP cells mainly through modulation of two related pathways: (i) p53 stabilization by specific phosphorylation and downregulation of murine double minute 2 protein (MDM2), mediated by the tumor suppressor p14ARF; (ii) negative regulation of NFκB activity, which results in reduction of the proapoptotic Bcl2 family protein expression. EGCG-induced p53 stabilization causes upregulation of its downstream targets WAF1/p21 and Bax; consequently, EGCG produces a change in the Bax/Bcl2 ratio, shifting the balance between pro-/antiapoptotic proteins in favor of apoptosis. This first event triggers the caspases cascade activation, followed by poly-ADP-Ribose polymerase (PARP) cleavage and chromatin fragmentation. Inactivation of p53, by using Small Interfering RNA, renders LnCap cells more resistant to EGCG-mediated apoptosis. On the other hand, stable transfection of PC3 cells (that are endowed with a mutated and inactivated p53 protein) with a cDNA encoding wild-type p53, allows to by-pass their resistance to EGCG-mediated apoptosis [24]. Ablation of p21 or Bax confers a growth advantage to the cells through inhibition of the mitochondrial pathway of caspase-dependent apoptosis.

Tumour necrosis factor-related apoptosis-inducing ligand/Apo2L ligand (TRAIL/Apo2L) is believed a promising candidate for cancer therapy, even if emergence of drug resistance limiting its potential use occurs.

Siddiqui et al. [25] reported that EGCG treatment sensitizes TRAIL-resistant LNCaP cells to TRAIL-mediated apoptosis through modulation of apoptotic pathways. TRAIL/Apo2L, when combined with EGCG, exhibited enhanced cells apoptotic activity, characterized by three major molecular events: (i) increase of PARP cleavage; (ii) proantiapoptotic Bcl2 family proteins modulation, favoring apoptosis; (iii) synergistic inhibition of apoptosis inhibitors and concomitant increase in caspase activation.

2.3. Anti- or Pro-Oxidant Effects and Activation of Phase II Detossifying Enzymes. GTCs were shown to suppress cell growth and induce apoptosis in DU145, through increasing ROS formation and mitochondrial depolarization. Although the molecular mechanisms are still not clear, GTCs-induced apoptosis is not related to the members of Bcl2 family, as EGCG did not alter Bcl2, BclX(L) and BAD expression, in this cell line [26].

Nuclear factor-E2-related factor 2 (Nrf2) is a transcription factor that plays a pivotal role in the antioxidant response and oxidative stress, through activation of phase II detoxifying or antioxidant enzymes [27, 28]. EGCG, as well as other electrophile natural compounds, are able to activate a core antioxidant responsive element (EpRe), present in the promoter region of many genes involved in the cellular response to oxidative stress [28, 29]. A widely accepted model for induction of EpRe-mediated antioxidant gene expression, plausible also for EGCG, involves phosphorylation of Nrf2, leading to enhanced Nrf2 accumulation and subsequent EpRe binding [28].

Activator Protein-1 (AP-1) is a redox-sensitive transcription factor that transduces modifications of the cellular redox status, modulating the expression of genes, including pro-survival genes, in responses to oxidative and electrophilic stresses.

Nair et al. [30] demonstrated that EGCG, when administered to PC3 cells together with sulforaphane (SFN) is able to reduce AP-1 induction. The authors also confirmed, by "*in silico*" analyses, the presence of conserved transcription factor binding site in the Nrf2 and AP-1 promoter region, suggesting that gene expression changes induced by SFN and EGCG, could be mediated via concerted modulation of the Nrf2 and AP-1 pathways [30].

2.4. Modulation of the NFκB Signalling and Inhibition of Inflammation Pathways. NFκB is a redox sensitive transcription factor, often overexpressed in tumor and cancer cell lines, that has been suggested to regulate a variety of cellular functions, including inflammation, immune response, growth, and cell death. NFκB resides in the cytoplasm, bound to its inhibitor IκB; once the inhibitor is released, NFκB phosphorylation occurs, followed by its translocation to the nucleus.

EGCG has been shown to decrease the DNA binding activity of NFκB, and reduce the expression of the p65 subunit of NFκB in LNCaP cells, stimulated by tumour necrosis factor alfa. NFκB over-expression is an important target in PCa due to the regulation of various downstream targets that include the cylcooxygenase-2 proteins (COX-2) [23].

In the LNCaP (androgen-dependent) and PC3 (androgen independent) prostate cancer cells, EGCG was shown to inhibit mitogen stimulated COX-2 expression through a mechanism probably involving the regulation of transcription factors, like NFκB, and not the direct binding to the enzyme [31]. Moreover, LnCaP and PC3 cells treatment with a combination of EGCG and COX-2 inhibitors resulted in: (i) enhanced cell growth inhibition; (ii) caspase-dependent induction of apoptosis and PARP cleavage; (iii) inhibition of peroxisome proliferator-activated receptor gamma; (iv) NFκB inhibition, when compared with the effects of the two singularly employed agents, suggesting that they play a synergistic role [32].

Matrix metalloproteinases (MMPs), generally involved in extracellular matrix degradation, result overexpressed in PCa and play an important role in tumor progression and invasion. NFκB is the key transcription factor involved in the regulation of MMPs genes.

In DU145 cells Vayalil and Katiyar [33] demonstrated that EGCG inhibits MMP-2 and MMP-9 (inactive and active form expression) through a dose-dependent phosphorylation inhibition of the extracellular signal-regulated kinase (ERK1/2) and p38 pathways. Inhibition of the activation of transcription factors c-jun and NFκB also occurs.

Siddiqui et al. [25] observed that EGCG, administered in combination with TRAIL, can inhibit LNCaP cells invasion and migration potential. The authors found the effect is mediated through inhibition of the expression of the following factors: vascular endothelial growth factor (VEGF), urokinase plasminogen activator (uPA), and angiopoietin 1 and 2. A significant inhibition in both MMP-2 and -9 protein expression and activity occurs, in the presence of up-regulation of the tissue inhibitor of metalloproteinases 1.

2.5. Insulin-Like Growth Factor (IGF) Axis, Mitogen-Activated Protein (MAP) Kinases Pathway, Phosphoinositide-3 Kinase (PI3K/AKT) Pathway. It has been suggested that the IGF axis plays a relevant role in PCa onset and development. Binding of IGF1 to its cognate receptor activates the intracellular tyrosine kynase domain, that produces phosphorylation of many protein substrates, including members of the MAP kinase cascade and PI3K/AKT. MAP kinases and the PI3K/AKT pathway are both involved in the complex modulation of signalling pathways, which regulates cellular processes like proliferation, survival/death, and motility, usually altered in carcinogenesis.

It has been reported that EGCG inhibits IGF-1 receptor activity with an IC_{50} of 14 μM [34]. Treatment of DU145 and LNCap cells with subapoptotic EGCG doses reduces IGF-induced growth [35].

Siddiqui et al. [36] found that EGCG is able to: (i) decrease PI3K and phospho-Akt levels and (ii) increase ERK1/2 level in both DU145 and LNCaP cells. Treatment of PC3 cells with EGCG results in activation of the ERK1/2 pathway, that is, not dependent by mitogen-extracellular signal-regulated kinase (MEK), the immediate upstream kinase responsible for ERK1/2 activation, suggesting an MEK-independent signalling mechanism. Pretreatment of PC3 cells with a PI3K inhibitor partially reduced both EGCG-induced ERK1/2 phosphorylation and cell proliferation inhibition. These results suggest that ERK1/2 activation via a MEK-independent and PI3-K-dependent signalling pathway is partially responsible for the antiproliferative EGCG effects in PC3 cells [20].

2.6. Androgen Receptor (AR) and Prostate-Specific Antigen (PSA). Experimental evidences suggest that androgens are

involved in PCa development and progression, being AR the essential mediator for androgen action. Detailed mechanisms of AR activation and modified function in PCa are reviewed in [37–39]. Briefly, the AR is a nuclear receptor activated through binding of its cognate ligands, such as testosterone and 5alfa-dihydrotestosterone, and consequent dissociation by the heat shock proteins (normally bounded to it in the resting state). The activation process involves several coactivators recruitments. Activated AR up regulates the transcription of genes containing androgen response elements in their promoters as PSA gene that, specifically expressed in prostate, has been widely utilized for PCa screening, in the last 20 years [40]. A very recent report by means of a fluorescence resonance energy transfer (FRET) based assay provides evidence that EGCG is a direct antagonist of androgen action.

EGCG is capable to physically interact with the ligand-binding domain of AR by replacing a high-affinity-labeled ligand (IC_{50}: 0.4 μM) [41].

In different LNCaP sublines, EGCG suppresses cell proliferation, PSA expression, and AR transcriptional activity, at concentration comprised in the 10–20 μM range [42, 43]. The effect on PSA expression might be related to reduction of AR activity, but it should also be considered, that EGCG, *in vitro,* can down regulate PSA by direct action on transcription and translation mechanisms [44, 45].

3. PCa Chemoprevention by Green Tea Polyphenols in Transgenic Mouse Model

Progress toward understanding the PCa biology has been slow due to the few animal research models of tumour onset and progression, available to study the spectrum of this uniquely human disease. Genetically engineered mice are being increasingly employed for delineating the molecular mechanisms of PCa development and the potential of new compounds as chemotherapeutic/chemopreventive drugs against it. Animal models present a rapid tumor growth comparing to the long latency of human PCa, which, on the other hand, makes the disease an ideal target for chemoprevention strategies. Mouse dorsolateral prostate lobe is functionally equivalent to human prostate peripheral zone, from where the majority of human cancer originate [46]. Preclinical studies with GTCs or with pure EGCG, administered at a human achievable doses, have been conducted in both transgenic animals and xenograft tumor models, in which murine and human cell lines, derived from primary tumor or metastasis, have been implanted subcutaneously [47, 48] or injected intraprostatically [49].

To the aim of studying human CaP, autochthonous murine models appear more suitable than orthotopic cell lines transplantation. In fact, transgenic mice exhibit sets of interactions between the different cellular, tissue and hormonal compartments appropriate to human prostate. Among the several lines of transgenic mice generated models, the transgenic adenocarcinoma of mouse prostate (TRAMP) has been well characterized and employed for a number of pre-clinical trials. Mice expressing the transgene display progressive forms of prostatic disease that histologically resemble human PCa, from mild intraepithelial neoplasia (PIN) to poor differentiate adenocarcinoma phase (PD) and finally to metastatic spread [50]. In the TRAMP model, the SV40 early genes (T and t antigens, Tag) are under the control of the minimal rat Probasin promoter −426/+28—fragment [50], which renders the transgene expression androgen dependent, restricting it to the epithelial cells of the dorsolateral and ventral prostate lobes, thus abrogating p53 and Rb function [51] and inactivating protein phosphatase 2A (PP2A), specifically in this tissue [52].

In many experimental studies conducted by Gupta et al. [53] and by other authors [54–57], TRAMP mice aged from 8 to 32 weeks, received 0.1% oral infusion of a 95% GTCs enriched mixture. The animals, when compared to water-fed TRAMP mice, presented a significant delay in primary tumor incidence and almost complete metastases inhibition; prostate and genitourinary tract weight, a well-known tumor growth index, was decreased (64% and 72%, resp.), correlating with the reduced expression of Proliferating Cell Nuclear Antigen (PCNA).

The insulin-like growth factor pathway IGF/IGFBP-3 has been suggested to regulate PCa growth and development through its gradually increased activation during cancer progression.

After GTCs administration TRAMP mice showed a significant decrease in the IGF/IGFBP-3 ratio [54], accompanied by an inhibition of the downstream signaling cascade that involves both PI3K/AKT and MAPK pathways. Also, a parallel inhibition of vascular endothelial growth factor (VEGF), matrix metalloproteinases MMP-2, and MMP-9 expression were demonstrated [55]. Furthermore, the authors showed that metastasis-promoting Mts1 (S100A4) level, that, as a rule increases in cancer development, resulted in markedly decreased, E-cadherin level, that is progressively lost during cancer progression, where restored [56]. Also, the NFκB pathway activity, generally activated as a function of tumor grade, was reduced after 32 weeks of EGCG treatment, at a time when a shift in balance between Bax and Bcl2, favoring apoptosis, also occurred [57].

Under similar experimental conditions, we observed that, while 100% of TRAMP mice underwent PCa at 24 weeks of age, exhibiting tumor cell transendothelial passage in the absorbing lymphatic vessels, only 20% of the animals receiving 0.3% of GTCs (oral infusion), developed the neoplasm [19, 58–60]. In TRAMP mice presenting tumor growth arrest, a sequence of events were demonstrated, such as downregulation of H3 histone (a process usually affecting chromatin structure and gene expression), upregulation of growth arrest-Specific gene 1 (GAS1) and suppression of Mini-Chromosome Maintenance protein 7 (MCM7), a marker of DNA synthesis, essential for its replication, that is aberrantly expressed in various cancer types.

Interestingly, the level of the secretory protein clusterin (CLU) and mRNA, dramatically downregulated with the disease onset and development, resulted to accumulate progressively in the prostate after GTCs administration and remain undetectable in the 20% of animals that presented PCa, in spite of receiving GTCs solution. In correlation with

these observations, when tumor progression was inhibited, organelles committed to protein synthetic and secretory activities, as endoplasmic reticulum and Golgi apparatus, appeared significantly reduced in the prostate, suggesting possibly protein posttranslational processes alterations [60]. We suggest that CLU might participate in the chemopreventive action exerted by GTCs in TRAMP mice [19].

Harper et al. [61], after treating a cohort of TRAMP mice with almost pure 0.06% EGCG in drinking water, demonstrated that the compound can act only by slowing PCa progression. EGCG inhibited early (12-week-old mice), but not late (28-week-old mice) PCa stage. The treatment resulted in many various effects: AR, IGF1, and its receptor decreased level, apoptosis-reduced cell proliferation, and phospho-extracellular signal-regulated kinases 1/2, cyclooxygenase-2, and inducible nitric oxide synthase reduced activities. To a better interpretation of the data, it is worthwhile noting that green tea polyphenols bioavailability and transformation are key factors that can limit the compound activities *in vivo*; EGCG, as a single agent, may present a low bioavailability and/or slow its rapid metabolism, when stabilized by the naturally occurring mixture of green polyphenols. In addition, attention has to be paid when comparing experimental works on TRAMP mice colonies obtained through different background strains. In fact, the genetic background may have a profound effect on some aspects of tumor initiation/progression in this animal model.

The latest experimental work from Adhami et al. [62] confirmed the efficacy of 0.1% GTCs in drinking water to TRAMP mice, starting at defined stages of PCa onset and progression: 6 weeks (normal prostate), 12 weeks (PIN), 18 weeks (well-differentiated adenocarcinoma, WD), 28 weeks (poorly-differentiated adenocarcinoma, PD), finally cancer development. Tumor-free survival was indeed extended to 38 weeks in the 6 weeks group mice, but only to 24 weeks in the 18 weeks group, compared with 19 weeks in water-fed controls. Additionally, IGF and its downstream targets were significantly inhibited only when intervention was initiated at early stages.

The study design was conceived as a response to the outcomes of human clinical trials, based on alternative and complimentary green tea therapy, showing minimal effect on hormone-refractory PCa stage (CRPC) [63, 64], but lower incidence on high grade PIN stage [65, 66].

Extensive laboratory studies in multiple animal models consistently show the GTCs inhibitory activities against prostate carcinogenesis [14] and suggest the importance of identifying the most vulnerable PCa stages towards GTCs chemoprevention in humans.

4. Possible PCa Chemoprevention by Green Tea Polyphenols in Humans

4.1. PCa: Statistics and Importance for Chemoprevention Strategies. PCa is the second most common cancer in American men with a 1 in 6 lifetime risk of developing it. The National Cancer Institute (NCI) estimated that approximately 217,730 new cases PCa would have been diagnosed in 2010 and there would have been approximately 32,050 PCa deaths [67]. In Europe, PCa has become the most common non-skin cancer neoplasm among men, with an estimated 382,000 cases occurred in 2008 [68]. Incidence has increased rapidly over the past two decades, and rates are dramatically influenced by PSA testing, as well as by latent cancer detection in prostate surgery.

According to a recent report from the prostate, lung, colorectal, and ovarian cancer screening trial, six annual PCa screening programs (10 years followup results) led to increased number of diagnosed cancers, in the screening cohorts, but no cancer-specific survival advantage was seen for the group. Therefore, the real possibility to "overdiagnose" and "over-treat" cancers which are not life threatening, must be carefully taken in account [69]. A reasonable explanation for the low efficacy of invasive radical intervention, which are the gold standard in clinical practice after a diagnosis of confined PCa, relies on the fact that the disease is biologically heterogeneous and its natural history is almost unpredictable. Some cancers growth slowly, showing indolent course, while others are very aggressive, quickly progressing to advanced metastatic stage, that is almost an incurable disease [70]. Therefore, it is important and urgent to search for prevention strategy, effective when the disease is still at an early and potentially curable stage. PCa is an ideal candidate for chemoprevention, because it has a high prevalence, a long latency, and it is potentially lethal. Preventive strategies could carry a high economic benefit on the healthcare system reducing the costs associated with PCa diagnosis and therapy, moreover, it might have a deep positive impact on the patients quality of life, reducing the morbidity associated with radical surgery (incontinence and impotence) [71].

4.2. Epidemiological Evidence of Green Tea Efficacy in PCa Chemoprevention. PCa etiology is multifactorial, but the marked disparity in its incidence between "Eastern" and "Western" cultures suggests that dietary and lifestyle factors play an important role in the disease development and progression. This is strongly supported by migratory studies showing that Asian men, who relocate to the United States of America and adopt a western lifestyle, have a significantly higher PCa risk, when compared to their native Asian counterparts [72, 73].

During the last two decades, the relationship between tea consumption and cancer has been a subject of research interest for many investigators. Unfortunately at present, many epidemiological studies present conflicting results about the green tea role in cancer prevention. Recently, an exploratory meta-analysis of observational studies supported the hypothesis that green, but not black tea, may have a protective effect against PCa. A total of six epidemiological studies, including two case-control studies as well as four cohort studies, evaluated green tea role in reducing developing PCa risk [74–79]. A borderline statistical significant decrease in the disease development is presented with increasing green tea intake and, moreover, only case-control studies (odds ratio, OR = 0.43; 95% CI: 0.25–0.73), but not

prospective cohort studies (OR = 1.00; 95% CI: 0.66–1.53) reached a statistically significant result [80].

Negative and conflicting results in epidemiological data may be due to study design pitfalls and to many hardly controllable variables, like tea infusion composition and way of preparation (temperature), GTCs bioavailability, diet and the lifestyle of the people included in the study, and last but not least, genetic differences in the ability to metabolize GTCs.

4.3. Pharmacokinetic Studies, Phase I Clinical Study, GTCs Metabolism and Tissue Distribution. Polyphenon E is a GTCs-enriched and defined product, virtually caffeine free (<0,5%) produced by Mitsui Norin Co. Ltd, a new drug investigated by the Food and Drug Administration. It contains 80% to 98% total catechins by weight, with EGCG main component, accounting for 50% to 75% of the material.

Chow et al. [81, 82] performed several pharmacokinetic phase I studies (in healthy volunteers) utilizing Polyphenon E (capsules) to determine the systemic tea polyphenols availability after a single or multiple dose and various dosing condition (200, 400, 600, 800 mg of EGCG). In the single-dose study, Polyphenon E was compared to pure EGCG for differences in pharmacokinetic parameters [81]; after its administration, at the four dose levels above mentioned, the average EGCG peak plasma concentration (C_{max}) was 72.7 ± 66.4, 125.3 ± 50.4, 165.7 ± 126.9, and 377.6 ± 149.8 ng/mL. Similar results were obtained after pure EGCG administration, at the same concentration, suggesting that GTCs and Polyphenon E do not have any effect on the EGCG pharmacokinetics. It should be noted that tea catechins bioavailability is quite low in humans, resulting in plasma concentrations 5 to 50 times less than concentrations shown to exert biological activities *in vitro* [14].

In the multiple dose study, the same authors evaluated effects and safety following chronic Polyphenon E administration [82], concluding GTCs do not accumulate in the body and are safe for human health.

A third pharmacokinetic study from the same authors [83], evaluated the role of fasted or fed state on Polyphenon E bioavailability (400, 800, or 1200 mg of EGCG), concluding that C_{max} of free catechins (EGCG, EGC, EC) can be increased of about 3-folds, when administered in a fasted state. The first study demonstrating that GTCs can be detected in the prostate tissue after green tea consumption has shown that after a short period (5 days) of green tea continuous intake (1.42 L of brewed tea divided in 5 daily doses), the prostate GTCs concentration were 0.1, 0.043, 0.040, 0.0021 (nmol/g tissue) for EGC, EC, EGCG, and ECG, respectively [84].

4.4. Proof of Principles and Phase II Studies of Green Tea Efficacy for PCa Chemoprevention and Treatment. There have been 5 intervention studies evaluating the GTCs effect on PCa treatment or prevention [63–65, 85, 86]. Three were single-arm open-label phase II trials, performed in patients diagnosed with a castration resistant PCa (CRPC) [63, 64, 86] and one was a pilot proof of concept study, performed to evaluate GTCs ability to reduce cancer incidence in a well-defined cohort of patients, bearing premalignant lesions (HGPIN) [65].

The most recent study, a randomized, double-blind, placebo-controlled phase II trial evaluated the effect of short-term Polyphenon E administration in men with PCa scheduled to undergo radical prostatectomy [85].

The primary endpoint of the trial reported in [64] was to determine the capacity of nonstandardized green tea powder to produce a PSA base level decline in 42 men with clinical CRPC evidence. Only 1 patient, among 42, manifested a transitory 50% decreased PSA level from baseline, during the 6 months followup. The negative result of this study needs to be critically considered; it should be taken into account that the enrolled population comprised men with an advanced cancer stage, that acquired resistance to standard hormone deprivation therapy, and that a nonstandardized GTCs preparation was employed.

Choan et al. [63] published the results of a study aimed to evaluate the effect of a standardized green tea extract (250 mg GTCs/day) on PSA level or measurable marks of the disease progression, after a minimum of 2 months therapy. Only 15, out of the enrolled 19 patients, completed at least 2 months therapy and all of them exhibited a progressive disease in the first 4 months. In addition, a very small population with an advanced cancer stage, unresponsive to previously administered hormonal therapy, was considered.

On the basis of encouraging results obtained by us and others in the PCa chemoprevention with animal models [19, 53, 62], our research group performed a proof of concept trial in a well-selected patients cohort at high risk to develop PCa [65]. We enrolled 60 patients with HGPIN diagnosis, that were randomly assigned to receive, according to a double-blind procedure, a standardized GTCs formulation (600 mg/day), or identical placebo capsules for 1 year. GTCs composition, virtually caffeine free, was total catechins 75.7%; EGC, 5.5%; EC, 12.2%; EGCG, 51.9%; ECG. All the patients, during the study, were subjected to regular prostate biopsy (6 and 12 months after the study beginning) to asses differences in PCa values between the two arms. Total serum PSA concentration was measured at 3, 6, 9, and 12 months, to check whether GTCs administration would reduce the value. Because concomitant benign prostate hypertrophy (BPH) was very common among the patients enrolled, we also evaluated the International prostate symptom score (IPSS) and quality of life (QoL) after 3 months of GTCs administration in a subcohort of patients with low urinary tract symptoms (LUTSs), as a further secondary end point. We found that 9 out of 30 patients receiving placebo developed PCa, while only 1 out of 30 patients receiving GTCs was found PCa positive after prostate biopsy. We did not find any significant difference in PSA values between the 2 arms, but we found a significant IPSS and QoL scores improvement.

Our results point the attention on the fact that GTCs should be considered interesting and promising natural compounds for PCa chemoprevention, while they are almost ineffective in case of full-developed metastatic neoplasia (CRPC). We also found that PCa incidence remained low

in patients belonging to the GTCs-arm, even two years after therapy suspension [66]. This result may suggest that chemoprevention activity and clinical benefits achieved with GTCs are stable over time. We hope that our data will be confermed by a large phase II study, now ongoing, sponsored by NCI. This trial is aimed to enroll about 300 HGPIN patients to be given 400 mg/day of Polyphenon E for PCa prevention. (Study of Polyphenon E in men with high-grade prostatic intraepithelial neoplasia, Protocol IDs: MCC-15008, R01 CA12060-01A1, NCT00596011).

McLarty et al. [86] published the results of a study aimed to evaluate the effect of short term (median treatment period 34.5 days) GTCs administration in a population of PCa diagnosed patients, scheduled for radical prostatectomy. The supplementation, performed with an high, well-tolerated, Polyphenon E dose (1.3 g GTCs/day) produced a significant decrease of biomarkers relevant for PCa development, like HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor), IGF-1 (insulin growth factor-1) and IGFBP-3 (insulin growth factor binding protein-3). These results support a potential GTCs role in PCa chemoprevention and treatment in early confined stage.

Disappointingly, the positive Polyphenon E influence resulted not statistically significative according to the results of a randomized, double-blind, placebo-controlled trial, having a study design strictly close to that of McLarty [85]. The reason(s) for the discrepancy between the two studies remains to be elucidated, but it should be considered that the lack of a control group in the single arm study made it easier to gain statistical significance. On the other hand, it may indicate that future studies would need a larger population to show a statistically significant difference in systemic biomarkers and Polyphenon E, or generally standardized GTCs formulation, should be preferentially tested in longer-term intervention studies and in a precancerous model, where its effects have a best chance to be demonstrated.

5. Molecular Mechanisms for Green Tea Polyphenols Anticancer Activity in PCa: An Emerging Role for Epigenetics

5.1. Modulation of Epigenetic Mechanisms. In the last five years, GTCs have been shown to be able to modulate all the principal epigenetic mechanisms: DNA methylation, regulation of chromatin structure (through histone posttranslational modifications), and alterations of noncoding miRNAs. Epigenetics is defined as changes in gene expression that do not involve changes in the DNA sequence. Importantly, these changes are both reversible and heritable through division of somatic cells, making epigenetic regulation of gene expression a dynamic process that plays a crucial role in a vast number of biological processes including development, cell differentiation, stem cell maintenance, and tissue homeostasis [87]. Deregulation of epigenetic mechanisms is found in numerous diseases and in virtually every kind of cancer. This observation, together with the dynamicity, and therefore the potential reversibility, of epigenetic mechanisms sparked the interest in developing epigenetic drugs capable to modulate DNA methylation and chromatin structure in cancer cells. In the last few decades, several epigenetic drugs have been developed and are now either tested in clinical trials or already established in the clinical care, even if the drugs, besides modulating epigenetic mechanisms, appear to exert a certain degree of cytotoxicity.

The observation that GTCs, together with other dietary polyphenols, can regulate specific epigenetic features of premalignant and malignant cells opens new possibilities for epigenetic therapy

5.2. DNA Methylation. DNA methylation is an important epigenetic determinant in gene expression. As a matter of fact, it participates in the maintenance of DNA integrity and stability, in chromosomal modification, and development of mutations. Generally, DNA hypermethylation is associated with genes inactivation and global genomic hypomethylation is associated to chromosomal instability induction. The DNA methylation occuring on the carbon-5 position of cytosine residues within a CpG dinucleotide sequence, represents the most studied epigenetic marker. In normal tissues, the process shows a bivalent function: CpG sites clustered in regulatory regions, within promoters and enhancers (CpG islands), are usually unmethylated, allowing for genes to be expressed; while sparse CpG sites (throughout the genome) are usually methylated, contributing to genome stability [88]. DNA methylation is crucially dysregulated in the vast majority of cancers, where CpG islands become hypermethylated, causing silencing of many genes, involved in cell cycle regulation, tumor suppression, DNA repair enzymes, receptors activity, and apoptosis, while the sparse CpG sites are usually subject to hypomethylation, favoring genomic instability, a common feature of cancer etiology [89].

Epigenetic analysis offers a potential noninvasive blood marker, complementary to PSA, for a preliminary PCa diagnosis. Since inhibition of DNA methyltransferases (DNMTs) may prevent hypermethylation and silencing of tumor suppressor key genes, it is reasonable to suppose that enzyme inhibition, along with histone deacetylase activation, may contribute to cancer treatment and or carcinogenesis prevention.

In human prostate cancer PC3 cells, as well as in other cell lines, Fang et al. [90] demonstrated that EGCG decreases total DNMTs activity, leading to reactivation of several genes silenced by methylation, such as retinoic acid receptor beta (RARb), which expression results increased by lowering the methylation levels of its promoter. This is the first demonstration of such an activity of a commonly consumed dietary constituent.

Recently, it has been reported that exposure of human prostate cancer LnCaP cells to GTCs causes a time-and concentration-dependent reactivation of the expression of the gluthathione-S-transferase p1 gene (GSTP1) [91]. The gene, mostly studied for methylation in PCa, results hypermethylated in almost 90% of the tumors [92]; in fact, it is recognized as a molecular PCa hallmark. Cells treatment with GTCs results in GSTP1 promoter demethylation, associated with a significant reduction of DNMT1 activity. Interestingly, the treatment does not cause global sparse CpG sites

hypomethylation, contributing to maintain genome stability. The authors present GTCs as excellent candidates for epigenetic chemoprevention against PCa, even more favorable than the most commonly DNMT inhibitors employed, such as 5-aza-2′deoxycitidine.

There is growing evidence that the epigenetic mechanisms that impact DNA methylation and histone status, also contribute to genomic instability. Instead, GTCs administration lacks of toxicity, do not reactivate the prometastatic gene S100P, as a reverse response of 5-aza-2′deoxycitidine administration, and is able to alter chromatin modeling by histone acetylation (the second global epigenetic mechanism of gene regulation, see below) [91].

Almost at the same time, Morey Kinney et al. [93] showed that GTCs administration to TRAMP mice (spontaneously developing prostate adenocarcinoma) had very little effect on DNA methylation. They have found that 5-methyldeoxycitosine level, together with methylation levels of B1 repetitive elements and the Mage-a8 gene, that are correlated to the phenomenon, remained unchanged in wild-type and TRAMP mice prostate. Also, they performed a genome-wide DNA methylation profiling of the HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) [94] and found no significant hypomethylation. Their study, however, arises some doubts on the interpretation of the data: most of the analysis is directed towards sparse CpG site, rather than CpG islands. Indeed, the lack of global hypomethylation in GTCs fed mice agree with the above results obtained in LnCaP cells [91]. Second, but most important, in their study, GTCs treatment did not cause chemopreventive effects on PCa development, in contrast with previous reports by other authors [19, 53]. The different composition of the GTCs preparation used by Morey Kinney et al. [93] might account for this discrepancy, since it contained only about half of the ECGC concentration (35%) relative to the other polyphenols, when compared to the preparations used in other studies (51.9% in [19] and 62% in [53]).

Among the still growing number of experiments aimed to clarify the GTCs power as epigenetic drugs against PCa, there is plenty of evidence that the GTCs can interfere with DNA methylation pathways *in vitro*, leading, also, to the reactivation of the tumor suppressor p16, known to be involved in the cell cycle regulation [95].

Three different molecular mechanisms, at least, have been proposed to explain GTCs effects on the DNA methylation process (Figure 2). In human models, GTCs are demonstrated to be readily methylated, by catechol-O-methyltransferase (COMT) which utilizes S-Adenosyl-L-methionine (SAM) as a methyl donor, producing equimolar concentration of S-adenosyl-L-homocysteine (SAH). It should be noted that SAM is an indispensable methyl donor for DNMTs and SAH is a potent inhibitor of DNMTs activity. Since GTCs administration causes both decreased SAM and increased SAH cellular concentrations, the hypothesis is proposed that GTCs may act by perturbing the regulatory physiological homeostasis of the two molecules [96].

This mechanistic explanation is supported by Lee et al. [97]. The authors, utilizing multiple modelling tools, have shown that the inhibitory effect exerted by several polyphenols on human DNMT1 is increased after *in vitro* COMT addition. In these experiments, EGCG resulted the much more potent DNMT1 inhibitor, able to act also independently of the COMT presence. In addition to the indirect action on DNA methylation demonstrated by [96], EGCG is able to inhibit DNMT1 by directly binding to the enzyme [90], with the gallic acid moiety of the molecule playing a crucial role in the interaction (Figure 2).

In human cell lines, EGCG is shown to act as an antifolate compound and disturb the folic acid metabolism by inhibiting dihydrofolate reductase. Since folic acid physiologically modulates DNA methylation, Navarro-Peran et al. [95] hypothesize that many molecular effects imparted by EGCG administration might be explained by this kind of interference with DNA methylation (Figure 2).

5.3. Chromatin Structure. Changes in the chromatin condensation, a crucial mechanism to control gene expression in eukaryotic cells [98], is regulated by at least eight posttranslational modifications of histones, such as acetylation, methylation, phosphorylation, poly-ADP-ribosylation, ubiquitination, glycosylation, and so forth, predominantly occurring on lysine residues [99]. Combinations of the different modifications constitute the histone code that defines the actual or potential transcriptional state [100] through proteins-DNA interaction, which in turns regulates gene expression.

Among the most studied processes, histone acetylation/deacetylation is finely regulated by balancing the activity of acetyl-transferases (HATs) and histone deacetylases (HDACs) enzyme families; inhibition of the latter rapidly results in histone hyperacetylation. Acetyl groups added to lysine residues by HATs, mainly on H3 and H4 histones, neutralizes aminoacid positive charge and consequently loosens DNA binding to histone complexes, ultimately resulting in chromatin relaxation and gene activation [99].

Being HDACs overexpression implicated in protecting cancer cells from genotoxic insults, many HDAC inhibitors synthesized in order to favor tumor suppressor genes re-expression and silence DNA repair pathways, are gaining a momentum as a novel cancer therapy. Raiendran et al. [101], however, indicate how dietary phytochemicals, by affecting the epigenome, and also can trigger DNA damage and repair mechanisms.

With regard to PCa, Pandey et al. [91] have observed a time-dependent inhibition of the total HDACs activity, after GTCs administration to LnCaP cells, correlating with mRNA and HDAC1-2-3 classes decrease.

Recently, GTCs administration was shown to inhibit HDAC1 activity and protein expression in PCa cell lines as LnCaP (harboring wild-type p53) and p53-null PC3 (lacking p53). When administered together a proteasome inhibitor, GTCs effect resulted prevented, indicating that HDAC1 is proteasoma degradated. HDAC1 inhibition was followed by up regulation of the cell cycle inhibitors WAF1/p21, ultimately inducing G_0/G_1 phase cell cycle arrest and apoptosis in both cell lines, irrespective of their p53 status [102].

A sustained DNA damage response, coupled with insufficient repair mechanisms, may be a pivotal mechanism

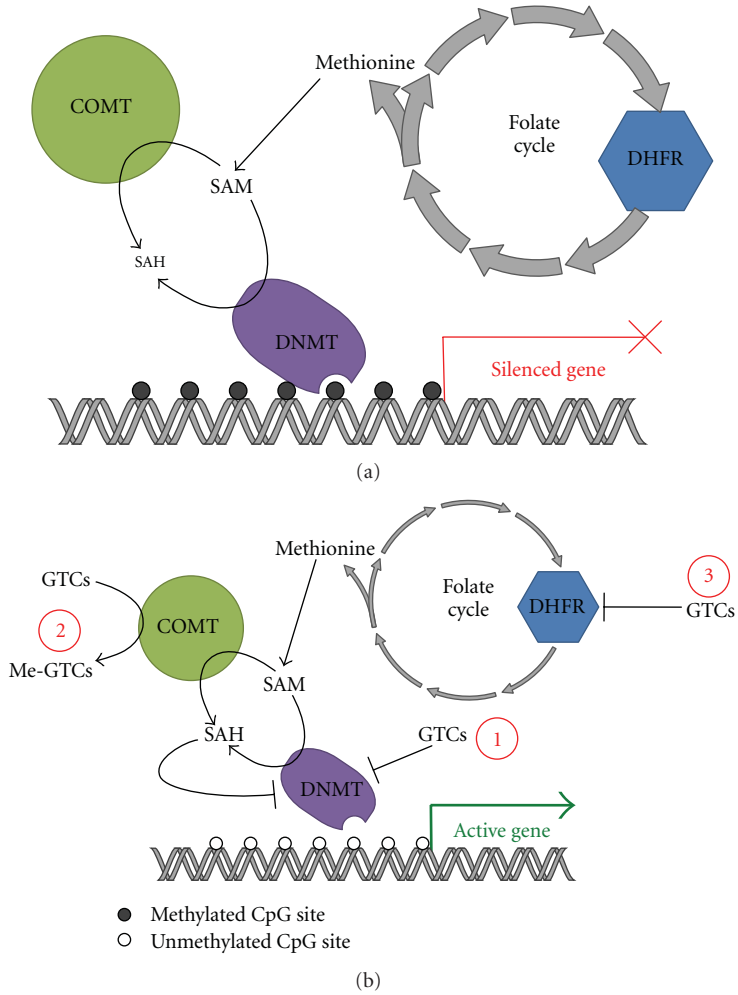

Figure 2: Mechanisms of inhibition of DNMTs by GTCs. (a) In a cancer environment, specific genes are silenced by hypermethylation of their promoters. DNA hypermethylation is catalysed by DNMTs, which use as a substrate SAM and release SAH as a by-product. (b) Within this context GTCs are able to inhibit DNMTs through three distinct mechanisms. (1) Direct inhibition. (2) GTCs are methylated by COMT, resulting in a depletion of SAM and accumulation of the DNMT inhibitor SAH. (3) Direct inhibition of DHFR, resulting in disruption of the folate cycle that influences negatively the levels of SAM. Inhibition of DNMTs ultimately results in DNA hypomethylation and re-expression of previously repressed genes.

for apoptosis induction in cancer cells exposed to dietary phytochemicals. The above findings provide new insight into the GTCs mechanisms, suggesting a novel approach to prevention and/or therapy.

5.4. microRNAs. microRNAs (miRNAs) comprise a novel class of endogenous, small, noncoding RNAs that control gene expression by acting on target mRNAs for degradation and/or translational repression. Transcription of miRNA genes and protein-coding genes shares common regulatory mechanisms: miRNA genes can be embedded in the introns of protein coding genes or can derive from their own transcript units in intergenic regions of the genome. When miRNA genes are located within introns of protein-coding genes, primary miRNA biogenesis is controlled by the same transcriptional mechanisms as the parent gene. In contrast, an independent miRNA gene will have its own transcriptional controls. Interestingly, multiple miRNAs can be produced within a single transcript, each of which can act independently [103, 104]. The long primary nuclear miR transcript ("pri-miRNA") undergoes maturation by the RNase-III Drosha/Dgcr8 enzyme complex, generating a precursor miRNA ("pre-miRNA") that is exported from the nucleus by exportin-5 [105]. A second cleavage takes place in the cytoplasm, involving the action of a complex containing another RNAase III enzyme named Dicer [106], to generate a single-strand mature miRNA. Mature miRNA, is usually incorporated in RNA-Induced Silencing Complex, a complex of proteins that is responsible for silencing the target mRNA [107]. Translational inhibition or degradation of targeted mRNA transcripts is due to imperfect or perfect base pairing between positions 2 to 8 from the 5′miRNA (also known as the seed sequence), with the 3′Un-Translated Region of their target mRNAs [104].

miRNAs are emerging as important regulators of gene expression and we expect them to be key players in intracellular signalling, thus enabling to close gaps of knowledge in molecular pathways. Pathogenic roles of miRNAs were initially described in cancer and miRNAs have now become a hot topic in medical research [108].

5.5. microRNA in PCa. To our knowledge, by employing miRNA microarray analysis, 7 miRNAs (miR-145, miR-141, miR-125, miR-1, miR-133, miR-106b, and miR-16) have been found downregulated in PCa [109–111]. In addition, the chromosomal region containing the miR-15/16 cluster and miR-101 is often lost during PCa progression. Table 1 summarizes recent publications on miRNAs in PCa.

Bonci et al. [112] demonstrated that reduced levels of miR-15/16 are associated with PCa growth and due to an increase in the protein levels of their target genes Bcl2, cyclin D1, and WNT3A. Conversely, overexpression of miR-15/16 suppresses tumor growth and induces its regression. Expression of miR-101 inversely correlates with upregulation of its target, enhancer of zezte homolog 2 (Ezh2), which is highly expressed in CRPC [113].

miR-331-3p and miR-449a are downregulated in PCa, contributing to cancer growth by overexpressing their targets ErbB2 [114] and HDAC1, respectively [115]. Poliseno et al. [116] demonstrated that miR-22 and the miR-106b~25 cluster are overexpressed in PCa, and potentiate cellular transformation both *in vitro* and *in vivo*. Intronic miR-106b~25 cluster cooperates with its host gene *MCM7* in cellular transformation both *in vitro* and *in vivo*.

Other tumor suppressor miRNAs in PCa are miR-34 expecially in p53-deficient PCa cells [117] and miR-330, which suppresses E2F-1 and E2F-1-mediated AKT phosphorylation [118]. In PCa cells, miR-21 is induced by stimulation of androgen receptors and mediates hormone-dependent and -independent cell growth [119]; in contrast, miR-221 increases in androgen-independent tumors [120]. MiR-146a, which is reduced in androgen-independent PCa cells, inhibits proliferation and cell invasion by targeting of RhO-activated protein Kinase 1 [121].

miRNAs that have been detected in human serum and plasma specimens, and circulating miRNA profiles, have now been associated with cancer, as an emerging class of diagnostic and prognostic biomarkers. Specifically in PCa, the expression of miR-141 has been found to be elevated in the plasma of PCa patients [122], where levels of miR-141 in PCa were able to predict tumor progression, when compared with other validated prostate biomarkers [123]. Finally, miR-141, miR-298, and miR-375 were also found to be upregulated at differential levels in the serum of men with CPRC [124].

5.6. microRNAs in PCa and Polyphenols. Recently, studies support a growing interest in the chemopreventive role of dietary agents such as polyphenols, as modulators of miRNA profiles in cancer progression and prevention [125]; (Table 2).

Table 1: miRNAs involved in PCa.

miRNAs	Target genes	References
miR-15/16	Bcl2, CCD1, WNT3a	[112]
miR-101	Ezh2	[113]
miR-331-3p	Erb2	[114]
miR-449a	HDAC1	[115]
miR-146a	ROCK1	[121]
miR-106b/25	PTEN	[116]
miR-330	E2F1	[118]

Table 2: miRNAs modulated by polyphenols in PCa.

miRNAs	Polyphenols	Regulation	References
miR-21	EGCG	down	[41]
miR-330	ECGC	up	[41]
miR-1296	Genistein	up	[129]
miR-17/92	Resveratrol	down	[130]
miR-106a/b	Resveratrol	down	[130]

Although the chemopreventive effects of EGCG, as well as other polyphenols, have been largely demonstrated in PCa by us [19, 59, 126] and other groups (reviewed in [127]), only few reports discussed the regulatory effect of miRNA expression by dietary components in PCa. At the moment, the first and only study that clearly correlated miRNA-regulation with catechins treatment in PCa is performed by Siddiqui et al. [41]. The authors studying the role of androgen receptor (AR) in both early and advanced stage of PCa etiology, showed that the androgen-regulated miRNA-21 and the tumor suppressor miRNA-330, commonly regarded to play a role in PCa, are, respectively, down- and upregulated in a xenograft mice model for PCa, following EGCG treatment. These findings strengthen EGCG as an androgen receptor signalling antagonist, which can block AR gene expression and cell growth in the human PCa cells. EGCG is suggested as a chemotherapeutic agent against CPRC.

Basing on experiments performed in pancreatic cells with the phytochemical substance curcumin by Bao et al. [128], it appears worthwhile investigating the EGCG effect on miR-200 and miR-21 expression in PCa cells. Interestingly, the increase of the two above miRNAs is correlated to the induction of the critical tumor suppressor gene PTEN, frequently defective in PCa.

A study examined the effect of the phytochemical compound genistein on minichromosome maintenance (MCM) genes, commonly dysregulated in cancer, showing that MCM2 expression genes is higher in PCa samples, whereas miR-1296 was significantly downregulated [129]. Genistein induced miR-1296 expression and subsequently downregulated the MCM2 expression, along with cell cycle arrest in S-phase. This study is worth of our attention, since we demonstrated a dramatic MCM7 genes suppression by GTCs in the prostate of TRAMP mice [59]; analyses of therapeutic effect of catechins on miR-1296 and on miR-106b~25 cluster, request further investigations.

Table 3: Principal progressive phases in human PCa development.

Definition	Acronyms	STAGE of PCa development	STAGE where CTCs could be possibly used
Proliferative Inflammatory Atrophy	PIA	Precursor of cancer initiation	YES
Prostatic Intraepithelial Neoplasia	MILD PIN	PreMalignancy	YES
	HIGH PIN	Malignancy	YES
Adenocarcinoma	WD	Well-differentiated Cancer	NO
	PD	POOR differentiated Cancer	NO
Fully Developed Metastatic Neoplasia	CRPC	Hormone refractory castration resistant Terminal cancer	NO

Finally, utilizing miRNA microarrays, Dhar et al. [130] found that miR-17-92 and miR-106ab clusters, with well-recognized oncogenic properties, were significantly downregulated after treatment with the phytochemical compound resveratrol.

To corroborate the hypothesis that EGCG may act as a negative tumorigenesis process, downregulating oncogenes miRNAs and/or upregulating tumor suppressor genes miRNAs, more definitive informations are needed.

6. Conclusion and Future Perspective

Despite the routinary employment of intermediate-risk prognosticators such as PSA, Gleason score, and T-category, PCa remains a complicate malignancy, exhibiting high heterogeneity features both when present in latent, clinically indolent, and in progressively more aggressive stages, leading to the "hormone refractory state" (CRPC). This is the most devastating PCa form, representing the terminal stage of transition from the androgen dependence stage, against which, at the moment, no curative therapy exists.

Although radical prostatectomy is curative in the majority of patients with clinically localized PCa, up to 40% of them fail to respond to local therapy and develop PSA recurrence, as a sign of metastatic growth; ultimately, many of these patients die from their disease. While latent PCa appears similarly frequent in men with different ethnic background, residing in culturally diverse geographic locations, experimental evidences show that the quality of diet and lifestyle factors may carry the mechanisms triggering the transition to the "hormone-refractory state" and, at the same time, may be the target for the action of anticancer prevention.

These considerations support the increasing use of complementary and/or alternative therapies such as developing a diet-based combinatorial approach. Chemopreventive action of a naturally occurring, nontoxic agent, such as green tea polyphenols, could be useful in the PCa management. Just a postponement of the "hormone-refractory state" or the maintenance of the androgen dependence would produce chronic instead of terminal PCa. On the other hand, there is growing consensus that a large subset of patients do not require aggressive treatment. Recent preclinical evidences showing that GTCs can inhibit cell cycle, induce apoptosis, and modulate several signalling pathways, strengthened by experiments with animal models, have demonstrated the possible utilization of these compounds in selected human PCa stages for preventing its development (Table 3).

To date, although additional investigations are needed, a real cancer preventive activity by tea polyphenols has not been consistently observed in the few studies with humans versus the several studies with animal models so far achieved. Table 4 suggests two main differences between humans and animals studies: (1) relatively low quantities of GTCs intake by patients, as compared to TRAMP mice (it is conceivable that GTCs availability in the prostate is different in mouse than in humans; on the other hand pharmacokinetics studies demonstrate EGCG limited systemic concentrations); (2) various confounding factors (as genetic differences, diet, lifestyle and, etc.) present during the patients treatment, unlike the controlled conditions to which animals are subjected to optimize cancer prevention effect.

In the future, efforts will be needed to monitor GTCs administration to humans starting from the standardization of optimal GTCs compositions and doses, and continuing by enhancing GTCs bioavailability through nanocapsules or liposome deliver (nanochemoprevention). Simultaneous check of plasmatic and bioptic EGCG levels will be indispensable; it remains to be determined whether EGCG undergo autooxidation in the prostate gland, as it occurs in cell culture.

Certainly novel biomarkers focused not only on PCa epithelial cells but also on cell interaction with the extracellular microenvironment, that will improve the ability to detect PCa, predict lethality, and monitor response to therapies (many markers validated in experimental studies are not yet introduced as routine diagnostics).

Substantial information on tea polyphenols chemopreventive activity will emerge from long-term and rigorous clinical trials on well-designed cohorts of patients. Although it may appear simplistic that clinical PCa heterogeneity is attributable to underlying molecular heterogeneity, clinical trials still have to consider the need of cohorts with definite cancer risk, classified by their gene expression signature, that represents a clinically relevant genetic biomarker, independent of the current diagnostic variables. PCa develops via a limited number of alternatively preferred genetic pathways, providing genetic subtype (molecular classification) that may constitute a framework for investigating PCa and possibly explain the clinical heterogeneity of the disease with regard to both tumor progression and therapeutic response.

Table 4: Studies on the effect of GTCs administration to TRAMP mice and humans with PCa.

Experimental groups	GTCs dose g/100 mL [oral administration]	Formulation (%)	PCa phases age-dependent (weeks)	PCa inhibition (%)	References
TRAMP mice	0.06	**GTCs**[a] EGCG (93)	Start 5; end 12	83	[61]
	0.1	**GTCs**[b]: EGCG (62) ECG (24) EGC (5) EC (6)	Start 8; end 32	42	[53]
	0.1	**GTCs**[b]: EGCG (62) ECG (24) EGC (5) EC (6)	Start 6; end 38	~50	[62]
			Start 18; end 24	~20	
	0.3	**GTCs**[c]: EGC (5.5), EC (12.2) EGCG (51.9) ECG (6.1)	Start 8; end 24	80	[19]

	GTCs dose mg/day [oral administration]	GTCs providers	PCa phases	Percentage (%) of PCa inhibition	References
HUMANS	500	Sabinsa Corporation	CRPC	No effect	[63]
	6000	Unilever	CRPC	No effect	[64]
	1300	Polyphenon E Matsui Norin,	CRPC	Mild effect	[86]
	600	Polyphenon E Matsui Norin	HGPIN	33	[65]
	800	Polyphenon E Matsui Norin	CRPC	No effect	[85]

GTCs[a]: Roche, GTCs[b]: natural resources and products, GTCs[c]: isolated by the investigators; CRPC (castration resistant prostate cancer), HGPIN (high grade prostatic intraepithelial neoplasia).

Conclusively, to avoid difficulties in the interpretation of the data, the future tea polyphenols protocols should take into account the following: (1) standardization in the preparation of the substances employed, choice of their doses and concentrations, systematically tested in plasma as well in biopsies; (2) more accurate stratification of a large number of the patients utilized for the analysis and selection of an appropriate treatment duration.

Abbreviations

AR: Androgen receptor
PCa: Prostate cancer
EC: Epicatechin
ECG: (-)-Epicatechin-3-gallate
EC: (-)-Epicatechin
EGC: (-)-Epigallocatechin
EGCG: (-)-Epigallocatechin-3-gallate
GTCs: Green tea catechins
miRNA: MicroRNAs
ROS: Reactive oxygen species
PSA: Prostatic specific antigen
TRAMP: Transgenic adenocarcinoma of mouse prostate.

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Review Article

Flavonoids Inhibit the Respiratory Burst of Neutrophils in Mammals

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Neutrophils represent the front-line defence cells in protecting organisms against infection and play an irreplaceable role in the proper performance of the immune system. As early as within the first minutes of stimulation, neutrophilic NADPH oxidase is activated, and cells release large quantities of highly toxic reactive oxygen species (ROS). These oxidants can be highly toxic not only for infectious agents but also for neighboring host tissues. Since flavonoids exhibit antioxidant and anti-inflammatory effects, they are subjects of interest for pharmacological modulation of ROS production. The present paper summarizes contemporary knowledge on the effects of various flavonoids on the respiratory burst of mammalian neutrophils. It can be summarized that the inhibitory effects of flavonoids on the respiratory burst of phagocytes are mediated via inhibition of enzymes involved in cell signaling as well as via modulation of redox status. However, the effects of flavonoids are even more complex, and several sites of action, depending upon the flavonoid structure and way of application, are included.

1. Introduction

Phagocytes, including neutrophils, play a key role in host defense against invading pathogens and play a crucial role in inflammatory processes. Neutrophils infiltrate inflamed tissues, degranulate their secretory vesicles, and release large amounts of bioactive compounds. As early as within the first minutes of stimulation, neutrophilic cells release large quantities of highly toxic reactive oxygen species (ROS), during the so-called "respiratory burst." ROS are known to belong to the most efficient microbicidal mechanisms. Thus, neutrophils represent the front-line defence cells in protecting organisms against infection and play an irreplaceable role in the proper performance of the immune system. However, excessive ROS production can further promote the inflammatory process and contribute to damaging the body's own cells and tissues. Accumulating evidence suggests that an abnormal, ineffective, or absent regulation of ROS production participates in the pathogenesis of chronic inflammatory disorders such as asthma, rheumatoid arthritis, allergic rhinitis, chronic obstructive pulmonary diseases, or inflammatory bowel disease [1, 2].

2. Respiratory Burst of Neutrophils

The respiratory burst of neutrophils is primarily characterized by the production of the superoxide anion radical, the first ROS produced by neutrophils upon their contact with a variety of stimuli (e.g., cytokines, growth factors, fragments of bacterial membranes, opsonins, and others). The significant source of the superoxide anion radical after stimulation was shown to be the NADPH oxidase multicomponent enzyme complex [3, 4]. NADPH oxidase includes the membrane-bound cytochrome b558, consisting of gp91phox and p22phox, and the cytosolic components p47phox, p67phox, and Rac1. In resting neutrophils, the NADPH oxidase complex is unassembled, and specific granules carrying flavocytochrome *b* can serve as a storage pool and a mechanism

by which flavocytochrome *b* is recruited to the plasma membrane and phagosomes. Upon activation, the cytosolic components associate with the membrane components, and the newly formed enzyme complex actively catalyzes the production of the superoxide anion radical [1, 5].

Interestingly, some agents do not directly induce a strong activation of phagocytes, but instead induce the so-called "priming" of phagocytes, accompanied with only limited degranulation, however with a significant potentiation of a respiratory burst in response to consequent stimulation with other activators. Thus, these priming agents—including proinflammatory cytokines, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha, and lipopolysaccharide (LPS)—are known to induce weak ROS production by neutrophils, but they strongly enhance ROS formation after exposure of phagocytes to a second, activating stimulus [6, 7].

Neutrophil-derived ROS modulate both the extra- and intracellular redox environments that play a key role in the regulation and potentiation of inflammatory responses. On the other hand, oxidants produced by NADPH oxidase can be highly toxic, not only for infectious agents but also for neighboring host tissues. Therefore, the tight regulation of the enzyme complex is necessary to control their production. The intracellular redox status can be pharmacologically modulated by chemical antioxidants that act by donating an electron to a free radical and converting it to a nonradical form, or by the inhibition of enzymes involved in ROS production, including inhibitors of NADPH oxidase. Since polyphenols exhibit antioxidant and anti-inflammatory effects, they are subjects of interest as potential compounds for such use [8, 9].

3. Polyphenols and Flavonoids

Polyphenols represent a wide variety of compounds derived from plants, for which they play a protective role. They form a vast family of many thousands of molecules found in everyday foods such as fruits and vegetables and chocolate. Polyphenols are also used in the food and cosmetics industries, as additives and supplements. Although there are limited data on specific polyphenols, polyphenol-rich foods have previously been associated, in multiple studies, with a decreased risk of cardiovascular and inflammatory diseases [10–12].

Flavonoids are the most abundant and most studied class of polyphenols. In the last decades, flavonoids have been considered promising plant secondary metabolites with antioxidative and immunomodulatory properties. Foods and beverages rich in flavonoids have been associated, in several epidemiologic studies, with a decreased risk of age-related diseases, and the concept that flavonoids and other phenolic compounds are responsible is supported by several animal and *in vitro* studies [13, 14]. The aim of the present paper is to summarize contemporary knowledge on the effects of various flavonoids on the respiratory burst of mammalian neutrophils.

4. Effects of Flavonoids on the Production of ROS by Neutrophils *In Vitro*

4.1. The Effect on the Activation of NADPH Oxidase. The inhibitory effect of flavonoids (kaempferol, morin, quercetin, and fisetin) on the respiratory burst of neutrophils was observed by Pagonis et al. [15] early as in 1986. While the distribution of flavonoids in nitrogen-cavitated neutrophils paralleled their respective hydrophobic characteristics, all the studied flavonoids inhibited hydrogen peroxide generation. Similarly, Zielinska et al. [16] approved the antioxidative properties of natural flavonoids (quercetin, kaempferol, and isorhamnetin) against the respiratory burst of polymorphonuclear neutrophils from healthy human donors *in vitro*, as measured by flow cytometry using dichlorofluorescein diacetate and luminol-dependent chemiluminescence. The authors found that the studied compounds decreased neutrophil hydrogen peroxide production in a concentration-dependent manner. They also asserted that the antioxidative activity of flavonoids depended on the number of hydroxyl groups. Wang et al. [17] investigated the cellular localization of the inhibitory effect of a natural flavonoid cirsimaritin against a formyl-methionyl-leucyl-phenylalanine- (fMLP-) induced respiratory burst in rat neutrophils. Cirsimaritin, in a concentration-dependent manner, inhibited the superoxide anion radical generation and the oxygen consumption of neutrophils. On the other hand, cirsimaritin did not reduce, but slightly enhanced, the superoxide anion radical generation in phorbol 12-myristate 13-acetate- (PMA-) activated or arachidonic acid-stimulated NADPH oxidase.

The results of these authors indicate that it is likely that the inhibition of the fMLP-induced respiratory burst by cirsimaritin in rat neutrophils is mainly through the blockade of the phospholipase D (PLD) signaling pathway. As observed in the following experiments [18], fMLP-induced superoxide anion radical generation and oxygen consumption in rat neutrophils were also inhibited by 2′,5′-dihydroxy-2-furfurylchalcone (DHFC), in a concentration-dependent manner. However, DHFC was less effective in the inhibition of both PMA-activated neutrophil NADPH oxidase activity and arachidonic acid-induced NADPH oxidase activation. The authors also confirmed that fMLP-induced cellular PLD activation was markedly inhibited by DHFC. On the basis of further experiments, the authors concluded that, in all probability, the suppression of the fMLP-induced respiratory burst by DHFC is mainly attributable to the inhibition of PLD activation via the blockade of protein kinase C- (PKC-) alpha, ADP-ribosylation factor (Arf), and RhoA membrane association [18]. This suggestion is in very good agreement with the results of Chang et al. [19], who reached similar results with a potential synthetic anti-inflammatory drug 2-benzyl-3-(4-hydroxymethylphenyl) indazole (CHS-111). Schematic diagram showing the possible mechanisms underlying the inhibition of ROS production by neutrophils using flavonoids is shown in Figure 1.

Selloum et al. [20] compared the effects of three aglycone flavonols (myricetin, quercetin, and kaempferol) and the natural glycoside rutin on superoxide anion radical generation. All tested flavonols inhibited the pholasin luminescence

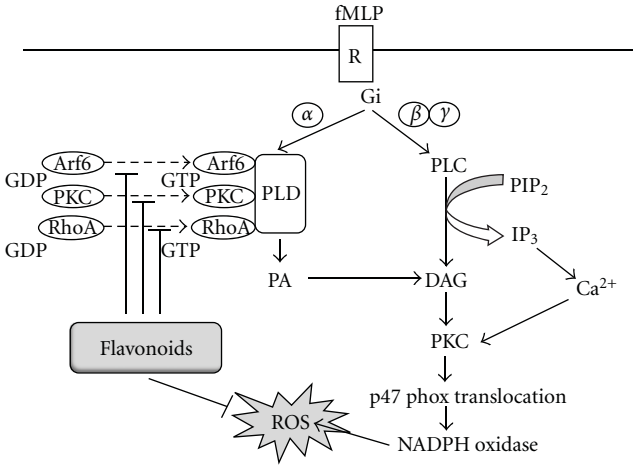

Figure 1: Schematic diagram showing the possible mechanisms underlying the inhibition of ROS production by neutrophils using flavonoids. The signaling molecules generally employed in mediating the activation of PLD are shown (some additional signaling molecules in these pathways are omitted). Blunt lines indicate the possible sites of the action of flavonoids (the blockade of PKC, Arf, and RhoA, as well as the scavenging of ROS generated by neutrophils).

of fMLP-stimulated neutrophils. Rutin influenced the oxidative burst of neutrophils in the same way as wortmannin and LY294002, two inhibitors of the phosphoinositide 3-kinase gamma, whereas the three other flavonols showed no effect. Lee et al. [21] observed that luteolin attenuated neutrophil respiratory burst but had a negligible effect on superoxide anion generation during PMA stimulation. Furthermore, luteolin effectively blocked MAPK/ERK kinase 1/2 and Akt phosphorylation in fMLP- and LPS-stimulated neutrophils.

Thus, an inhibition of enzymes involved in signaling rather than a scavenging of superoxide anion radicals dominates in fMLP-stimulated neutrophils exposed to flavonoids in these particular studies.

Flavonoids are also the basic constituents of various plant extracts. For example, Pastene et al. [22] studied the effects of a standardized extract of apple peel (60% of total polyphenols, 58% of flavonoids) with regard to the intra- and extracellular production of ROS in human neutrophils stimulated by *Helicobacter pylori*, PMA, or fMLP. The extracellular and intracellular production of ROS was evaluated using chemiluminescence, with the isoluminol-horseradish peroxidase and luminol-superoxide dismutase/catalase systems, respectively. Apple-peel extract inhibited the respiratory burst of neutrophils induced by all three activators, in a concentration-dependent manner. Interestingly, this effect was observed on both the intra- and extracellular chemiluminescence of neutrophils. This result suggests that apple-peel flavonoids could attenuate the damage to gastric mucosa caused by neutrophil-derived ROS, particularly when *Helicobacter pylori* displays its evasion mechanisms [22].

Kenny et al. [23] demonstrated that certain flavonoids and related compounds isolated from cocoa could moderate a subset of signaling pathways derived from the LPS stimulation of neutrophils, mainly neutrophil oxidative bursts and activation markers. They hypothesized that flavonoids could decrease the impact of LPS on the fMLP-primed neutrophil ability to generate ROS by partially interfering in the activation of the MAPK pathway [23].

A *Ginkgo biloba* extract containing flavonoids, among other compounds, was tested by Pincemail et al. [24] for its effect on the release of ROS (superoxide anion radical, hydrogen peroxide, and hydroxyl radical) during the stimulation of human neutrophils by a soluble agonist. The extract slowed down the oxygen consumption (respiratory burst) of the stimulated cells by its inhibitory action on NADPH-oxidase. The extract was also able to reduce the activity of myeloperoxidase contained in neutrophils. Moreover, it had free radical scavenging activity. According to Ciz et al. [25], a higher number of hydroxyl substituents are an important structural feature of flavonoids in respect to their scavenging activity against ROS, while C-2,3 double bond (present in quercetin and resveratrol) might be important for the inhibition of ROS production by phagocytes.

4.2. Scavenging of ROS Generated by Neutrophils. Flavonoids have powerful antioxidant activities *in vitro*, being able to scavenge a wide range of reactive oxygen, nitrogen, and chlorine species, such as superoxide anion radical, hydroxyl radical, peroxyl radicals, hypochlorous acid, and peroxynitrous acid. They can also chelate metal ions, often decreasing metal ion prooxidant activity [9, 26–28]. Therefore, the effects of various flavonoids on the respiratory burst of phagocytes can, at least partially, also be explained by their antioxidative properties. This means that, besides inhibiting the production of ROS by neutrophils, flavonoids can diminish the adverse oxidative stress by scavenging the reactive oxygen species that have already been produced.

Daels-Rakotoarison et al. [29] prepared a rose-hip extract deprived of vitamin C (thus excluding its ROS scavenging activity) and studied its effects on polymorphonuclear neutrophils production of ROS such as superoxide anion radicals, hypochlorous acid, and hydrogen peroxide, which may possess deleterious effects for the organism. The extract contained mainly proanthocyanidins and flavonoids. The results showed that the extract inhibited ROS in acellular and cellular systems equally. They concluded that the extract did not present an effect on neutrophil metabolism, and that its effect was due to the antioxidative effects of rose-hip polyphenolics.

5. Effects of Flavonoids on Neutrophils *In Vivo*

Most papers dealing with the effects of polyphenols on biological systems are based on *in vitro* experiments. However, when compared to experiments in isolated cells, the observations within whole organisms revealed controversial results. Ellinger et al. [30] observed that daily consumption of red wine or dealcoholized red wine (red wine is well known for its high content of flavonoids) did not induce any changes in the respiratory burst of the phagocytes of healthy adult volunteers. They concluded that other mechanisms than a modulation of phagocytosis and respiratory burst of phagocytes

by polyphenols present in red wine might explain the protective effects of red wine against coronary heart disease that have been observed in epidemiological studies [30]. Henson et al. [31] observed that quercetin supplementation for 3 weeks before and 2 weeks after the ultramarathoners competing in the 160 km run had no effect on decreases in neutrophil respiratory burst activity. Rutin supplementation to the healthy controls and Golden Syrian hamsters fed a high-fat diet had no immunomodulatory effects on the neutrophil oxidative metabolism assessed by whole blood luminol-enhanced chemiluminescence and 2′,7′-dichlorofluorescein diacetate-dependent flow cytometry [32].

On the other hand, López et al. [33] tested whether dealcoholized red and white wines with high content of flavonoids can decrease the oxidative stress associated with the development of a granuloma induced in rats by subcutaneous administration of carrageenan. The results indicate that the nonalcoholic compounds of wines not only improve antioxidant status in an inflammatory situation, but also limit cell infiltration into the granuloma pouch, and moreover, neutrophils from the inflammatory exudate of rats fed dealcoholized wines showed decreased superoxide anion radical (and increased nitric oxide) production. Thus, the delivery of flavonoids directly to the inflamed area seems to be an important issue in the treatment of inflammatory diseases.

Many of the *in vitro* studies did not take bioavailability and metabolism factors into consideration, and the effects reported in those studies do not necessarily occur *in vivo*. It is clear that under *in vivo* conditions food components must be bioavailable in some form to exert biological effects. There have been major advances in the past few years in our knowledge regarding polyphenol absorption and metabolism. According to published studies reviewed by Williamson and Manach [34], it is apparent that most classes of polyphenols are sufficiently absorbed while crossing the intestinal barrier and reaching micromolar concentrations in the bloodstream that have been shown to have the potential to exert biological effects in *in vitro* studies.

Manach et al. [12] have reviewed studies where the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of various classes of polyphenol (anthocyanins, flavonols, flavanones, flavonol monomers, proanthocyanidins, isoflavones, hydroxycinnamic acids, and hydroxybenzoic acids) were analyzed. It is apparent from the studies reviewed that gallic acid and isoflavones are most well absorbed in humans followed by catechins, flavanones, and quercetin glucosides, with different kinetics. The least well-absorbed polyphenols were the proanthocyanidins, the galloylated tea catechins, and the anthocyanins.

It has to be noted that the intestinal absorption of flavonoids can be regulated using new delivery carriers with good biocompatibility, biodegradability, and safety. This is especially advantageous in the treatment of inflammatory bowel disease resulting from the transmural infiltration of neutrophils, macrophages, lymphocytes, and mast cells, ultimately giving rise to mucosal disruption and ulceration. Flavonoids can be encapsulated into a carrier (e.g., coated chitosan pellets) which rapidly passes the pylorus, regardless of the feeding state of the patient, and the influence of the gastric emptying rate on their upper gastrointestinal transit time is minimised [35].

Although most polyphenols are absorbed to some extent, this is highly dependent on the type of polyphenol. Because physiologic concentrations do not exceed 10 mol/L, the effects of polyphenols *in vitro* at concentrations of 10 mol/L are generally not valid, with the possible (but unproven) exception of the intestinal lumen. Furthermore, absorption is accompanied by extensive conjugation and metabolism, and the forms appearing in the blood are usually different from the forms found in food. This indicates that *in vitro* experiments with the form of polyphenols found in food (the aglycone) are not necessarily relevant to the *in vivo* situation [36].

There are now many intervention studies in the literature that demonstrate significant biological effects of polyphenol consumption among humans, with the use of many different biomarkers. Some of the papers described intervention studies involving consumption of foods, and, in many of those cases, it was not proved that the observed effects were attributable to the polyphenol component.

6. Summaries

Results of various studies suggest that the inhibitory effects of flavonoids on the respiratory burst of phagocytes are mediated via various mechanisms. It can be concluded that the effects of flavonoids on mammalian neutrophils are complex, and that several sites of action depending upon the flavonoid structure, flavonoid's subcellular distribution, and pathway of stimulation are included.

Abbreviations

Arf:	ADP-ribosylation factor
CHS-111:	2-benzyl-3-(4-hydroxymethylphenyl) indazole
DMFC:	2′,5′-dihydroxy-2-furfurylchalcone
ERK:	Extracellular-signal-regulated kinase
fMLP:	Formyl-methionyl-leucyl-phenylalanine
LPS:	Lipopolysaccharide
MAPK:	Mitogen-activated protein kinase
PKC:	Protein kinase C
PLD:	Phospholipase D
PMA:	Phorbol 12-myristate 13-acetate
ROS:	Reactive oxygen species.

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Research Article

Mate Tea Prevents Oxidative Stress in the Blood and Hippocampus of Rats with Acute or Chronic Ethanol Administration

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Objective. The aim of this study was to evaluate the influence of acute and chronic intake of mate tea on the effects elicited by acute and chronic administration of ethanol. *Methods*. Oxidative stress was evaluated by measuring thiobarbituric acid-reactive substances (TBARS), as well as the activities of the antioxidant enzymes, catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in the hippocampus and blood of rats. Male Wistar rats were randomly assigned to four groups, for both acute and chronic treatment: (1) control group, (2) treated group, (3) intoxicated group, (4) and intoxicated group treated with mate tea. *Results*. Both ethanol administrations significantly increased TBARS in plasma and hippocampus of rats and altered antioxidant enzyme activities, changes which were reverted by mate tea administration. *Conclusions*. Data indicate that acute and chronic ethanol administration induced oxidative stress in hippocampus and blood and that mate tea treatment was able to prevent this situation.

1. Introduction

The cellular metabolism of all aerobic organisms produces reactive oxygen species (ROS), which are neutralized by non-enzymatic defenses (α-tocopherol, carotene, ascorbic acid, etc.) and the activity of antioxidant enzymes. The imbalance between the production and the elimination of ROS represents a situation named oxidative stress characterized by molecular injuries induced by oxidative reactions with proteins, carbohydrates, and lipids [1]. The lipid content of the cellular membrane is the first structure to be attacked by ROS and, thus, lipid peroxidation represents the most frequent injury resulting from the action of ROS [2].

ROS have been associated with the physiopathology of several diseases, such as cellular dysfunction of patients with atherogenesis, cardiopathy, senility, carcinogenesis, rheumatic disease, and diabetes type 2, and are also responsible for the ageing process [3, 4]. Moreover, ROS are involved in the pathogenesis of neurologic conditions such as epilepsy, multiple sclerosis, dementia, and neurodegenerative diseases such as Alzheimer and Parkinson. This is understandable since the central nervous system (CNS) is highly sensitive to oxidative stress because of its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of its antioxidant defenses [5–7].

Much evidence indicates that antioxidant ability decreases with ageing, leading to the increase of macromolecule oxidation and, consequently, related diseases [8–11]. The cellular redox state might be modified by the diet, since food components can increase or decrease antioxidant cellular ability. Chronic ethanol intoxication, for example, may lead to a high generation of ROS, while the consumption of dietary antioxidants contributes to the enhancement of the

endogenous mechanisms of defense (enzymatic antioxidant system) [12, 13]. In literature, there are several strategies for induction of oxidative stress; in this work we used the experimental model described by Kasdallah-Grissa et al. [14], which consists in an intraperitoneal injection of 35% ethanol at a dose of 3 g/kg.

There are many ways by which the diet may interfere in the formation and defense against free radicals [15–17]. Fruits, vegetables, and whole grains possess significant amounts of bioactive phytochemicals capable of promoting great health benefits, beyond basic nutrition [18]. Other natural antioxidants sources include teas, since their consumption may represent a safe alternative to increase the ingestion of dietetic antioxidants. Tea is the second most consumed beverage in the world, with a per capita human ingestion of approximately 120 mL/day [19]. In this context, the aqueous extract of yerba mate (*Ilex paraguariensis*) is consumed at a rate of over 1 liter per day by millions of individuals in South America, representing the mainly alternative to coffee and tea [20].

Ilex paraguariensis infusion is a potential source of polyphenols. Its aqueous extract is a typical antioxidant-containing beverage that is largely consumed in several South American countries, including Southern Brazil, Uruguay, Paraguay, and Argentina. Moreover, yerba mate presents a healthy nutritional profile, containing the vitamins A, C, and E; B complex; the minerals potassium, magnesium, calcium, manganese, iron, selenium, phosphorus, and zinc; several aminoacids; various polyphenols, mainly caffeoyl derivatives (such as chlorogenic acid); and some flavonoids (rutin, quercetin, kaempferol) [21].

Polyphenols are a class of compounds containing a benzene ring bound with one or more hydroxyl groups which exert antioxidant activity [22]. Consumption of mate tea significantly contributes to the overall antioxidant intake and provides high amounts of caffeoylquinic acid derivatives, with biological effects potentially beneficial for human health. The compound that may be primarily responsible for this activity is chlorogenic acid [23]. Chlorogenic acids are a family of esters formed between certain *trans* cinnamic acids and (−)-quinic acid and are also major phenolics compounds in coffee, strawberries, pineapples, apples, sunflower, and blueberries. Chlorogenic acids are free radical and metal scavengers, may interfere with glucose absorption, and have been shown to modulate gene expression of antioxidant enzymes, among other biological activities [24]. Roasted extracts of *Ilex paraguariensis* keep the same components with the addition of melanoidins, which have some bioactive properties of their own [21].

Reports that demonstrate the ability of antioxidant nutrients to revert or prevent conditions initiated by the increased production of free radicals are extremely important to promote healthy food habits, combating NTCD and even precocious ageing through the diet. As such, this study evaluated the effects of acute and chronic ethanol administration on an important parameter of oxidative stress, namely, thiobarbituric acid-reactive substances (TBARS), as well as on the activities of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in the blood and hippocampus of rats and the influence of acute and chronic intake of mate tea, a product derived from yerba mate, on the effects elicited by acute and chronic administration of ethanol on these parameters. We used hippocampus because the brain is exceptionally susceptible to oxidative stress [6, 7] that may be caused by xenobiotics such as ethanol. In addition, after ethanol intoxication, an increase in the level of aldehydes is observed in the brain as well as the serum of rats [25] and aldehyde oxidation by some chemicals and enzymatic systems leads to the generation of free radicals [26].

2. Methods and Materials

2.1. Animals. Wistar rats of 60 and 45 days of age (180–200 g), obtained from the Central Animal House of the Regional University of Blumenau, Blumenau, Brazil, were used in the experiments. The animals from our own breeding stock were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1°C), with free access to water and commercial protein chow. The "Principles of Laboratory Animal Care" (NIH publication 85–23, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the local Institution. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Mate Tea Preparation. Mate tea used in this study was from the same batch and was obtained from Leão Jr., Curitiba, PR, Brazil. The tea beverage was prepared accordingly to the instructions of the label.

2.3. Acute Treatment of Ethanol and Mate Tea. Twenty male rats aged 60 days were randomly assigned to four groups, according to the intervention: (1) control group received a single intraperitoneal injection of 0.9% NaCl and pure water by intragastric gavage; (2) treated group received a dose of mate tea (0.5 g/kg) [27] by intragastric gavage and an intraperitoneal injection of 0.9% NaCl; (3) intoxicated group received intraperitoneal injection of 35% ethanol at a dose of 3 g/kg [14] and pure water by intragastric gavage; (4) intoxicated group treated with mate tea received a dose of mate tea (0.5 g/kg) [27] by intragastric gavage and intraperitoneal injection of 35% ethanol at a dose of 3 g/kg [14]. The mate tea dose used was equivalent to that found in 0.75 liters mate tea/day [13]. Animals were sacrificed by decapitation without anaesthesia 1 h later, and the hippocampus removed and blood collected.

2.4. Chronic Treatment of Ethanol and Mate Tea. Twenty male rats aged 45 days were randomly assigned to four groups, according to the intervention, as described previously for the acute treatment. Each group received the respective intervention daily, for 15 days. Animals were sacrificed by decapitation without anaesthesia 12 hours after the last administration, and the brain removed and blood collected.

2.5. Preparation of Brain Tissue. Animals were killed by decapitation and the hippocampus was removed and dissected immediately on an ice-cooled glass plate. Time elapsed between decapitation and dissection was less than 1 min. After dissection, the hippocampus was homogenized with appropriate buffer for each assay with a Potter-Elvehjem glass homogenizer. The maximum period between homogenate preparation and enzyme analysis was always less than 3 days. All procedures were carried out at 0–4°C.

2.6. Erythrocyte and Plasma Preparation. Erythrocytes and plasma were prepared from whole blood samples obtained from rats. Whole blood was collected and transferred to heparinized tubes for erythrocyte separation. Blood samples were centrifuged at 1,000 ×g, and plasma was removed by aspiration and frozen at −80°C until determination. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride). Lysates were prepared by the addition of 1 mL of distilled water to 100 μL of washed erythrocytes and frozen at −80°C until determination of the antioxidant enzyme activities.

For antioxidant enzyme activity determination, erythrocytes were frozen and thawed three times and centrifuged at 13,500 ×g for 10 min. The supernatant was diluted in order to contain approximately 0.5 mg/mL of protein.

2.7. Thiobarbituric Acid Reactive Substances (TBARS). TBARS measure malondialdehyde (MDA), a product of lipoperoxidation caused mainly by hydroxyl free radicals. For measurements, homogenates and plasma in 1.15% KCl were mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS were determined by the absorbance at 535 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the supernatants. TBARS were calculated as nanomole of malondialdehyde formed per milligram of protein [28].

2.8. Catalase Activity Assay (CAT). Hydrogen peroxide (H_2O_2) disappearance was continuously monitored with a spectrophotometer at 240 nm for 90 s. One unit of the enzyme is defined as 1 μmol of hydrogen peroxide consumed per minute, and the specific activity is reported as units per mg protein [29].

2.9. Glutathione Peroxidase Activity Assay (GSH-Px). GSH-Px activity was measured by the method of Wendel [30], except for the concentration of nicotinamide adenine dinucleotide phosphate (NADPH), which was adjusted to 0.1 mM after previous tests performed in our laboratory. Tert-butylhydroperoxide was used as substrate. NADPH disappearance was continuously monitored with a spectrophotometer at 340 nm for 4 min. One GSH-Px unit is defined as 1 μmol of NADPH consumed per minute, and specific activity is reported as units per mg protein.

2.10. Superoxide Dismutase Assay (SOD). This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on oxygen (O_2), which is a substrate for SOD [31]. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can then be indirectly assayed spectrophotometrically at 420 nm. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as units/mg protein.

2.11. Protein Determination. Protein was measured by the method of Lowry et al. with bovine serum albumin used as standard [32].

2.12. Statistical Analysis. Data were analyzed by Student's *t*-test and by analysis of variance (ANOVA) followed by the Duncan multiple range tests when the *F*-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. $P < 0.05$ was considered significant.

3. Results

We first studied the effects of acute ethanol, mate tea, and mate tea plus ethanol administration on an important parameter of lipoperoxidation, namely, TBARS and on the antioxidant enzymes activities of CAT, GSH-Px, and SOD in the hippocampus and blood of rats. Figure 1 shows that acute ethanol administration significantly increased TBARS (81%) (a) as well as decreased CAT (40%) (b) and SOD (40%) (c) activities in the hippocampus of rats and that acute mate tea administration prevented the increase in TBARS ($F(3, 16) = 12.895$; $P < 0.001$), as well as the reduction of CAT ($F(3, 16) = 4.795$; $P < 0.01$) and SOD ($F(3, 16) = 11.825$; $P < 0.001$) activity, caused by acute ethanol administration, when compared to the control group. However acute ethanol administration had no effect on GSH-Px ($t(8) = 0.490$; $P > 0.05$) activity, when compared to the control group (result not shown). As can be observed in Figure 2, acute ethanol administration significantly increased TBARS levels (35%) (a) and CAT (61%) (b) activity in plasma and erythrocytes of rats, respectively, while acute mate tea administration was able to prevent the increase in TBARS ($F(3, 16) = 11.898$; $P < 0.001$), caused by ethanol, in the plasma of rats. In contrast, such prevention was not observed for the increased CAT activity observed in the erythrocytes of rats ($F(3, 16) = 5.809$; $P < 0.01$). In addition, GSH-Px ($t(8) = 0.60$; $P > 0.05$) and SOD ($t(8) = -1.714$; $P > 0.05$) activities did not undergo any significant alteration by acute ethanol administration, when compared to the control groups (results not shown).

Subsequently, the effects of chronic ethanol, mate tea, and mate tea plus ethanol administration on TBARS and on the antioxidant enzymes activities of CAT, GSH-Px, and SOD were also evaluated in the hippocampus and plasma/erythrocytes of rats. As can be observed, Figure 3 shows that chronic ethanol administration significantly increased TBARS (48%) (a) and decreased CAT (21%) (b) and GSH-Px (8%) (c) activities in the hippocampus of rats and that mate tea pretreatment prevented the increase in TBARS ($F(3, 16) = 10.958$; $P < 0.001$), as well as the

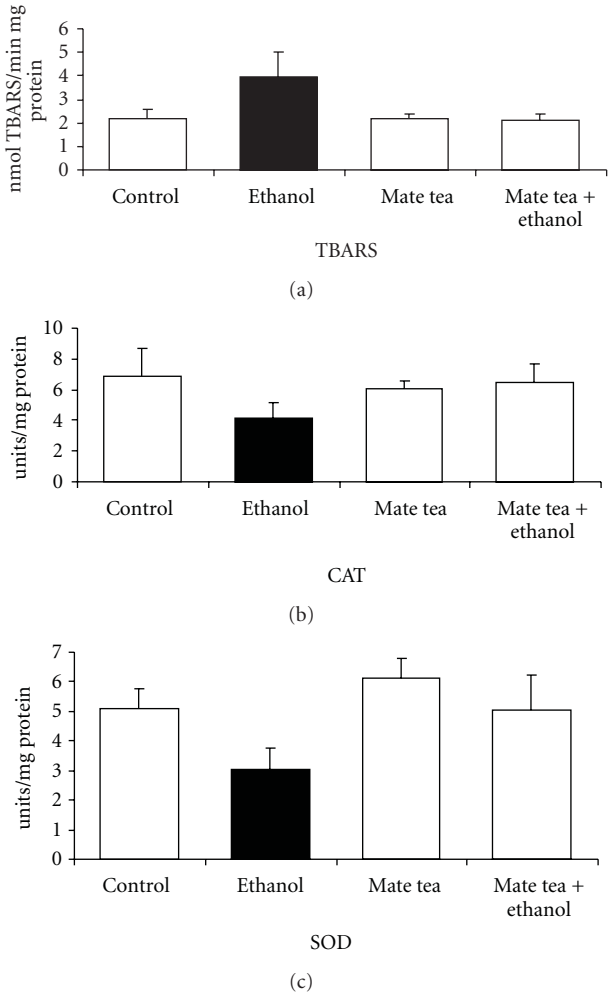

Figure 1: Effects of acute administration of ethanol, mate tea, and mate tea plus ethanol on thiobarbituric acid reactive substances (TBA-RSs) (a), on catalase (b) and superoxide dismutase (c) activities in rat hippocampus. Data are mean ± SD for 5 independent experiments (animals) performed in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control group (Duncan multiple-range test). One catalase (CAT) unit is defined as 1 μmol of H_2O_2 consumed per minute. One superoxide dismutase (SOD) unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation.

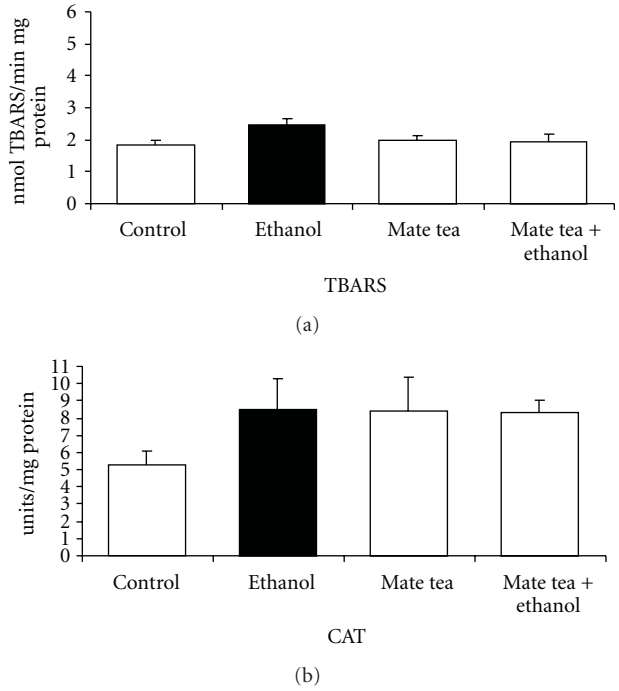

Figure 2: Effects of acute administration of ethanol, mate tea, and mate tea plus ethanol on thiobarbituric acid reactive substances (TBA-RSs) (a) and on catalase (b) activity in rat blood. Data are mean ± SD for 5 independent experiments (animals) performed in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control group (Duncan multiple-range test). One catalase (CAT) unit is defined as 1 μmol of H_2O_2 consumed per minute.

decrease in CAT ($F(3,16) = 3.247$; $P < 0.05$) and GSH-PX ($F(3,16) = 4.882$; $P < 0.01$) activities in the rat hippocampus. SOD ($t(8) = 1.471$; $P > 0.05$) activity did not undergo any significant changes by chronic ethanol administration (result not shown). With regard to the chronic ethanol, mate tea and mate tea plus ethanol administration in the plasma/erythrocytes of rats, Figure 4 shows that chronic ethanol administration significantly increased TBARS (52%) (a) and SOD (38%) (b) activity as well as decreased CAT (42%) (c) and GSH-PX (13%) (d) activities in blood of rats. In addition, pretreatment with mate tea prevented the increase in TBARS ($F(3,16) = 9.043$; $P < 0.001$) in the plasma of rats, prevented the increase in SOD ($F(3,16) = 9.032$; $P < 0.001$) activity in erythrocytes, and also abolished the reductions in CAT ($F(3,16) = 10.261$; $P < 0.001$) and GSH-Px ($F(3,16) = 28.783$; $P < 0.01$) activities in erythrocytes, caused by chronic ethanol administration, respectively. Statistical analyses demonstrated that acute and chronic mate tea administration *per se* did not interfere in most of the parameters studied.

4. Discussion

Growing evidence suggests that the major killers, cardiovascular disease and cancer, can be prevented or delayed to some extent by dietary changes, such as a reduction in fat intake and increased consumption of fruits, grains, and vegetables [33–35]. We obtain several compounds from a healthy diet that may act to diminish oxidative damage in vivo. Since our endogenous antioxidant defenses are not 100% efficient, it is reasonable to propose that dietary antioxidants are important in diminishing the cumulative effects of oxidative damage over the long human lifespan, and that they account for some of the beneficial effects of fruits, grains, and vegetables [36].

In this study, we verified the effects of acute and chronic ethanol, mate tea, and mate tea plus ethanol administration on TBARS and on the antioxidant enzymes activities of CAT, GSH-Px, and SOD in the hippocampus and blood of rats, in order to verify whether the dose chosen of ethanol

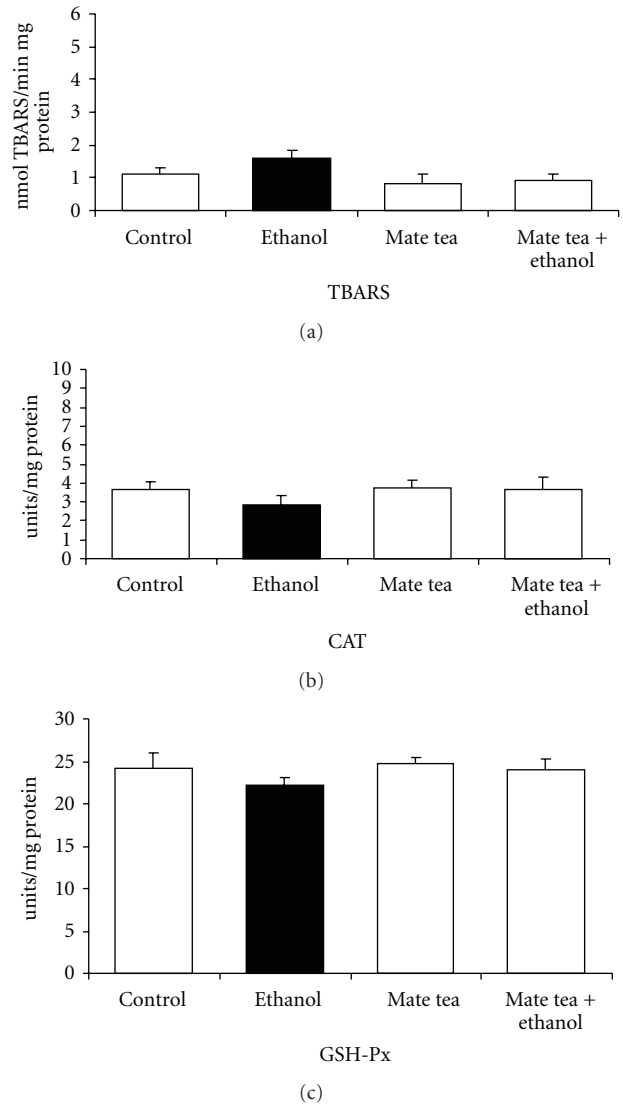

Figure 3: Effects of chronic administration of ethanol, mate tea, and mate tea plus ethanol on thiobarbituric acid reactive substances (TBA-RSs) (a), on catalase (b) and glutathione peroxidase (c) activities in rat hippocampus. Data are mean ± SD for 5 independent experiments (animals) performed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control group (Duncan multiple-range test). One catalase (CAT) unit is defined as 1 μmol of H_2O_2 consumed per minute. One glutathione peroxidase (GSH-Px) unit is defined as 1 μmol of NADPH consumed per minute.

could induce oxidative stress and the possible antioxidant properties of mate tea, since yerba mate possesses many bioactive compounds, such as caffeine, phenolic acids, and saponins, which are absorbed by the body and may act as antioxidants, and since mate tea is a product derived from this herb.

Our results showed that acute and chronic ethanol administration provoked an oxidative stress, as demonstrated by the elevated TBARS levels in the hippocampus and plasma of rats. With regard to antioxidant enzyme activities, we observed that acute ethanol administration significantly reduced CAT and SOD activity in the hippocampus and increased CAT activity in the erythrocytes of rats. Chronic ethanol administration significantly reduced CAT and GSH-Px activities in the hippocampus of rats. In rat erythrocytes, we observed a decrease in CAT and GSH-Px, activity, despite an increase in SOD activity. The different results caused by acute or chronic ethanol administration on the antioxidant enzyme activities probably occur due to the fact that in acute treatment rats were sacrificed 1 h after the administration of ethanol, when ethanol levels were high, while in chronic treatment rats were killed 12 h after the last injection, suggesting an effect that was promoted by secondary metabolites, and not by ethanol itself.

Although we cannot precisely establish the mechanisms by which an increase in CAT activity in erythrocytes was detected following ethanol acute administration, in addition to the increase in SOD activity in erythrocytes, caused by ethanol chronic administration, it is possible that this occurred due to a higher stimulation of enzyme synthesis through gene transcription, as a compensatory effect for the increased generation of free radicals provoked by ethanol. Previous data have shown that antioxidant enzymes may respond to oxidative stress by increasing their activity in order to reduce damages [37–39]. It is important to point out that enzymes investigated in this study seem to have a different response to ROS depending on the tissue investigated [40].

Our results suggest that acute and chronic ethanol administration induces oxidative stress, since it provoked lipoperoxidation as well as modified antioxidant enzyme activities in the rat hippocampus and blood, allowing us to verify the possible antioxidant effects of mate tea. Results showed that acute and chronic mate tea administration prevented the increase in TBARS, caused by acute and chronic ethanol administration in the hippocampus and plasma of rats.

With regard to antioxidant enzyme activity, results demonstrate that acute mate tea administration prevented the decrease in CAT and SOD activities in the rat hippocampus, caused by acute ethanol administration, although it was not capable of preventing the increase in CAT activity in rat erythrocytes, caused by acute ethanol administration. In addition, chronic mate tea administration prevented the reduction in CAT activity in the hippocampus and erythrocytes of rats, as well as preventing the reduction in GSH-Px activity in the hippocampus and erythrocytes and, finally, the increase in SOD activity in rat erythrocytes.

Our results corroborate previously data found in the literature [13, 20, 27, 41, 42] showing the antioxidant properties of mate tea, where acute and chronic mate tea administration was found to prevent lipoperoxidation as well as prevent the modifications in antioxidant enzymes activities caused by ethanol in the hippocampus and blood of rats.

Miranda et al. demonstrated that mate tea increases DNA resistance against H_2O_2-induced damage [13], while other authors have observed a decrease in lipoperoxidation in the plasma and liver of rats, after mate tea consumption [27]. Yerba mate was shown to inhibit the progression of

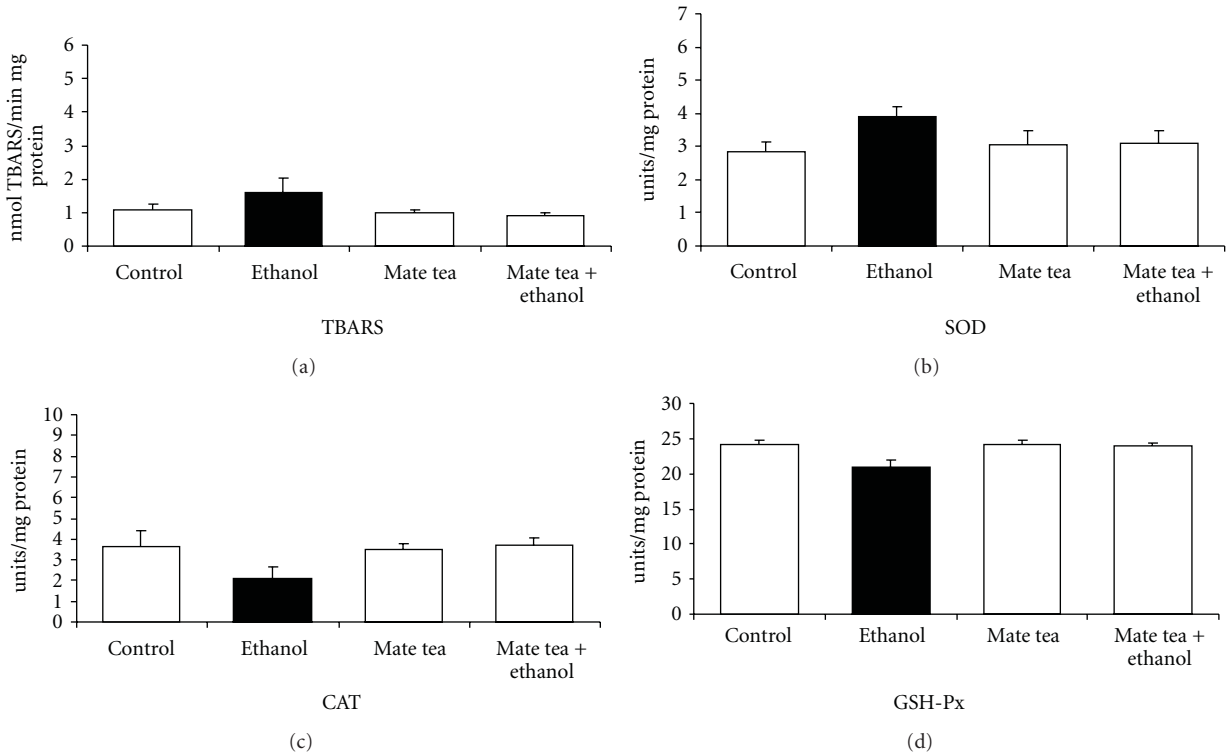

Figure 4: Effects of chronic administration of ethanol, mate tea, and mate tea plus ethanol on thiobarbituric acid reactive substances (TBA-RSs) (a), on superoxide dismutase (b), catalase (c), and glutathione peroxidase (d) activities in rat blood. Data are mean ± SD for 5 independent experiments (animals) performed in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control group (Duncan multiple-range test). One superoxide dismutase (SOD) unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation. One catalase (CAT) unit is defined as 1 μmol of H_2O_2 consumed per minute. One glutathione peroxidase (GSH-Px) unit is defined as 1 μmol of NADPH consumed per minute.

atherosclerosis in cholesterol-fed rabbits, although it did not decrease the serum cholesterol or aortic TBARS and antioxidant enzymes [20]. The plasma concentration and tissue distribution of antioxidant compounds of a hydroethanolic extract of *Ilex paraguariensis*, before and after enzymatic hydrolysis, were accessed in rats. Both extracts presented high antioxidant activity and phenolic content. Rats given the hydrolyzed extract showed increased plasma antioxidant activity and higher plasma levels of caffeic acid. However, the hydrolyzed extract of *Ilex paraguariensis* did not altered skin and brain antioxidant activity in rats, 20 minutes after administration, suggesting that antioxidant compounds in the extract were not effectively distributed to these tissues or that the time to reach measurable concentrations was different from that in the sample collection [43].

Studies that involve yerba mate antioxidant effects in humans are still limited. In a study with healthy adults, a lower susceptibility of plasma and LDL-c to lipid peroxidation was found associated with a higher antioxidant capacity after acute ingestion of a mate infusion [41]. In another survey, healthy young women received 5 g of instant mate tea in 500 mL of water for a week and results showed that acute and prolonged consumption of mate tea resulted in a significant reduction in TBARS levels, in parallel to a significant increase in plasma resistance to peroxidation by Cu^{2+}. The gene expression of the antioxidant enzymes SOD, GSH-Px, and CAT also increased [42].

Mate tea potential was also tested on oxidative stress biomarkers and LDL oxidisability in normolipidaemic (NL) and hyperlipidaemic (HL) subjects. Chronic treatment with mate tea for 60 days (2.5 g of instant mate tea to 200 mL of cold water) resulted in the marked increase in SOD activity in erythrocytes and serum total antioxidant status for both groups. Moreover, the lag time of copper-induced LDL peroxidation, a measure of resistance to oxidation, increased 53% and 69% for the NL and HL groups, respectively, after mate tea ingestion. The serum TBARS levels were significantly reduced in the HL group after treatment. On the other hand, GSH-Px activity and diene production (nmol/min/mg LDL) did not reach statistical significance for either group after supplementation [44].

There is growing evidence that oxidative stress may play an important role in the pathogenesis of Alzheimer's disease, Parkinson disease, and Amyotrophic lateral sclerosis, since the brain, which consumes large amounts of oxygen, is particularly vulnerable to oxidative damage [45]. Evidence also shows that nutritionally derived sources of brain-accessible antioxidants may provide an approach to slow the onset and progression of neurodegenerative disorders [46]. However, one possible limitation of the neuroprotective strategy

(including antioxidant administration) might be consequent to the fact that when neurologic symptoms occur, a certain amount of neuronal death has already occurred. Thus, the neuroprotective agents (including antioxidants) can, at best, only rescue the surviving neurons, an effect that might not be sufficient to attenuate the neurologic symptomology. It is, therefore, important to start therapeutic intervention at an early stage in the disease process, suggesting that dietary habits may exert influence on the progression of neurodegenerative disorders [45]. Thus, mate tea intake might be an alternative to delay or prevent neurological disease, since our results showed that it provides antioxidant protection in CNS tissue.

In summary, in the present study we demonstrate that acute and chronic mate tea administration prevented the alterations in some parameters of oxidative stress in the hippocampus and blood of rats, caused by the *in vivo* administration of ethanol. This suggests that mate tea may have a high antioxidant capacity, probably due to its bioactive components and that mate tea ingestion could prevent oxidative stress-related disease. However, further studies are necessary to evaluate whether mate tea could be useful as a potential adjuvant therapy to neurologic patients.

Abbreviations

CAT: Catalase
CNS: Central nervous system
GSH-Px: Glutathione peroxidase
H_2O_2: Hydrogen peroxide
MDA: Malondialdehyde
NADPH: Nicotinamide adenine dinucleotide phosphate
NTCD: Nontransmissible chronic disease
O_2: Oxygen
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TBARS: Thiobarbituric acid-reactive substances.

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