Original Article

Thai Journal of Pharmaceutical Sciences

Antiproliferative effects of *Pereskiopsis diguetii*, *Caralluma speciosa* and *Euphorbia ritchiei* hydroalcoholic extract

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Received: Oct 10, 2017
Accepted: May 04, 2018
Published: Jun 06, 2018

ABSTRACT

To date, cancers remain to be global health problem. The toxicity and the emergence of chemotherapy resistance of currently prescribed anticancer agents highlight the need for the discovery and the development of novel anticancer drugs. The previous successes in the discovery of anticancer agents and other biologically active compounds from diverse types of flora support the value of plants as natural resources for the screening of novel drugs. However, the pharmacological data of succulent plants are largely limited. **Objectives:** Here, we screened hydroalcoholic extracts of selected succulent plants for potential anticancer activities against four cancer cell lines including colorectal cancer cell line HT29, hepatocellular carcinoma cell line HepG2, head-and-neck cancer cell line HN22, and cervical cancer cell line HeLa. **Materials and Methods:** Crude extracts were obtained from fresh specimens using 95% ethanol as solvent, concentrated using a rotary evaporator, and dissolved in dimethyl sulfoxide. 50–500 mg/ml of extracts were first evaluated for cytotoxicity through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay at 48 or 72 h. The extracts showing cytotoxicity were further examined through flow cytometry for cell cycle distribution. **Results:** *Pereskiopsis diguetii* (F.A.C. Weber) Britton and Rose, a member of Cactaceae family, exhibited cytotoxic effects against HT29, HepG2, and HN22. *Caralluma speciosa* (N.E.Br.) N.E.Br., a member of Apocynaceae family, exhibited cytotoxic activities toward HT29 and HN22 cells. *Euphorbia ritchiei* (P. R. O. Bally) Bruyns exhibited cytotoxic effects against HepG2, HN22, and HeLa. Flow cytometry analysis revealed that these extracts altered cell cycle profile of the majority of the susceptible cancer cell lines, suggesting that these extracts likely exhibited antiproliferative or pro-apoptotic activities. **Conclusion:** Together, our results pinpointed these succulents as potential candidates for further investigations which may lead to the discovery of novel anticancer agents.

Keywords: Antiproliferative, cytotoxic, pro-apoptotic, succulent

INTRODUCTION

Cancer is a group of complex diseases resulted from a profound change within the genome of various types of cells which enables an uncontrolled growth.[1] Cancer is a major health problem and a leading cause of death worldwide. To date, pharmacological treatment remains to be one of the standard therapies for various types of cancer. Despite the variety of anticancer medications currently available, there exists a need for novel anticancer agents. First, some anticancer drugs are often associated with serious side effects,[2] limiting their usage. Second, cancer cells often adapt and gain tolerance to prescribed anticancer agents[3,4] which forces a switch to other pharmacological regimen. The increase in the diversity of anticancer agents will provide alternatives for the management of the disease and will ultimately improve the overall quality of life of cancer patients.[3]

Plants, as sources of biologically active compounds with diverse molecular structures, are useful natural resources for the screening of new compounds with desired biological activities.[5] Over the years, many plant-derived compounds have reached clinical trials or been approved by the USFDA for the management of diverse pharmacological conditions including infectious diseases and cancers.[6,7] The search for

anticancer agents from plants which began in the 1950s has proved fruitful since it led to the discovery of the anticancer vinca alkaloids, vinblastine, and vincristine and the isolation of podophyllotoxins,[8] the natural precursor of etoposide. Other naturally derived anticancer agents include irinotecan from *Camptotheca acuminata* and taxol from *Taxus brevifolia*.[7] Naturally derived anticancer agents from plants, marine, and microorganisms are estimated to constitute up to 60% of currently used anticancer drugs.[8]

Succulent plants or succulents are drought-tolerant plants with certain parts, i.e., root, stem, or leaf developed for water storage, giving them unique fleshy appearances. Succulents encompass diverse species of flora from many different botanical families including Aizoaceae, Crassulaceae, Apocynaceae, Euphorbiaceae, and Cactaceae, to name but a few. Only a number of succulents have been characterized for their pharmacological properties. *Euphorbia ritchiei* (ER) (P. R. O. Bally) Bruyns., *Pereskiopsis diguetii* (PD) (F.A.C. Weber) Britton and Rose, and *Caralluma speciosa* (CS) (N.E.Br.) N.E.Br. are classified in the botanical family Euphorbiaceae, Cactaceae, and Apocynaceae, respectively. Despite not being indigenous species of Thailand, these succulents are collected and cultivated as decorative plants by Thai enthusiasts. Direct anticancer effects of PD, *CS*, and ER have not been described; however, anticancer properties of certain member of the family Euphorbiaceae,[9] Cactaceae,[10] and Apocynaceae[11-13] have been documented.

The aim of this study is to assess anticancer potentials of succulent plants cultivatable in Thailand. The succulent plants selected are PD, CS, and ER, each a member of distinct botanical family. Hydroalcoholic crude extract, prepared from freshly cut specimen, was analyzed for cytotoxic effects against four human cancer cell lines: Head-and-neck cancer HN22, liver cancer HepG2, colon cancer HT29, and cervical cancer HeLa cells. The detected cytotoxicities were then further examined through cell cycle distribution analysis using propidium iodide staining and flow cytometry.

Figure 1: Photograph of live plant specimen. (a) *Pereskiopsis diguetii* (F.A.C. Weber) Britton and Rose, (b) *Caralluma speciosa* (N.E.Br.) N.E.Br., (c) *Euphorbia ritchiei* (P. R. O. Bally) Bruyns

Table 1: Weight and total volume of 95% ethanol used for the extraction

Plant	Weight (g)	Volume of ethanol (ml)
PD	624	600
C. speciosa	395	400
ER	743	250

PD: *Pereskiopsis diguetii,* CS: *Caralluma speciosa*, ER: *Euphorbia ritchiei*

MATERIALS AND METHODS

Preparation of Hydroalcoholic Extracts

Fresh specimens were collected from plants propagated and grown in our greenhouse in Bangkok, Thailand. Picture of each plant is shown in Figure 1. Plant specimens were rinsed with water to remove soil and grit, if necessary. Afterward, plants are weighted, ground in 95% ethanol, and allowed to stand for 2 days. Debris and coarse particles were then removed through filtration using No. 4 Whatman filter paper. The filtrates were then concentrated using a rotary evaporator at 50°C and dried on a water bath at 60°C. The dried extracts were then dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 100 mg/ml. The weight of each specimen and the total volume of ethanol used for the extraction are described in Table 1.

Cell Culture

Hepatocellular carcinoma cell line HepG2, colorectal cancer cell line HT29, head-and-neck cancer cell line HN22, and cervical cancer cell line HeLa were kindly gifted from Professor Praneet Opanasopit, Faculty of Pharmacy, Silpakorn University**.** HepG2 was maintained in Dulbecco's modified Eagle's medium (Gibco, Waltham, MA, USA) supplemented with 10% FBS (Gibco). HT29 was maintained in Dulbecco**'s** modified Eagle's medium supplemented with 10% FBS, 1% non-essential amino acids (Gibco), and 1% GlutaMAX (*Gibco*). HN22 was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% GlutaMAX. HeLa was maintained in minimum essential media (*Gibco*) supplemented with 10% FBS, 1% non-essential amino acids, and 1% GlutaMAX. All cells were cultured in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Evaluation of Cytotoxic Effects by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Assay

Cells were plated at a density of 1 × 10^4 cells/well onto 96-well plate and incubated with varying concentration

of the extracts for 48 h (HN22 and HeLa) or 72 h (HepG2 and HT29) or 0.5% DMSO (vehicle) for negative control. DMSO concentration was maintained at 0.5% for all groups. Afterward, cells were washed with phosphate buffer saline (PBS) solution and then incubated with 1 mg/ml Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich, St. Louis, MO, USA) for 4 h during which the purple formazan crystals are formed. 100 μl of 100% DMSO were then added to each well to dissolve formazan crystals. Absorbance at 550 nm was measured using a microplate reader and used to calculate the percentage of cell viability. Percentage cell viability was calculated using the following equation:

% cell viability = average A550 of experimental group/ average A550 of control group

All experiments were performed in triplicate. Student's t-test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Cell Cycle Analysis by Flow Cytometry

Cells were treated with 500 μg/ml of the extracts or 0.5% DMSO vehicle control for 48 h (HN22 and HeLa) or 72 h (HepG2 and HT29). Cells were then washed, harvested, and fixed with 70% ice-cold ethanol. Afterward, cells were washed twice with ice-cold PBS and treated with 100 μg/ml of DNase-free RNase A (Bio Basic, Amherst, NY, USA) in PBS containing 0.1% v/v Triton-X 100 (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature. Cells were then stained with 20 μg/ml propidium iodide (Life Technologies, Carlsbad, CA, USA) in PBS containing 0.1% v/v Triton-X 100 for 15 min at room temperature in the dark. Cell cycle distribution was then analyzed with a flow cytometer (Facscanto, BD Biosciences, San Jose, CA, USA). Data were analyzed using ModFitLT V3.0 software (BD Biosciences). All experiments were done in triplicate. Student's t-test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Cytotoxic Activities of PD Hydroalcoholic Extract

PD hydroalcoholic extract exhibited a modest, yet statistically significant, cytotoxic effect against hepatocellular carcinoma cell line HepG2, colorectal cancer cell line HT29, and head–and-neck cancer cell line HN22 [Figure 2], which was more pronounced at high concentration (500 μg/ml). At this concentration, cell viability of HepG2, HT29, and HN22 was decreased by approximately 13%, 15%, and 17%, respectively. However, cervical cancer cell line HeLa appeared to be insensitive to PD, as cell viability was unperturbed even when treated at 500 μg/ml.

Next, we sought to explore the mechanism of PD-mediated cytotoxic effects by analyzing cell cycle distribution of PD-sensitive cancer cell lines treated with a maximal concentration of PD (500 μg/ml) using propidium iodide staining and flow cytometry. Propidium iodide is a fluorogenic dye which binds to DN, allowing the DNA content of the stained cells to be analyzed by flow cytometry[14] Cells can then be classified into specific populations according to phases of the cell cycle, i.e., G0/G1, S, and G2/M based on DNA content. Moreover, apoptotic cells can be simultaneously quantified - since apoptosis is associated with a degradation and subsequent loss of degraded DNA from the cell, these cells cluster in a sub-G1 or hypodiploid peak.[14,15]

Treatment of HepG2 with 500 μg/ml of PD resulted in a statistically significant increase in sub-G1 population [Figure 3a and b], suggesting that the observed cytotoxic effect of PD in HepG2 cells was likely due to the pro-apoptotic effect of PD in this cell line. Similarly, PD-treated HN22 exhibited an increase in sub-G1 population and a marked increase in the population of cells in S-phase [Figure 3c and d], suggesting that the cytotoxicity of PD in HN22 cells was likely due to a combination of antiproliferative activity (S-phase cell cycle arrest) and pro-apoptotic effect. Intriguingly, HT29 treated with 500 μg/ml of PD exhibited a prominent increase in G0/G1 and G2/M population [Figure 3e and f], indicating that PD elicited an antiproliferative activity through an induction of cell cycle arrest of HT29 cells at G0/G1 and G2/M phase. In addition, a modest increase in sub-G1 population was also detected in PD-treated HT29 [Figure 3f, boxed], suggesting that PD might also induce apoptosis in this cell line.

Cytotoxic Activities of CS Hydroalcoholic Extract

CS hydroalcoholic extract exhibited a statistically significant and dose-dependent cytotoxic effect against HT 29 and HN22 cells [Figure 4]. At the highest concentration tested (500 μg/ml), cell viability of HT 29 and HN22 was decreased by approximately 38% and 31%, respectively. CS treatment did not elicit any detectable cytotoxic effect from HepG2 and HeLa cells even at the highest concentration tested, suggesting that HepG2 and HeLa were likely insensitive to CS.

Cell cycle analysis of HT29 treated with 500 μg/ml of CS revealed a significant increase of cells in S-phase of the cell cycle [Figure 5c and d], suggesting that cytotoxic effect of CS might result from an antiproliferative effect due to an S-phase cell cycle arrest. On the contrary, CS treatment of HN22 cells was associated with a robust increase in sub-G1 population, suggesting that the cytotoxic effect of CS in HN22 cells was likely mediated by apoptosis induction.

Cytotoxic Activities of ER Hydroalcoholic Extract

ER hydroalcoholic extract exhibited a statistically significant cytotoxic effect against HepG2, HeLa, and HN22 cells [Figure 6]. The maximal inhibition of cell viability of ER-treated HepG2 (24%) and HN22 (47%) was observed when cells were treated at the highest dose (500 μg/ml), whereas the maximal inhibitory effect of ER against HeLa (26%) was reached at 100 μg/ml. Strikingly, an approximately 50% decrease in cell viability was observed in HN22 treated with ER at 500 μg/ml, whereas HT29 appeared to be insensitive to ER.

ER-treated HepG2 cells exhibited a marked increase in G2/M and sub-G1 populations [Figure 7a and b], indicating that the cytotoxic effect of ER in HepG2 might result from its antiproliferative (G2/M cell cycle arrest) and pro-apoptotic activity. Similarly, ER treatment elicited G2/M cell cycle arrest

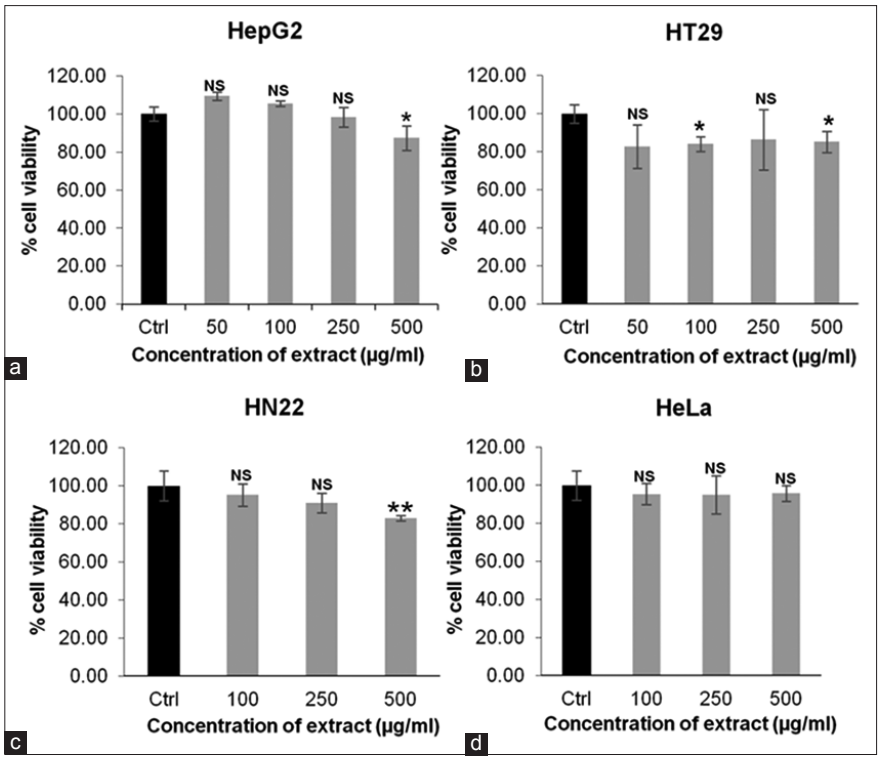

Figure 2: Cytotoxic effects of *Pereskiopsis diguetii* (PD) hydroalcoholic extract. Cytotoxic effects of PD hydroalcoholic extract against (a) HepG2, (b) HT29, (c) HN22, and (d) HeLa cells. Cells were treated with various concentrations of PD in triplicate or 0.5% dimethyl sulfoxide for control group. Viable cells were quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay. Data were expressed as mean ± standard deviation. Non-significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$ versus control (Ctrl) group

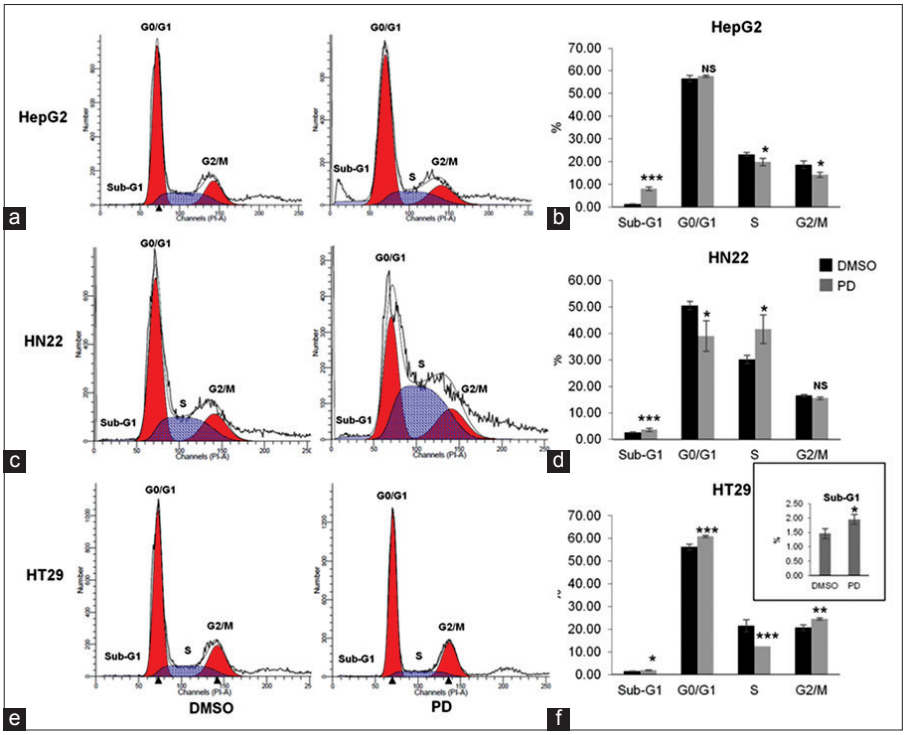

Figure 3: Cell cycle distribution of HepG2, HN22, and HT29 cells treated with PD. Cells were treated with 500 μg/ml of PD or 0.5% dimethyl sulfoxide (DMSO) vehicle control in triplicate and stained with propidium iodide as described in material and methods. Cell cycle profile of HepG2 (a), HN22 (b), and HT29 (c) was analyzed by flow cytometry. Pictures depicted were representative images of the triplicate. Note the differences in Y-axes scales among figure b, d and f. The mean percentage of cells in sub-G1, G0/G1, S, and G2/M phase of the cell cycle of HepG2 (b), HN22 (d), and HT29 (e and f) cells treated with PD or DMSO were analyzed and shown in graph as mean ± standard deviation. Non-significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ versus control group (DMSO)

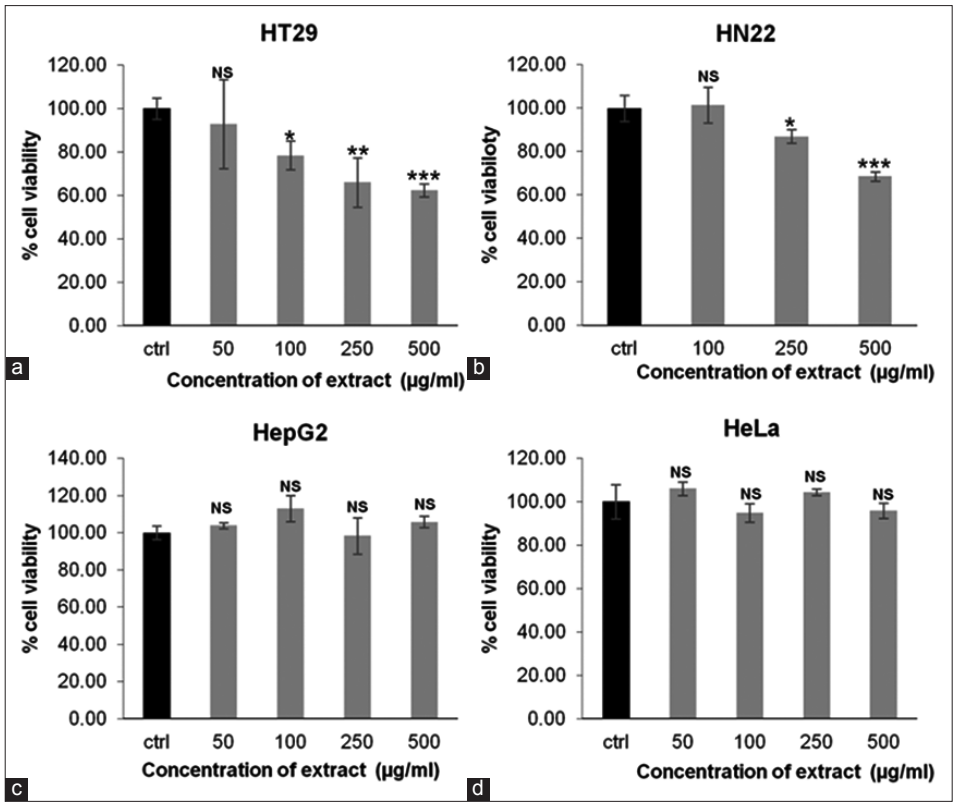

Figure 4: Cytotoxic effects of *Caralluma speciosa* (CS) hydroalcoholic extract. Cytotoxic effects of CS hydroalcoholic extract against (a) HT29, (b) HN22, (c) HepG2, and (d) HeLa cells. Cells were treated with various concentrations of CS in triplicate or 0.5% dimethyl sulfoxide for control group. Viable cells were quantified with MTT assay. Data were expressed as mean ± standard deviation. Non-significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ versus control (Ctrl) group

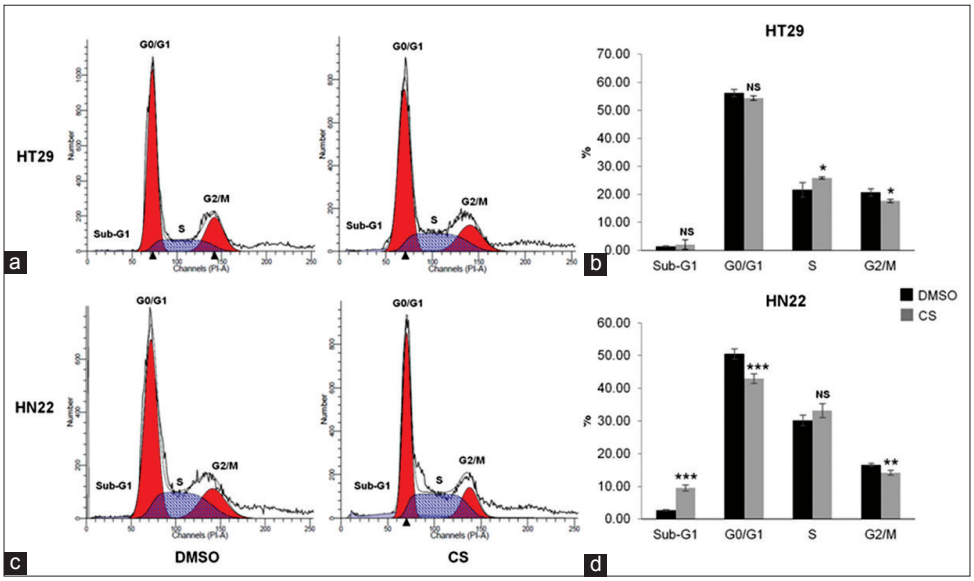

Figure 5: Cell cycle distribution of HepG2, HT29, and HN22 cells treated with CS. Cells were treated with 500 μg/ml of CS or 0.5% dimethyl sulfoxide (DMSO) vehicle control in triplicate and stained with propidium iodide as described in material and methods. Cell cycle profile of HT29 (a) and HN22 (c) was analyzed by flow cytometry. Pictures depicted were representative images of the triplicate. Note the differences in Y-axes scales between figure b and d. The average percentage of cells in sub-G1, G0/G1, S, and G2/M phase of cell cycle of HT29 (b) and HN22 (d) cells treated with PD or DMSO was analyzed and shown in graph as mean ± standard deviation. Non-significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ versus control group (DMSO)

in HeLa cells, as indicated by an increased in G2/M population [Figure 7c and d]. In contrast to HepG2, HeLa cells treated with ER did not exhibit an increase in sub-G1 population, suggesting that ER likely exhibited cytotoxicity toward HeLa

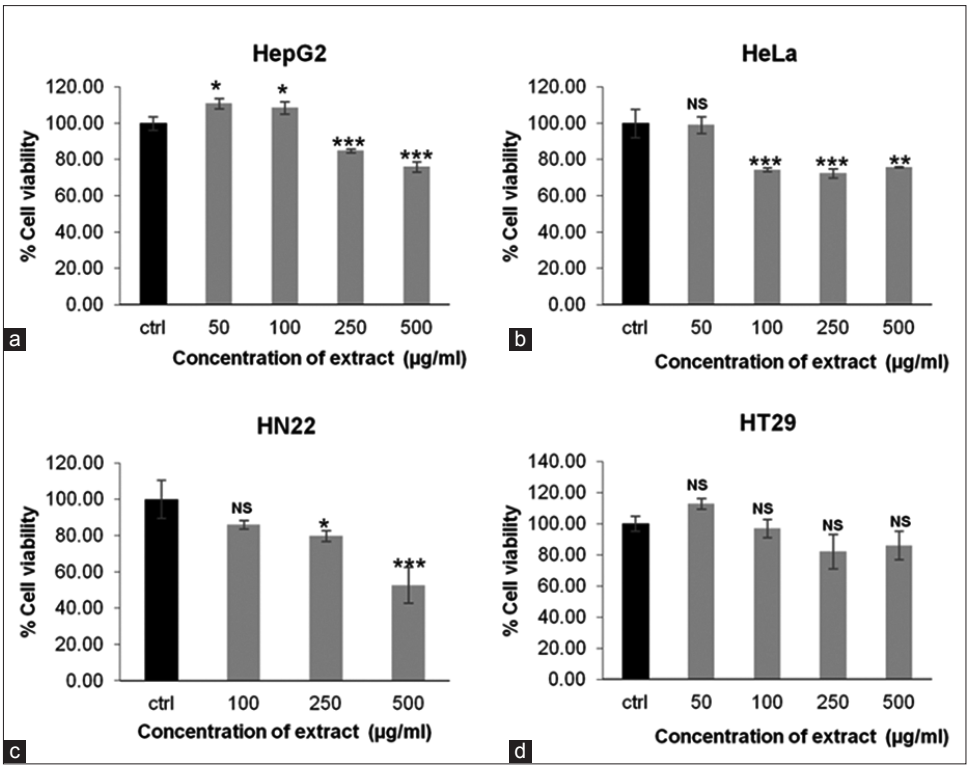

Figure 6: Cytotoxic effects of *Euphorbia ritchiei* (ER) hydroalcoholic extract. Cytotoxic effects of ER hydroalcoholic extract against (a) HepG2, (b) HeLa, (c) HN22,l and (d) HT29 cells. Cells were treated with various concentrations of ER in triplicate or 0.5% dimethyl sulfoxide for control group. Viable cells were quantified with MTT assay. Data were expressed as mean ± standard deviation. Non-significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ versus control (Ctrl) group

cells solely through an antiproliferative activity, at least at the concentration tested. Intriguingly, despite the readily detectable cytotoxic effect through MTT assay, no significant change in cell cycle distribution was detected in ER-treated HN22 cells [Figure 7e and f].

DISCUSSION

Given that succulents have undergone a unique evolution to tolerate extreme environment, it is likely that these plants produce unique molecules for their survival advantages. Driven by this reason, we sought to screen selected succulent plants for potential anticancer properties, using cytotoxicity testing by MTT assay for the screening due to its relatively simplistic procedure followed by flow cytometry cell cycle profiling to gain mechanistic insight. In this study, we detected cytotoxic effects of extracts from selected succulent plants including PD, CS, and ER.

In this report, we detected cytotoxic activity of CS against the colorectal cancer cells HT29 and head-and-neck cancer cells HN22. To the best of our knowledge, the direct cytotoxic activity of *C. speciosa* against cancer cells has not been described. Concordance with our findings, other groups have reported cytotoxic properties of other caralluma species, i.e., *Caralluma tuberculata* against colorectal cancer cell lines Caco-2.[11] Cytotoxic effects against mammary cancer cell lines have been detected in several other caralluma species including *C. quadrangular,*[12,13] *C. Russelliana,*[12] *and C. tuberculata.*[11,12] Flow cytometry revealed that the cytotoxicity of CS against HT29 and HN22 might be mediated by an S-phase cell cycle arrest and apoptosis, respectively. In line with our findings, pro-apoptotic activity has been reported in glycosides isolated from *C. tuberculata*.[11]

The cytotoxic effects of cacti toward cancer cells have been detected in cell lines derived from various cancer types including cancer of the head and neck,[16] liver,[17] colon and rectum,[16-18] from cacti in the genus Pereskia,[16] Opuntia,[17] Hylocereus,[19] and Myrtillocactus.[18] However, the direct cytotoxic effect of PD has remained obscure. Here, we detected a weak, but statistically significant, cytotoxic effect of PD against hepatocellular carcinoma cell line HepG2, colorectal cancer cell line HT29, and head-and-neck cancer cell line HN22, suggesting that PD may possess anticancer effects as other cacti species. Moreover, the dietary supplement of *Opuntia humifusa* fruits inhibited carcinogenesis induced by chemical carcinogens[20] and UVB irradiation[21] in mouse skin suggesting that, in addition to cancer-curative properties, cacti may also have cancer-preventive effects. Interestingly, our result indicated that PD exhibited a weak pro-apoptotic activity toward all susceptible cancer cell lines and induced cell cycle arrest in HT29 and HN22. In accordance with our result, alterations of cell cycle have been reported in cancer cell lines treated with extracts or isolated compound from *O. humifusa*[22,23] and *Opuntia ficus-indica,*[24] whereas apoptosis induction activities have been reported in extracts or isolated compound from *O. ficus-indica*[25,26] and *Pereskia bleo*.[27]

Our result indicated that ER displayed cytotoxic activity toward hepatocellular carcinoma cell line HepG2, cervical cancer cell line HeLa, and head-and-neck cancer

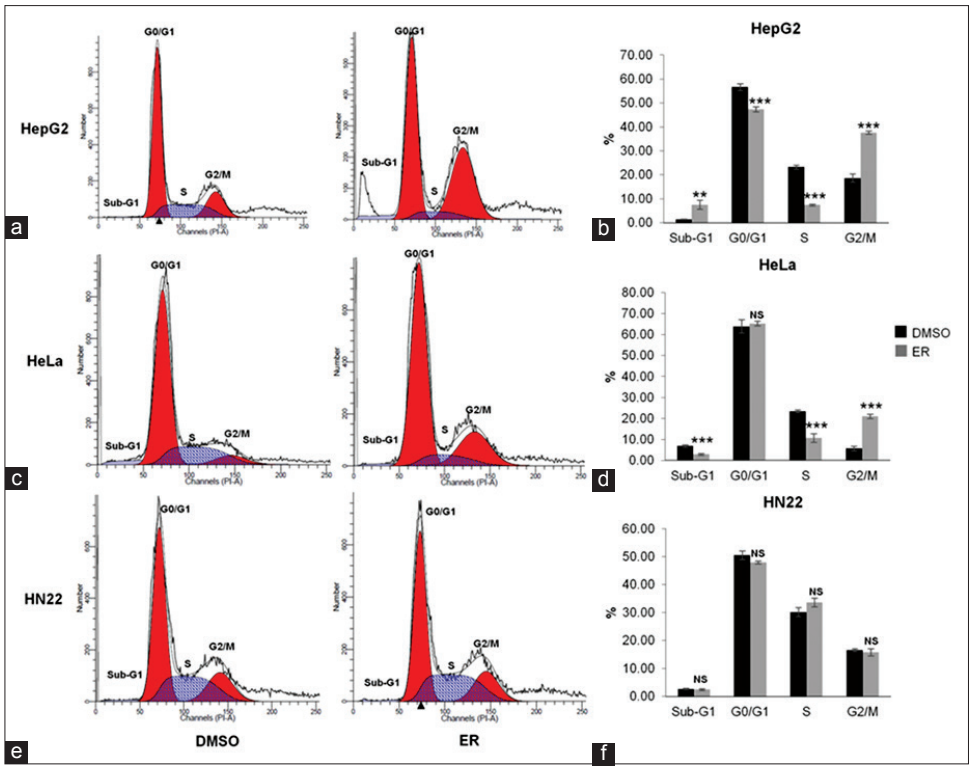

Figure 7: Cell cycle distribution of HepG2, HeLa, and HN22 cells treated with ER. Cells were treated with 500 μg/ml of ER or 0.5% dimethyl sulfoxide (DMSO) vehicle control in triplicate and stained with propidium iodide as described in material and methods. Cell cycle profile of HepG2 (a), HeLa (c), and HN22 (e) was analyzed by flow cytometry. Pictures depicted were representative images of the triplicate. Note the differences in Y-axes scale among figure b, d and f. The average percentage of cells in sub-G1, G0/G1, S, and G2/M phase of the cell cycle of HepG2 (b), HeLa (d), and HN22 (f) cells treated with PD or DMSO was analyzed and shown in graph as mean ± standard deviation. Non-significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ versus control group (DMSO)

cell line HN22. Of the three groups of succulents tested, the pharmacological properties of Euphorbias appear to be the most extensively characterized for diverse types of ailments including cancers.[28] In line with our findings, *in vitro* cytotoxic activities against hepatocellular carcinoma,[29-31] cervical cancer,[32] head–and-neck cancer,[33] and other cancer types including ovarian, breast, prostate, stomach, lung, and pancreas have been detected in other Euphorbia species, for example, *E. Turcomanica,*[32] *E. Pekinensis,*[29] *E. Tirucalli,*[33,34] *E. Kansui,*[30] and *E. supine.*[31] Interestingly, ER appeared to induce cell cycle arrest primarily at the G2/M phase, at least in HepG2 and HeLa. In addition, apoptosis was detected in HepG2 treated with PD. In line with our report, G2/M phase cell cycle arrest was detected in cancer cell lines treated with 7,13-diacetyl-5-angeloyl-20-nicotinyl-3-propionyl-1,2,6,7-tetrahydroingenol (DANPT), a diterpene compound isolated from *E. erythradenia*,[35] and 13-Oxyingenol dodecanoate (13OD) isolated from *E. kansui.*[36] Many Euphorbia-derived compounds and extracts have been reported to induce apoptosis of diverse types of cancer cell lines.[36-39]

In this study, we did not detect cytotoxic activity against hepatocellular and cervical cancer from CS, colorectal cancer from ER, or cervical cancer from PD; however, these effects have been detected in other species within the same genus.[12,16,19,23,32,40] This discrepancy may be attributable to the differences in the extraction methods or the intrinsic differences in the species of plant specimens. We also observed that the cytotoxic properties of these plants are cell-type dependent, which may be expected given that different types of cancers are associated with different underlying genetic profile.[41] A similar effect has been reported as the extract from *Euphorbia triaculeata* exhibited a significant cytotoxic effect against MCF7 and PC-3 cell lines while HepG2 appeared insensitive to the extract.[42] Therefore, it may be worthwhile to evaluate the cytotoxic effects of these extracts against other cancer cell lines.

The cytotoxic effect of chemical compounds is usually expressed as half-maximal inhibitory concentration (IC_{50}), which is the concentration of the compound which decreases the viability of tested cells by half. However, we were unable to determine the IC_{50} of most of the extracts since the solubility of the extracts does not permit testing at higher concentrations. Before the present study, we tested all four cancer cell lines for the maximal tolerable concentration of DMSO. The result indicated that these cells can tolerate DMSO up to 0.5%. Given the solubility of these crude extracts, treatment of cells at a concentration higher than 500 μg/ml will exceed the maximal tolerable DMSO concentration. Further refinement of the extracts by polarity-based fractionation may enable the analysis of cytotoxic effects of these extracts at higher concentrations.

Given that both the identity and concentration of the biologically active compounds in these extracts are not known, the potency and pharmaceutical value of these compounds

cannot be assessed. On the one hand, the plants may contain chemicals with potent effects at a low concentration, in which condition the identification of the active compound may lead to the discovery of a new lead for anticancer agents. Alternatively, the active compounds may be abundant but have a relatively weak effect, in which condition the identification of the compounds and their derivatization may still hold promises for the development of novel anticancer agents. Future characterization of these extracts by bioassay-guided fractionation is warranted to shed light on the cytotoxic properties of these succulents. In support of this notion, active principles with anticancer activities have been isolated from plants in the genus *Caralluma,*[11,13] *Opuntia* (Cactaceae),[43,24,25] *Myrtillocactus* (Cactaceae),[18] *Pereskia* (Cactaceae),[16] and *Euphorbia.*[9,44,45] An example of succulent-derived compound which has been approved by the USFDA for the treatment of cancer is ingenol mebutate which is an active principle isolated from *Euphorbia peplus.* Ingenol mebutate has been approved as a treatment for actinic keratosis, a pre-cancerous lesion which, untreated, could develop into skin cancer (reviewed in the study of Ogbourne and Parsons[46] Keating[47]).

In this report, flow cytometry analysis of DNA content was utilized to further characterize the general cytotoxic effects detected by MTT assay. Flow cytometry allows a simultaneous detection of cell cycle distribution of cells treated with the extracts as well as a quantitation of sub-G1 population which generally reflects cells undergoing apoptosis - a common mode of cell death initiated by anticancer agents.[15] Alternatively, plant-derived compounds may activate different cell death pathway such as necrosis.[22] However, in contrast to apoptosis, necrotic cells, in general, do not exhibit an immediate decline in DNA content.[15] This might explain the cytotoxic effect of ER toward HN22, which manifested as a robust decline in cell viability which was not accompanied by any detectable change in cell cycle distribution. Cytotoxic responses accompanied by changes in cell cycle distribution without an increase in sub-G1 peak likely reflect antiproliferative activities, possibly through cell cycle arrest at various stages of the cell cycle. Nevertheless, these effects should be further validated using specific assays. For example, extracts exhibiting pro-apoptotic activity should be further confirmed through annexin V/propidium iodide double staining or the analysis of expression level or activities of proteins involving in the apoptotic process such as Bcl-2 proteins and Caspases. Similarly, cell cycle arrests can be confirmed through the expression and activity of cell cycle-specific proteins such as cyclins and cyclin-dependent kinases. Intriguingly, PD and ER displayed dual pro-apoptotic and cell cycle arrest activities toward certain cell lines. At this point, we cannot conclude if these dual effects are mediated independently by different compounds within these extracts or resulted from an effect of one compound exhibiting both activities. Notably, many anticancer agents elicit both cell cycle arrest and pro-apoptotic activity in cancer cell lines, suggesting that in some cases, these effects are codependent. Moreover, prolonged cell cycle inhibition can lead to apoptosis.[48] In summary, our results provide fundamental insights into the cytotoxic, antiproliferative, and pro-apoptotic activities of crude ethanolic extracts from *P. diguetii*, *C. speciosa*, and *E. ritchiei* and highlight these succulent plants as promising candidates for further studies and potential development of novel anticancer agents.

ACKNOWLEDGMENTS

We thank Professor Praneet Opanasopit for cell lines and MTT reagents. We thank Associate Professor Wisit Tangkeangsirisin for propidium iodide. We thank the Faculty of Pharmacy, Silpakorn University research fund for financial support.

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