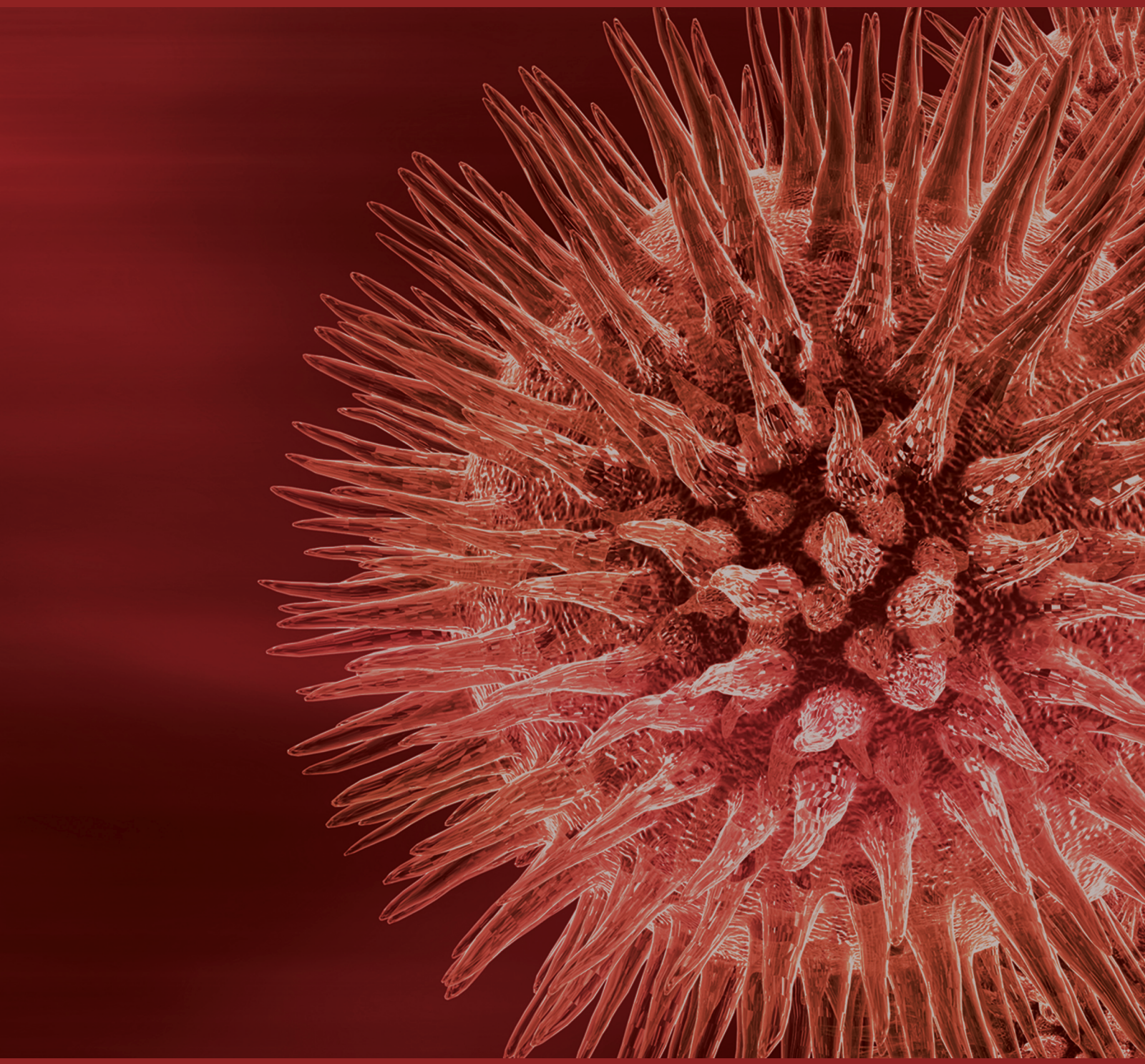
Journal of Biomedicine and Biotechnology

Natural Products for Medicine

Guest Editors: Masa-Aki Shibata, Ikhlas A. Khan, Munekazu Iinuma, and Tomoyuki Shirai



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Contents

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Editorial
Natural Products for Medicine

Masa-Aki Shibata,[1] Ikhlas A. Khan,[2] Munekazu Iinuma,[3] and Tomoyuki Shirai[4]

[1] *Laboratory of Anatomy and Histopathology, Faculty of Health Science, Osaka Health Science University, Osaka 530-0043, Japan*
[2] *Department of Pharmacognosy, School of Pharmacy, University of Mississippi, MS 38677, USA*
[3] *Laboratory of Pharmacognosy, Gifu Pharmaceutical University, Gifu 501-1196, Japan*
[4] *Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan*

Correspondence should be addressed to Masa-Aki Shibata, masaaki.shibata@ohsu.ac.jp

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Throughout human history, natural products—including terrestrial plants, animal products, marine organisms, and products of microorganismal fermentation—have been used in traditional medicines. This historical experience with natural products as therapeutic agents has evolved to sophisticated isolation of active chemical entities from ethnopharmacological plants with the effect that, in modern medicine, natural products are increasingly the primary sources in early drug discovery.

The population is aging in many modern societies and is linked to steadily increasing morbidity rates of cancer and cerebrovascular disease, thus mandating the importance of better preventive as well as therapeutic options. Many medically advanced societies are exploring the adjunctive use of western and oriental medicine alternatives, enhancing the demand for natural products. While evidence-based data is still scant in the field of alternative medicines, in this special issue we have the pleasure of sharing with our readers many scientific articles on natural products with potential medicinal use.

This special issue contains 6 review articles and 19 original peer-reviewed papers. The reviews included summarize such diverse topics as the biological and pharmacological effects of ginsenoside Rb1, isolated from red ginseng root, on skin damage (Y. Kimura et al.); tumor suppression in animals by a fruit pulp containing high amounts of β-cryptoxanthin and hesperidin (T. Tanaka et al.); the favorable effects of purple corn flower in treatment of infectious diseases (J. B. Hudson); the biomechanisms and clinical outcome of *Rhus verniciflia* extract in patients (W. Choi et al.); the safety of cruciferous plant extracts (O. Scott et al.); and the antitumor activity of artemisinin and its analogs (M. P. Crespo-Ortiz and M. Q. Wei). The original articles embrace a wide variety of topics, such as apoptosis, suppression of cell proliferation and tumorigenesis (Y. Hu et al., H. Kurose et al., A. Lerner et al., Booth et al., Kita et al., Mohd et al., and G. M. Geetha et al.), the antibacterial/antifungal/anti-inflammation/antioxidative characteristics of natural extracts (E. Biazar et al., Y.-S. Lee et al., Y. Hu et al., A. Cha et al., H. Sun et al., and P.-F. Kao et al.), cardiovascular activity of specific natural products (K. Awang et al.), the effects of extracts on blood parameter levels in diabetic patients (T. Klangjareonchai and C. Roongpisuthipong), examination of isolation techniques (N. A. Mahyudin et al.), enzymatic activity (B. Elya et al.) and metabolism of certain extracts (Y. Shimoda et al.); and specific effects of some natural products on aryl hydrocarbon receptor expression (H. M. Korashy et al.).

We would like to thank the authors for their timely contributions. Finally, we also thank all reviewers for their hard work questioning, pruning, and refining these articles.

Masa-Aki Shibata
Ikhlas A. Khan
Munekazu Iinuma
Tomoyuki Shirai

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Research Article

Alterations in Cell Cycle and Induction of Apoptotic Cell Death in Breast Cancer Cells Treated with α-Mangostin Extracted from Mangosteen Pericarp

Hitomi Kurose,[1] Masa-Aki Shibata,[2] Munekazu Iinuma,[3] and Yoshinori Otsuki[1]

[1] *Division of Life Sciences, Department of Anatomy and Cell Biology, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki, Osaka 569-8686, Japan*
[2] *Laboratory of Anatomy and Histopathology, Faculty of Health Science, Osaka Health Science University, 1-9-27 Temma, Kita-ku, Osaka 530-0043, Japan*
[3] *Laboratory of Pharmacognosy, Faculty of Pharmacy, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan*

Correspondence should be addressed to Yoshinori Otsuki, an1001@art.osaka-med.ac.jp

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The development of molecularly targeted drugs has greatly advanced cancer therapy, despite these drugs being associated with some serious problems. Recently, increasing attention has been paid to the anticancer effects of natural products. α-Mangostin, a xanthone isolated from the pericarp of mangosteen fruit, has been shown to induce apoptosis in various cancer cell lines and to exhibit antitumor activity in a mouse mammary cancer model. In this study, we investigated the influence of α-mangostin on apoptosis and cell cycle in the human breast cancer cell line MDA-MB231 (carrying a p53 mutation, and HER2, ER, and PgR negative) in order to elucidate its anticancer mechanisms. In α-mangostin-treated cells, induction of mitochondria-mediated apoptosis was observed. On cell-cycle analysis, G1-phase arrest, increased $p21^{cip1}$ expression and decreases in cyclins, cdc(s), CDKs and PCNA were observed. In conclusion, α-mangostin may be useful as a therapeutic agent for breast cancer carrying a p53 mutation and having HER2- and hormone receptor-negative subtypes.

1. Introduction

Various molecularly targeted drugs against a range of cancers, including breast cancer, have recently been developed. Trastuzumab is a monoclonal antibody against human epidermal growth factor (HER/ErbB) receptor 2 (HER2/ErbB2). Around 15–20% of patients with breast cancer have HER2-positive tumors, and overexpression of HER2 is observed in these patients [1]. Trastuzumab has been shown to induce tumor regression in such patients. Sunitinib, sorafenib, and bevacizumab are multitargeted tyrosine kinase inhibitors that inhibit tumor neovascularization and are currently in clinical trials [2, 3]. These drugs are associated with serious problems such as adverse effects, drug resistance, and low efficacy of single therapy, particularly against metastatic or recurrent breast cancer. Hormone therapy has also been used against hormone receptor-positive breast cancer. However, about 10 to 15% of breast cancers do not express either estrogen or progesterone receptor (ER and PgR, resp.) and do not overexpress the HER2 gene [4].

Mangosteen (*Garcinia mangostana* Linn) pericarp contains various phytochemicals, primarily xanthones, and the resin extracts have long been used for medicinal purposes in Southeast Asia [5]. α-Mangostin is a one of xanthones present in mangosteen pericarp (78% content). A recent study has shown that α-mangostin induces cell-cycle arrest and apoptosis in various types of human cancer cells [5–8]. We previously reported that α-mangostin significantly inhibits both tumor growth and metastasis in a mouse model of mammary cancer [9, 10]. In addition, α-mangostin treatment significantly decreased the levels of phospho-Akt-threonine 308(Thr308) in a human mammary carcinoma cell line and mammary carcinoma tissues *in vivo* [10].

Here, we investigated the antitumor potential of α-mangostin on apoptosis and cell cycle arrest in a human breast cancer cell line carrying a p53 mutation and having HER2-, ER-, and PgR-negative status.

2. Materials and Methods

2.1. Experimental Regimen. Mangosteen (*Garcinia mangostana* Linn) pericarps were dried, ground, and successively extracted in water and 50% ethanol. After freeze-drying the 50% ethanol extract, the resultant dried powder was suspended in water partitioned with ethyl acetate. The ethyl acetate extract was then purified by chromatography on silica gel with the n-hexane-ethyl acetate system and recrystallized to give α-mangostin at >98% purity. For *in vitro* use, crystallized α-mangostin was dissolved in dimethyl sulphoxide (DMSO), and aliquots of stock 20 mM solution were stored at −20°C.

2.2. Cell Line. The MDA-MB231, a human mammary carcinoma cell line stably expressing the green fluorescence protein (GFP) [11], was maintained in RPMI-1640 medium containing 10% fetal bovine serum with streptomycin/penicillin in an incubator under 5% CO_2. MDA-MB231 cells have a p53 mutation [12, 13] and HER2-, ER-, and PgR, negative feature [14].

2.3. Cell Viability. MDA-MB231 cells were grown in RPMI-1640 medium supplemented with 10% bovine serum under an atmosphere of 95% air and 5% CO_2 at 37°C. These cells were plated into 96-well plates (1×10^4 cells/well) one day before α-mangostin treatment. They were subsequently incubated for 24 h with culture medium containing DMSO vehicle alone (control) or with medium containing α-mangostin at various concentrations up to 48 μM. Cell viability was determined using a Cell-Titer-Bule Cell Viability Assay (Promega Co., Madison, WI, USA). The IC_{50} under these conditions was found to be 20 μM α-mangostin for 24 h treated and 16 μM for 48 h treated in MDA-MB231 cells; thus, all *in vitro* studies were performed using 20 μM α-mangostin.

2.4. Time-Lapse Imaging. Cells were grown on 35 mm culture dishes under the above-mentioned condition. These cells were subsequently incubated for 24 h with culture medium containing DMSO vehicle alone (control) or with medium containing 20 μM α-mangostin. Imaging has started when the reagents were added into dishes. Time-lapse images were taken using the fluorescence microscope BZ8000 (Keyence, Osaka, Japan).

2.5. Nuclear Staining. For nuclear staining, DAPI with mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) was used after immunofluorescence staining. For morphological examination of apoptotic changes, Hoechst33342 (Lonza Walkersville, Inc., Walkersville, MD, USA) was added to cultured medium at a concentration of 5 μg/mL.

2.6. Caspase Activity. MDA-MB231 cells were plated into 96-well plates at a concentration of 1×10^4 cells/well one day before α-mangostin treatment. Cells were treated with 20 μM α-mangostin or DMSO alone for 24 h. The activities of caspase-3, caspase-8, caspase-9, and caspase-4 were measured using a Fluorometric Protease Assay Kit (MBL, Inc., Nagoya, Japan) in which cells were lysed with Cell Lysis Buffer contained in this kit and the protein concentration adjusted to 50 μg in each sample. Caspase activity was measured in terms of fluorescence intensity produced by caspase cleavage of the corresponding substrate, using Fluoroskan Ascent (Thermo Election Co., Helsinki, Finland).

2.7. Cell-Cycle Distribution. Flow cytometric analysis was conducted on trypsinized MDA-MB231 cell suspensions that were harvested after a 24 h treatment with 20 μM α-mangostin and fixed in cold 70% ethanol. The cells were stained with a 50 μg/mL propidium iodide solution containing 100 μg/mL RNase A for 20 min at 37°C and then placed on ice just prior to flow cytometric analysis (EPICS Elite ESP; Coulter Co., Miami, FL, USA). The percentage of cells in each phase of the cell cycle was determined using a Multicycle Cell-Cycle Analysis program (Coulter Co.).

2.8. ssDNA Analysis. MDA-MB231 cells were plated into 96-well plates at a concentration of 1×10^4 cells/well one day before α-mangostin treatment. Cells were treated with 20 μM α-mangostin or DMSO alone for 24 h. Single-Strand break DNA (ssDNA) levels were detected by using ApoStrand ELISA Apoptosis Detection Kit (Enzo Life Sciences International, Inc., Butler Pike Plymouth Meeting, PA, USA) and measured using Microplate reader (Corona ELECTRIC Co. Ltd., Ibaraki, Japan).

2.9. Cytochrome c Release. Cytochrome *c* leaving the mitochondrial membrane was measured using the InnoCyte Flow Cytometric Cytochrome *c* Release Kit (Merck; Darmstadt, Germany). MDA-MB231 cells were harvested after a 24 h treatment with 20 μM α-mangostin or DMSO alone and 1×10^6 cells were resuspended in 300 μL Permeabilization Buffer to remove the cytosolic cytochrome *c*. The cells were fixed 4% paraformaldehyde and washed. After treatment with blocking buffer, the cells were treated with anticytochrome *c* mouse monoclonal antibody (clone 7H8, Santa Cruz, Biotechnology, CA, USA), followed by secondary antibody conjugated to FITC. Then, the cells were analyzed using a flow cytometer, BD FACSAria (Becton Dickinson, Franklin Lakes, NJ, USA).

2.10. Immunofluorescence Staining. MDA-MB231 cells were grown on 24 mm × 24 mm cover glasses and fixed in 4% formaldehyde solution in phosphate buffer. Immunofluorescence staining was performed with anti-PCNA mouse monoclonal antibody (clone PC10; Cell Signaling Technology, Danvers, MA, USA), cyclin D1 rabbit monoclonal antibody (clone 92G2; Cell Signaling Technology), $p21^{cip1}$ rabbit polyclonal antibody (clone C-19; Santa Cruz Biotechnology). These antibodies were also used in Western blotting.

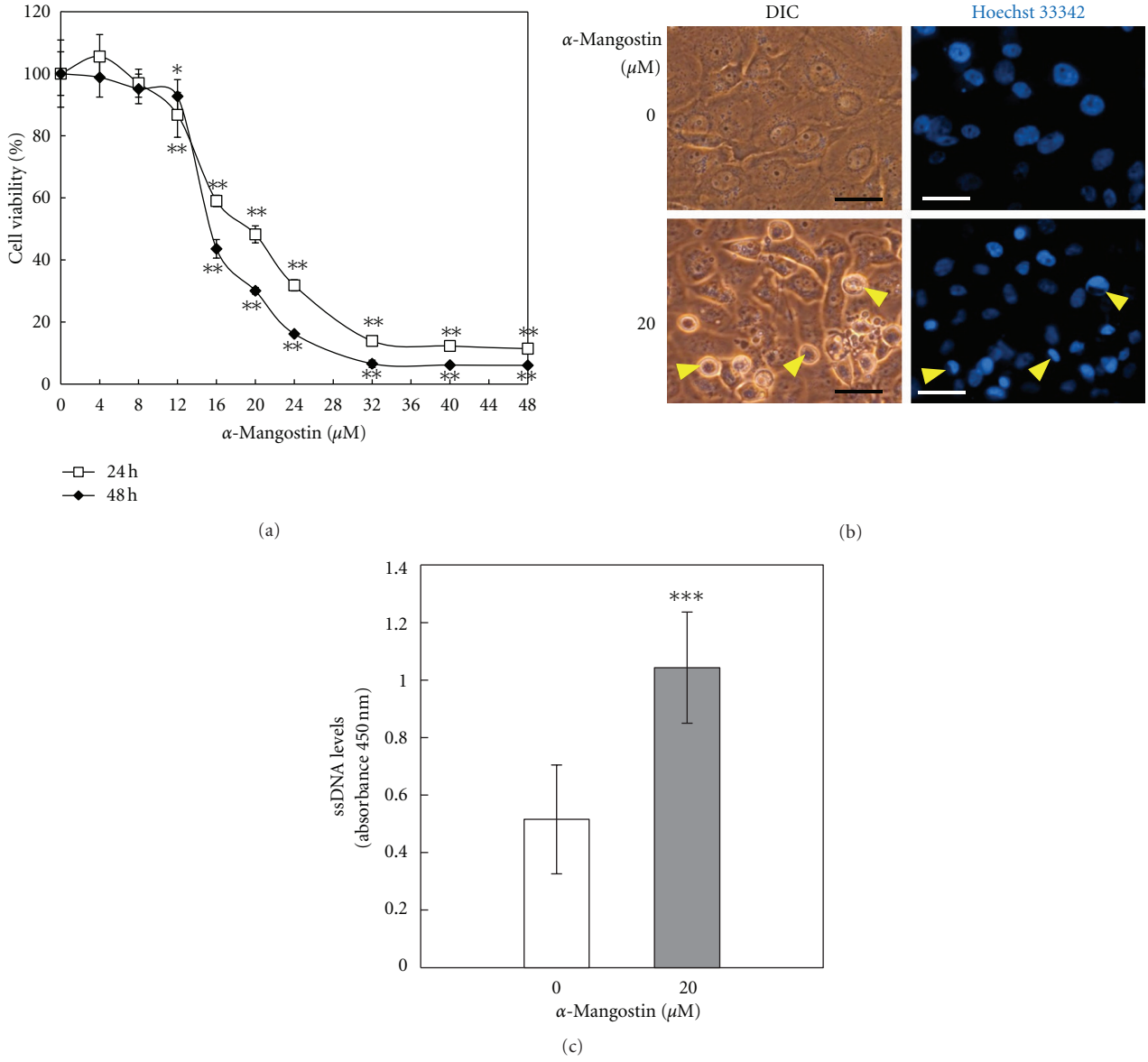

Figure 1: Cell viability and apoptosis detection in MDA-MB231 cells after α-mangostin treatment. (a) Cell viability was significantly lower in human mammary carcinoma MDA-MB231 cells treated with more than 12 μM α-mangostin for 24 or 48 h (*$P < 0.05$, **$P < 0.01$). Five samples from each of α-mangostin dosage were examined. The IC_{50} concentration was determined to be 20 μM for 24 h; therefore, 20 μM α-mangostin was used for all *in vitro* studies. (b) Morphological changes in MDA-MB231 cells treated with 20 μM α-mangostin for 24 h, as compared to controls. Upper two panels show controls and lower two panels show α-mangostin-treated cells. α-Mangostin-treated cells appeared shrunken and chromatin condensation was observed (yellow arrow heads in lower right panel). Scale bars = 50 μm. (c) ssDNA levels were determined by ELISA and were significantly elevated in cells treated with α-mangostin for 24 h, as compared to control levels (***$P < 0.001$). Data are presented as mean ± SD. For all analyses, five samples from control and α-mangostin-treated cells were examined.

2.11. Western Blotting. Total protein was extracted from whole cell lysates of MDA-MB231 cells treated with DMSO or α-mangostin according to the IC_{50} data previously stated. Total protein (40 μg) was fractionated on 16% SDS-PAGE mini gels (TEFCO, Tokyo, Japan) under reducing conditions and transferred to PVDF membranes (Immobilon - P Transfer Membrane, Millipore; Billerica, MA, USA). The membranes were activated in 100% methanol for 15 seconds and incubated with primary antibodies for the following proteins: cytochrome *c*, Bid, PCNA, cyclin D1, $p21^{cip1}$, and β-actin. Membranes were then incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP). β-Actin (clone N-21) and Full-length Bid (clone FL-195) antibodies were from Santa Cruz Biotechnology. Antibody recognizing cleaved Bid was obtained from R&D Systems (R&D Systems, Inc, Minneapolis, MN, USA). Antibody binding was subsequently visualized by exposure to an enhancing chemiluminescence reagent (Amersham ECL;

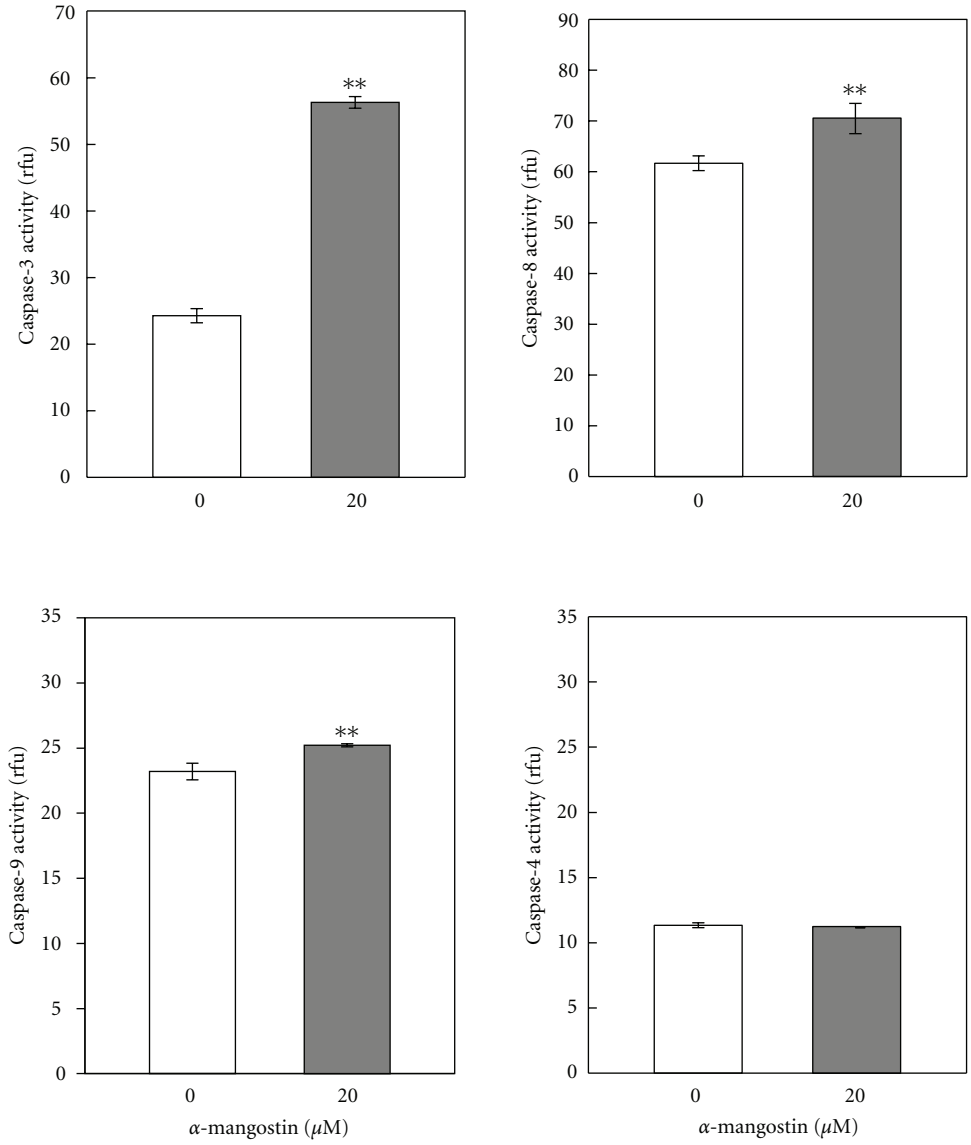

Figure 2: Caspase activity of MDA-MB231 cells after treatment with α-mangostin. Caspase activity was evaluated by luminescence assay. Activity of caspase-3, caspase-8, and caspase-9, but not caspase-4, was significantly elevated in MDA-MB231 cells treated with 20 μM α-mangostin for 24 h (**$P < 0.01$). Data are presented as mean ± SD. Five samples from control and α -mangostin-treated cells were used for measurement of caspase activity.

GE Healthcare UK Ltd., Buckinghamshire, UK). Blots were visualized using a LAS-3000 image analyzer (Fujifilm, Co., Tokyo, Japan).

2.12. Real-Time PCR. MDA-MB231 cells were treated with DMSO or 20 μM α-mangostin for 6, 12, and 24 h. Protein was extracted using cell lysis buffer containing protease and phosphatase inhibitor cocktail. Total RNA was extracted from these cells with FastPure RNA kit (Takara Bio Inc., Shiga, Japan) and cDNAs were synthesized with PrimeScript RT reagent kit (Takara Bio Inc.). Primers involved in the cell-cycle regulation were used containing PrimerArray Cell cycle (human) (Takara Bio Inc.) and real-time PCR reaction was performed with Thermal Cycler Dice Real Time (Takara Bio Inc.). Data were corrected against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values and expressed as mean ± SD.

2.13. Statistical Analysis. Significant differences in the quantitative data between groups were analyzed using Student's *t*-test via the method of Welch, and *P* values less than 0.05 were considered to represent a statistically significant difference.

3. Results and Discussion

3.1. Cell Viability. Viability analyses of MDA-MB231 human mammary cancer cells showed significantly lower viability

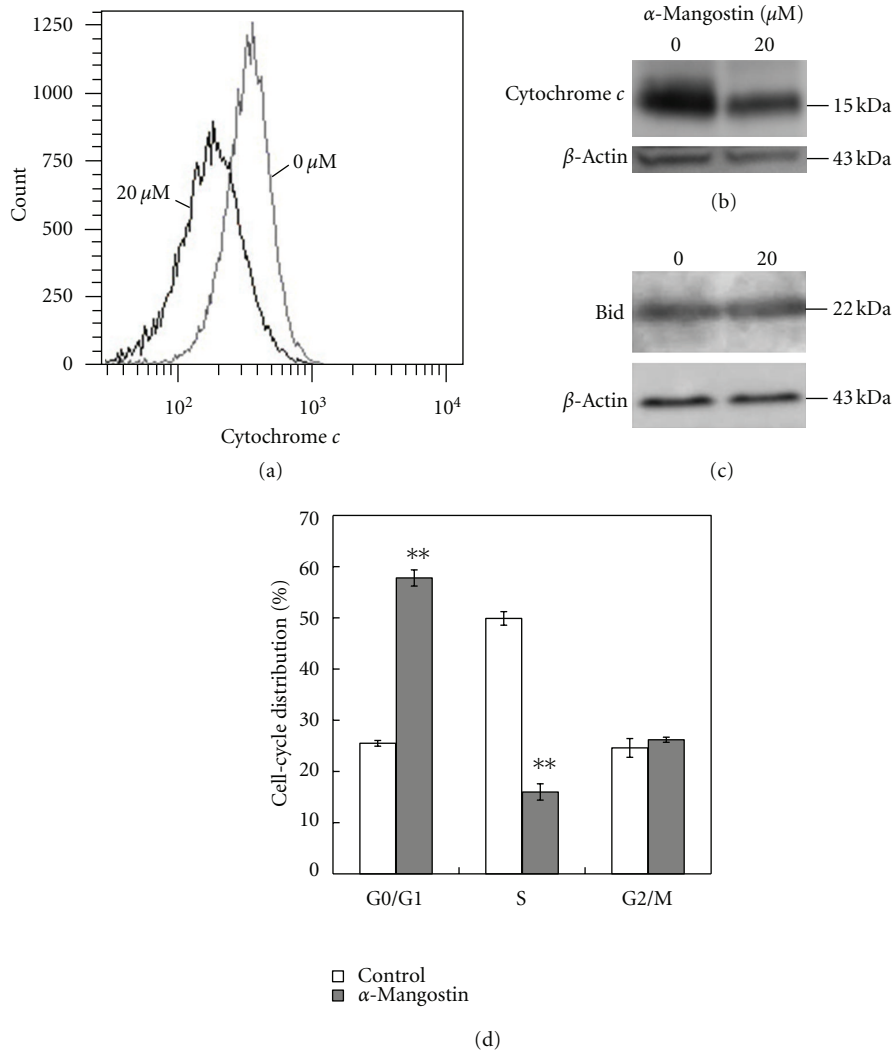

Figure 3: Cytochrome *c* expression, Bid cleavage, and cell-cycle distribution of MDA-MB231 cells after α-mangostin treatment. (a) Cytochrome *c* in mitochondria, as determined by flow cytometry (black line indicates α-mangostin-treated cells and gray line indicates controls). The levels of cytochrome *c* protein in mitochondrial fractions were significantly lower in cells treated with α-mangostin for 24 h (b). Western blots of cytochrome *c* (15 kDa) showed significant decreases in cells treated with α-mangostin for 24 h, as compared to controls (upper panel). In α-mangostin-treated cells, cytochrome *c* was released from mitochondria, leading to decreases in concentration. β-Actin served as an internal control (lower panel, same in (c)). (c) Western blots of Bid (22 kDa) in control cells and cells treated with α-mangostin for 24 h were similar (upper panel). Cleaved Bid was not observed after α-mangostin treatment. Three samples were used for all analyses in (a)–(c). (d) Cell-cycle analysis confirmed that α-mangostin induced arrest in the G1-phase and inhibition of cells entering the S-phase in MDA-MB231 cells ($^{**}P < 0.01$). Data are presented as mean ± SD of triplicate, independent measurements.

after 24 and 48 h of treatment with more than 12 μM α-mangostin (Figure 1(a)). Based on the IC_{50} data, 20 μM was determined to be the optimal concentration of α-mangostin for *in vitro* studies.

3.2. Morphological Changes. For morphological examination of apoptotic changes, MDA-MB231 cells were stained with Hoechst 33342 (Figure 1(b)). After 20 μM α-mangostin treatment for 24 h, cells appeared shrunken and chromatin condensation was observed (yellow arrow heads in lower right panel). These changes suggest that the antiproliferative effects of α-mangostin are associated with apoptosis in MDA-MB231 human mammary cancer cells. Furthermore, we performed time-lapse imaging from the start of 20 μM α-mangostin treatment for 24 h. After 6 h, cell proliferation started to decrease significantly and cell shape had changed after 12 h (data not shown).

3.3. Apoptosis Studies

3.3.1. ssDNA Analysis. In order to confirm that the morphological changes observed after α-mangostin treatment occurred as a consequence of apoptosis, we measured ssDNA levels of α-mangostin treated cells, as compared to controls. In α-mangostin-treated cells, ssDNA levels were significantly elevated ($P < 0.001$, Figure 1(c)). This suggests that the

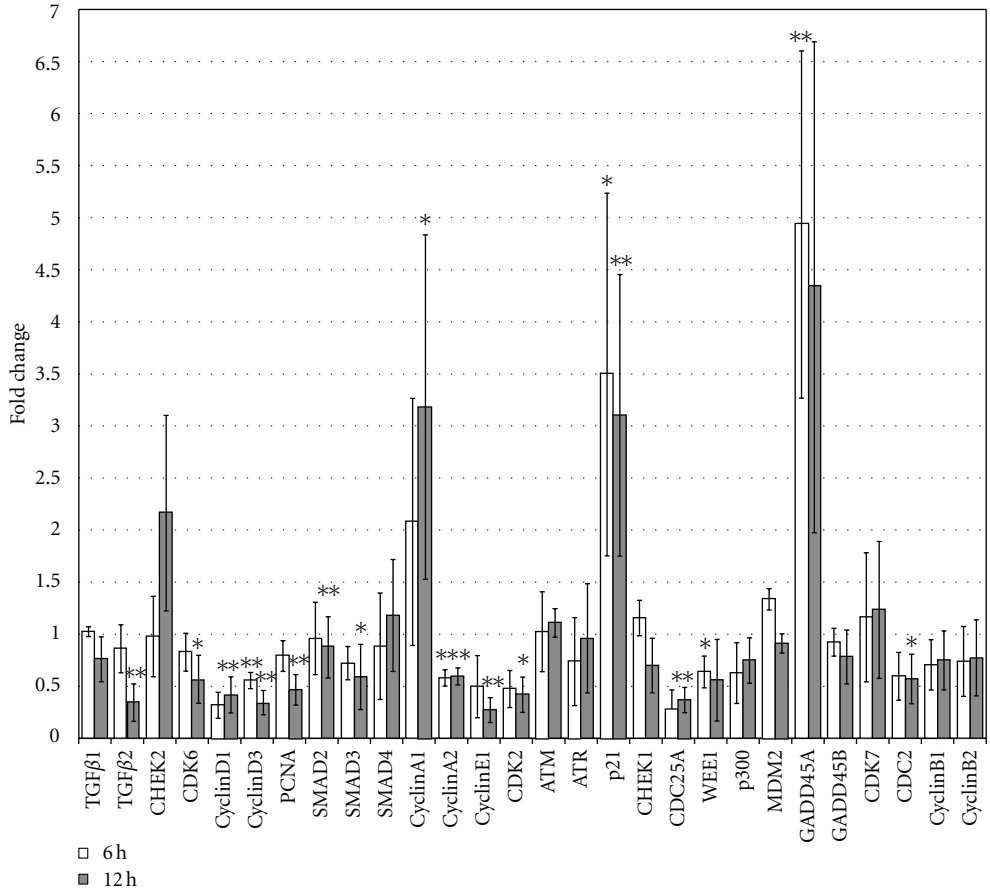

Figure 4: Real-time PCR analysis of gene expression associated with cell cycle. mRNA expression of cell-cycle-related genes, such as cyclins, cdc(s), CDKs, and PCNA, was examined by real-time PCR assay in MDA-MB231 cells after treatment with α-mangostin, as compared with controls. White bars and gray bars show the results of analysis for 6 h and 12 h after treatment with 20 μM α-mangostin, respectively ($^*P < 0.05$, $^{**}P < 0.01$). Relative levels of target gene expression were calculated using the $\Delta\Delta$ CT method. GAPDH was used as a reference gene. Data are presented as means $\pm$ SD of triplicate independent measurements.

morphological changes in human mammary cancer cells that occurred after α-mangostin treatment were caused by apoptosis.

3.3.2. Caspase Activity. Significantly elevated caspase-3, caspase-8, and caspase-9 activity was observed in MDA-MB231 cells treated with α-mangostin for 24 h (Figure 2), as compared to controls. The activity of caspase-4 did not differ significantly between control cells and α-mangostin-treated cells (Figure 2). These results indicate that α-mangostin-induced mitochondria mediated apoptosis.

3.3.3. Cytochrome c Release. In order to confirm mitochondria-mediated apoptosis, levels of cytochrome *c* were measured by flow cytometry and Western blot. The levels of cytochrome *c* protein in mitochondrial fractions were significantly lower in cells treated with α-mangostin for 24 h (Figure 3(a)). Cytochrome *c* protein was released from mitochondria, leading to decreases in concentration (Figure 3(b)). These results were strongly suggesting engagement of the mitochondria-mediated apoptotic pathway.

3.3.4. Bid Cleavage. As caspase-8 activity was elevated, we examined whether the mitochondrial pathway was activated via caspase-8-Bid cleavage by performing Western blots for Bid. Full-length Bid (22 kDa) was equally detected in control cells and in cells treated with α-mangostin for 24 h (Figure 3(c)); this indicates that no Bid cleavage occurred. In addition, no cleaved Bid (15 kDa) was observed when using cleaved Bid-detectable antibody in any of the groups (data not shown). These results suggest that apoptosis was induced by α-mangostin via mitochondria, but was not accompanied by Bid cleavage. We previously reported that α-mangostin decreased phospho-Akt-Th308 in MDA-MB231 cells and mammary carcinoma tissues [10]. Akt is a serine/threonine protein kinase that mediates the downstream effects of phosphatidylinositol 3-kinase (PI3K) by phosphorylating multiple targets involved in regulating diverse cellular functions, including proliferation, growth, and survival. Therefore, Akt phosphorylation by therapeutic agents leads to growth inhibition, cell-cycle arrest, and apoptosis in cancer cells. A previous study in human colon cancer cells showed that α-mangostin inhibited Erk1/2

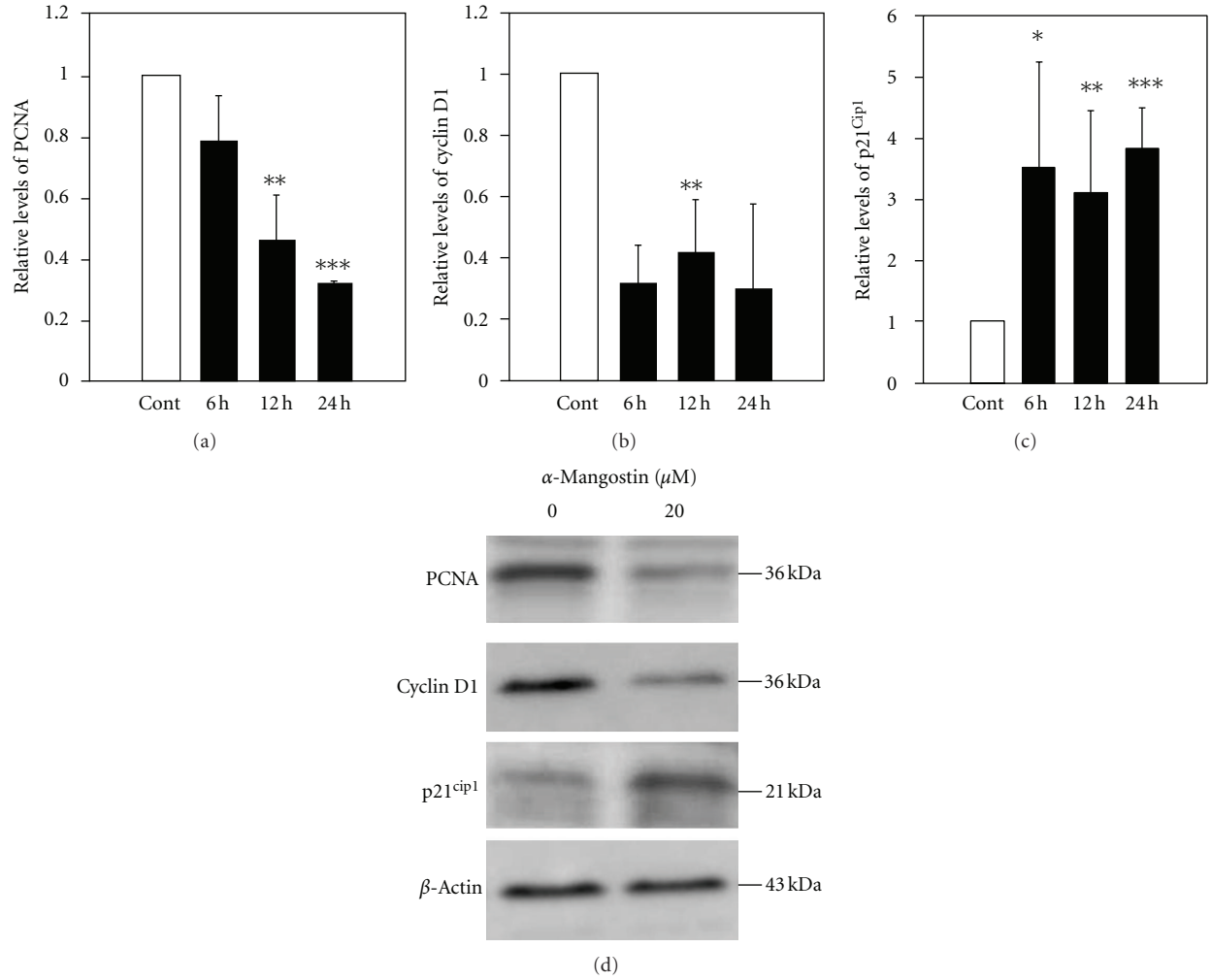

Figure 5: Changes in expression of genes involved in G1 phase regulation. (a)–(c) mRNA expressions of cell-cycle regulators for G1 phase, PCNA (a), cyclin D1 (b), and $p21^{cip1}$ (c), were examined using real-time PCR assays in MDA-MB231 cells after treatment with α-mangostin, as compared to controls. White bars indicate the results for controls and black bars indicate the results of analysis at 6 h, 12 h, and 24 h after treatment with α-mangostin ($^*P < 0.05$, $^{**}P < 0.01$). PCNA and cyclin D1 expressions were significantly lower and $p21^{cip1}$ was significantly higher in cells treated with α-mangostin for 24 h, as compared to controls. Relative target gene levels were calculated using the $\Delta\Delta$CT method. GAPDH was used as a reference gene. Data are presented as means ± SD of triplicate independent measurements. (d) Western blots of PCNA (36 kDa) and cyclin D1 (36 kDa) showed significantly lower levels, and $p21^{cip1}$ (21 kDa) showed significantly higher levels in cells treated with α-mangostin for 24 h, as compared to controls (left lane). These results were similar to those for PCR analysis, as shown in (a)–(c). β-Actin served as an internal control (lowest panel). Three samples from control and α-mangostin-treated cells were used for measurement.

and Erk5 phosphorylation involving the mitogen-activated protein kinase (MAPK) signaling pathway [5]. PI3K/Akt and Erk signaling target Bcl-2-associated agonists of cell death (Bad), a member of the Bcl-2 family. Bcl-2 family members are known to be regulators of programmed cell death. Bad promotes apoptosis by displacing Bax, another Bcl-2 family member, from binding to Bcl-2 and Bcl-xL, causing cytochrome *c* release from mitochondria [15]. These data suggest that apoptotic cell death caused by α-mangostin treatment in human mammary carcinoma cells occurs via mitochondria-mediated apoptosis, followed by inhibition of PI3K/Akt signaling pathway, and may include Bad activation.

3.4. Cell-Cycle Distribution. As measured by flow cytometry, 24 h exposure to 20 μM α-mangostin induced a significant elevation in the number of cells in the G1-phase, as compared with control cells (Figure 3(d)). There was also a significant reduction in the S-phase population in α-mangostin-treated cells (Figure 3(d)). These results suggest that G1-phase arrest was the result of α-mangostin inhibiting entry into S-phase.

3.5. Expression of Cell-Cycle Regulatory Genes. As α-mangostin treatment induced cell-cycle arrest, we investigated the expression of cell-cycle regulatory genes. Real-time analysis revealed that the expression of $p21^{cip1}$ was up-regulated and CHEK2 expression tended to increase for more than 6 h after α-mangostin treatment (Figure 4). $p21^{cip1}$ is a cyclin-dependent kinase inhibitor and the encoded protein binds to and inhibits the activity of cyclin-CDK2 (CDK: cyclin-dependent kinase) or -CDK4 complexes, thereby functioning

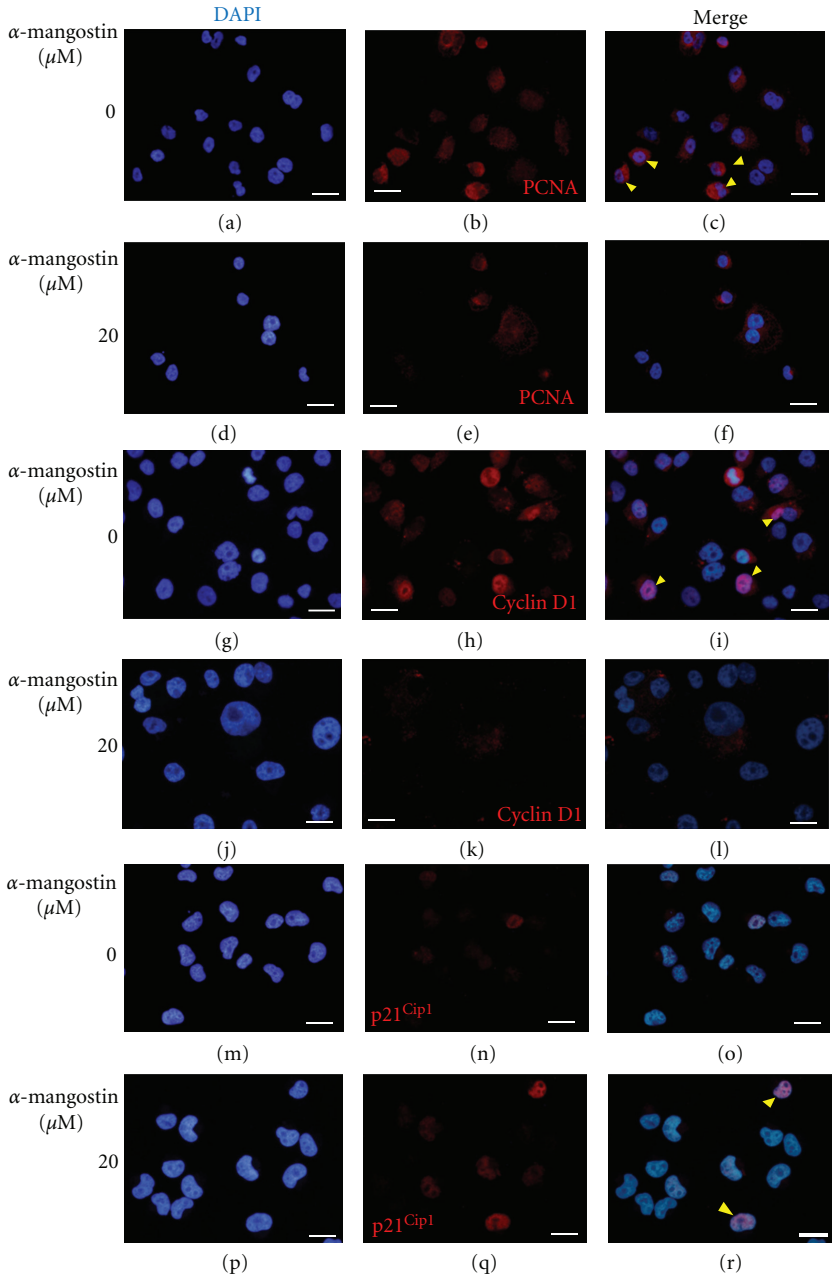

Figure 6: Expression of PCNA, cyclin D1 and $p21^{cip1}$ proteins after α-mangostin treatment. Immunofluorescence of PCNA (a)–(f), cyclin D1 (g)–(l), and $p21^{cip1}$ (m)–(r) in MDA-MB231 cells after 24 h of treatment with α-mangostin ((d)–(f), (j)–(l), (p)–(r)), as compared with controls ((a)–(c), (g)–(i), (m)–(o)). Yellow arrowheads in panels (c), (i), and (r) indicate higher expression of each protein in nuclei. PCNA and cyclin D1 expression was reduced and $p21^{cip1}$ expression were elevated in α-mangostin-treated cells, as compared to controls. Scale bars = 25 μm.

as a regulator of cell-cycle progression at the G1-phase [16]. CHEK2 is one of the cell-cycle checkpoint regulators and putative tumor suppressors that lead to G1-phase arrest through CDK phosphatase and cell division cycle 25 homolog A (cdc25A) phosphorylation [17]. Increases in $p21^{cip1}$ and CHEK2 expression lead to decreases in CDKs and cyclins, and G1-phase arrest and inhibition of cell proliferation, followed by decreases in proliferating cell nuclear antigen (PCNA). Furthermore, we confirmed the expression of G1/S-phase-related molecules, particularly $p21^{cip1}$, PCNA, and cyclin D1 by using Western blotting and immunofluorescence staining (Figures 5 and 6). On Western blotting analysis, PCNA (36 kDa) and cyclin D1 (36 kDa) protein were significantly lower, and $p21^{cip1}$ (21 kDa) protein was significantly elevated in cells treated with α-mangostin for 24 h, as compared to controls (Figure 5(d)). These results agreed with those of the real-time PCR analysis shown in Figures 4 and 5(a)–5(c). Immunofluorescence staining

revealed that PCNA and cyclin D1 expression were lower and that $p21^{cip1}$ expression was elevated in the nuclei of α-mangostin-treated cells, as compared to controls (Figure 6). These results suggest that α-mangostin induces G1-phase arrest and S-phase suppression by altering the expression of cell-cycle-related molecules, such as $p21^{cip1}$, CHEK2, cyclins, cdc(s), CDKs, and PCNA.

4. Conclusion

In conclusion, our results demonstrated that the therapeutic effects of α-mangostin are mediated by mitochondria-mediated apoptosis under control of the PI3K/Akt signaling pathway. α-Mangostin may be useful as a therapeutic agent for breast cancer carrying a p53 mutation and including HER2/hormone-negative subtypes.

Acknowledgments

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Research Article

Cardiovascular Activity of Labdane Diterpenes from *Andrographis paniculata* in Isolated Rat Hearts

Khalijah Awang,[1] Nor Hayati Abdullah,[2] A. Hamid A. Hadi,[1] and Yew Su Fong[3]

[1] *Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2] *Medicinal Plants Division, Forest Research Institute of Malaysia (FRIM), Kepong, 52109 Kuala Lumpur, Malaysia*
[3] *Department of Pharmacy, Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia*

Correspondence should be addressed to Khalijah Awang, khalijah@um.edu.my

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The dichloromethane (DCM) extract of *Andrographis paniculata* Nees was tested for cardiovascular activity. The extract significantly reduced coronary perfusion pressure by up to 24.5 ± 3.0 mm Hg at a 3 mg dose and also reduced heart rate by up to 49.5 ± 11.4 beats/minute at this dose. Five labdane diterpenes, 14-deoxy-12-hydroxyandrographolide (**1**), 14-deoxy-11,12-didehydroandrographolide (**2**), 14-deoxyandrographolide (**3**), andrographolide (**4**), and neoandrographolide (**5**), were isolated from the aerial parts of this medicinal plant. Bioassay-guided studies using animal model showed that compounds, (**2**) and (**3**) were responsible for the coronary vasodilatation. This study also showed that andrographolide (**4**), the major labdane diterpene in this plant, has minimal effects on the heart.

1. Introduction

Andrographis paniculata Nees, family Acanthaceae, has been used since time immemorial in Ayurvedic medicine, mainly for liver problems and dysentery [1]. The plant is also known as "Indian Echinacea" and "King of bitter." Phytochemical screening on this herbal plant showed that it contains a lot of flavonoids and terpenoids while moderate in alkaloids and tannins compounds [2]. This plant has been featured in at least 26 Ayurvedic formulae, whereas in traditional chinese medicine, *A. paniculata* is an important "cold property". In Malaysia, *A. paniculata* is more commonly known as "hempedu bumi" and is widely used in traditional medicine, especially for the treatment of cardiovascular disorders.

Previous researches have shown that *A. paniculata* extract and its labdane diterpenes have a broad range of pharmacological effects such as the ability to inhibit replication of the HIV virus [3, 4], prevent common cold [5–7], antimalarial [8], prevent diarrhea [9], antibacterial [10], anti-inflammatory [11–13], antihyperglycemic effect [14, 15], suppress various cancer cells [16–18], and antifertility and pregnancy-terminating effects [19].

In earlier cardiovascular studies, *A. paniculata* extracts significantly reduced atherosclerotic artery stenosis and lowered restenosis rates after angioplasty in rabbits [20] decreases platelet aggregation *in vitro* [21] and were reported to be antihypertensive in rats [22]. Nevertheless, the scientific basis for the use of *A. paniculata* in treating "heart problems" is still unclear. To our knowledge, its direct effects on the isolated heart of an animal model are not known. Therefore, in this study, the dichloromethane (DCM) extract of *A. paniculata* was tested against coronary vessels, cardiac muscle contractility, and heart rate in Langendorff perfused rat hearts. Our preliminary study showed that the dichloromethane extract caused coronary vasodilation, reduced heart rate while not affecting the cardiac contractility of isolated perfused rat hearts. Further isolation work was performed on the dichloromethane extract in order to identify the active compound(s) responsible for the cardiovascular activity observed.

2. Materials and Method

2.1. General. All spectral data were obtained on various instruments; infrared (IR) spectrum was recorded on the Perkin Elmer FT-IR (fourier transform) spectrometer RX1, ultraviolet (UV) spectrum was taken on a Shimadzu UV-160A UV-visible recording spectrophotometer, nuclear magnetic resonance spectrum (NMR) was obtained from JEOL JNM-LA400 FT-NMR spectrometer (400 MHz), and mass spectrum (MS) was recorded on a Shimadzu GC-MS (gas chromatograph—mass spectrometry) spectrometer (HP 6890 Series Mass Selective Detector and HP 6890 Series GC System). The cardiovascular activity was measured using polygraph model 7D Grass, peristaltic pump model Compact Drive MasterFlex, and pressure transducer model GOULD Statham USA.

2.2. Plant Material. The aerial parts of *Andrographis paniculata* were supplied by the Herbarium Unit, Department of Botany, University of Malaya, with herbarium series number KL4930.

2.3. Extraction and Isolation. Dried and ground aerial parts (650.0 g) of the plant were extracted with dichloromethane for 6 h using a Soxhlet apparatus at 45°C. The extract was concentrated under reduced pressure to yield 26.5 g of dichloromethane (DCM) crude extract. DCM (3 g) was chromatographed on a silica gel column with a CH_2Cl_2-MeOH gradient solvent system to give 80 fractions, which were combined into 4 subfractions (A–D) based on TLC (thin-layer chromatography) patterns and ^{1}H NMR spectra. Subfraction B was subjected to column chromatography on silica gel and gave 14-deoxy-11,12-dihydroandrographolide (**2**) (264.6 mg, CH_2Cl_2/MeOH, 98 : 2) and 14-deoxyandrographolide (**3**) (352.8 mg, CH_2Cl_2/MeOH, 99 : 1). Subfractions A, C, and D underwent further isolation by column chromatography analyses over silica gel. Sub fraction C gave andrographolide (**4**) (882.3 mg, CH_2Cl_2/MeOH, 96 : 4), sub fraction D gave neoandrographolide (**5**) (44.1 mg, CH_2Cl_2/MeOH, 90 : 10), and sub fraction A gave 14-deoxy-12- hydroxyandrographolide (**1**) (176.4 mg, CH_2Cl_2/MeOH, 97 : 3). The structure of each compound was confirmed by comparison of its NMR, IR, UV, and MS data with literature values [12].

2.4. Cardiovascular Activity on Isolated Rat Hearts. The extracts and isolated compounds were screened for their cardiovascular effects in isolated perfused rat hearts by using the Langendorff-perfused model and method, with modification [23]. The Polygraph model (Grass Instrument Co. model 7D) was calibrated to measure heart rate, developed tension, and coronary perfusion pressure of the heart. The modified Krebs-Henseleit solution (37°C) was pumped to flow at a constant rate (10 mL/min) by using a peristaltic pump (Compact Drive Masterflex), and the gas (95% O_2 and 5% CO_2) was allowed to pass through this solution. The Krebs-Henseleit solution contained the following: NaCl 118 mM, D-glucose 11.6 mM, $NaHCO_3$ 25 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, and $CaCl_2$ 1.23 mM.

Sprague Dawley rats (250–300 g) were anaesthetized with sodium pentobarbitone (72 mg/1000 g) and killed by cervical dislocation. The heart was isolated and put in a Petri dish containing cold Krebs-Henseleit solution to stop the heartbeat. After the fat and connective tissues had been removed from the heart, the aorta was cannulated and perfused with the Krebs-Henseleit solution. The coronary vessel tone was indicated by coronary perfusion pressure, which was measured with a pressure transducer (GOULD Statham USA) and monitored with the Grass Model 7D Polygraph.

The apex of the left ventricle was then attached with a hook and connected to the isometric tension transducer with a piece of thread, which passed through a pulley system. The resting heart tension was adjusted to 2 g to the optimum contractile force. The contractility was monitored by an isometric tension transducer and recorded as developed tension. Heart rate was also detected through this transducer.

The perfused isolated rat heart (PIH) was stabilised for about 20 min before any drug samples were injected. All injections were made at the rubber tubing near the cannula into the Krebs-Henseleit buffer. The labdane diterpenes compounds of *A. paniculata* were prepared as 1 mg/mL solutions. The first compound at 100 μL was injected into the PIH soon after its heart rate, contractility, and coronary perfusion pressure stabilized. The effect of the first compound/dose was allowed to disappear, and then administered with the second compound until the last compound followed by 40% ethanol as a vehicle control and finally 1000 nmoles isoprenaline and sodium nitroprusside as positive controls, all in the same heart. The compounds administred to the next heart were arranged randomly in order to avoid bias.

2.5. Statistical Analyses. Data obtained were expressed as mean standard error (± S.E.M.). The data were analyzed for statistical significance using Student's *t*-test. *P* values less than 0.05 were considered to be significant ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

3. Results and Discussion

The DCM extract of *A. paniculata* [24] significantly reduced the coronary perfusion pressure by up to 24.5 ± 3.0 ($P < 0.05$) and 29.4 ± 8.5 mm Hg ($P < 0.05$) at doses of 3 mg and 1 mg, respectively, and the heart rate by up to 49.5 ± 11.4 beats/minute ($P < 0.05$) at the 3 mg dosage. Four isolated rat hearts were used to investigate the effect of the DCM. Further isolation work was performed on the dichloromethane extract in order to identify the compound(s) responsible for the cardiovascular activity. Fractionation and purification of the DCM extract led to the isolation and purification of five labdane diterpenes, identified as 14-deoxy-12-hydroxyandrographolide (**1**), 14-deoxy-11,12-dihydroandrographolide (**2**) (*trans* isomer), 14-deoxyandrographolide (**3**), andrographolide (**4**), and neoandrographolide (**5**) (Figure 1). The structure of each compound was identified by comparison of their NMR, IR, UV, and MS data with literature values [25]. At least seven rat hearts were used to investigate the effect of each diterpenoid

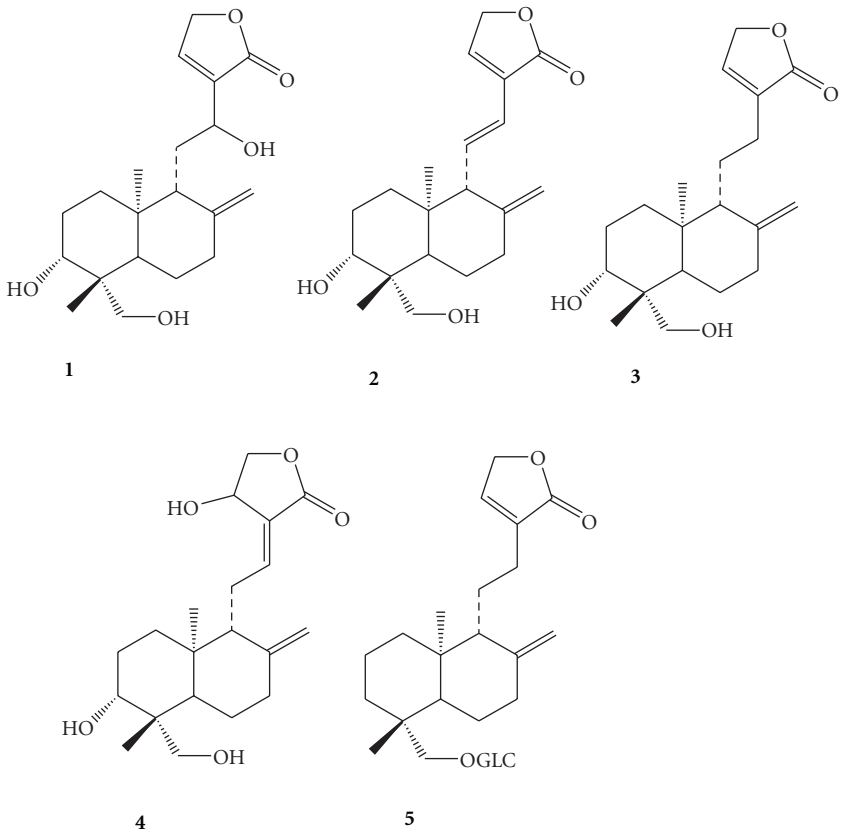

Figure 1: Chemical structure of compounds **1–5**.

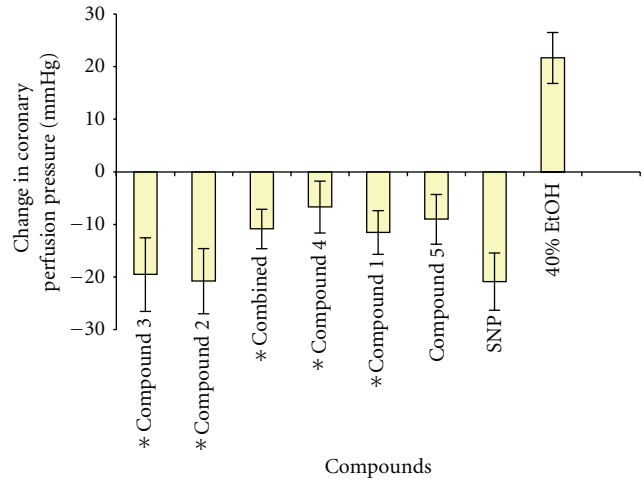

Figure 2: The effects of diterpenoids isolated from the dichloromethane extract of *A. paniculata*, sodium nitroprusside (SNP), and 40% EtOH, on coronary perfusion pressure of isolated rat hearts. The results are expressed as mean ± s.e.m., $^*P < 0.05$, student's paired *t*-test, compounds versus vehicle (40% ethanol), $n = 7$. Basal coronary perfusion pressure = 72.1 ± 5.6 mm Hg.

on coronary perfusion pressure (a measure of coronary tone), developed tension (a measure of cardiac contractility), and heart rate.

All compounds were prepared as 1 mg/mL solutions in 40% ethanol. The dose used for each compound was 0.1 mg. All of these diterpenoids reduced coronary perfusion pressure (CPP) significantly with compound **3** showing the largest reduction (−19.5 ± 7.0 mm Hg, $P < 0.05$) (Figure 2). This value was comparable with sodium nitroprusside (positive control), which reduced CPP by 22.0 ± 5.4 mm Hg ($P < 0.05$). The combination of all five diterpenoids in the same amounts reduced CPP by 10.8 ± 3.7 mm Hg ($P < 0.05$).

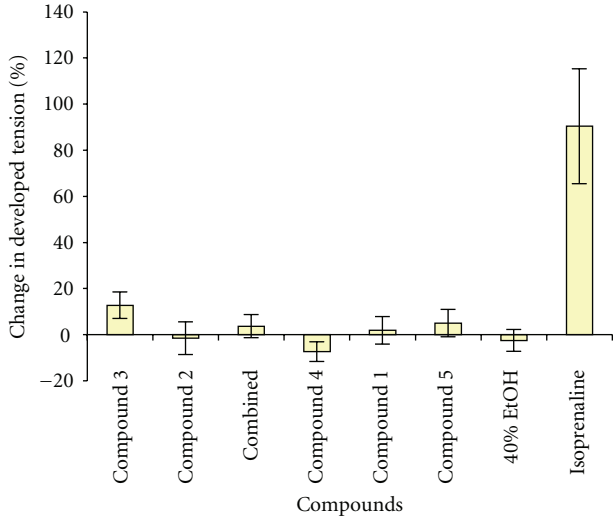

Figure 3: The effects of diterpenoids isolated from the dichloromethane extract of *A. paniculata*, 40% EtOH and isoprenaline on developed tension of isolated rat hearts. The results are expressed as mean ± s.e.m, $n = 7$. Basal developed tension = 3.9 ± 0.3 g.

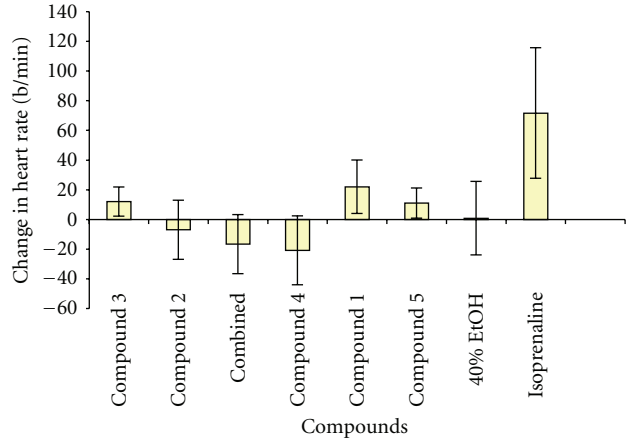

Figure 4: The effects of diterpenoids isolated from the dichloromethane extract of *A. paniculata*, 40% EtOH and isoprenaline on heart rate (beats/min) of isolated rat hearts. The results are expressed as mean ± s.e.m, $n = 7$. Basal heart rate = 251 ± 13 beats/min.

None of the five diterpenoids showed any significant effect on cardiac contractility and heart rate as compared with the positive control, isoprenaline (Figures 3 and 4).

Many studies have been reported on the cardiovascular activity of *A. paniculata* [20–28] and on other pharmacological aspects [3–19]. However, no cardiovascular activity study involving a direct effect on the isolated hearts of rats has been published previously. In this current study, the DCM crude extract showed potent cardiovascular activity on the isolated perfused rat heart, except that it did not affect cardiac contractility. Fractionation and purification of the DCM extract led to the isolation of five active compounds (**1–5**), with 14-deoxy-11,12-dihydroandrographolide (**2**) and 14-deoxyandrographolide (**3**) showing the largest reduction in coronary perfusion pressure (−18.96 ± 6.19 and −19.5 ± 7.00 Mm Hg, resp.) ($P < 0.05$). The results from our study support the previous finding by Zhang and Tan [26] that showed 14-deoxy-11,12-dihydroandrographolide (**2**) and 14-deoxyandrographolide (**3**) caused significant falls in mean arterial pressure (MAP) in the hearts of anaesthetized rats. However, none of the five diterpenes showed any significant effect on heart rate. This observation suggests that compound/s other than compounds **1–5** may be responsible for this activity.

The decreased coronary perfusion pressure is a measurement for coronary artery vasorelaxation of an isolated rat heart. Due to the constant perfusion of Krebs-Henseleit into the heart during the experiment, any change in coronary perfusion pressure therefore would reflect change in vascular resistance [28]. Activation of β-adrenoceptors caused vasorelaxation of coronary channel.

Another mechanism involved in the cardiovascular activity of 14-deoxy-11,12-dihydroandrographolide (**2**) and 14-deoxyandrographolide (**3**) has been reported [22–24, 26]. The ability of *A. paniculata* extract to dilate coronary vessels has not been reported before, but both 14-deoxy-11,12-dihydroandrographolide (**2**) and 14-deoxyandrographolide (**3**) have been reported to dilate aortic rings [26]. The compound 14-deoxy-11,12-dihydroandrographolide (**2**) exhibited hypotensive effect in anaesthetized rats [29]. Both compounds exert their vasorelaxant activity by the release of nitric oxide (NO) and activation of the guanylate cyclase pathway, as well as the blockade of Ca^{2+} influx through both voltage- and receptor-operated Ca^{2+} channels [30].

4. Conclusions

In conclusion, the ability of 14-deoxy-11,12-dihydroandrographolide (**2**) and 14-deoxyandrographolide (**3**) to dilate coronary vessels and aortic rings [23] supports the traditional use of *A. paniculata* in the treatment of cardiovascular disorder and also in alleviating hypertension. These results also suggest that both compounds (**2**) and (**3**) might be good drug candidates for the treatment of angina and hypertension. Therefore, further *in vivo* pharmacological, pharmacokinetic, and toxicological studies need to be undertaken. This result also shows that the major compound, andrographolide (**4**), is not the major contributor to the cardiovascular activity of this plant.

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Research Article

Comparative Study of the Effect of Baicalin and Its Natural Analogs on Neurons with Oxygen and Glucose Deprivation Involving Innate Immune Reaction of TLR2/TNFα

Hui-Ying Li,[1] Jun Hu,[1] Shuang Zhao,[1] Zhi-Yi Yuan,[1] Hong-Jiao Wan,[2] Fan Lei,[1] Yi Ding,[3] Dong-Ming Xing,[1] and Li-Jun Du[1]

[1] *Protein Science Laboratory of the Ministry of Education, Laboratory of Pharmaceutical Sciences, School of Life Sciences and School of Medicine, Tsinghua University, Beijing 100084, China*
[2] *Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China*
[3] *Drug Discovery Facility, School of Life Sciences, Tsinghua University, Beijing 100084, China*

Correspondence should be addressed to Li-Jun Du, lijundu@mail.tsinghua.edu.cn

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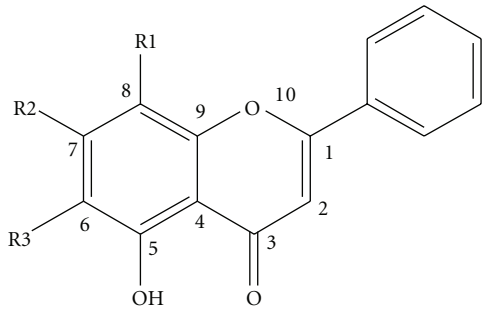
This work is to study the baicalin and its three analogs, baicalin, wogonoside, and wogonin, on the protective effect of neuron from oxygen-glucose deprivation (OGD) and toll-like receptor 2 (TLR2) expression in OGD damage. The results showed that baicalin and its three analogs did protect neurons from OGD damage and downregulated protein level of TLR2. D-Glucopyranosiduronic acid on site 7 in the structure played a core of cytotoxicity of these flavonoid analogs. The methoxyl group on carbon 8 of the structure had the relation with TLR2 protein expression, as well as the anti-inflammation. In addition, we detected caspase3 and antioxidation capability, to investigate the effect of four analogs on cell apoptosis and total antioxidation competence in OGD model.

1. Introduction

Scutellariae radix, a Chinese medicine, has various pharmacological effects such as antibacterial, antivirus, anti-inflammation, antioxidation, and antistroke in clinic [1–4]. Its active ingredient is a kind of flavones, consisting of baicalin, baicalein, wogonin, and wogoniside briefly (Figure 1) [5]. It is reported that Scutellariae radix protects neurons from the injury of ischemia and reperfusion [6]. Baicalin, the major component of the flavones, was confirmed with the neuroprotection of the damage by ischemia and reperfusion and the action on central nerve system [7–10]. Recently, baicalein was studied with a promising effect of neuroaction on multiple sites [11–17]. However, no report presented the effect of baicalin and TLR2 expression on neurons. A few researches reported that wogonin and wogonoside acted on the neurons [18–20]. Baicalin and baicalein were studied as an antioxidant; in a few models baicalein was even stronger than baicalin in reducing several free redials [21]. Comparing them, baicalein and baicalin significantly attenuated the cellular injury induced by hydrogen peroxide, but wogonin and wogonoside acted more weakly [22]. It is suggested that there must be other mechanisms of wogonin and wogonoside on neuroprotection.

As the anti-inflammation of baicalin and baicalein was confirmed, the researches on the innate immune response were studied in cerebral ischemia reperfusion and presented the reason partly why the anti-inflammation of baicalin and baicalein was one of the major assays for the protection of brain from the ischemia reperfusion damage [23–27]. Several receptors were indicated as the key targets of ischemic reperfusion injury in the glia, such as toll like receptors (TLRs) and the NODs (nucleotide-binding oligomerisation domain) [28–30]. Our prior experiment showed baicalin attenuated the expression of NOD2 and TNFα in neurons with the ischemia and reperfusion damage *in vivo* and



Compounds	R1	R2	R3
Baicalin	–H	–O–Glu	–OH
Baicalein	–H	–OH	–OH
Wogonoside	$-OCH_3$	–O–Glu	–H
Wogonin	$-OCH_3$	–OH	–H

Figure 1: Chemical structures of baicalin and its natural analogs, baicalein, wogonoside, and wogonin.

in vitro [31]. Wogonoside was reported with inhibition of lipopolysaccharide-induced angiogenesis via toll-like receptor 4 (TLR4) signal transduction [32]. However, TLR2 has not been investigated by baicalin and its natural analogs.

Based on the previously mentioned information, we investigated the regulation behavior of baicalin and its analogs through one kind of neuron cell PC12 in order to explore the possible targets of baicalin and its analogs in innate immune reactions during the oxygen and glucose deprivation (OGD) and the relations between the chemical structure and the effect among baicalin and its natural analogs. Since TLR2 is one of the initial receptors in innate immunity and the inflammatory activation, as well as TNFα, can be inhibited by baicalin in lung cells [33, 34], the protein expression of TLR2 and TNFα were tested to find out the role of baicalin and its analogs in this nerve injury models. Meanwhile, caspase3 is a well-known index of apoptosis, and several studies reported that a few flavones could induce apoptosis of inflammatory cells [35–37], so we detected the expression of caspase3 protein. Additionally, the effect of the four analogs on the total antioxidation capability of cells in OGD model was also detected in this paper, which further compared the whole competence of these flavones [38].

2. Materials and Methods

2.1. Chemicals and Materials. Baicalin (purity was 98%) was presented by Dr. Lujun Zhang, Laboratory of Pharmaceutical Sciences, Tsinghua University. Wogonoside (purity was 98%) was presented by Dr. Xiuwei Yang, School of Pharmaceutical Science, Peking university. Baicalein and wogonin, all with purity of 98% (batch number 111595-200604 and 111514-200403), were purchased from China National Institute for the Control of Pharmaceutical and Biological Products. Deionised water was used throughout the experiment. Other organic solvents and reagents were of analytical-reagent grade. PBS buffer with pH7.4 was prepared in our laboratory. PC12 cells were provided by the Cell Bank of the Institute of Fundamental Medicine, Chinese Academy of Medical Science (Beijing, China). Fetal bovine serum (FBS) and RPMI 1640 medium was purchased from GIBCO. Anti-TLR2/TNFα/caspase3/β-actin antibodies were purchased from Santa Cruz Company (USA). The secondary antibody was purchased from Zhongshan Company (Beijing, China). The Total antioxidation capability (T-AOC) detection kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Cell Culture. PC12 cells were adjusted to about 1×10^6 cells/mL with 2 mL inoculate into each well of 6-well plates (Costar) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 5% horse serum and penicillin/streptomycin (100 U/mL each). Cells were cultured in a humidified incubator (5% CO_2) (Sanyo, Japan) at 37°C and allowed to attached for at least 48 h.

2.3. Cytotoxicity Assays In Vitro. The safe dosages of baicalin and its three analogs to the cells were evaluated by MTT (methylthiazol tetrazolium) assay *in vitro*. After 24 h of cells incubation in 96-well plates, baicalin and its analogs were added with the dosage from 10 mg/mL to 0.001 mg/mL. 24 h later of the compounds added, culture medium in 96-well plates were incubated with MTT (5 mg/mL, 200 μL per well) at 37°C for 4 h. Then the medium was carefully aspirated, and 200 μL dimethylsulfoxide (DMSO) per well was added to dissolve the blue formazan products. The values of absorbance at 490 nm were measured using a microplate reader (Model 550Bio-RadUSA). The results of the absorbance of the test wells were expressed as cell alive. The 50% cytotoxicity concentration (CC_{50}) was calculated [39].

2.4. Cells for Oxygen-Glucose Deprivation. For the oxygen-glucose deprivation (OGD) [40], we replaced the growth medium with glucose-free culture medium (2 mL per well) and put the plates into an incubator (YCP-30Q, Changsha, China) full of 95% N_2/5% CO_2 at 37°C for 120 min. Then cells were returned to the normal feeding medium and incubated under normal conditions at 37°C (Sanyo, Japan) for the specific time for later experiments. Control cell cultures not deprived of oxygen and glucose were incubated under normal conditions in the medium with glucose.

For protective effect of the compounds from OGD damage, the cell living assay was carried out as previously described. After 24 h of cells incubation in 96-well plates, baicalin and its analogs were added with the dosages from 10 mg/mL to 0.001 mg/mL. 24 h later from adding the compounds, culture medium in 96-well plates was incubated with MTT (5 mg/mL, 200 μL per well) at 37°C for 4 h. The medium was carefully aspirated, and 200 μL DMSO per well was added to dissolve the blue formazan products. The values of absorbance at 490 nm were measured using a microplate reader. The results of the absorbance of the

test wells were expressed as cell alive. The 50% effective concentration (EC_{50}) was calculated [39]. Combined with cytotoxicity (CC_{50}) of the compounds, the safety indices (SIs) were calculated by EC_{50}/CC_{50} [41].

For TLR2/TNFα and caspase3 study, the minimum safety concentration of baicalin and its analogs was ordered as 10 μg/mL. These compounds (10 μg/mL) were added when culture medium was exchanged with glucose solution and cells were cultured in normal condition. The cells were picked up for protein experiment at the specific reperfusion time (0.5, 1, 3, 6 h) after baicalin and its analogs added. In control, medium was used as a vehicle and the cells were picked up at the reperfusion time of 6 h.

2.5. Preparation of Samples. Two groups without medicines were employed as control. One group of the cells was seeded in the medium with normal growth medium during all the experimental process without oxygen-glucose deprivation. The second group was seeded in the medium with process of oxygen-glucose deprivation as the model control. At each time point, the cell sample was prepared the same as previous description. The medium was drawn out from each well, and cells were washed three times with cold PBS (pH 7.4) and used with 100 μL RIPA to gather total protein for Western blotting.

2.6. Western Blot. Western blot assay for protein expression of β-actin, TLR2, TNFα, and Caspase3 was referenced in [42] with minimodification as follows: loaded samples into the gel (10%), was run until the marker (purchased from Fermentas Republic of Lithuania) and went to the end of the gel. The transfer should be semidry for 60 min at 12 volts and 280 mA. Then the membrane was blocked with 10% milk in PBST ($1 \times$ PBS + 0.1% Tween20) for 60 min shaking slowly at room temperature. The membrane was put in 1 mL PBST with antibody in a 1 : 1000 dilution, incubated for 60 min at room temperature on the shaker, and washed for 3 times using PBST after the primary antibody incubation. After that, the membrane was incubated for 60 min in PBST with the secondary antibody with the concentration 1 : 3000. The membrane was washed 3 times with PBST at last.

2.7. Total Antioxidation Capability Detection. The principle of this experiment was to detect the color change following the reduction of Fe^{3+} to Fe^{2+} by the reducing components in the samples. The reducing components might include the enzyme and nonenzymatic molecules such as lipid-soluble antioxidant vitamin E and water-soluble antioxidants vitamin C, bilirubin, and uric acid. Then the optical density was measured at 520 nm with a microplate reader [43]. The cell sample was prepared the same as previous description and picked at the specific reperfusion time (6 h) after baicalin and its analogs added. The medium was drawn out from each well, and the cells were washed three times with cold PBS (pH 7.4). These cells in the plate were resolved with PBS (1 mL/well) by repeated freezing and thawing. The supernatant was collected for T-AOC test after centrifuge 12 000 rpm/min.

2.8. Data Analysis. All values were expressed as mean ± S.D. Data were statistically analyzed by ANOVA. The Newman-Keuls comparisons were used to determine the source of significant differences where appropriate. P values below 0.05 were considered statistically significant. The software of CALC 2.0 (The China Association of Pharmacology) was employed for CC_{50} and EC_{50} calculation.

3. Results

3.1. Cell Growing Profile. The cells were seeded in the plates of the medium with 10% FBS for 48 h. They would not be used for the experiments till they grew well and attached to each other tightly in the flat of multiwell plates.

3.2. MTT Tests of Cytotoxicity. In order to examine the cytotoxicity and determine the safe dosages of baicalin and its analogs, MTT assay was conducted on PC12 cells *in vitro*. Based on the experiments, 10 μg/mL of baicalin and wogonoside was the highest in safety concentration on normal PC12 cells, and 1 μg/mL of baicalein and wogonin was the highest in safety concentration on normal PC12 cells (Figure 2).

In PC12 cells treated with oxygen-glucose deprivation, only less than 40% cells survived with comparison of normal control ($P < 0.05$). Compared with model control, the minimum effective concentration (MEC) of baicalin and wogonin was 10 μg/mL, while the MEC of both wogonoside and baicalein was the same at 1 μg/mL. The maximum effective concentration (MAXEC) of baicalin and woronin was the same at 1 mg/mL, implying the same safety of them. The MAXEC of wogonoside was 1 mg/mL, but the MAXEC of baicalein was only at 10 μg/mL. It was suggested that baicalein was more cytotoxic than that of wogonoside. All these compounds were not shown in the concentration-dependent manner distinctly (Figure 3).

The CC_{50} of baicalin (188.4 μg/mL or 0.422 mmol/L) showed large differences from other three compounds. Baicalein showed more cellular toxicity with CC_{50} 8.9 μg/mL (equal to 0.0329 mmol/L). Wogonin and wogonoside displayed toxicity similar to CC_{50} 10.6 μg/mL (equal to 0.0373 mmol/L) and 13.7 μg/mL (equal to 0.0298 mmol/L), respectively. However, baicalein was more effective of protection of the cells from OGD damage. The minimum effective dosage of baicalein was 1 μg/mL (equal to 0.0037 mmol/L). The EC_{50} of baicalin was 1.2 μg/mL (equal to 0.0027 mmol/L), and the EC_{50} of Wogonin and wogonoside was 4.3 μg/mL (equal to 0.0151 mmol/L) and 7.4 μg/mL (equal to 0.0161 mmol/L). Comparing between the toxicity and the effect, the safety indices of the four compounds were 156 (baicalin), 8.89 (baicalein), 2.47 (wogonin), and 1.85 (wogonoside), respectively (Table 1).

3.3. Effect on the Expressions of TLR2, TNFα, and Caspase3. Injured by OGD, the proteins of TLR2 and TNFα in the cells were expressed distinctly which means the OGD stimulated the innate immune reaction, causing inflammation. The caspase3 protein in OGD model was upregulated in

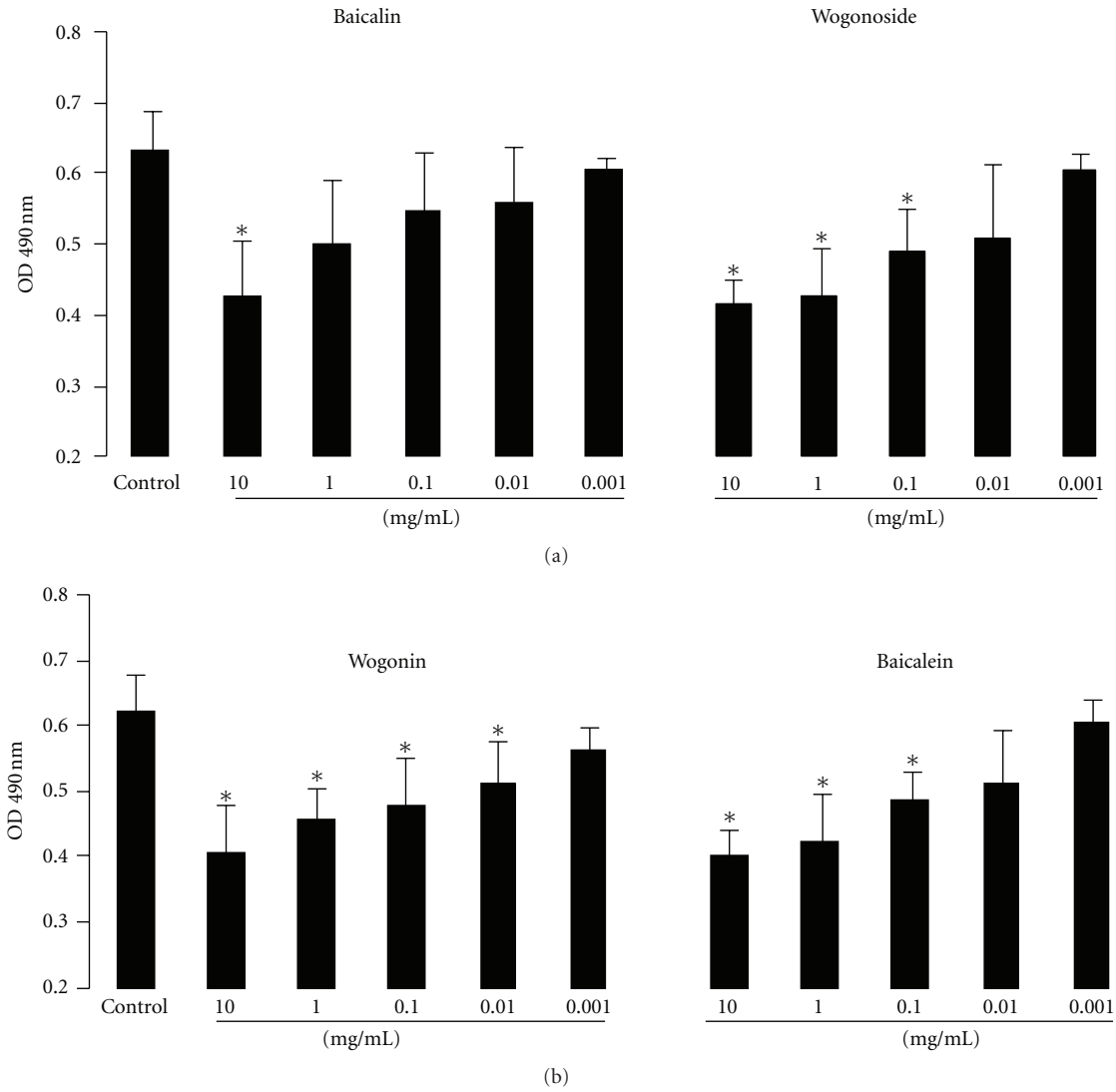


Figure 2: Cytotoxcity of baicalin, wogonoside, baicalein, and wogonin in normal PC12 cells (MTT assay). After 24 h of cells incubation in 96-well plates, the chemicals were added with the dosage from 10 mg/mL to 0.001 mg/mL. $^*P < 0.05$ versus normal control. Data were presented as mean ± S.D. from seven independent experiments.

Table 1: Values of CC_{50} and EC_{50} of baicalin, baicalein, wogonoside, and wogonin.

Compounds	CC_{50} μg/mL (mmol/L)	EC_{50} μg/mL (mmol/L)	SI
Baicalin	188.4 (0.422)	1.2 (0.0027)	156
Wogonoside	13.7 (0.0298)	7.4 (0.0161)	1.85
Baicalein	8.9 (0.0329)	1.0 (0.0037)	8.89
Wogonin	10.6 (0.0373)	4.3 (0.0151)	2.47

CC_{50} means the 50% cytotoxic concentration.
EC_{50} means the 50% effective concentration.
SI (the safety index) was calculated by CC_{50}/EC_{50}.

some degree, but there was no significant statistical value (Figure 4). Baicalin attenuated the protein expression of TLR2 and TNFα 3 hours after the administration. When baicalin took effect for 0.5 h, the expression of TLR2 showed statistical significance no matter when compared with the normal or comparing with the model ($P < 0.05$), and the TLR2 expression of 1 h increased (comparing with the normal, $P < 0.05$); however, its expression of 3 h or 6 h showed no obvious difference comparing with the normal (comparing with the model, $P < 0.05$). The expression of TNFα of 0.5 h was still higher than the control ($P < 0.05$), and its expressions of 1 h, 3 h, and 6 h were downregulated obviously, which showed significant difference from the model level ($P < 0.05$). Baicalin did not apparently affect the expression of protein caspase3, which was shown in Figure 4(a).

After the addition of wogonoside (10 μg/mL), TLR2 and TNFα were downregulated apparently. The expression of TLR2 was much lower than that of the model, even than the normal level ($P < 0.05$). Accordingly, the expression of

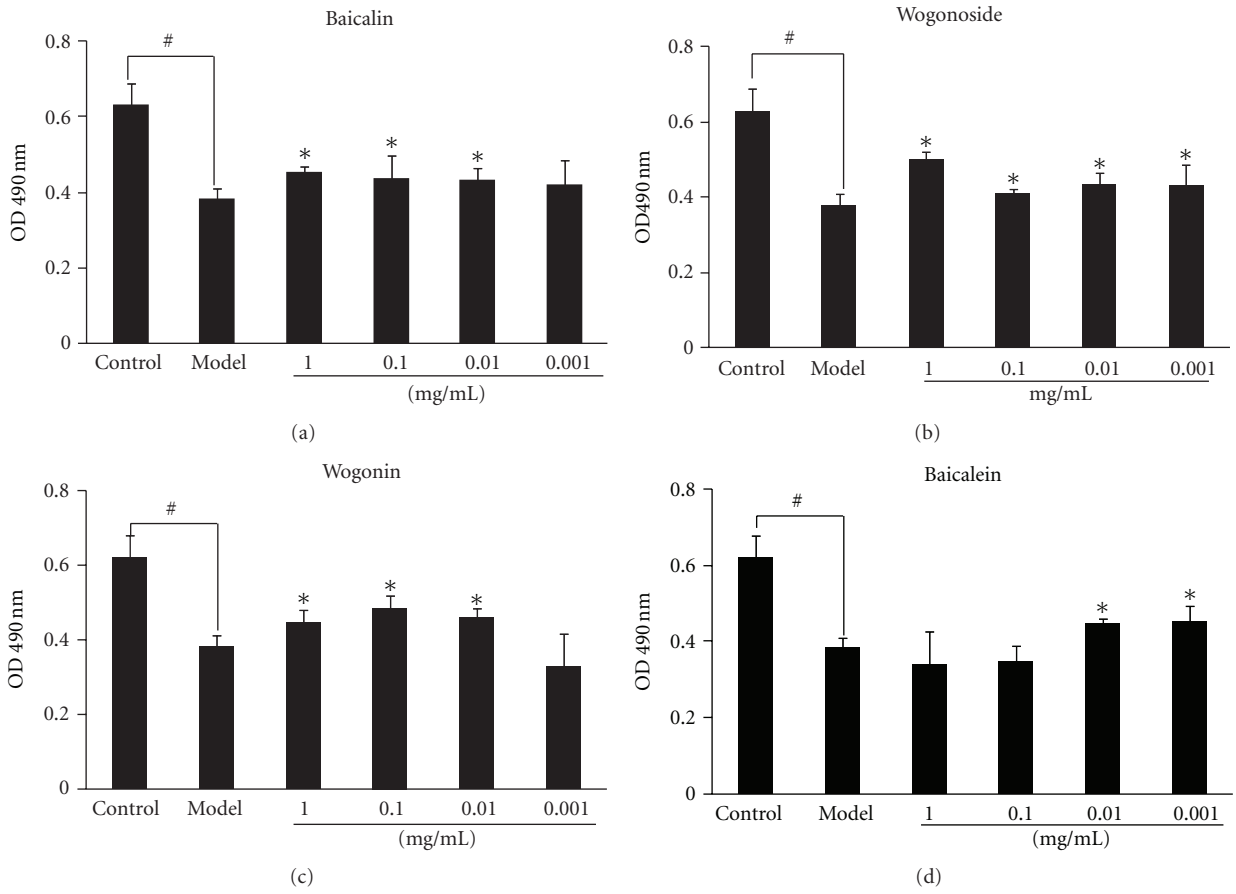


Figure 3: Protection of baicalin, wogonoside, baicalein, and wogonin in PC12 cell with oxygen-glucose deprivation (OGD) (MTT assay). After 24 h of cells incubation in 96-well plates and 2 h oxygen-glucose deprivation, the compounds were added with the dosage from 1 mg/mL to 0.001 mg/mL. $^{\#}P < 0.05$ versus normal control. $^{*}P < 0.05$ versus model. Data were presented as mean ± S.D. from seven independent experiments.

TNFα was inhibited by wogonoside apparently from 0.5 h after its administration till the timepoint of 6 h ($P < 0.05$). Wogonoside also did not show obvious effect on the expression of caspase3 (Figure 4(b)).

After baicalein administration, TLR2 was still upregulated significantly 0.5 h and 1 h after the addition of baicalein ($P < 0.05$), and it decreased down to the normal at 3 h and 6 h. Comparing with the model, TNFα was upregulated at 0.5 h and decreased gradually later. Like TLR2, it was expressed down to the normal at 3 h and 6 h (Figure 4(c)).

In wogonin group, the two factors decreased apparently. TLR2 decreased to the normal after 0.5 h of the addition of wogonin (comparing with the model, $P < 0.05$), and it stayed at a lower level during the experiment course. In the 0.5 h and 1 h time points, TNFα was expressed more than the normal ($P < 0.05$) but less than the model ($P < 0.05$), and at 3 h and 6 h, it came close to the normal level like TLR2 expression (Figure 4(d)).

Similar to baicalin and wogonoside groups, the expression of caspase3 showed no significant change in baicalein and wogonin groups (Figures 4(c) and 4(d)).

3.4. Assay of Total Antioxidation Competence (T-AOC). Utilizing the T-AOC kit, we found that the antioxidation competence of the normal cells was about 2.5 U/mg, and one of the cells treated by oxygen-glucose deprivation was far below 0.5 U/mg. In baicalin group, the detection value was 1.8 U/mL, showing a significant difference no matter comparing with the normal or comparing with the OGD model. In wogonoside, baicalein, and wogonin groups, the values were 1.6, 0.8, and 0.6 U/mg, respectively, which all showed a significant difference comparing with the normal or the model (Figure 5).

4. Discussions

Baicalin is a glycosylated compound derived from baicalein, and it has a functional group of D-glucopyranosiduronic acid on site 7 of baicalein (Figure 1), so that it will exhibit more biological function. The basic structure of baicalin consists of benzopyran with three hydroxyl groups at site 5, 6, and 7 and a phenyl group at the other side of the benzopyran (site 2). Baicalin also has an enol structure on the benzopyran ring to maintain the conjugation. The two differences between baicalin and wogonin lie in methoxyl group on carbon 8 of wogonin and hydroxyl group on carbon 6 of baicalein. The constitution of three hydroxyl groups in baicalin may account for more hydrophilic characters.

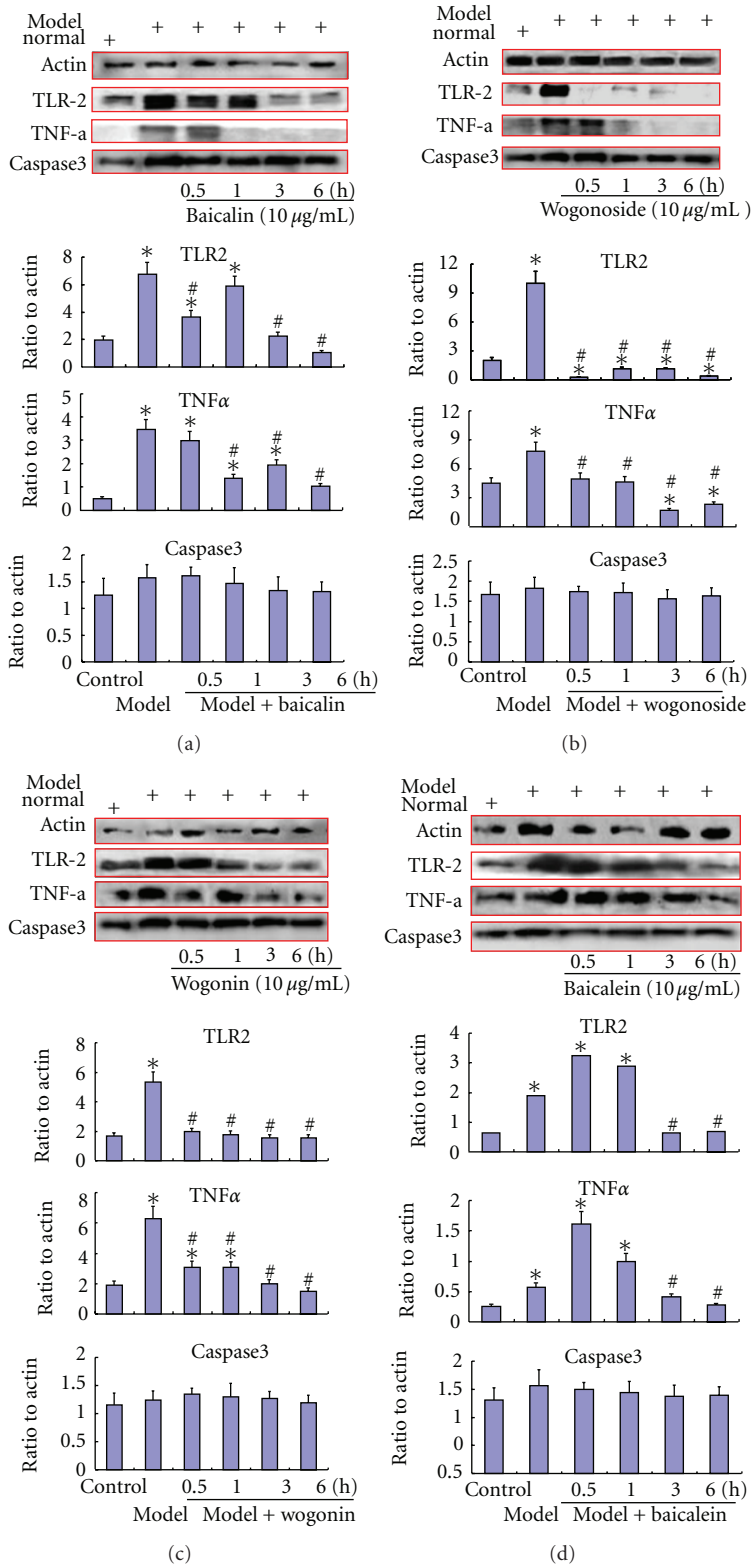


Figure 4: Effect of the four analogs on the protein expressions of TLR2, TNFα, and caspase3 in PC12 cells with oxygen-glucose deprivation (OGD). The protein level was measured by Western blot. Model means treatment with oxygen-glucose deprivation. 0.5, 1, 3, and 6 h mean the time of reperfusion process after OGD. (a) stands for baicalin effect; (b) stands for wogonoside effect; (c) stands for baicalein effect; (d) stands for wogonin effect. Baicalin, wogonoside, and wogonin were used in a concentrating of 10 μg/mL, and the baicalein was used in a concentrating of 1 μg/mL. $^{*}P < 0.05$ versus normal control. $^{\#}P < 0.05$ versus model group. Data were presented as mean ± SD from three independent experiments.

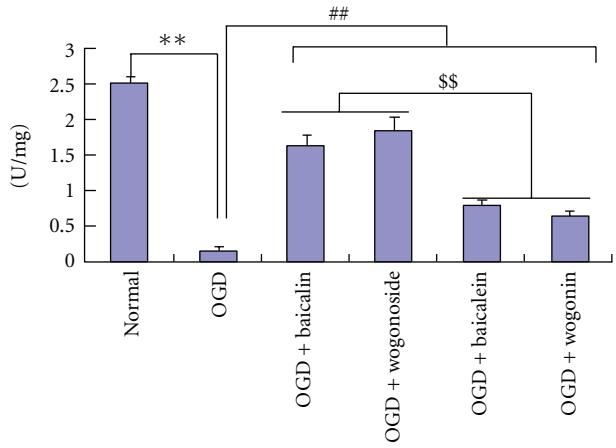


Figure 5: Effect of baicalin and its three analogs on the total antioxidation competence of the PC12 cells treated with oxygen-glucose deprivation. Model means treatment with oxygen-glucose deprivation. Baicalin, wogonoside, and wogonin were used in a concentrating of 10 μg/mL, the baicalein was used in a concentrating of 1 μg/mL. $^{**}P < 0.01$ versus normal control. $^{\#\#}P < 0.01$ versus model group. $^{\$\$}P < 0.01$ versus baicalin and wogonoside groups. Data were presented as mean ± S.D. from eight independent experiments.

Moreover, wogonoside is a glycosylated form of wogonin with D-glucopyranosiduronic acid on carbon 7. All four compounds share similar carbon skeleton with a benzopyran structure and also a hydroxyl group on carbon 5; baicalein and baicalin have hydroxyl groups on carbon 6, while wogonin and wogonoside does not have; baicalein and wogonin have hydroxyl groups on carbon 7, while wogonoside and baicalin are both glycosylated on the hydroxyl group at site 7; last, wogonin and wogonoside have methoxyl groups on carbon 8, but baicalein and baicalin does not have the functional groups [44, 45].

By the result of cytotoxicity and neuroprotection, D-glucopyranosiduronic acid on site 7 was important for reducing cytotoxicity, in which baicalin was less cytotoxic than baicalein and wogonoside was less cytotoxic than wogonin. Methoxyl group on carbon 8 and hydroxyl group on carbon 6 were the major factor influencing the neuroprotective effect, by which the EC_{50} of baicalin and baicalein was less than that of wogonoside and wogonin (Figure 1 and Table 1) after they were administered.

In this study, we found that TLR2 was highly expressed during the oxygen and glucose deprivation (OGD) damage in PC12 cells, which suggested that the receptor was activated in the damaged neurons (Figure 4). TLR2 had been identified as a key mediator of immune responses and inflammatory reactions, and it mediated proinflammatory responses through activating of TNFα [46–48]. The receptor was significantly up-regulated comparing with the normal level, indicating it joined neuron injury induced by OGD.

By the literature of research, TLR2 in the central nerve system (CNS) is realized as the direct source of the damaging signal and an important potential therapeutic target [49]. In our results, the expressions of TLR2 and TNFα decreased apparently a few hours after we added the compounds. In the same profile, the expressions of TLR2 and TNFα with wogonin and wogonoside were more inhibited than those with baicalin and baicalein. The inhibition of the expression started from 0.5 h after wogonin and wogonoside administration; however the inhibition of TLR2 and TNFα expression appeared later at 3 h after baicalin and baicalein administration. Therefore, wogonin and wogonoside were realized to have preference with TLR2. Combined with their chemical structures, all the results implied that methoxyl group on carbon 8 in the structure is the pharmacophore hypothesis of the effect of TLR2 inhibition, relating to inflammation inhibition.

Conditioning the oversuppression on TLR2 and TNFα by these analogs, we detected caspase3 to confirm whether there existed apoptosis with the addition of the analogs. Comparing with the normal, the expression of caspase3 protein in OGD model indeed increased in some degree, but showed no statistical difference. With the administration of the four analogs, the level caspase3 protein showed no obvious change, although it showed the tendency to decrease at 6 h time point and near to the normal control (comparing with the normal or the model, $P > 0.05$). The above results suggested the injured PC12 cells in OGD model had no apparent apoptosis, and the four analogs also could not induce apoptosis in the injured PC12 cells. According to the literature, baicalin attenuated global cerebral ischemia reperfusion injury in gerbils via antioxidative and antiapoptotic pathways [50], and baicalein and wogonin could both induce the apoptosis in human pancreatic cancer cells and HL-60 leukemia cells [51, 52]. Therefore, our results of apoptosis by these compounds need to be studied further.

Referring to the T-AOC detection, we found that the total antioxidation capability sequence was baicalin and wogonoside > baicalein and wogonin, indicating that a glycosylated form on site 2 of baicalin and wogonoside played a key role in overall antioxidation.

Taken together, baicalin and its three analogs presented comprehensively the protection of neuron cells from OGD damage and the inhibition of TLR2 expression and TNFα (the factor in the downstream), suggesting the possibility of these compounds used in stroke therapy by targeting TLR2, one of the major mechanisms of the cerebral damage. There is a structure-activity relationship among them with safety, efficacy, and specificity of drug targeting of TLR2. This is the first time to study baicalin and its natural analogs on molecular targets of TLR2 and TNFα, as well as T-AOC. All that will have benefit in elucidating and developing them for the new drugs.

Authors' Contribution

The first two authors contributed equally.

Acknowledgments

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Research Article

Metabolomics Analysis of *Cistus monspeliensis* Leaf Extract on Energy Metabolism Activation in Human Intestinal Cells

Yoichi Shimoda,[1] Junkyu Han,[1,2] Kiyokazu Kawada,[1,2] Abderrazak Smaoui,[3] and Hiroko Isoda[1,2]

[1] *Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan*
[2] *Alliance for Research on North Africa, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan*
[3] *Laboratory of Extremophile Plants, Center of Biotechnology of Borj-Cedria, 2050 Hammam-Lif, Tunisia*

Correspondence should be addressed to Hiroko Isoda, isoda.hiroko.ga@u.tsukuba.ac.jp

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Energy metabolism is a very important process to improve and maintain health from the point of view of physiology. It is well known that the intracellular ATP production is contributed to energy metabolism in cells. *Cistus monspeliensis* is widely used as tea, spices, and medical herb; however, it has not been focusing on the activation of energy metabolism. In this study, *C. monspeliensis* was investigated as the food resources by activation of energy metabolism in human intestinal epithelial cells. *C. monspeliensis* extract showed high antioxidant ability. In addition, the promotion of metabolites of glycolysis and TCA cycle was induced by *C. monspeliensis* treatment. These results suggest that *C. monspeliensis* extract has an ability to enhance the energy metabolism in human intestinal cells.

1. Introduction

A lot of natural herbs distributed around the Mediterranean Sea have been traditionally used by local people [1]. Most of the studies on plants to grow for Mediterranean climate have been investigated mainly in tolerance for the drying and relationship with environmental stress. However, the physiological function of natural herb is still poorly understood though many species grow allover the place. In an area of North Africa, the several species of natural herb were employed in traditional medicine as active against *Helicobacter pylori*, oxidative stress, hypertension, and hypoglycaemic [2–4]. *Cistus monspeliensis* is a perennial plant which in widely distributed from South Europe to North Africa. The genus *Cistus* is popular on tea and spice but not utilized for the antiaging effects. *C. monspeliensis* has been reported to have an antioxidation, antibacterial, and anti-inflammatory effects [5].

In the small intestine, goblet cells secrete mucus that forms a coating over the epithelial layer. The main function of epithelial layer includes absorption of food compounds [6]. The ingested capsaicin was carried into the intestinal epithelium, which is in contact with a high concentration of food ingredients [7, 8]. Considering the relevance of the food components and the effects of the oral route for human exposure, we have investigated the effect of capsaicin on the energy metabolism of intestine, by using Caco-2 cell line, a well-known *in vitro* model of intestinal epithelium [6].

The intracellular ATP accumulation is important for optimal integrity of the mucosa and has been suggested to play a specific role in the regulation of absorption and barrier functions [9, 10]. From these reports, it was supposed that the intracellular ATP accumulation contributes to the homeostasis of Caco-2 cells, because the regulation of absorption and barrier functions are necessary for Caco-2 cells differentiation [11].

In this study, the energy metabolism underlying the effect of *C. monspeliensis* extract on intestinal epithelium was clarified by performing the capillary electrophoresis time-of-flight mass spectrometry (CE-ToF/MS) analysis. Moreover, we performed real-time PCR to quantitate mRNA using the primers, related with ATP production, and the luciferase

assay was performed to measure intracellular ATP production in the intestinal epithelium. This is the first report that the extract of *C. monspeliensis* was induced by the activation of energy metabolism in human intestinal epithelial cells.

2. Materials and Methods

2.1. Cell Culture. Human intestinal epithelial Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were used between passages 10 to 30. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (Hyclone Co., Ltd.), 1% (v/v) penicillin-streptomycin (Lonza, Walkersville, MD USA), and 1% (v/v) nonessential amino acids (Cosmo Bio Co., Ltd., Tokyo, Japan) and incubated in an atmosphere of 5% CO_2 at 37°C.

2.2. Plant Materials and Extracts Preparation. 23 plants were collected between June and July in 2008 from Jendouba, Kairouan, and Kasserine areas in Tunisia and air-dried in the shade at room temperature and ground to powder with an electrical blender and stored at room temperature. Each plant sample (1 g) was extracted with 10 mL of distilled water at 105°C for 15 minutes or with 10 mL of 70% (w/v) ethanol at room temperature for 1 week. The extract water was filtered and sterilized using 0.22 μm membrane filter and stored at −80°C.

2.3. DPPH Assay. The antioxidant effect of plants was determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay performed. DPPH (2 mg) was dissolved in ethanol (12.76 mL). The ethanol solution (6 mL), 400 mM 2-morpholinoethanesulfonic acid monohydrate (MES) solution (1.5 mL), and MilliQ water (4.5 mL) were mixed in one tube. Furthermore, plants extract (10 μL) and the mixing solution (190 μL) were added in 96-well plates and incubated for 10 min at room temperature. The absorbance was spectrophotometrically determined at 520 nm using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, USA).

2.4. Total RNA Isolation and Real-Time PCR. After incubating seeded plates for 24 h, total RNA was purified using the ISOGEN kit (Nippon Gene Co., Ltd., Japan.) Total RNA was quantified by measurement at 260 nm with a UV spectrophotometer and was also measured at 280 nm to assess purity. Only RNA with a 260/280 ratio higher than 1.8 was used for the real-time PCR. Template cDNA was obtained from total RNA using the SuperScript reverse transcriptase system (Invitrogen). Briefly, RNA was denatured at 65°C for 5 min and incubated with 1 mL oligo (dT) 12–15 primers and chilled at 4°C. After adding SuperScript II reverse transcriptase (200 units), the reaction mix was incubated at 42°C for 60 min, then 10 min at 70°C. For the quantification of mRNA, nested primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Quantitative PCR reactions were performed in a MiniOpticon instrument (Bio-Rad, USA). Briefly, the RT mix (2 mL) was used as template for the real-time PCR mix containing 0.5 mM forward and reverse nested primers (2 μL each) and 2 × SYBR Green supermix (10 μL). The primers used were checked using the BLASTn program of the GeneBank; their sequence (TPI: forward: 5′-CTTGGCTGAGAGATGGAAGG-3′, reverse: 5′-CAGTGAAGGCAGACAAACCA-3′; PGM: forward: 5′-GCGGAGAACTTCATCCAGAG-3′, reverse: 5′-TGTCAGAATGATCCCACCAA-3′; ATP synthase: forward: 5′-CTGGAGGACCTGTTGATGCT-3′, reverse: 5′-TGGGGTTTTTCGATGACTTC-3′) was based on the known sequences in the cording region of the human genes. The amplification conditions were 3 min at 95°C, 10 sec at 95°C, 30 sec at 62°C, and 30 sec 72°C for 41 cycles. At the end of the reaction, a melting curve analysis was carried out to check for the presence of primer dimers.

2.5. Metabolomics Analysis. After incubating seeded plates for 24 h, the extract was added at 0.1% (w/v) concentration and the cells were incubated for 12 h. After treatment, 5% (w/w) mannitol solution was added at 10 mL and was removed. Once again, 5% (w/w) mannitol solution was added at 2.0 mL and was removed. Cells were scraped in 1.3 mL of methanol which includes 10 μM each of 2 internal standards (ISC1 and ISA1), and methanol including scraped cells was transferred at 1.0 mL to centrifuge tube. 1 mL of chloroform and 400 μL of MilliQ water were added to the solution and then thoroughly mixed. Followed by centrifugation at 2,300 g for 5 min at 4°C, the 250 μL of water layer was removed and centrifugally filtered through a 5-kDa-cutoff filter (Millipore, USA) to remove proteins. The filtrate was desiccated and dissolved in 20 μL of MilliQ water prior to injection. The capillary electrophoresis time-of-flight mass spectrometry (CE-ToF/MS) experiments were performed using an Agilent CE-ToF/MS system (Agilent Technologies Co.) unit 5. Separations were carried out on a fused silica capillary (50 μm i.d. × 80 cm total length). CE-ToF/MS conditions for anionic metabolites were followed. Run buffer: anion buffer solution (p/n: H3302-1021), rinse buffer: anion buffer solution (p/n: H3302-1022), sample injection: pressure injection 50 mbar, 25 sec, CE voltage: positive, 30 kV, MS ionization: ESI negative, MS capillary voltage: 3,500 V, MS scan range: *m/z* 50–1,000, sheath liquid (p/n: H3312-1020) [12].

2.6. ATP Measurement. ATP was assessed by firefly bioluminescence using the luminescence luciferase assay kit (TOYO Ink, Tokyo, Japan). Caco-2 cells were plated in 96-well plates at 1.0×10^6 cells/mL in 100 μL. After 24 h incubation, the extract was added at 0.1, 0.01, and 0.001% (w/v) concentration, and the cells were incubated for 3, 6, and 12 h. After treatment, 100 μL of luciferin-luciferase solution was added and stirred for 3 min using a microplate genie, 100 V (Scientific industries, Inc., USA). The luminescence was determined using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, USA).

2.7. MTT Assay. The viability of cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) assay. Briefly, Caco-2 cells were plated in 96-well plates at 1.0 × 106 cells/mL in 100 μL. After 24 h incubation, extract diluents with medium were added to obtain final concentrations from 0.1% to 0.001% (w/v), and the cells were cultured for 24 h, followed by the addition of 10 μL of 5.0 mg/mL of MTT. After 12 h incubation, 150 μL of 10% sodium dodecyl sulfate (Wako) was added and incubated for 48 h. The absorbance was spectrophotometrically determined at 570 nm using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, USA).

3. Results

3.1. Effect of Antioxidant of 23 Plants Extracts. DPPH radical is well known as a stable organic free radical which has been used for estimation of the antioxidant capacity. DPPH radical is changed to nonradical to react antioxidants. We determined the antioxidation of 23 plants grow around the Mediterranean Sea. Especially, eighteen plants had higher antioxidation more than 75% (Table 1). We selected *C. monspeliensis* in twenty three plants that had high antioxidation to assess ATP production because this plant was used to traditional foods.

3.2. Effect of C. monspeliensis Extract on the Expression of TPI, PGM, and ATP Synthase mRNA by Caco-2 Cells. To investigate the effect of *C. monspeliensis* extract on the activation of energy metabolism in human intestinal epithelium (Figure 1), real-time PCR was employed to evaluate the mRNA expression of genes, related with glycolysis and TCA cycle. In this experiment, we used the primers of triosephosphate isomerase (TPI), phosphoglycerate mutase (PGM), and ATP synthase that have a deep relationship with intracellular ATP production and relatively higher expression in Caco-2 cells. Especially, to treat 0.1% (w/v) *C. monspeliensis* for 6 h, mRNA expression levels of TPI, PGM, and ATP synthase were upregulated by 170%, 161%, and 310%, respectively. PGM is an enzyme that catalyzes the internal transfer of a phosphate group from C-3 to C-2 which results in the conversion of 3-phosphoglycerate to 2-phosphoglycerate through a 2, 3-bisphosphoglycerate intermediate. TPI is an enzyme that catalyzes the reversible interconversion of the triose phosphate isomers, dihydroxyacetone phosphate, and D-glyceraldehyde 3-phosphate. TPI plays an important role in glycolysis and is essential for efficient energy production. ATP synthase is an important enzyme that creates energy for the cell to use through the synthesis of ATP in mitochondria. In Figure 1, mRNA expression of TPI and PGM, glycolytic enzymes, increased to treat with *C. monspeliensis* extract. Also, ATP synthase, electron transport chain enzyme, was increased.

3.3. Effect of C. monspeliensis Extract on the Metabolomics by Caco-2 Cells. As a result of the metabolomics analysis, *C. monspeliensis* extraction increased production of each metabolite in glycolysis and TCA cycle (Table 2). Glycolysis is a pathway of ATP production in anaerobic. In this result, three metabolites on rate-limiting step in glycolysis (glucose 6-phosphate, fructose 1,6-diphosphate, and pyruvic acid) were increased by *C. monspeliensis* extraction. Especially, pyruvic acid was the most increased in glycolytic metabolites. The production of pyruvic acid is related to promote function of ATPase that involved ATP synthesis [13]. ATP is not produced in TCA cycle; however, a lot of ATP is produced in electron transport chain. Antiaging on promoting energy metabolism is expected to promote function of glycolytic and electron transport chain enzymes that are pathways of ATP production.

Table 1: Effect of antioxidant of 23 plants extracts. The radical 2,2-diphenyl-1-picryhydrazyl (DPPH) was used for measuring the antioxidants. The hot water extract and the ethanol extract were diluted to 10 or 100 times by each solution. The values of antioxidant were calculated by the following formula: antioxidant (%) = $\{1-(\text{Absorbance}_{520}\ (\text{Sample})/\text{Absorbance}_{520}\ (\text{Control}))\}\times 100$.

Name of plants	EtOH extract	
	0.1% (w/v)	1% (w/v)
Ajuga iva	14	85
Artemisia campestris	38	91
Artemisia herba-alba	2	17
Cistus monspeliensis	24	93
Cyperus longus	10	50
Daphne gnidium	5	37
Erica multiflora	24	92
Globularia alypum	18	91
Laurus nobilis	10	54
Lavandula angustifolia	13	84
Lavandula officinalis	2	10
Lavandula stoechas	3	17
Marrubium vulgare	8	50
Mentha rotundifolia	8	43
Mentha viridis	12	71
Origanum majorana	13	85
Phillaria angustifolia	15	82
Pinus halepensis	11	53
Rhamnus lycioides	10	54
Teucrium polium	10	72
Thymus capitatus	13	87
Vitex agnus	13	78
Ziziphus lotus	15	76

3.4. Effect of C. monspeliensis on the Intracellular ATP Production by Caco-2 Cells. In the results of real-time PCR and metabolomics, *C. monspeliensis* was upregulated metabolite and mRNA expression involved ATP production. We determined ATP production of *C. monspeliensis* extract using the luminescence luciferase assay kit (Figure 2). 0.1, 0.01, and 0.001% (w/v) of *C. monspeliensis* extraction increased ATP production on Caco-2 compared with nontreated cells. Especially, to treat 0.01% (w/v) of *C. monspeliensis* for 3 h, ATP production was upregulated by 172%. As a result, we

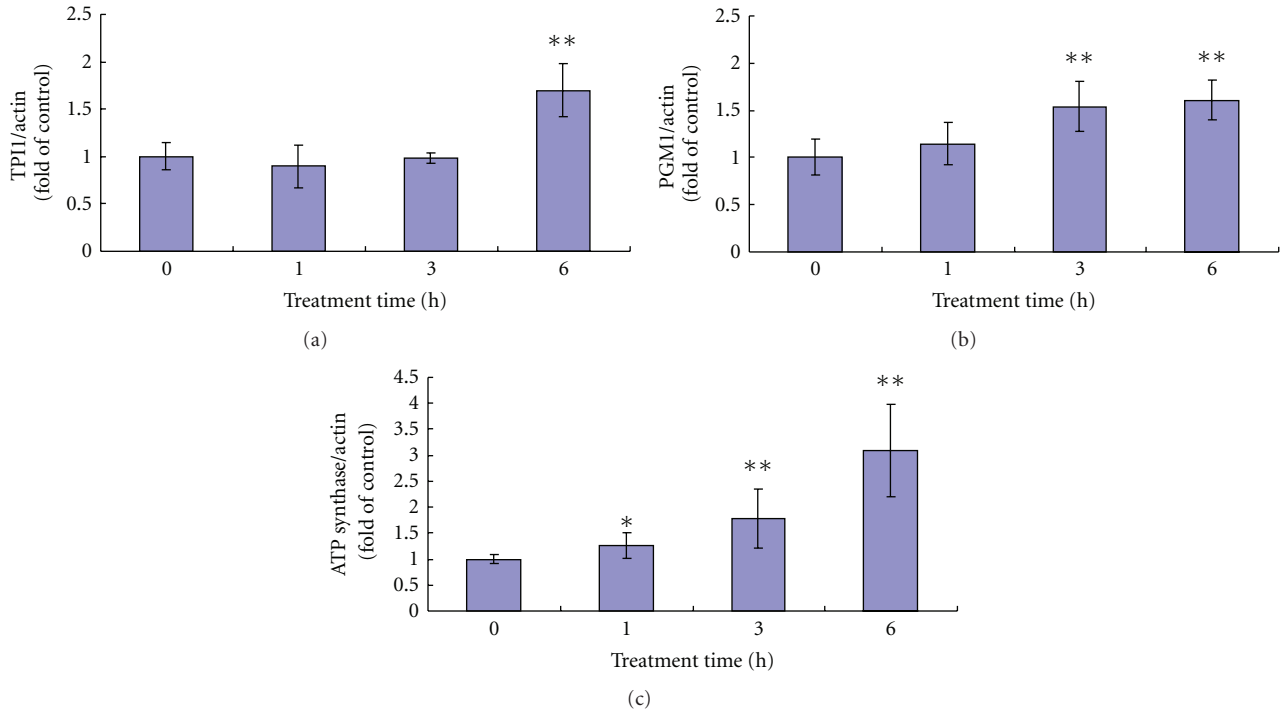

Figure 1: Effect of *C. monspeliensis* extract on the expression of TPI (a), PGM (b), and ATP synthase (c) mRNA by Caco-2 cells. β-actin was used as a house-keeping gene. The mRNA expression of TPI, PGM, and ATP synthase was normalized by β-actin mRNA expression. Each bar represents the mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ compared to the control as determined by the t-test.

Table 2: Effect of *C. monspeliensis* extract on the metabolomics by Caco-2 cells. Quantified levels of metabolites involved in glycolysis pathway were determined. Caco-2 cells were treated with 0.1% (w/v) *C. monspeliensis* extract for 12 h. The ratio of quantified levels of metabolites was calculated by Caco-2 cells treated or nontreated with *C. monspeliensis* extract.

Metabolite	Ratio
Glycolysis	
Glucose 6-phosphate	1.7
Fructose 6-phosphate	1.6
Fructose 1,6-diphosphate	1.5
Dihydroxyacetone phosphate	2.1
3-Phosphoglyceric acid	1.0
Phosphoenolpyruvic acid	0.8
Pyruvic acid	4.2
TCA cycle	
Acetyl CoA_divalent	0.7
Citric acid	1.5
Cis-aconitic acid	1.4
Isocitric acid	1.0
2-Oxoglutaric acid	1.6
Succinic acid	0.8
Fumaric acid	1.1
Malic acid	1.0

considered that *C. monspeliensis* had effect of ATP production and to promote energy metabolism on Caco-2.

3.5. Effect of C. monspeliensis on the Cell Viability by Caco-2 Cells. We determined cell proliferation on Caco-2 using MTT assay. From the result of MTT assay, 0.01 and 0.001% (w/v) of *C. monspeliensis* extraction were not changed cell proliferation on Caco-2 cells, whereas 0.1% (w/v) of *C. monspeliensis* extraction induced the increase of cell proliferation on Caco-2 cells compared with nontreated cells. As a result, we considered that 0.1 to 0.001% (w/v) *C. monspeliensis* extract had not toxic effect for Caco-2 cells.

4. Discussion

ATP is a multifunctional nucleotide that is the most important as a "molecular currency" of intracellular energy transfer. In this role, ATP transports chemical energy within cells for metabolism. The glycolytic and TCA cyclic enzymes played an important role in the intracellular ATP production [14, 15]. TPI enzyme is essential for energy production, allowing two molecules of glyceraldehyde 3-phosphate to be produced for every glucose molecule, thereby doubling the energy yield. PGM enzyme catalyzes 1,3-bisphosphoglycerate and plays an important role downstream of glycolysis. The activity of TPI and PGM was not influenced by age or caloric restriction [16], while the other glycolytic enzymes were influenced. Furthermore, the activity and expression of these two enzymes, however, are

decreased by oxidation and disorders (Alzheimer's disease, hemolytic anemia, erythrocyte destruction, etc.) [17, 18]. These observations indicate that the expression and activity of these glycolytic enzymes were upregulated in response to specific signals, such as *C. monspeliensis* extraction. Furthermore, ATP synthase contains a rotary motor involved in biological energy conversion. ATP synthase uses the proton motive force to make ATP from ADP and inorganic phosphate (Pi) in mitochondria.

However, these enzymes have contribution toward not only ATP production but also regulation of cellular function. TPI isomerizes dihydroxy acetone phosphate (DHAP) to glyceraldehydes 3-phosphate (GAP). Its deficiency has been known to cause a severe multisystemic disease with autosomal recessive inheritance [19] and neurodegeneration [20]. Decreased TPI activity induces the accumulation of DHAP, which produces methylglyoxal instead of GAP. The methylglyoxal induces oxidative damage to proteins and DNA and accumulation of advanced glycation end products (AGEs), which leads to structural degeneration and functional decline of brain cells [21].

Also PGM is related with the apoptosis of human prostate cancer cells, LNCaP, DU145, and PC-3 [22]. Monoubiquitination of phosphoglycerate mutase, as well as formation of a noncovalent complex containing ubiquitin and phosphoglycerate mutase, increased in colorectal cancer, which may suggest a potential pathophysiological event [23]. A decreased level of phosphoglycerate mutase isoenzymes was reported in breast carcinoma [24] indicating its differential expression.

Active regulation of the mitochondrial ATP-synthase (complex V) in response to the cellular energy demand has been demonstrated in several species like rat, dog, and humans and different types of tissue like heart muscle, skeletal muscle, fibroblasts, and brain. Regulation of the ATP-synthase seems to be a central physiological phenomenon which is presumably present in many other species and other organs. As energy supply via the mitochondrial ATP-synthase plays such a vital role in almost every cell of the body, more diseases will probably be identified where (primary or secondary) abnormalities of this enzyme occur [25].

In addition, one of the causes of aging, free radical, is focused on oxidative cytotoxic. Especially, oxidative cytotoxic in mitochondrion is related to aging [26]. Mitochondrion that is important as metabolism organ of ATP production caused impairment accompanied effect of aging and ROS. Also, it is known that function of SOD decreased [27]. From this paper, the effect of enhancement component on ATP production can make use of antiaging effector as well as functional foods for antiaging.

Our research showed that *C. monspeliensis* extract induced the expression of enzymes, related with intracellular ATP production, in human intestinal epithelial cells. And *C. monspeliensis* extract enhanced the production of intracellular ATP. From these results, we suggest that *C. monspeliensis* extract can be used as antiaging effector. It is known that species of *Cistus* contain quercetin, kaempferol, aesculin, myricetin, and flavan-3-ols [28]. For example, quercetin has

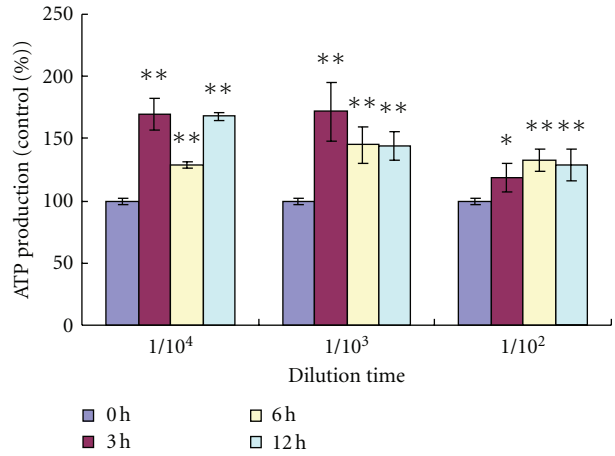

Figure 2: Effect of *C. monspeliensis* extract on the intracellular ATP production by Caco-2 cells. Caco-2 cells were treated with 0.1% to 0.001% (w/v) of *C. monspeliensis* extract for 3 h, 6 h, and 12 h. Each bar represents the mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ compared to the control as determined by the *t*-test.

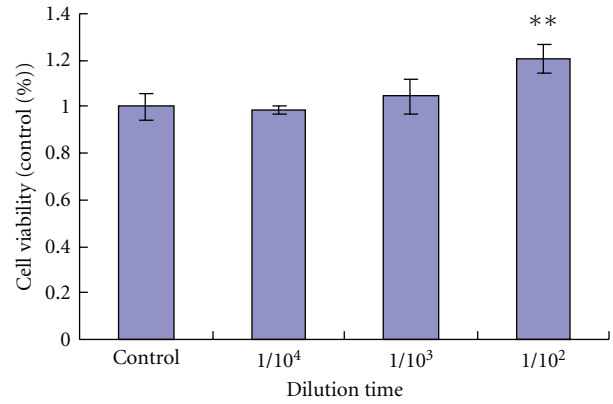

Figure 3: Effect of *C. monspeliensis* extract on the cell viability by Caco-2 cells. Caco-2 cells were treated with 0.1% to 0.001% (w/v) of *C. monspeliensis* extract for 24 h. Each bar represents the mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ compared to the control as determined by the *t*-test.

antioxidation and has a function to improve memory impairment that accompany Alzheimer's disease [29]. Moreover, it is known that an aging is caused for decline of intestinal absorption [30, 31]. We expect that *C. monspeliensis* extract can contribute to prevent several diseases like senescence, Alzheimer's disease, cardiosclerosis, stenocardia, cancer, and so forth and recover the function of impairment intestinal absorption.

0.001, 0.01% (w/v) of *C. monspeliensis* increased ATP production (Figure 2) and, however, did not changed cell viability as compared with control (Figure 3). On the other hand, 0.1% (w/v) of *C. monspeliensis* decreased ATP production as compared with low-concentration sample and, however, up-regulated cell viability in MTT assay. From these results, we considered that ATP was consumed for cell proliferation in 0.1% (w/v) of *C. monspeliensis*, and

ATP accumulation a little decreased as compared with low concentration (0.01 and 0.001% (w/v)). It was reported that the ATP accumulation was contributed to the proliferation and homeostasis on Caco-2 cells [10]. We expect that *C. monspeliensis* extract can induce the ATP accumulation and then the activation of proliferation and homeostasis on Caco-2 cells.

We considered that isolation of active component from *C. monspeliensis* extract should be fulfilled and further *in vivo* studies should focus on the confirmation of activation and safety of *C. monspeliensis* extract.

5. Conclusion

Our findings indicate that *C. monspeliensis* extract had high antioxidant ability. In addition, we show that *C. monspeliensis* extract has the function of promoting energy metabolism pathways, including glycolysis, TCA cycle, and electron transport chain, in human intestinal epithelial cells. Also, *C. monspeliensis* extract enhanced the production of intracellular ATP on intestinal epithelium. From these results, *C. monspeliensis* extract has an ability to enhance the energy metabolism. Further studies are in progress to elucidate the effect of *C. monspeliensis* on antiaging.

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Research Article

Camel Milk Modulates the Expression of Aryl Hydrocarbon Receptor-Regulated Genes, *Cyp1a1*, *Nqo1*, and *Gsta1*, in Murine hepatoma Hepa 1c1c7 Cells

Hesham M. Korashy,[1] Mohamed A. M. El Gendy,[2] Abdulqader A. Alhaider,[3] and Ayman O. El-Kadi[2]

[1] *Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, 11451 Riyadh, Saudi Arabia*
[2] *Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada T6G 2E1*
[3] *Department of Pharmacology, College of Medicine, King Saud University, 11461 Riyadh, Saudi Arabia*

Correspondence should be addressed to Ayman O. El-Kadi, aelkadi@pharmacy.ualberta.ca

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There is a traditional belief in the Middle East that camel milk may aid in prevention and treatment of numerous cases of cancer yet, the exact mechanism was not investigated. Therefore, we examined the ability of camel milk to modulate the expression of a well-known cancer-activating gene, Cytochrome P450 1a1 (*Cyp1a1*), and cancer-protective genes, NAD(P)H:quinone oxidoreductase 1 (*Nqo1*) and glutathione S-transferase a1 (*Gsta1*), in murine hepatoma Hepa 1c1c7 cell line. Our results showed that camel milk significantly inhibited the induction of *Cyp1a1* gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent Cyp1a1 inducer and known carcinogenic chemical, at mRNA, protein, and activity levels in a concentration-dependent manner. In addition, camel milk significantly decreased the xenobiotic responsive element (XRE)-dependent luciferase activity, suggesting a transcriptional mechanism is involved. Furthermore, this inhibitory effect of camel milk was associated with a proportional increase in heme oxygenase 1. On the other hand, camel milk significantly induced Nqo1 and Gsta1 mRNA expression level in a concentration-dependent fashion. The RNA synthesis inhibitor, actinomycin D, completely blocked the induction of Nqo1 mRNA by camel milk suggesting the requirement of *de novo* RNA synthesis through a transcriptional mechanism. In conclusion, camel milk modulates the expression of Cyp1a1, Nqo1, and Gsta1 at the transcriptional and posttranscriptional levels.

1. Introduction

The aryl hydrocarbon receptor (AhR), a cytosolic ligand-activated transcriptional factor, belongs to the basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription proteins, which are involved in regulation of cell differentiation and proliferation [1, 2]. Mechanistically, AhR is located in the cytoplasm bound with heat shock protein-90 (HSP90) and AhR interacting protein (AIP) forming inactive complex. Activation of AhR upon binding with its ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a polycyclic aromatic hydrocarbon (PAH), causes dissociation of HSP90 and AIP from the activated ligand-receptor complex, subsequently leading to translocation of the complex into nucleus. In the nucleus, the ligand-receptor complex dimerizes with AhR nuclear translocator (ARNT), which subsequently binds to xenobiotic-responsive element (XRE) located in the promoter region of so-called AhR-regulated genes resulting in promoting its transcription and protein translation processes [3, 4].

The AhR-regulated gene batteries include phase I xenobiotic metabolizing enzymes such as the cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1 and phase II enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase A1 (GSTA1), uridine diphosphate glucuronosyltransferase 1A6, and aldehyde dehydrogenase-3 [3, 4]. Among these genes, *CYP1A1* plays a particular role in bioactivating procarcinogens into carcinogen and toxic

metabolites and hence is considered as cancer-activating gene [5], whereas the *NQO1* and *GSTA1* catalyze reduction of several environmental contaminants and endogenous compounds that maintain endogenous antioxidants, such as ubiquinone and vitamin E, to protect tissues against mutagens, carcinogens, and oxidative stress damage [6, 7]. Accordingly, one of the strategies for protecting human cells and tissues from the toxic effects of carcinogenic and cytotoxic metabolites generally include attenuation of the carcinogen-activating genes, CYP1A1 signaling pathways, and/or enhancing the adaptive mechanisms by increasing the expression of detoxification and antioxidant genes, such as NQO1 and GSTA1 [8].

Chemoprevention by dietary constituents in the form of functional food has a well-established beneficial role in health promotion and emerged as a novel approach to control cancers [9]. Camel's milk is different from other ruminant milk, having low cholesterol, lactoferrin, low sugar, high minerals (sodium, potassium, iron, copper, zinc and magnesium), high vitamin C, B2, A, and E, low protein, and high concentrations of insulin. Recent studies have reported that camel milk is the most active milk among other species against *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium,* and rotavirus [10, 11]. In addition, it has been demonstrated that milk, in addition to secretory IgA and IgM, also contains numerous nonantibody components that possess antiviral activity, including lactoferrin [10].

Until recently, it is traditionally claimed that drinking camel milk may help to fight against serious diseases and cure numerous cases of cancer; however, this claim has never been exposed to scientific scrutiny investigation. Therefore, the main objective of the current study was to explore the capacity of camel milk to modulate the expression of *Cyp1a1*, *Nqo1*, and *Gsta1* genes as target for cancer prevention in murine hepatoma Hepa 1c1c7 cells.

2. Materials and Methods

2.1. Materials. 7-Ethoxyresorufin (7ER), Dulbecco's Modified Eagle's Medium (DMEM), protease inhibitor cocktail, sulforaphane (SNF), resveratrol (RES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and rabbit anti-goat IgG peroxidase secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). Resorufin and 100 × vitamin supplements were purchased from ICN Biomedicals Canada (Montreal, QC). TRIzol and Lipofectamine 2000 reagents were purchased from Invitrogen (Carlsbad, CA). The High-Capacity cDNA reverse transcription kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Actinomycin D (Act-D) was purchased from Calbiochem (San Diego, CA). Dual-Luciferase Reporter Assay System was obtained from Promega Corporation (Madison, WI). Chemiluminescence Western blotting detection reagents were obtained from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Cyp1a1 goat anti-mouse polyclonal primary antibody, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) rabbit anti-goat polyclonal antibody, and anti-rabbit IgG peroxidase secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON).

2.2. Milk Sample Collection and Preparation. Camel milk was collected aseptically from five healthy domestic camels (*Camelus dromedaries*). The camel milk was collected from farm and desert living animals. The collection of milk was usually conducted during the feeding time and was performed by experienced attendants. Milk was allowed to flow directly into sterile stainless steel containers and then transferred to glass vials. Camel samples were transported to the laboratory as soon as practical (<4 h) and were frozen at −80°C. Aqueous portion (fat-free, skimmed milk) was removed from the lipids (cream) as described before [12]. Briefly, aliquots of pooled milk samples were centrifuged at 1400 ×g for 30 minutes at 4°C, thereafter, the creamy layer consisting largely of fat was removed by filtration through a glass wool plug in a Pasteur pipette. Camel milk was collected and kept in −80°C freezer until use.

2.3. Cell Culture and Treatments. Murine hepatoma Hepa 1c1c7 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM, without phenol red supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 100 IU/mL penicillin, 10 μg/mL streptomycin, 0.1 mM nonessential amino acids, and vitamin supplement solution. Cells were grown in 75 cm^2 tissue culture flasks at 37°C under a 5% CO_2 humidified environment.

Hepa 1c1c7 cells were plated onto 96- and six-well cell culture plates in DMEM culture media for the mRNA, protein, and catalytic activity assays. In all experiments, the cells were pretreated for indicated time interval in serum-free media with various concentrations of camel milk in the presence or absence of TCDD as indicated. Stock solutions of TCDD were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C, in which the concentration of DMSO did not exceed 0.05% (v/v).

2.4. Cytotoxicity of Camel Milk. The effects of camel milk (fat-free) on Hepa 1c1c7 cell viability were determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT salt to formazan crystals as described previously [13]. Twenty-four hours after incubating the cells with the tested milk in the presence and absence of TCDD in a 96-well cell culture plate at 37°C under a 4% CO_2 humidified incubator, the media were removed and a 100 μL of serum-free medium containing 1.2 mM of MTT dissolved in phosphate-buffered-saline (PBS), pH 7.4, was added to each well. The plate was then incubated in a CO_2 incubator at 37°C for 2 h. The media were then decanted off by inverting the plate, and a 100 μL of isopropyl alcohol was added to each well, with shaking for 1 h to dissolve the formazan

Table 1: Primers sequences used for Real-Time PCR reactions.

Gene	Forward primer	Reverse primer
Cyp1a1	5′-GGT TAA CCA TGA CCG GGA ACT-3′	5′-TGC CCA AAC CAA AGA GAG TGA-3′
Ho-1	5′-GTG ATG GAG CGT CCA CAG C-3′	5′-TGG TGG CCT CCT TCA AGG-3′
Nqo1	5′-GGA AGC TGC AGA CCT GGT GA-3′	5′-CCT TTC AGA ATG GCT GGC A-3′
Gsta1	5′-CCC CTT TCC CTC TGC TGA AG-3′	5′-TGC AGC TTC ACT GAA TCT TGA AAG-3′
β-actin	5′-TAT TGG CAA CGA GCG GTT CC-3′	5′-GGC ATA GAG GTC TTT ACG GAT GTC-3′

crystals. The color intensity in each well was measured at wavelength of 550 using BIO-TEK Instruments EL 312e microplate reader, Bio-Tek Instruments (Winooski, VT). The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

2.5. Determination of Cyp1a1 Enzymatic Activity. Cyp1a1-dependent 7-ethoxyresorufin (7ER) O-deethylase (EROD) activity was performed on intact living Hepa 1c1c7 cells using 7ER as a substrate [14]. After incubation of the cells with increasing concentrations of fat-free camel milk for 24 h, media were aspirated and the cell monolayers were rinsed with PBS. Thereafter, 100 μL of 2 μM 7ER in assay buffer (0.05 M Tris, 0.1 M NaCl, pH 7.8) was then added to each well. Immediately, an initial fluorescence measurement ($t = 0$) at excitation/emission (545 nm/575 nm) was recorded from each well using Baxter 96-well fluorometer (Deerfield, IL). The plates were then replaced in the incubator, and additional set of fluorescence measurements of the wells were recorded every 5 min for 20 min interval. The amount of resorufin formed in each well was determined by comparison with a standard curve of known concentrations. The working solution was then aspirated, the cells were rinsed twice with PBS, and 50 μl of double de-ionized water was added to lyse the cells. After placing of the cell plates at −80°C for 30 min, the cell lysates were allowed to thaw, and protein levels were determined using a modified fluorescent assay [15]. The rate of resorufin formation was expressed as pmol/min/mg protein.

2.6. RNA Extraction and cDNA Synthesis. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. Thereafter, first strand cDNA was synthesized using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was added to a mix of 2.0 μL of 10x reverse transcriptase buffer, 0.8 μL of 25x dNTP mix (100 mM), 2.0 μL of 10x reverse transcriptase random primers, 1.0 μL of MultiScribe reverse transcriptase, and 3.2 μL of nuclease-free water. The final reaction mix was kept at 25°C for 10 min, heated to 37°C for 120 min, heated for 85°C for 5 s, and finally cooled to 4°C [16].

2.7. Quantification of mRNA Expression by Real-Time Polymerase Chain Reaction (RT-PCR). Quantitative analysis of specific mRNA expression was performed by RT-PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). The 25 μL reaction mix contained 0.1 μL of 10 μM forward primer and 0.1 μL of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μL of SYBR Green Universal Master Mix, 11.15 μL of nuclease-free water, and 1.25 μL of cDNA sample. The primers used in the current study (Table 1) [17, 18] were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The RT-PCR data was analyzed using the relative gene expression (i.e., $\Delta\Delta CT$) method, as described in Applied Biosystems User Bulletin [19]. The data are presented as the fold change in gene expression normalized to the endogenous housekeeping gene (β-actin) and was determined using the equation: fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(\text{target})} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(\text{treated})} - \Delta Ct_{(\text{untreated})}$.

2.8. Protein Extraction and Western Blot Analysis. Twenty-four hours after incubating the cells with increasing concentrations of camel milk (fat-free), the cells were washed once with cold PBS and collected by scraping in 100 μL of lysis buffer (50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μL/mL of protease inhibitor cocktail). The lysates were incubated on ice for 1 h with intermittent vortexing every 10 min, followed by centrifugation at 12,000 ×g for 10 min at 4°C. The supernatant was then stored at a −80°C freezer for later use in the Western blot analysis.

Western blot analysis was performed as described previously [20]. For Cyp1a1 immuno-detection, 30 μg of proteins from each treatment group were diluted with same amount (1 : 1) of 2X loading buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 1.5% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol), boiled and loaded onto a 10% SDS-polyacrylamide gel. Samples were electrophoresed at 120 V for 2 h, and the separated proteins were transferred to Trans-Blot nitrocellulose membrane (0.45 μm) in a buffer containing 25 mM Tri-HCl, 192 mM glycine, and 20% (v/v) methanol. Protein blots were blocked overnight at 4°C in a solution containing 5% skim milk powder, 2% bovine serum albumin, and 0.5% Tween-20 in TBS solution (0.15 M NaCl, 3 mM KCl, 25 mM Tris-base). Thereafter, the blocking solution was removed and the blots were rinsed three times

in a wash buffer (0.1% Tween-20 in TBS). Proteins were detected by incubation with a primary polyclonal goat anti-mouse Cyp1a1 antibody for 2 h at 4°C in TBS containing 0.01% sodium azide and 0.05% Tween-20. The primary antibody solution was removed and blots were rinsed three times with a wash buffer, followed by incubation with horseradish peroxidase-conjugate rabbit anti-goat secondary antibody for 1 h at room temperature followed by washing as previously described. Antibody detection was performed using the enhanced chemiluminescence method. The intensity of Cyp1a1 bands was quantified, relative to the signals obtained for Gapdh, using Java-based image-processing software, ImageJ (W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).

2.9. Transient Transfection and Luciferase Assay. Transient transfection and luciferase assay were carried out as described previously [21]. Briefly, Hepa 1c1c7 cells (3×10^4 cells per well) were plated onto 12-well cell culture plates. Each well was cotransfected with 1.5 μg of XRE-driven luciferase reporter plasmid pGudLuc 1.1 and 0.1 μg of the renilla luciferase pRL-CMV vector, used for normalization. The pGudLuc 1.1 plasmid was provided as a gift from Dr. Michael S. Denison (University of California, Davis, CA), while pRL-CMV vector was obtained from Promega Corporation (Madison,WI). Transfection procedure was carried out using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen), the luciferase assay was performed according to the manufacturer's instructions (Promega), and luciferase activity was reported as relative light unit of firefly luciferase to renilla luciferase (Fluc/Rluc).

2.10. Statistical Analysis. All results are presented as mean ± SEM. The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows, Systat Software Inc., (San Jose, CA). One-way analysis of variance (ANOVA) followed by Student-Newman-Keul's test was carried out to assess the significant difference between different groups. The differences were considered significant when $P < 0.05$.

3. Results

3.1. Effect of Camel Milk on Hepa 1c1c7 Cell Viability. To determine the cellular toxicity effects of camel milk (fat-free) in the presence and absence of TCDD on Hepa 1c1c7, cells were treated for 24 h with increasing concentrations of camel milk (0, 15, 25, 50, 100, and 200 μL/mL) in the presence and absence of TCDD (1 nM) and the cell viability was determined by MTT assay. Figure 1 shows that neither camel milk alone nor with TCDD were toxic to Hepa 1c1c7 cells at all concentrations of camel milk used, with the exception of mixture of TCDD plus camel milk (200 μL/mL) that significantly decreased cell viability by approximately 25%. Based on these results, concentrations of 0, 25, and 100 μL/mL of fat-free camel milk in the presence and absence of TCDD were chosen to be used in the subsequent experiments.

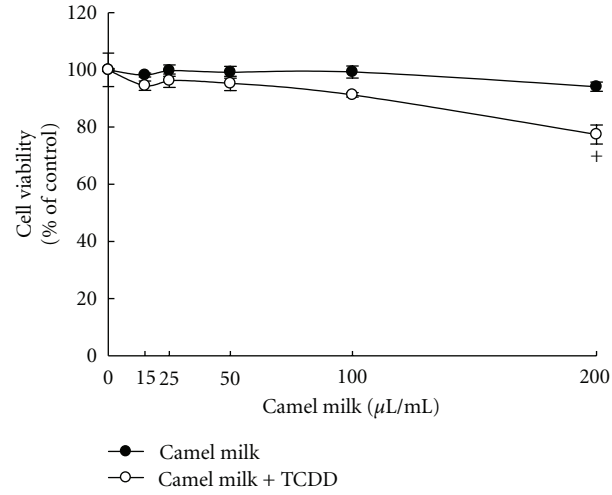

Figure 1: Effect of camel milk on Hepa 1c1c7 cell viability. Hepa 1c1c7 cells were incubated for 24 h with various concentrations of fat-free camel milk (0, 15, 25, 50, 100, and 200 μL/mL) in the presence and absence of TCDD (1 nM). Thereafter, cell viability was assessed using the MTT assay. Values are presented as percentage of the control (mean ± SEM, $n = 8$). $^*P < 0.05$ compared with control (concentration = 0 μL/mL, nontreated cells).

3.2. Effect of Camel Milk on the TCDD-Mediated Induction of Cyp1a1 Catalytic Activity in Hepa 1c1c7 Cells. To determine the capacity of camel milk to alter the induction of Cyp1a1 catalytic activity by TCDD, Hepa 1c1c7 cells were preincubated with increasing concentrations of camel milk (fat-free) (0, 25, and 100 μL/mL) or RES (25 μM), a positive control, for 30 min before the incubation with 1 nM TCDD for additional 24 h. The TCDD 1 nM concentration was selected from our previous concentration-response study that caused maximum induction of Cyp1a1 gene without significant cell toxicity [22]. Thereafter, Cyp1a1 activity was determined by EROD assay. Figure 2 shows that TCDD alone markedly induced Cyp1a1 enzymatic activity level by 180-fold. Furthermore, camel milk at all concentrations tested significantly inhibited the TCDD-mediated induction of Cyp1a1 activity by approximately 63% and 80% at the concentrations of 25 and 100 μL/mL, respectively, (Figure 2). On the other hand, RES, a well-known AhR antagonist, significantly reduced the Cyp1a1 induction by TCDD (Figure 2). The observed inhibition of Cyp1a1 by camel milk (fat-free) particularly at the highest concretion is due to a direct effect of camel milk and not because of any cell toxicity (Figure 1).

3.3. Effect of Camel Milk on the TCDD-Mediated Induction of Cyp1a1 mRNA Level in Hepa 1c1c7 Cells. To determine whether the inhibitory effect of camel milk (fat-free) on the TCDD-mediated induction of Cyp1a1 activity (Figure 2) is attributed to a transcriptional mechanism, Cyp1a1 mRNA levels were determined in Hepa 1c1c7 cells treated for 6 h with TCDD (1 nM) in the presence and absence of different concentrations of camel milk (0, 25, and 100 μL/mL) or RES (25 μM) as positive control. Our results showed that

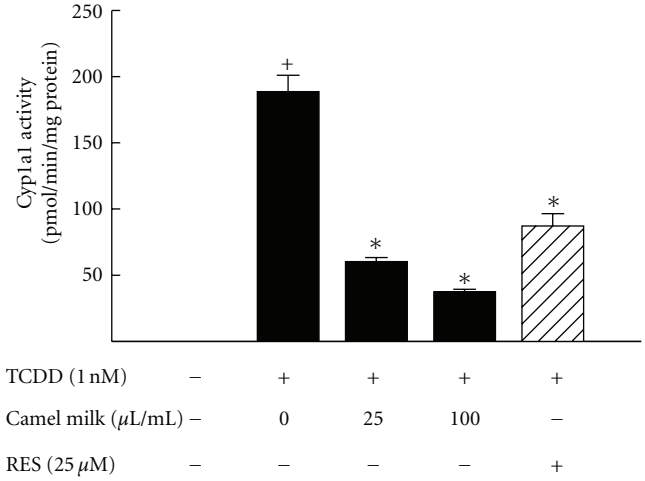

Figure 2: Effect of camel milk on the TCDD-mediated induction of Cyp1a1 activity. Hepa 1c1c7 cells were treated for 24 with TCDD (1 nM) in the presence and absence of camel milk (0, 25, and 100 μL/mL) or the positive control, resveratrol (Res, 25 μM). Cyp1a1 activity was measured in intact living cells using EROD assay. Values are presented as mean ± SEM ($n = 8$). $^{+}P < 0.05$ compared with control (sterile water-treated cells), $^{*}P < 0.05$ compared with TCDD-treated cells.

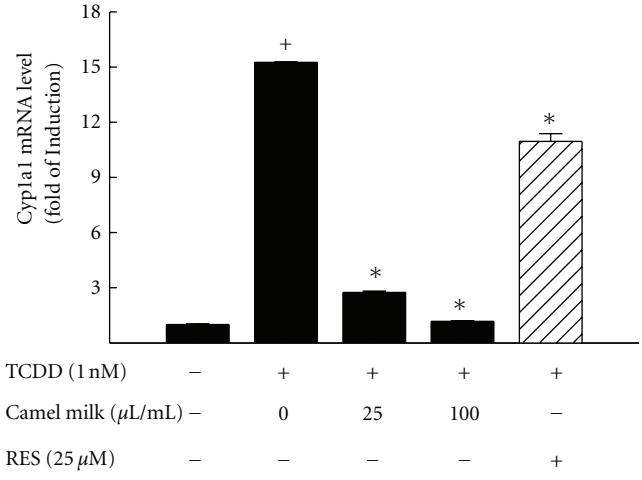

Figure 3: Effect of camel milk on the TCDD-mediated induction of Cyp1a1 mRNA. Hepa 1c1c7 cells were treated for 6 h with TCDD (1 nM) in the presence and absence of camel milk (fat-free) (0, 25, and 100 μL/mL) or the positive control, resveratrol (Res, 25 μM). The amount of Cyp1a1 mRNA was quantified using real-time PCR and normalized to β-actin housekeeping gene. Duplicate reactions were performed for each experiment, and the values represent mean of fold change ± SEM. ($n = 4$). $^{+}P < 0.05$ compared with control (sterile water-treated cells), $^{*}P < 0.05$ compared with TCDD-treated cells.

TCDD significantly induced Cyp1a1 mRNA level by approximately 15-fold (Figure 3). Importantly, incubation of Hepa 1c1c7 cells with camel milk (fat-free) significantly decreased the TCDD-mediated induction of Cyp1a1 mRNA in a concentration-dependent manner (Figure 3). The maximum inhibition (90%) was observed at the highest concentration tested (100 μL/mL) (Figure 3). Similar to Cyp1a1 activity, the positive control RES significantly decreased the TCDD-mediated induction of Cyp1a1 mRNA (Figure 3).

3.4. Effect of Camel Milk on the TCDD-Mediated Induction of Cyp1a1 Protein in Hepa 1c1c7 Cells. To further investigate whether the obtained inhibition on TCDD-mediated induction of Cyp1a1 mRNA levels by camel milk is translated into a functional Cyp1a1 protein, Western blot analysis was carried out. Total protein was isolated from Hepa 1c1c7 cells treated for 24 h with TCDD (1 nM) in the presence and absence of different concentrations of camel milk (fat-free) (0, 25, and 100 μL/mL). Figure 4 shows that the induction of Cyp1a1 protein by TCDD (72-fold) was significantly reduced by camel milk (fat-free) by approximately 30% and 65% at concentrations of 25 and 100 μL/mL, respectively. This pattern of inhibition is similar to what was observed at the activity and mRNA level (Figures 2 and 3).

Taken together, these results showed that camel milk stands prominently in its inhibition of the induction of *Cyp1a1* gene expression at the activity, mRNA, and protein levels.

3.5. Effect of Camel Milk on XRE-Dependent Luciferase Activity. To explore the ability of fat-free camel milk to inhibit the AhR-dependent reporter gene expression, Hepa 1c1c7

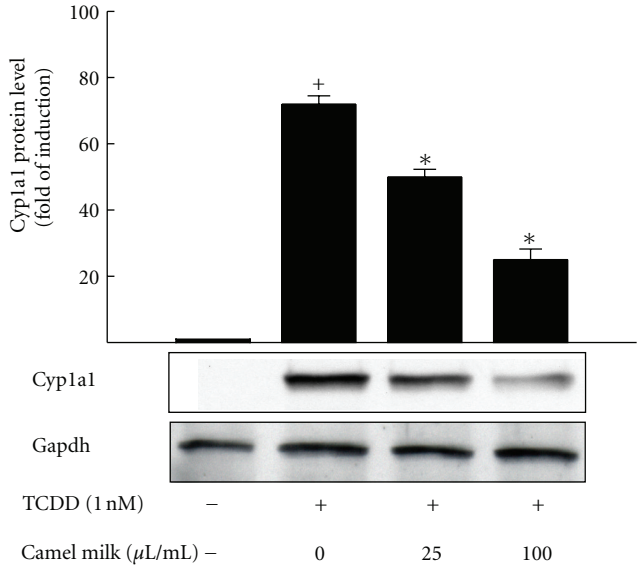

Figure 4: Effect of camel milk on the TCDD-mediated induction of Cyp1a1 protein. Hepa 1c1c7 cells were treated for 24 with TCDD (1 nM) in the presence and absence of camel milk (fat-free) (0, 25, and 100 μL/mL); thereafter, Cyp1a1 protein level was determined by Western blot analysis and was detected using the enhanced chemiluminescence method. The intensity of Cyp1a1 protein bands was quantified relative to the signals obtained for Gapdh protein, using ImageJ. One of the three representative experiments is shown and values represent mean of fold change ± SEM. ($n = 3$). $^{+}P < 0.05$ compared with control (sterile water-treated cells), $^{*}P < 0.05$ compared with TCDD-treated cells.

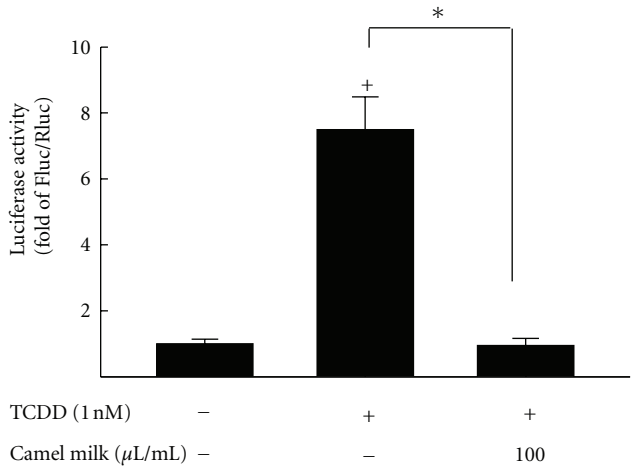

Figure 5: Effect of camel milk on XRE-dependent luciferase activity. Hepa 1c1c7 cells were transiently cotransfected with XRE-driven luciferase reporter plasmid pGudLuc 1.1. and renilla luciferase control plasmid pRL-CMV. Cells were treated with sterile water or camel milk (fat free, 100 μL/mL) 30 min before the addition of TCDD (1 nM) for an additional 24 h. Cells were lysed and luciferase activity was reported as relative light unit (RLU) of firefly luciferase to renilla luciferase (Fluc/Rluc) (mean ± S.E.M., $n = 4$). $^{+}P < 0.05$ compared control (sterile water-treated cells), $^{*}P < 0.05$ compared with TCDD-treated cells.

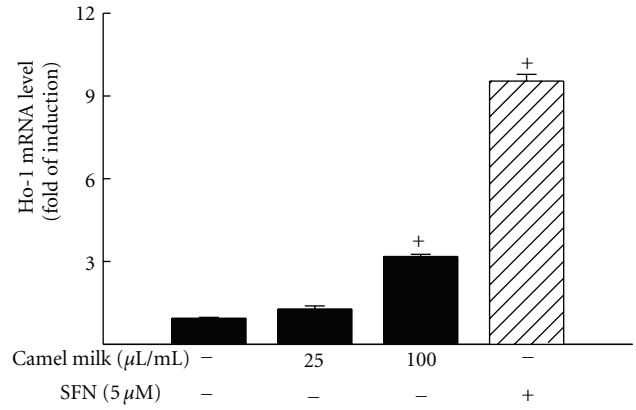

Figure 6: Effect of camel milk on Ho-1 mRNA expression level. Hepa 1c1c7 cells were treated for 6 h with increasing concentrations of fat-free camel milk (0, 25, and 100 μL/mL) or SFN (5 μM), as a positive control. The amount of Ho-1 mRNA was quantified using real-time PCR and normalized to β-actin housekeeping gene. Duplicate reactions were performed for each experiment, and the values represent mean of fold change ± SEM. ($n = 4$). $^{+}P < 0.05$ compared with control (sterile water-treated cells).

cells were transiently cotransfected with the XRE-dependent luciferase reporter gene and renilla luciferase vector, which was used for normalization of transfection efficiency. Cells were then incubated for 24 h with TCDD (1 nM) in the presence and absence of camel milk (100 μL/mL). Our results showed that TCDD significantly induced XRE-dependent luciferase activity by approximately 7-fold (Figure 5). Importantly, camel milk (fat-free, 100 μL/mL) completely blocked the induction of XRE-dependent luciferase activity by TCDD to its control level (Figure 5).

3.6. Effect of Camel Milk on Ho-1 mRNA in Hepa 1c1c7 Cells. The capacity of camel milk to inhibit TCDD-mediated induction of Cyp1a1 at the activity level more than at the protein levels prompted us to investigate the possible role of Ho-1, a rate-limiting enzyme of heme degradation, in this inhibitory effect. For this purpose, Ho-1 mRNA level was determined in Hepa 1c1c7 cells treated with increasing concentrations of camel milk (0, 25, and 100 μL/mL) or SFN (5 μM), as a positive control, for 6 h. Our results show that camel milk (fat-free) significantly increased the Ho-1 mRNA expression level by the highest concentration tested (100 μL/mL) by approximately 3-fold (Figure 6). Similarly, SFN caused a 9-fold induction in Ho-1 mRNA level. These data may suggest a role for Ho-1 in camel milk-mediated effects.

3.7. Effect of Camel Milk on Nqo1 and Gsta1 mRNA Levels in Hepa 1c1c7 Cells. To further investigate whether camel milk (fat-free) is able to increase the expression of chemoprotective genes, *Nqo1* and *Gsta1* mRNA levels were measured in Hepa 1c1c7 cells treated for 6 h with camel milk (0, 25, and 100 μL/mL) or SFN (5 μM), as a positive control. Figure 7 shows that camel milk (fat-free) significantly induced Nqo1 and Gsta1 mRNA levels by approximately 2.5- and 8-fold, respectively, at the highest concentration tested (100 μL/mL). A similar induction was observed with SFN, strong inducer of Nqo1 and Gsta1 (Figure 7).

3.8. Effect of Transcription Inhibitor, Act-D, on Camel-Milk-Induced Nqo1 mRNA Levels. To further investigate whether camel milk (fat-free) is able to increase the *de novo* Nqo1 RNA synthesis, Hepa 1c1c7 cells were treated with Act-D (5 μg/mL, RNA synthesis inhibitor) immediately before the addition of camel milk (fat-free, 100 μL/mL) for additional 6 h. Thereafter, Nqo1 mRNA was quantified by real-time PCR. If camel milk increases the amount of Nqo1 mRNA through a transcriptional mechanism, a decrease in the Nqo1 mRNA level would be expected. Figure 8 shows that Act-D slightly but significantly inhibited the constitutive expression of Nqo1 mRNA, whereas markedly blocked the camel milk-induced Nqo1 mRNA by approximately 70% (Figure 8).

4. Discussion

The current study provides the first mechanistic evidence of the ability of camel milk to significantly inhibit the induction of *Cyp1a1*, a cancer-activating gene, and to induce *Nqo1* and *Gsta1*, cancer protecting genes in Hepa 1c1c7 cells at the transcriptional and posttranscriptional levels.

One of the strategies for protecting human cells and tissues from the toxic effects of carcinogenic and cytotoxic metabolites include attenuation of the carcinogen-activating

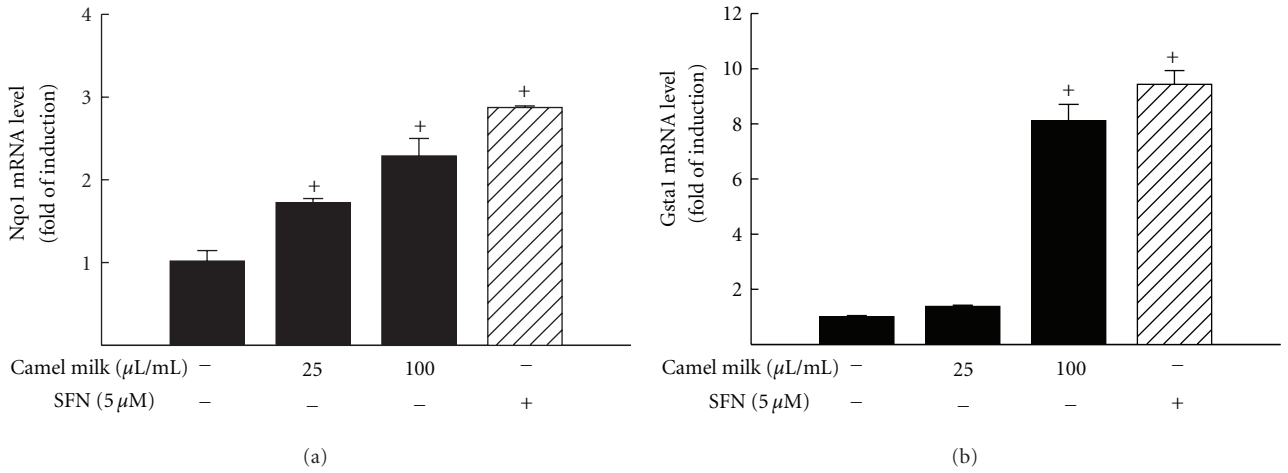

Figure 7: Effect of camel milk on Nqo1 (a) and Gsta1 (b) mRNA expression levels. Hepa 1c1c7 cells were treated for 6 h with fat-free camel milk (0, 25, and 100 μL/mL) or SFN (5 μM), as a positive control. The amount of Nqo1 and Gsta1 mRNA levels was quantified using real-time PCR and normalized to β-actin housekeeping gene. Duplicate reactions were performed for each experiment and the values represent mean of fold change ± SEM. ($n = 4$). $^{+}P < 0.05$ compared with control (sterile water-treated cells).

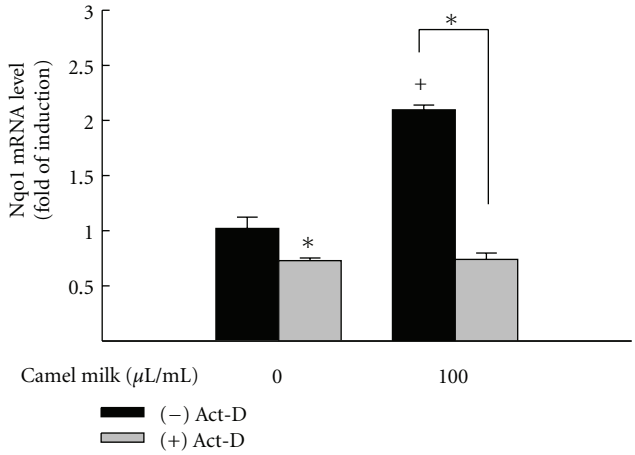

Figure 8: Effect of transcription inhibitor, Act-D, on the induction of Nqo1 mRNA by camel milk. Hepa 1c1c7 cells were incubated with the transcription inhibitor, Act-D (5 μg/mL), immediately before the addition of fat-free camel milk (100 μL/mL) for 6 h. The amount of Nqo1 mRNA was quantified using real-time PCR and normalized to β-actin housekeeping gene. Duplicate reactions were performed for each experiment, and the values represent mean of fold change ± SEM. ($n = 4$). $^{+}P < 0.05$ compared with control (sterile water-treated cells), $^{*}P < 0.05$ compared with corresponding treatment in the absence of Act-D.

genes signaling pathways and/or enhancing the adaptive mechanisms by increasing the expression of detoxification and antioxidant genes. Accordingly, we hypothesize that camel milk exhibits anticancer effects by inhibiting the expression of *Cyp1a1* and/or inducing *Nqo1* and *Gsta1* genes. Therefore, to test our hypothesis we examined the capacity of camel milk to (a) inhibit the induction of Cyp1a1 at the mRNA, protein, and activity levels, (b) induce the expression of Nqo1, and Gsta1 mRNA levels in Hepa 1c1c7 cells, and (c) explore the underlying molecular mechanism.

Several lines of evidence showed that induction of CYP1A1 is strongly correlated with increased incidence of several human colon, rectal, and lung cancers [23, 24]. In addition, studies on the carcinogenicity and mutagenicity of PAHs have demonstrated a significant role for the induction of CYP1A1 in bioactivating these environmental toxicants into their ultimate carcinogenic forms [25]. Thus Cyp1a1 induction is considered a useful biomarker of exposure to carcinogenic substances [26]. Accordingly, we have first examined the potential effect of camel milk on the expression of *Cyp1a1* gene after induction by TCDD using EROD as a probe for Cyp1a1 activity [27]. Our results showed that camel milk (fat-free) at all concentrations tested reduced Cyp1a1 EROD activity in a concentration-dependent manner (Figure 2). Importantly, the modulation of Cyp1a1 activity by camel milk is attributed to a decrease in transcriptional and translational regulation of Cyp1a1 gene. This was evidenced by the ability of camel milk to alter the expression of Cyp1a1 at the mRNA and protein levels (Figures 3 and 4).

The transcriptional regulation of *Cyp1a1* and *Nqo1* genes by camel milk (fat-free) was demonstrated by several lines of evidence. First, the inhibition of XRE-dependent luciferase assay that occurs only through the AhR activation suggests an AhR-dependent transcriptional control (Figure 5). Second, the ability of the transcription inhibitor, Act-D, to significantly block the newly synthesized Nqo1 mRNA (Figure 7) suggests a requirement of *de novo* RNA synthesis for the induction of Nqo1 mRNA by camel milk. Taken together, these results strongly suggest that the inhibition of Cyp1a1 and induction of Nqo1 genes by camel milk is mediated, at least in part, at the transcriptional level.

Interestingly, the observation that camel milk-mediated inhibition of Cyp1a1 induction by TCDD at the activity

levels is higher than those observed at the protein levels (Figure 2), suggest that a possible involvement of a post-translational mechanism, such as phosphorylation, proteasomal degradations, modulation of *HO-1* gene expression, could be involved [22, 28]. Among these mechanisms, *HO-1* gene expression, a rate-limiting enzyme in heme catabolism, has been shown to alter cellular heme, the prosthetic group of CYP450, content and hence the enzyme activity [29]. To test this hypothesis, we have examined the effect of camel milk (fat-free) on the expression of Ho-1 mRNA level. Our results showed that the camel milk increases the level of Ho-1 mRNA levels (Figure 6). Taken together, we postulated here that the capacity of camel milk to induce the expression of Ho-1 mRNA resulted in a decrease in Cyp1a1 activity levels through degrading its heme content. This postulation is supported by our previous observations that in mesoporphyrin, competitive Ho-1 inhibitor or knockdown of Ho-1 using siRNA significantly restored the inhibition of Cyp1a1 activity by heavy metals [30, 31].

The chemo protective effect of camel milk was further supported, in addition to inhibition of Cyp1a1, by the ability to upregulate antioxidant genes, particularly Nqo1 and Gsta1 mRNA levels in a concentration-dependent manner (Figure 7). Our results are in agreement with previous studies that reported the ability of camel milk to induce GST levels in healthy and schistosoma-infected mice [32]. Thus increased expression of *Nqo1* and *Gsta1* by camel milk will increase the levels of several antioxidant enzymes which prevent the formation of highly reactive oxygen radicals and hence reduce DNA adduct and cell damage [33]. In addition, overexpression of *NQO1* in several human solid tumors and cancer cells has been shown to activate bioreductive chemotherapeutic agents in tumor cells that allow tumor cytotoxicity without corresponding toxicity to normal cells [34].

Although the potential mediators in camel milk involved in the downregulation of Cyp1a1 and induction of Nqo1 and Gsta1 were not examined in this study, several previous studies have reported that camel milk contains considerably higher amounts of antioxidant vitamins, such as E and C, in comparison to cow milk [35], lysosomes [11], lactoferrins [11, 36], and immunoglobulines [36]. In addition, ongoing research in our laboratory has shown the presence of several compounds in camel milk that could be involved in milk-mediated effect (unpublished data). Among these mediators, lactoferrin, an iron-binding glycoprotein, is known to exert *in vitro* and *in vivo* antitumor activity [37]. In this context, it has been recently reported that lactoferrin inhibits the development of cancer through inhibiting CYP1A1 activation in 7,12-dimethylbenz[a]anthracene- (DMBA-) induced hamster buccal pouch carcinoma model. Taken together the results obtained from our laboratory and previously published reports, we speculate that lactoferrin could be responsible for camel-milk-mediated effect. In addition, the main components of the camel milk have been already determined [38]. Ongoing research in our laboratory focuses on identifying and characterizing the most effective component of camel milk using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and one-dimensional gel electrophoresis, where several proteins were found to be relatively abundant in camel milk (data not shown).

In conclusion, the results of current study suggest that camel milk could protect against or decrease the deleterious effects of many environmental toxicants and carcinogens such as PAHs, probably through modulation of AhR-regulated genes of *Cyp1a1*, *Nqo1*, and *Gsta1* at the transcriptional and posttranscriptional mechanisms. These results are of potential clinical significance to humans in that it uncovers the molecular mechanism involved and could explain the anecdotal evidence for the successful use of camel milk in the treatment and/or prevention of various medical conditions.

Abbreviations

AhR: Aryl hydrocarbon receptor
Act-D: Actinomycin D
CYP1A1: Cytochrome P450 1A1
DMSO: Dimethyl sulfoxide
7ER: 7-ethoxyresorufin
EROD: 7-ethoxyresorufin O-deethylase
Gapdh: Glyceraldehyde-3-phosphate dehydrogenase.
Gsta1: Glutathione S-transferase a1
MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
Nqo1: NAD(P)H:quinone oxidoreductase
PAHs: Polycyclic aromatic hydrocarbons
RES: Resveratrol
SFN: Sulforaphane
TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
XRE: Xenobiotic responsive element.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

The Safety of Cruciferous Plants in Humans: A Systematic Review

Ori Scott,[1] Elaine Galicia-Connolly,[2] Denise Adams,[2] Soleil Surette,[2] Sunita Vohra,[2,3] and Jerome Y. Yager[1]

[1] *Pediatric Neurosciences, Department of Pediatrics, Stollery Children's Hospital, University of Alberta, Room 3-469, Edmonton Clinic Health Academy (ECHA), 11405-87 Avenue, Edmonton, AB, Canada T6G 1C9*
[2] *CARE Program, Department of Pediatrics, University of Alberta, Edmonton Clinic Health Academy (ECHA), 11405-87 Avenue, Edmonton, AB, Canada T6G 1C9*
[3] *School of Public Health, University of Alberta, Edmonton Clinic Health Academy (ECHA), 11405-87 Avenue, Edmonton, AB, Canada T6G 1C9*

Correspondence should be addressed to Jerome Y. Yager, jyager@ualberta.ca

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Some cruciferous plants may serve as preventive treatments for several medical conditions; our objective was to systematically investigate their safety in humans. Four electronic databases were searched, and, of 10,831 references identified, 50 were included. Data were extracted by two independent reviewers, whereafter the association between interventions and adverse events was assessed. Adverse events in 53 subjects were identified through clinical trials; of these, altered drug metabolism was rated as certainly/likely caused by cruciferous plants. Adverse events in 1247 subjects were identified through observational studies, of which none received high causality ratings. Adverse events in 35 subjects were identified through case reports, of which allergies and warfarin resistance were rated as certainly/likely caused by cruciferous plants. We conclude that cruciferous plants are safe in humans, with the exception of allergies. Individuals treated with warfarin should consult their physician. Further investigation of uses of cruciferous plants in preventative medicine is warranted.

1. Introduction

In the last decade, a rapid rise in the demand for natural health products has become evident. This prominent trend stems from increasing awareness of the potential for significant adverse effects caused by pharmacologic interventions, along with a growing interest in preventive medicine strategies [1].

A group of plants that has been shown to possess strong anti-inflammatory and antioxidative abilities is the cruciferous plants of the *Brassica* genus. Brassica is a genus of plants in the Brassicaceae family. Vegetables of the family Brassicaceae (also called Cruciferae) are generally referred to as cruciferous vegetables. These vegetables are widely cultivated, with many genera, species, and cultivars being raised for food production. The most common Brassica vegetables eaten by people are in a single species (*B. oleracea*), including kale, collard greens, cabbage, Brussels sprouts, kohlrabi, broccoli, and cauliflower. Numerous other species in the genus are also edible such as mustard (*B. nigra*), Chinese cabbage (*B. rapa*), and oilseed rape (*B. napus;* sometimes referred to as "rapeseed oil" or "canola") [2].

Some members of this group have received wide acclaim as potential natural preventers or attenuators of several health conditions such as coronary artery disease, gastritis, and cancer [3–8]. Recent studies reveal that these therapeutic effects may extend to the fetus as well: an international case-control study showed that maternal consumption of cruciferous vegetables during pregnancy decreases the risk of childhood anaplastic astrocarcinomas [9]; a study in rats found that offspring of spontaneously hypertensive rats that consumed broccoli sprouts during gestation had lower blood pressure and reduced markers of oxidative stress and inflammation, compared to controls [10].

The impetus for this systematic review stems from work done in our laboratory that has shown the potential benefit of supplementing the diet of pregnant dams with broccoli sprouts in preventing perinatal brain injury. In this regard,

Table 1: Eligibility criteria and data extracted[1].

PICOS	Eligibility criteria	Data extracted
Patient	All human subjects were included	Age, concurrent medical conditions and treatments, reason for intervention (where relevant)
Intervention	All types of exposure (oral, topical, or respiratory) to cruciferous plants, their derivatives, or their constituents	Plant or substance exposed to, route of exposure, duration of exposure, dose (if available)
Comparators	Reports with or without a comparator group. Reports without control groups were included in order to include all potential adverse events	Numbers in the intervention and comparator groups (when relevant)
Outcome	Reports of presence or absence of adverse events	Presence or absence of adverse events, description of adverse event, acute management of adverse event (for case reports), outcome (when available), and causality
Study design	All study designs which were relevant to the assessment of safety were included. Studies in all languages were included and translated when necessary	Type of study design and setting

[1] PICOS: patient, intervention, comparators, outcome, study design.

we have established a model of chronic placental insufficiency that results in fetal growth restriction and damage to the white matter of the brain, reminiscent of periventricular leukomalacia, the anatomic hallmark of cerebral palsy. Supplementing the maternal diet during the last trimester of pregnancy and first weeks of infant nutrition prevented the white matter injury and accompanying behavioural deficits in the offspring. These findings suggest, for the first time, a safe and efficacious approach to the prevention of developmental disability and cerebral palsy [11]. In order to provide the background in which to determine whether this therapy would be acceptable for humans, a thorough determination of the safety of the cruciferous species of plants in humans is necessary.

Researchers now believe that the key to the therapeutic abilities of cruciferous plants is their high content of phase-2 enzyme inducers such as sulforaphane, which induce the transcription of genes found under the control of the antioxidant response element (ARE). This causes an upregulation of the endogenous antioxidant glutathione, crucial to the cell's ability to withstand oxidative stress [12–14].

Several reviews evaluated the safety and health benefits of some components of cruciferous plants, such as indole-3-carbinol and isothiocyanates, and found them safe for humans [15, 16]. However, to our knowledge, a systematic review evaluating the safety of cruciferous plants, namely, the degree of certainty that they would not cause any adverse effects, has not yet been published.

The purpose of this paper is to systematically collect and synthesize all published reports of human adverse events associated with exposure to cruciferous plants. This approach of analyzing safety of a multi-ingredient natural product is especially important when considering consumption of the whole plant as a therapeutic means, as opposed to treatment with an isolated component or metabolite of a plant.

2. Methods

This systematic paper was undertaken in line with the relevant criteria of the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement [17]. The following methods used in the systematic review, including identification, screening, eligibility criteria, inclusion, and data extraction, were agreed upon by the authors *a priori*.

References were identified through comprehensive search strategies, which were developed in conjunction with a research librarian. An electronic search of the following four databases was performed: Medline, Embase, Pascal, and IPA (International Pharmaceutical Abstracts), from inception to July 2010, irrespective of language. The following search terms were used: isothiocyanate, Brassica, cruciferous, glucoraphanin, sulforaphane, broccoli, kale, cabbage, cauliflower, collard green, Brussels sprout. MESH headings and keywords were searched, and truncation was used as needed. A list of the search strategies is available as supplemental material available online at doi: 10.1155/2012/503241.

The research question and eligibility criteria were developed by using PICOS (patient, intervention, comparators, outcome, study design). The inclusion criteria were any studies reporting original data, addressing safety of cruciferous plants, their derivatives, or their constituents, to humans. Details of the eligibility criteria are reported in Table 1.

The references were imported into a bibliographic database (RefWorks). Titles and abstracts of identified studies were independently screened by two reviewers. Full texts for potentially relevant studies were obtained and reviewed for inclusion based on predetermined criteria. Disagreement was resolved by consensus, and if necessary a third party was consulted (JYY).

Data was extracted by two independent reviewers, using standardized forms. Disagreement was resolved by consensus. Data relating to the patient or group, the intervention,

the comparator group (where relevant), the outcomes measured (adverse events or lack thereof), and the study design were extracted as detailed in Table 1.

The degree of association between the intervention and the adverse event was independently assessed by the two reviewers using the Causality Algorithm used by Health Canada and the WHO Collaborating Centre for International Drug Monitoring (Health Canada 2009). Categories for assessment were certainly, likely, possibly, unlikely, conditional/unclassified, and Unassessible/Unclassifiable. Disagreement between reviewers was resolved by discussion. Guidelines used to assess causality are available as supplemental material.

The studies were finally categorized into 3 groups: (1) trials, (2) observational studies, and (3) case reports. Studies were classified as controlled clinical trials when involving one or more test treatments, at least one control treatment, specified outcome measures for evaluating the studied intervention, and when the method for assigning patients to the test treatment was not a mathematical randomization technique. Randomized controlled trials, on the other hand, were defined as studies in which randomization using mathematical techniques, such as the use of a random numbers table, is employed to assign patients to test or control treatments. Studies were classified as single-case experimental design trials when the subjects served as his/her own control, rather than using another individual or a group.

We did not perform a meta-analysis as the inclusion criteria for subjects, interventions, clinical outcomes, and study designs were very heterogeneous; conducting a meta-analysis using these data would not have been appropriate.

3. Results

3.1. Study Characteristics. Searches resulted in a total of 10,831 references. After screening of titles and abstracts, the full texts of 367 articles were obtained. Of these, 50 articles met all inclusion criteria. Of the total included publications, 45 were published in English, 2 were published in German, and one each was published in Italian, French, and Spanish.

Articles were excluded for the following reasons: not human subjects (210), not evaluating adverse effects or lack thereof (21), intervention or substance exposure does not include cruciferous plants (2), not presenting original data (47). Thirty-seven articles discussing toxic oil syndrome (37) were excluded post hoc for reasons presented below.

Of the 50 included studies, 13 were trials (6 single-case experimental design trials, 4 controlled clinical trials, and 3 randomized control trials (RCTs)), 13 were observational studies (7 cohort studies, 3 case-control studies, 2 cross-sectional studies, and 1 qualitative survey), and 24 were case reports (Figure 1).

3.2. Trials. Thirteen trials were included, reporting adverse events in 53/496 patients (10.7%) [18–30]. Three of them were randomized controlled trials (RCTs), 4 were controlled clinical trials, and 6 were single-case experimental design (SCED) trials. Six of the trials were classified as safety trials, meaning that their stated primary aim was to assess safety of interventions [18–23], whereas 7 were nonsafety trials which evaluated efficacy of interventions but also reported adverse events [24–30] (Table 2).

The safety trials [18–23] included 1 RCT, 1 controlled clinical trial, and 4 SCED trials. The only safety RCT was a double-blinded trial in 12 healthy subjects, ages 28–57, of whom 9 consumed daily doses of broccoli sprout extract combined with myrosinase (an enzyme found in all Brassica which converts glucosinolates to active isothiocyanates) over a period of 7 days. Extracts contained 25 μmol glucosinolate, 100 μmol glucosinolate, or 25 μmol isothiocyanate. No adverse events were reported [18].

The second safety trial was a double-blind controlled clinical trial in 76 subjects, ages 3–39. Thirty-eight subjects had a reported mustard allergy, and 38 age- and gender-matched controls suffered from dust-mite allergy but had no mustard allergy. Both groups underwent food challenges with mustard and a variety of other allergens. Of the 38 patients, 14 had a positive mustard challenge, of which 12 had oral allergy syndrome (pruritus, mild angioedema of lips, tongue, and throat), 1 had more severe angioedema with bronchial asthma, and 1 had systemic anaphylaxis. It is important to mention that of the 38 patients, 35 were atopic (including rhinitis, and/or bronchial asthma, atopic dermatitis) [19].

The last four safety trials were all single-case experimental design trials (SCED). The first was a trial in 10 healthy subjects, ages 21–30, which strived to determine the effect of cruciferous vegetables on phenacetin and antipyrine metabolism. Subjects started by consuming a control diet for 13 days, after which they were placed on a diet containing 300 g/day of Brussels sprouts and 200 g/day of cabbage over a period of 7 days, and finally returned to the control diet for the last 10 days. In parallel, every morning the subjects were given either 900 mg of phenacetin or 1.8 mg/kg of antipyrine. The mean plasma concentrations of either drug were measured at different intervals after administration. Researchers found that the cruciferous diet enhanced the phenacetin metabolism in the gastrointestinal tract and/or during its first pass in the liver, increasing its rate of elimination. No changes in antipyrine metabolism were noted [20]. It is worth noting that the US Food and Drug Administration ordered the withdrawal of drugs containing phenacetin in November 1983, owing to its carcinogenic and kidney-damaging properties (Federal Register of October 5, 1983 (48 FR 45466)).

The second SCED trial included 10 healthy subjects, ages 23–35, and aimed to determine the effect of cruciferous vegetables on acetaminophen and oxazepam metabolism. Subjects started by consuming a control diet for 10 days, after which they were put on a diet containing 300 g/day of Brussels sprouts and 200 g/day of cabbage over a period of 10 days, and finally returned to the control diet for the last 10 days. In parallel, every morning the subjects were given either 45 mg of oxazepam or 1500 mg of acetaminophen. The mean plasma concentrations of either drug were measured at different intervals after administration, and a 24 h urinary recovery of conjugates was performed. Researchers found that the cruciferous diet enhanced the acetaminophen metabolism and glucuronide conjugation; however, they mention

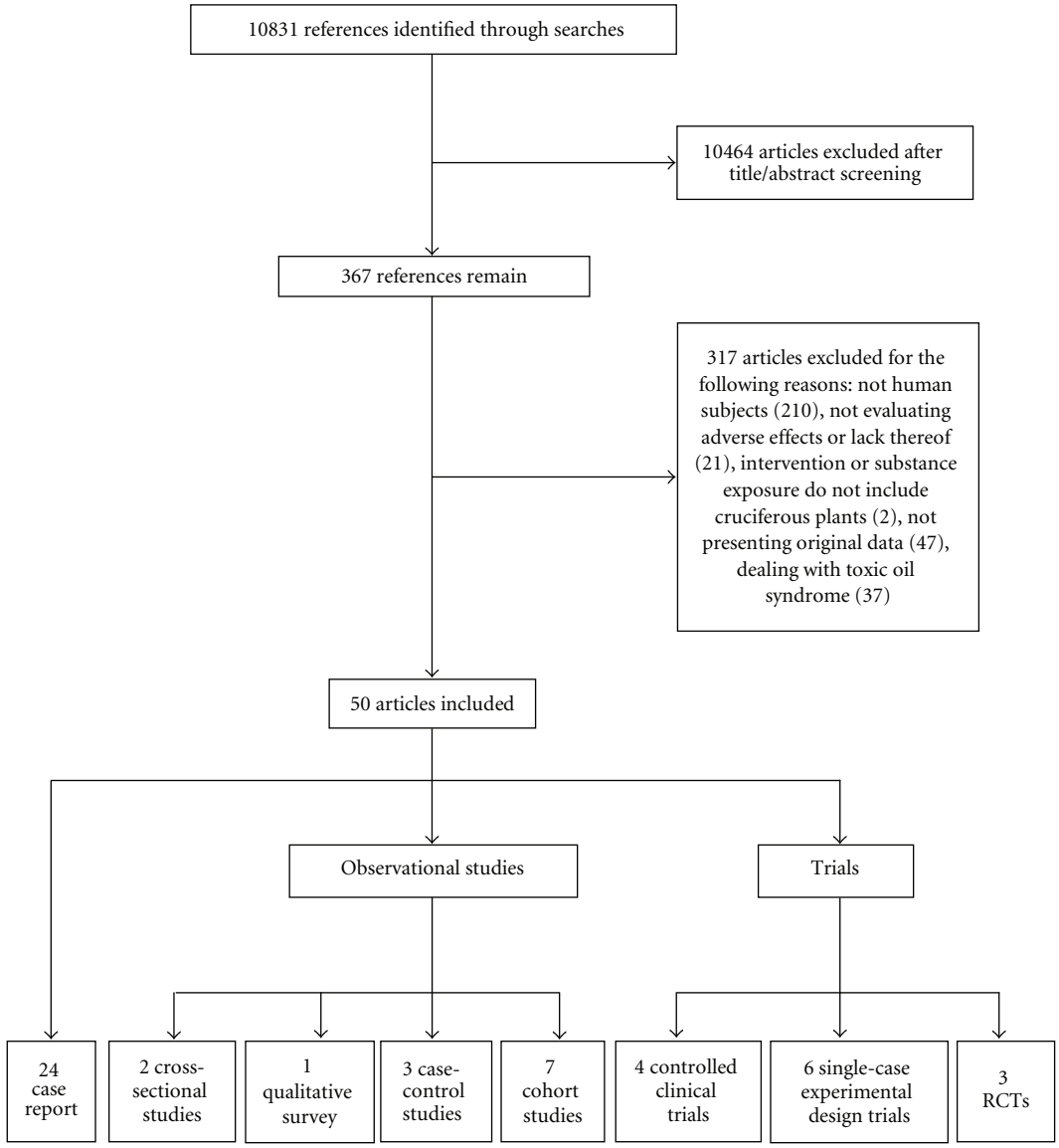

Figure 1: PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram of study identification, inclusion, and exclusion.

that the decreased levels of cysteine conjugate in urine actually indicate reduced toxicity. No changes in oxazepam metabolism were noted [21].

The third safety SCED trial included 10 healthy subjects, ages 22–40; the goal of this study was to determine the effect of a diet rich in Brussels sprouts on warfarin metabolism. Subjects consumed their customary diets for 3 days and were then instructed to consume 400 g/day of Brussels sprouts for the next 18 days. Warfarin administration (20 mg) was provided twice on days 1 and 18. Mean plasma concentrations of warfarin were measured at different time periods, as well as prothrombin activity, and clearance rates were calculated. Researchers showed that the diet had resulted in accelerated warfarin metabolism and decreased anticoagulation [22].

The last safety SCED trial included 6 subjects, ages 20–38, with a history of recurrent allergic reactions to consumption of raw cabbage. This study aimed to determine the sensitivity profile of such patients to other cruciferous vegetables (cauliflower, mustard, and broccoli), as well as to cooked versus raw cabbage. Skin prick tests were performed for a variety of allergens; later, food challenges were performed with cooked cabbage, and levels of cabbage-specific IgE were measured in patients' sera. All patients were found to be atopic (5 with allergic rhinitis); all had positive skin prick tests to mugwort (*Artemisia vulgaris*) pollen and other aeroallergens (for instance, pollen of plants from the genera *Parietaria* and *Olea*), and 5 had positive skin prick tests to hazelnuts, walnuts, and peanuts. Mustard sensitivity (cross-reactivity with cabbage) was detected in all patients. Patients demonstrated no allergic reaction to cooked cabbage. All patients were found to have cabbage-specific IgE antibodies in their sera [23].

Table 2: Trials of cruciferous plants, their derivatives, or their constituents in humans.

Study details			Patient details			Intervention				Outcome		
Reference	Study design[1]	Setting	No. of patients/ comparators[2]	Age range	Concurrent conditions/ treatments	Plant/ substance	Route[3]	Duration[4]	Dose	Adverse events	Outcome[5]	Causality[6]
Safety trials Shapiro et al. 2006 [18]	RCT	Hospital	9/3	28–57 yrs	Healthy	Broccoli sprout extract	Oral	7 days	3 doses a day, 25 μmol glucosinolate 100 μmol glucosinolate, or 25 μmol isothiocyanate	No adverse events	NA	NA
Figueroa et al. 2005 [19]	CCT	Hospital	38/38	3–39 yrs	Reported mustard allergy. Atopy and allergies to a variety of other allergens	Mustard and a variety of other allergens	Oral	Acute exposure	Oral: increasing doses of mustard (80–6480 mg)	14 showed positive mustard challenge (12 had oral allergy syndrome, 1 had more severe angioedema with bronchial asthma, and 1 had anaphylaxis)	Resolved after symptomatic treatment	Possibly
Pantuck et al. 1979 [20]	SCED	Multisite hospital and homes	10	21–32 yrs	Healthy	Cabbage and Brussels sprouts	Oral	7 days	200 g/d cabbage and 300 g/d Brusselss sprouts	All showed accelerated phenacetin metabolism	Resolved after diet was discontinued	Likely
Pantuck et al. 1984 [21]	SCED	Multisite hospital and homes	10	23–35 yrs	Healthy	Cabbage and Brusselss sprouts	Oral	10 days	200 g/d cabbage and 300 g/d Brusselss sprouts	All showed accelerated acetaminophen metabolism, enhanced glucuronide conjugation	Resolved after diet was discontinued	Likely
Ovesen et al. 1988 [22]	SCED	Multi-site hospital and homes	10	22–40 yrs	Healthy	Brusselss sprouts, lightly steamed	Oral	2 weeks	400 g/d	All showed accelerated warfarin metabolism	Resolved after diet was discontinued	Likely

Table 2: Continued.

Study details			Patient details		Intervention				Outcome		
Reference	Study design[1]	Setting	No. of patients/ comparators[2]	Age range	Concurrent conditions/ treatments	Plant/ substance	Route[3]	Duration[4]	Dose	Adverse events	Outcome[5]	Causality[6]
Vovolis et al. 2009 [23]	SCED	Hospital	6	20–38 yrs	Recurrent allergic reactions after consuming cabbage. Atopy	Cabbage (raw versus cooked) and a variety of other allergens	Oral and topical allergy tests	Acute exposure	NA	All showed positive skin prick tests and IgE tests to raw cabbage; positive skin prick tests to a variety of allergens	NA	Possibly
Nonsafety trials Rosen et al. 1998 [24]	CCT	Hospital	18	2.5–61 yrs	Recurrent respiratory papillomatosis	Indole-3-carbinol supplement	Oral	9–24 months	200 mg twice a day for adults; for kids based on weight	Imbalance and tremor in one adult who was given twice the original dose by the researchers and unsteadiness with nausea in two pediatric patient at who by mistake took higher doses	Full resolution: in adult after returning to original dose. In pediatric cases spontaneously	Possibly
Kensler et al. 2005 [25]	RCT	Community	100/100	25–65 yrs	Healthy	Broccoli sprout infusion	Oral	12 days	Not specified	No adverse events	NA	NA
Singh et al. 1997 [26]	RCT	Multisite hospital and homes	120/118	Mean: 48 yrs	Acute myocardial infarction	Mustard oil	Oral	1 year	20 g/d	No adverse events	NA	NA
Jood et al. 2001 [27]	CCT	Community	33/33	10–12 yrs	Nutritional deficits: low serum hemoglobin and retinol	Cauliflower leaves powder, in biscuits, or shakarpara	Oral	4 months	Not specified	No adverse events	NA	NA
Rosen and Bryson 2004 [28]	CCT	Multi-site hospital and homes	33	5–71 yrs	Recurrent respiratory papillomatosis	Indole-3-carbinol supplement	Oral	10–86 months	200 mg twice a day for adults; pediatric dosage was determined by weight	No adverse events	NA	NA

Table 2: Continued.

Reference	Study details		Patient details			Intervention				Outcome		
	Study design[1]	Setting	No. of patients/ comparators[2]	Age range	Concurrent conditions/ treatments	Plant/ substance	Route[3]	Duration[4]	Dose	Adverse events	Outcome[5]	Causality[6]
Riedl et al. 2009 [29]	CCT	Single site	59/5	>18 yrs	Healthy	Broccoli sprout homogenate	Oral	3 days (once per day)	25–200 g broccoli per ingestion	No adverse events	NA	NA
Dinkova-Kostova et al. 2007 [30]	SCED	Single site	17	25–51 yrs	Healthy	Broccoli sprouts extract with daikon myrosinase, dissolved in 80% acetone and 20% water	Topical	Applied twice	Up to 40 nmol	No adverse events	NA	NA

[1] RCT, randomized controlled trial; CCT, controlled clinical trial; SCED, single-case experimental design trials.
[2] Comparator numbers appear only for controlled trials.
[3] Route of exposure.
[4] Duration of exposure.
[5] Degree of resolution in cases were adverse events were reported; NA, not applicable.
[6] The degree of association between the intervention and the adverse event, as rated by reviewers; NA, not applicable.

The nonsafety trials [24–30] included 2 RCTs, 4 controlled clinical trials, and 1 single-case experimental design trial, of which only one identified adverse events.

The trial which reported the adverse events was a controlled clinical trial in 18 subjects, ages 2.5–61, who suffered from recurrent respiratory papillomatosis and underwent complete surgical removal. All subjects received oral indole-3-carbinol supplementation for a period of 9–24 months in order to examine whether the supplements reduced the occurrence of the papillomas. Doses were 200 mg twice a day for adults, and individually calculated for pediatric patients based on body weight. Adverse events were reported in 3 patients: 1 adult, who was receiving a higher dose (400 mg twice a day) for 10 days according to the researchers' recommendation, suffered from imbalance and tremor and 2 girls, 2.5 and 12 years old, who took an overdose by mistake, suffered from unsteadiness and nausea. The symptoms in the adult resolved after returning to original dose and resolved spontaneously in the girls within a few hours to a day. According to the authors, the supplement did not cause any other side effects or complications (including acceleration of the disease) and was generally well tolerated [24]. It is unclear from this paper which other medications these specific individuals were taking, and whether they had any other acute health conditions which may have contributed to the adverse events described.

All other non-safety trials reported no adverse events with oral consumption of broccoli sprout infusion/homogenate, mustard oil, cauliflower leaves powder or indole-3-carbinol supplements, or with topical application of broccoli sprouts extract [25–30]. These studies included both adult and pediatric populations, whose concurrent medical conditions included none (healthy), nutritional deficits (haemoglobin and retinol), recurrent respiratory papillomatosis, and acute myocardial infarction (Table 2).

3.3. Observational Studies. Thirteen observational studies were included, reporting adverse events related to cruciferous plants in 1,247 patients [31–43]. Seven of them were cohort studies, 3 were case-control studies, 2 were cross-sectional studies, and 1 was a qualitative survey (Table 3).

Three cohort studies, one case-control study, and one cross-sectional survey examined prevalence of allergic reactions to oilseed rape [31–35]. In the first cohort, 7/1478 subjects who were naturally exposed to oilseed rape had either a positive skin test, a positive radio-allergosorbent test, or nasal sensitivity in response to oilseed rape exposure, whereas the proportion of such allergic reactions was 14/37 in those who were occupationally exposed to oilseed rape [31].

In the second cohort, only 147/4468 subjects with suspected oilseed allergy showed positive skin prick test to oilseed rape; most of the subjects in this study were allergic to a variety of other antigens [32].

In the third cohort, 12/22 village residents reported increased allergic symptoms during a year when oilseed rape surrounded the village, compared with a year when another crop surrounded the village. However, authors mention that the symptoms reported did not correlate with oilseed rape pollen levels measured. They were therefore not sure what the true cause could be [33].

In a case-control study, 37 people complained of seasonal allergic symptoms and bronchial reactivity in response to an unknown allergen, of whom 23 were tested. However, only 2 were found to be allergic to oilseed rape and only 10 (including those 2) were found to be atopic. The authors concluded that the symptoms could not be attributed to oilseed rape in most of these cases of seasonal allergy [34].

In the cross-sectional survey, 683/869 of village residents who were exposed to oilseed rape complained of seasonal cough, wheeze, and headaches. However, the authors concluded that the proportion of people who suffered from such symptoms was not much higher in subjects living in close proximity to oilseed rape in comparison to control subjects who do not; this suggests that the seasonal symptoms in rural areas cannot be attributed to oilseed rape allergy alone [35].

Yet another observational study dealing with allergic reactions was a cohort in 259 individuals with suspected contact allergy to foods containing allyl-isothiocyanate. Of 259 subjects who underwent allergy skin tests, 43 had a questionable reaction, of whom 15 had irritation and 3 had follicular reaction. Only two showed a true positive reaction, but one was lost to followup [36].

Three studies suggested a possible connection between cruciferous vegetables and cancer. The first was a cohort study in 64,327 women in Japan, ages 40–79, which examined the possible connection between dietary habits and risk of ovarian cancer death, based on food-frequency questionnaires. Whereas no adverse events were reported with consumption of cabbage or green leafy vegetables, a positive association was established between moderate-high consumption of Chinese cabbage and ovarian cancer. The authors suggest that this might be attributed to the fact, that, in many cases, Chinese cabbage is eaten pickled, as pickled food was proven to increase the risk of cancer [37]. The researchers did not adjust for total energy consumption, or for comalignancies such as breast/endometrial cancer; the first is important as several studies found an association between total energy consumption and increased risk for various types of cancer [38, 39]. The latter is crucial, as many women with a history of such comalignancies are at a higher risk of developing ovarian cancer than the general population [40, 41].

The second suggested a connection between cruciferous vegetables and cancer in a case-control study from Kuwait in which 313 thyroid cancer patients were paired with age- and gender-matched controls; the study's aim was to examine the relationship between different sociodemographic, medical or dietary factors and thyroid cancer, based on questionnaires. Whereas no adverse events were associated with broccoli consumption, a nonstatistically significant positive association was established between moderate-high consumption of cauliflower or cabbage and thyroid cancer ($P = 0.08$ and $P = 0.16$, resp.). The authors conclude that no clear association between consumption of cruciferous vegetables and thyroid cancer could be established [42].

The last study bringing up a possible association between cruciferous vegetable consumption and cancer is a case-control study which paired 246 thyroid cancer patients from

Table 3: Observational studies of adverse events associated with human exposure to cruciferous plants, their derivatives or their constituents.

	Study details			Patient details		Intervention				Outcome		
Reference	Study design[1]	Setting	No. of patients/ comparators[2]	Age range	Concurrent conditions/ treatments	Plant/ substance	Route[3]	Duration[4]	Dose[5]	Adverse events[6]	Outcome[7]	Causality[8]
Fell et al. 1992 [31]	Cohort	Multi-site	1515 (1478: natural exposure, 37: occupational exposure)	Adults	Atopy in some subjects	Oilseed rape	Respiratory and topical allergy tests	Acute to chronic	NA	In naturally exposed, 4/1478 showed positive skin test and 3/1478 showed positive RAST and nasal sensitivity. In occupationally exposed, 9/37 showed positive skin test and 5/37 showed positive RAST and nasal sensitivity	Not specified	Possibly
Hemmer et al. 1997 [32]	Cohort	Single site	4468	Not given	Suspected inhalant allergy to oilseed rape. Multiple allergies to other pollen allergens	Oilseed rape	Respiratory	1 year	NA	147 showed positive skin prick test to oilseed rape	NA	Possibly
Parrat et al. 1995 [33]	Cohort	Community	22	Adults	Not given	Oilseed rape	Respiratory	Seasonal	NA	Allergy (sneezing, coughing, eye irritation) in 10	Improvement when not exposed	Possibly
Lerbaek et al. 2004 [36]	Cohort	Hospital	259	Not given	Suspected contact allergy to foods containing allyl isothiocyanate	Allyl isothiocyanate 0.1% in petrolatum	Topical allergy test	Acute	NA	In 43patients: ?+ reaction, of whom 15 had irritation and 3 had follicular reaction. Two showed a true + reaction but one lost to follow up	Spontaneous resolution	Possibly

Table 3: Continued.

Reference	Study details			Patient details		Intervention				Outcome		
	Study design[1]	Setting	No. of patients/ comparators[2]	Age range	Concurrent conditions/ treatments	Plant/ substance	Route[3]	Duration[4]	Dose[5]	Adverse events[6]	Outcome[7]	Causality[8]
Sakauchi et al. 2007 [37]	Cohort	Community	64327 women	40–79 yrs	Not given	Cabbage, Chinese cabbage, green leafy vegetables, and other foods	Oral	Chronic (exact time frame given)	Varies: 0 times a week to almost every day	No adverse events with consumption of cabbage or green leafy vegetables. Of 100 women who reported moderate-high consumption of Chinese cabbage, 46 had ovarian cancer	Death	Possibly
Michnovicz and Bradlow 1997 [44]	Cohort	Community	12	22–48 yrs	No history of recent or chronic illness, drug use, or recent changes in weight	Indole-3-carbinol	Oral	7 days	5–7 mg/kg/day	No adverse events	NA	NA
Michaud et al. 2002 [45]	Cohort	Community	27111	50–69 yrs	Male smokers with no history of cancer, not using vitamins A, E or beta-carotene in excess	Cruciferous vegetables	Oral	chronic (reported intake in last 12 months)	NA	No adverse events	NA	NA
Soutar et al. 1995 [34]	CCS	Hospital	37/24	17–54 yrs	Seasonal allergic symptoms and bronchial reactivity	Oilseed rape	Respiratory	Seasonal	NA	Of the 23 cases tested, only 2 were found to be truly allergic to oilseed rape, and only 10 (including these 2) were atopic	Not mentioned	Possibly (for 2 who are allergic) Unlikely (for the rest)

Table 3: Continued.

Reference	Study details			Patient details		Intervention				Outcome		
	Study design[1]	Setting	No. of patients/ comparators[2]	Age range	Concurrent conditions/ treatments	Plant/ substance	Route[3]	Duration[4]	Dose[5]	Adverse events[6]	Outcome[7]	Causality[8]
Memon et al. 2002 [42]	CCS	Kuwait cancer control center	313/313	5–70 yrs	Cases: thyroid cancer. Either cases or controls: asthma, diabetes mellitus, gall bladder disease, hyper-tension, lupous, polyposis coli, skin allergy, skin disease (not specified)	Cabbage, cauliflower, broccoli	Oral	Chronic (exact time frame given)	Varies: 0 to 7 days a week	No adverse events with consumption of broccoli. 63/101 people with high cabbage consumption and 55/91 people with high cauliflower consumption had thyroid cancer; however, *P* trends were not statistically significant (0.08 and 0.16, resp.)	Not mentioned	Possibly
Galanti et al. 1997 [43]	CCS	Community	246/440	18–75 yrs	Not given	White and red cabbages, cauliflower, broccoli, Brusselss sprouts	Oral	Chronic (exact time frame given)	Varies: <2 to >6 times a week	56/110 people who at anytime lived in areas in Sweden where goiter and iodine deficiency were endemic until the 1960's and who reported moderate-high consumption of cruciferous vegetables had thyroid cancer	Not mentioned	Possibly
Soutar et al. 1994 [35]	CSS	Community	869/867	14–50 yrs	Both villages: 448 smokers, 325 ex-smokers	Oilseed rape	Respiratory	Chronic: months	NA	683 of 869 who were exposed had seasonal cough, wheeze, and headaches	Not mentioned	Possibly

Table 3: Continued.

Reference	Study details			Patient details		Intervention				Outcome		
	Study design[1]	Setting	No. of patients/ comparators[2]	Age range	Concurrent conditions/ treatments	Plant/ substance	Route[3]	Duration[4]	Dose[5]	Adverse events[6]	Outcome[7]	Causality[8]
Sato et al. 2004 [46]	CSS	Community	438	39–60 yrs	No history of gastric cancer or gastric ulcer	Broccoli	Oral	Chronic (exact time frame given)	Varies: never to few times a week	46/186 people who consumed broccoli once or more a week had changes in enzymes which might indicate chronic atrophic gastritis	Ongoing	Possibly
Lust et al. 1996 [47]	QS	Community	273	<4 mo	Not given	Various foods, including cruciferous vegetables (specifically cabbage, cauliflower, broccoli)	Oral (through breast milk)	Unclear (mothers were asked whether they ate different items the previous week)	Not given	63/273 exhibited colic symptoms (abdominal pain, irritability, intense crying)	Not mentioned	Possibly

[1] CCS, case-control study; CSS, cross-sectional study; QS, qualitative survey.
[2] Comparator numbers appear only for case-control and cross-sectional surveys.
[3] Route of exposure.
[4] Duration of exposure.
[5] NA, not applicable, in cases where dose cannot be quantified.
[6] RAST, radioallergosorbent test.
[7] Degree of resolution in cases were adverse events were reported; NA, not applicable.
[8] The degree of association between the intervention and the adverse event, as rated by reviewers; NA, not applicable.

Sweden and Norway with 440 age- and gender-matched controls. The goal of the study was to evaluate the relationship between certain dietary habits and the risk of follicular and papillary thyroid carcinomas. With regard to cruciferous vegetables, researchers found that for people who, at any time, resided in areas of Sweden where goiter and iodine deficiency were endemic until the 1960's, there was an increased risk for thyroid cancer with consumption of cruciferous vegetables. These findings were not reproduced anywhere else in Sweden or Norway. Authors suggest that this dual effect might be related to interactions between cruciferous vegetables and other food components such as iodine [43]. They also expressed surprise, as their findings stood in contradiction to those of many research groups that found a protective effect of cruciferous vegetables against thyroid cancer [48–51].

The next observational study included in our paper was a cross-sectional study which aimed to determine the association between broccoli consumption and chronic atrophic gastritis in 438 men, ages 39–60 years. The authors found that consumption of broccoli once or more weekly increased the risk for chronic atrophic gastritis, based on serological tests for pepsinogen I and II. However, the authors acknowledge some major drawbacks to their work. First, *H. Pylori* infection, which increases the risk for chronic atrophic gastritis, was not measured. Second, serologic criteria were used for diagnosis, meaning that the results were prone to measurement errors. Moreover, it is unclear whether the changes in enzyme profiles were accompanied by any clinical manifestations [46].

The last observational study reporting adverse events was a qualitative survey which examined the relationship between consumption of certain foods by breast-feeding mothers, and the appearance of colic symptoms in their babies (4 months old or younger). The survey conductors found a positive association between consumption of cruciferous vegetables (cabbage, cauliflower, and broccoli) and colic symptoms: abdominal pain, irritability, and intense crying [47]. This study exhibited several major limitations: first, all determinants of colic symptoms, as defined by the authors (intense crying, irritability, and abdominal pain) can be subjected to subjective interpretation; the diagnosis of infantile colic, as established over 50 years ago, requires a healthy baby to exhibit "periods of intense, unexplained fussing/crying lasting more than 3 hours a day, more than 3 days a week for more than 3 weeks" [52]. In the study, however, no such objective temporal requirements were defined; any presentation of the symptoms, regardless of duration, was considered to constitute colic. Furthermore, since the questionnaire was administered over the course of one week only, instead of the 3 weeks which are required for diagnosis of colic, it is doubtful whether such diagnosis can be made at all. Moreover, since food intake and colic symptoms were reported only for a week, it is hard to establish proper temporal relationships between the two.

Two cohort studies included in our paper identified no adverse events with exposure to cruciferous plants or their constituents; the first study included 12 subjects who ingested 5–7 mg/kg/day of indole-3-carbinol [44]. The second study, which followed 27,111 male smokers over a period of 12 months, attempted to discover whether intakes of fruits, vegetables, carotenoids, or vitamins A, E, or C could be associated with risk of bladder cancer in these people. The study determined that no association between bladder cancer risk and chronic consumption of cruciferous vegetables could be established [45].

3.4. Case Reports. Twenty-four case reports were included in the review, reporting adverse events in 35 individuals. The patients ranged from 17 to 70 years of age, whose concurrent medical conditions included allergies, both to cruciferous plants and to a variety of other allergens, asthma, bronchitis, atopy, cardiovascular diseases, and different skin conditions (eczema, pruritus, eryhtema, dermatitis, dryness and scaling, and blisters) (Table 4). The adverse events occurred after topical, respiratory, or oral exposure to different cruciferous plants, their constituents, or their derivatives. Twenty-one case reports reported allergic or hypersensitivity reactions in 31 individuals, including allergic contact dermatitis/contact urticaria, contact hypersensitivity, aggravation of eczema, cutaneous lesions similar to pityriasis rosea, asthma, rhinoconjunctivitis, aggravation of cough and chest pain, local swelling and itching, and anaphylaxis. The suspected agents triggering these reactions were turnip seeds, cabbage, broccoli, oilseed rape (flour or pollen), cauliflower, mustard (a variety of preparation forms), isothiocyanates in paint, and *Diplotaxis erucoides* (*Brassica erucoides*) pollen. It is important to note that 18 of the individuals (approximately 50% of total) had previous risk factors such as atopy, allergies to allergens unrelated to cruciferous plants (for instance, grasses, dust mite, nuts), different skin conditions, or asthma. Furthermore, 12 of those people had been exposed to the allergens occupationally, for chronic periods of time, presumably leading to the adverse events [53–73].

Two case reports documented warfarin resistance in 3 patients with cardiovascular diseases. The first patient had a prosthetic aortic valve and a history of myocardial infarctions with prolonged prothrombin time [74]. The second had pulmonary embolism, and the third suffered from an unspecified cardiovascular disease [75]. The adverse event in the first patient was attributed to excessive consumption of lettuce and greens (turnip, mustard greens, broccoli); the patient was a 35 years old woman who intended to lose weight by consuming only the above-mentioned foods, which led to a consumption of 6000 μg/day of vitamin K (60 times higher than the recommended consumption). After 5 weeks of dieting, she felt substernal chest pain and was referred to the hospital, where a myocardial infarction was diagnosed. She was treated with heparin and nitroglycerine. The second and third cases were attributed to chronic consumption of up to 450 g/day of broccoli; one of them required treatment with Coumadin. All three cases ended up in full resolution of adverse events and a recommendation to restrict consumption of vitamin K-rich foods [74, 75].

The last case report reported a suspected toxic irritative dermatitis (nonallergic) in an individual who applied a home-made mustard wrap in order to relieve the symptoms of bronchitis; authors believe the wrap may have contained some toxic compounds and concluded that the reaction was

Table 4: Case reports of adverse events associated with human exposure to cruciferous plants, their derivatives or their constituents.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Blaiss et al. 1987 [53]	21	In the past allergic rhinitis to numerous inhalant allergens: grasses, milds, ragweed, and dust mite. Immunotherapy to grass and dust allergens stopped a year prior to reported events	Cabbage (in coleslaw)	Oral	2 acute ingestions	Not specified	Anaphylaxis: pain and swelling in mouth and throat with difficult breathing. Swelling of lip, tongue, soft palate. No diffuse urticaria, wheezing or hypotension	Subcutaneous epinephrine, discharged on oral antihistamines and steroids	Full resolution	Certainly	Skin test also revealed allergy to mustard, cauliflower, and broccoli. Reexposure to coleslaw via oral ingestion after 2 weeks triggered same symptoms
Brito et al. 2001 [54]	29 and 37	Not given	Diplotaxis erucoides (wall rocket) pollen	Respiratory	2 months every year	NA	Patient 1: rhinoconjunctivitis Patient 2: rhinoconjunctivitis and asthma. Patients were tested and found to be allergic to *Diplotaxis erucoides* pollen		Not mentioned	Likely	Occupational exposure
Compes et al. 2007 [55]	38	Ex-smoker, with a personal history of house dust mite allergic rhinitis and asthma, and a family history of atopy	Turnip seeds	Topical and respiratory	Chronic (exact duration not mentioned)	NA	Rhinitis and asthma. Patient was tested and found to be allergic to turnip seeds	Not mentioned	Not mentioned	Possibly	Bird fancier. Patient was also found to be allergic to avian antigens

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Caldan 1981 [56]	28	Eczema and hay fever. In the past contact with other vegetables caused itching	Cabbage	Topical	Not reported (unclear whether chronic eczema is related to cabbage exposure)	NA	Contact urticaria. Patch test revealed allergy to "green leaves" (cabbage and Brusselss sprouts)	Not mentioned	Not mentioned	Possibly	Paper lacks many details regarding both urticaria and eczema
Chakrabarti et al. 2003 [57]	56	3-year history of severe eczema and recurrent blisters on palms	Broccoli	Topical	Chronic (exact duration not mentioned)	NA	Allergic contact dermatitis (worsening of eczema)	Subcutaneous epinephrine, discharged on oral antihistamines and steroids	Partial resolution: eczema improved upon avoidance of topical contact with broccoli and other vegetables	Possibly	Patient refused patch tests to other vegetables, but it is possible that she is allergic not only to broccoli
Dannaker et al. 1987 [58]	38	2 year history of hand dermatitis; 6 months history of dryness and scaling at angles of mouth (Unclear whether her hand dermatitis is related to mustard exposure)	Mustard in salad dressings	Topical	Chronic, for the past 3 years	NA	Acute episode of allergic contact dermatitis	Not mentioned	Not mentioned	Possibly	Occupational exposure

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Di Giacomo et al. 1998 [59]	48	Not given	Oilseed rape flour	Respiratory	Chronic (exact duration not mentioned)	NA	Episodes of asthma and rhinoconjunctivitis Allergy tests showed that skin prick tests were only mildly positive for oilseed rape flour, and the peak respiratory flow only slightly decreased during exposure	Not mentioned	Not mentioned	Possibly	Occupational exposure
Hernandez et al. 2005 [60]	70	Allergies to some fruit and nuts, seasonal allergic rhinoconjunctivitis	Cauliflower	Oral	Acute ingestion	Not specified	Oropharyngeal itching, facial and hand swelling, severe bronchospasm	Required emergency room consult	Full resolution	Possibly	No details of what other components the dish included

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Jorro et al. 1995 [61]	43, 17, and 19	Patient 1: history of IgE-dependent rhinitis since childhood; urticaria angioedema episodes related with shellfish. Patient 2: history of IgE-dependent asthma and rhinitis and urticaria to latex. Patient 3: history of IgE-dependent rhinitis	Mustard sauce	Oral	Acute ingestions	Not specified	Patient 1: episodes of pruritus, swelling of tongue, dysphagia, dysphonia, facial edema, and progressive upper respiratory difficulty. On one occasion, he experienced hypotension, and on another urticaria and palpebral edema. Patient 2: episodes of pruritus, swelling of lips and tongue, and edema. Patient 3: dysphonia, dysphagia, progressive upper respiratory difficulty, and generalized urticaria. Upon allergy testing, patients were found to be allergic to mustard	Not mentioned	Full resolution	Likely	None
Lingelbach et al. 2003 [62]	40	For the past 11 years: episodes of exercise-induced anaphylaxis after eating, once or twice a year	Cabbage and mustard	Oral	Acute ingestions	NA	Anaphylaxis induced by exercise after ingestion of cabbage or mustard	IV adrenaline, antihistamine, and corticosteroids	Full resolution	Likely	Patient was also found to be allergic to other foods

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Meding 1985 [63]	40	Vesicular hand eczema for the past 9 years, sometimes worsened for no clear reason, and also after every mustard ingestion	Mustard and rapeseed	Oral (however, allergy testing was topical)	Acute ingestions	Not specified	Vesicular episodes (worsening of eczema). Positive skin prick test to crushed seeds of rapeseed	Not mentioned	Partial resolution: eczema improved upon avoidance of mustard and rapeseed	Possibly	Apart from ingestion, patient was also occupationally exposed to rapeseed

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Pasricha et al. 1985 [64]	35 and 47	Patient 1 had suffered from dermatitis ever since she was 15, which worsened when handling cattle food. Patient 2: itching and erythematous papules for the past 1.5 years, for which she was treated	Patient 1: mustard; Patient 2: mustard khal	Topical	Chronic (exact duration not mentioned)	NA	Patient 1: itching and erythmatous papular lesions on forearms, arms, neck, forehead, ear lobules, and sides of face for the past 8 months. Patient 2: itching and erythmatous papules on forearms, forehead, cheeks, ear lobules, neck, and dorsum of feet for the past 1.5 years. Upon allergy testing, patient 1 found to be allergic to mustard, jowar flour, and wheat flour. Patient 2 found to be allergic to mustard khal and maize	Not mentioned (it is mentioned that treatment was given, but no further details are provided)	Improved with treatment, but authors do not mention what happens when not exposed	Possibly (patient 1), unlikely (patient 2)	Patient 1 had been using mustard oil for years and only lately did she start experiencing adverse event. Patient 2 did not report any exposure to mustard
Quirce et al. 2005 [65]	41	Allergic rhinoconjunctivitis to pollen. For past 7 years: episodes of ocular and nasal itching, sneezing, watery nose, tearing, dry cough, chest tightness, and dyspnea after inhaling cauliflower or cabbage vapors	Cabbage	Oral	Acute ingestion	Not specified	Generalized urticaria, facial and oropharyngeal angioedema. Upon allergy testing (with cabbage or cauliflower): severe rhinoconjunctivitis and an early asthmatic reaction	Required emergency room consult	Full resolution	Possibly	Occupational exposure

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Rosenberg and Gervais 1986 [66]	42 and 34	Patient 1: asthma triggered by isocyanate paint. Patient 2: ex-smoker, asthma triggered by isocyanate paint	Patient 1: mustard and horseradish. Patient 2: mustard, radish, turnip	Oral	Acute ingestions	Not specified	Patient 1: face flushing, asthma attack. Patient 2: asthma attack	Patient 1: not mentioned; Patient 2: hospitalized, treated w/theophylline	Full resolution. In patient 2: recurrence when eating mustard/radish	Likely	Occupational exposure. No testing done for food allergies in either patients
Sanchez-Guerrero and Escudero 1998 [67]	36 and 54	Patient 1: for past 7 years: pruritus, erythema, vesicles, fissures, and peeling in both hands, as well as facial angioedema, within 6–8 h after handling broccoli. Patient 2: asthma; in past 4 years, papules and vesicles in both hands and eyelids 24–36 hours after exposure to cauliflower or broccoli	Broccoli or cauliflower	Topical	Chronic (exact duration not mentioned)	NA	Patient 1: acute episodes of contact dermatitis after handling broccoli; Patient 2: acute episodes of contact dermatitis after handling broccoli or cauliflower	Not mentioned	Patient 1: full resolution after avoiding broccoli. Patient 2: no resolution even when avoids allergens	Possibly	Occupational exposure
Schulze and Wollina 2003 [68]	19	Not given	Mustard (sauce and oil)	Oral and topical	varies (acute to chronic)	Not specified	At 4 years of age-topical exposure caused eye lacrimation and cauterization, and dyspnea. Upon ingestion at age of 19: angioedema and bronchospasm	Topical and oral antihistamines	Not mentioned	Possibly	Some mustard allergy tests were positive but rubbing test was negative. Cross-reactivity with other Brassica

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Suh et al. 1998 [69]	43	Smoker; cough and chest pain	Oilseed rape dust	Respiratory	Chronic	NA	Aggravation of cough and chest pain	Not mentioned	Not mentioned	Possibly	Occupational exposure; allergy testing not performed
Valero et al. 1995 [70]	34, 31, 25, 52, and 33	Patient 1: house dust allergy. Patient 2: seasonal rhinitis. Patient 3: peach allergy; seasonal rhinitis; familial atopy. Patient 4: seasonal rhinoconjuctivitis and bronchial asthma, family pollen allergy.	Mustard sauce or mustard pollen	Oral and respiratory	Acute (ingestion) to seasonal (pollen inhalation)	Not specified	Patient 1–3: urticaria, facial edema, rhinoconjunctivitis/rhinitis. Patient 4: facial edema, bronchospasm, rhinitis. Patient 5: urticaria, facial edema, and bronchospasm. All found to be allergic to mustard	Not mentioned	Full resolution	Possibly	None

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
van Ketel et al. 1975 [71]	43	Eczema in past 10 years	Cauliflower	Topical	Chronic (exact duration not mentioned)	NA	Aggravation of eczema. Upon allergy testing was found allergic to both cauliflower and other Brassica: Brusselss sprouts and red cabbage	Not mentioned	Not mentioned	Possibly	Occupational exposure. Aggravation of eczema also occurs after contact with onions, tulip bulbs, rubber gloves, and pesticides, but to a lesser extent
Widstrom and Johansson 1986 [72]	25	As a child, severe atopic dermatitis, rhinitis and swelling of throat in reaction to fish or egg. Currently, rhinitis when exposed to cats or dogs	Mustard (in mustard sauce or mayonnaise)	Oral	Acute ingestions	Not specified	Acute episodes of urticaria and angioneurotic edema of face and neck. Upon allergy testing was found to be allergic to mustard	Not mentioned	Full resolution	Possibly	None
Zawar 2005 [73]	25	Healthy	Mustard oil	Topical	Several acute applications	NA	Cutaneous lesions similar to pityriasis rosea (appeared after first application)	Corticosteroids and antihistamines	Full resolution followed by recurrence on re-exposure	Certainly	None
Walker 1984 [74]	35	Prosthetic aortic valve, history of myocardial infarctions with prolonged prothrombin time. Treated with warfarin and dipyridamole	Lettuce and greens (e.g., turnip, mustard greens, broccoli)	Oral	5 weeks	Not specified. Authors do mention, however, that vitamin K intake was 6000 μg per day	Diet-induced warfarin resistance which led to substernal chest pain and myocardial infarction	Referred to hospital, where she was treated with nitroglycerin and heparin	Full resolution	Likely	Vitamin K consumption was 60 times more than the norm (360 μg)

Table 4: Continued.

Reference	Patient details		Intervention				Outcome				
	Age	Concurrent conditions/ treatments	Plant/ substance	Route[1]	Duration[2]	Dose[3]	Adverse events	Acute management	Outcome[4]	Causality[5]	Comments
Kempin 1983 [75]	Not given	Patient 1: pulmonary embolism; Patient 2: cardiovascular disease (not specified which). Treated with heparin and Coumadin (=warfarin)	Broccoli	Oral	Chronic (exact duration not mentioned)	Up to 450 g/day	Warfarin resistance	Coumadin anticoagulation in patient 2	Full resolution	Likely	None
Geier 1991 [76]	45	Bronchitis	Home-made mustard wrap, containing ground mustard seeds and water	Topical	Acute application (20 minutes)	Not specified	Toxic irritative dermatitis	Topical corticosteroids	Full resolution	Possibly	Authors labeled case as toxic irritative dermatitis and NOT an allergic reaction and warn against use of home-made medications

[1] Route of exposure.
[2] Duration of exposure.
[3] NA, not applicable, in cases where dose cannot be quantified.
[4] Degree of resolution in cases here adverse events were reported; NA, not applicable.
[5] The degree of association between the intervention and the adverse event, as rated by reviewers; NA, not applicable.

not an allergic one. Hence, they advised against the use of home-made wraps. The patient was treated with corticosteroids with full resolution. This case was very brief and lacked a great deal of information regarding previous medical history and whether other medications had been used to treat the patient's bronchitis. Moreover, no test was conducted to verify the cause of the adverse event [76].

3.5. Excluded Papers. Of the 317 papers excluded, 280 did not fulfill the criteria for our systematic review (i.e., they did not evaluate adverse effects or lack thereof, intervention or substance exposure did not include cruciferous plants, or did not present original data). Another 37 articles were excluded since they dealt with toxic oil syndrome. In order to promote transparency in our decision-making process, we shall explain the rationale behind excluding this latter group of papers.

Toxic oil syndrome is the name given to a disease outbreak in Spain in 1981. Its first appearance was as an acute lung disease, which was followed by a range of other chronic symptoms affecting the lungs, the liver, the kidneys, the skin, the joints, the central nervous system, and the immune system [77–83]. The cause for the disease was traced to the consumption of cheap, refined, rapeseed oil that had been intended for industrial use rather than for human consumption. It was sold as "olive oil" and was therefore used for cooking. According to the World Health Organization, toxic compounds derived during the refinement process, used to remove the aniline and to denature oils intended for industrial use, were responsible for causing the disease. Hence, toxic oil syndrome has come to be considered a chemical incident, rather than a side effect associated with consumption of rapeseed oil, since consumption of non-industrial rapeseed oil does not cause the disease. In fact, the specific refinement process of the oil that caused the disease was so unique that experimental studies performed in a variety of laboratory animals have hitherto failed to reproduce the symptoms of human toxic oil syndrome [84].

4. Discussion

Research conducted in the last decade supports the notion that some cruciferous plants and their derivatives may serve to prevent or attenuate several medical conditions. To further this field of investigation, we conducted this systematic paper to determine the safety parameters surrounding the use of cruciferous plants. In this regard, our review identified adverse events in 1335 individuals, out of a total of 101,198 individuals who were included in all studies. Of these, 1292 adverse events were ranked as only possibly or unlikely to be caused by cruciferous plants. Only 43 were determined to have been certainly or likely caused by exposure to cruciferous plants. Adverse events, which could certainly or likely be attributed to members of the *Brassica* genus, included allergic reactions (including anaphylaxis), changes in metabolism of acetaminophen, phenacetin and warfarin, and warfarin resistance. Most adverse events reported in our paper were allergic or hypersensitivity reactions; however, their significance for the general healthy population is hard to infer, due to confounding medical histories or low causality ratings. Adverse events from both allergy trials and case reports were mostly in individuals who suffered from atopy, allergies to cruciferous plants and/or other allergens (for instance, grasses, ragweed, dust mite, pollen, dogs, cats and different fruit and nuts), asthma, or a variety of skin conditions such as eczema, pruritus, erythema, dermatitis, dryness and scaling, or blisters. A possible conclusion from these case reports, however, is that occupational exposure to Brassica plants may predispose to develop an allergy to these plants. With regards to observational studies which investigated allergies to cruciferous plants or their derivatives, all concluded that the cases could not be attributed to the suspected culprits.

A second category of adverse events included changes in metabolism of phenacetin, acetaminophen, and warfarin. While phenacetin has been banned for use by the FDA, acetaminophen and warfarin continue to be widely used. As such, it was important to understand if these changes in metabolism pose any risk to those who use these drugs. As for acetaminophen, the authors explain that the decreased levels of the cysteine conjugate of acetaminophen found in subjects' urine actually indicate decreased toxicity; thus, cruciferous plants may not pose any risk to individuals who consume acetaminophen. With regards to warfarin, however, the finding that cruciferous vegetables alter its metabolism might bear greater clinical relevance. It has been suggested that foods high in vitamin K, such as cruciferous vegetables, may interact with warfarin and its anticoagulant activity. In all case reports describing warfarin resistance, the patients had consumed very large amounts of cruciferous vegetables (up to 450 g/day or the equivalent of 6000 μg vitamin K/day), amounts not warranted for consumption by the general population. However, it is possible that lower doses would have some effects on anticoagulation as well. Similarly large amounts of cruciferous vegetables (400 g/day) were given to subjects in the SCED trial which examined changes in warfarin resistance. As many foods interact with warfarin, among which are mango, avocado, fish oil, soy milk, and foods high in vitamin K [84], it is imperative for physicians to discuss the consumption of such foods (including cruciferous vegetables) with their patients when starting treatment with anticoagulants.

Reports of other types of adverse events identified in our systematic review, including cancer, chronic atrophic gastritis, infantile colic, and toxicity-related events, could only be ranked as possibly or unlikely to be caused by exposure to cruciferous plants, as there was not enough evidence to establish a stronger causal relationship between the suspected culprits and the adverse events. Further research employing better methodology should be undertaken in order to establish whether positive association exists between consumption of Brassica vegetables and any of the above-mentioned adverse events.

Finally, a number of studies did not find any adverse events associated with consumption of cruciferous plants or their derivatives in subjects with a variety of health conditions, including history of myocardial infarction, nutritional deficits, or recurrent respiratory papillomatosis, or in healthy individuals.

In summary, to date, adverse events presumably related to cruciferous plants have been reported in 1335 individuals, identified through 50 studies which included a total of 101,198 individuals. However, the number of those which were certainly/likely caused by Brassica plants (including allergic reactions, changes in drug metabolism, and warfarin resistance) was much lower: only 43 of all reported adverse events received such high causality rating. For comparison, the literature shows that the pooled prevalence of adverse drug reactions ranges from 4.2 to 6% [85]. This frequency, if applied to cruciferous plants, would translate into 4250–6072 reports of adverse events out of the included 101,198 individuals, a value much higher than what was found in our current study.

When analysing our findings, one must bear in mind that the total number of individuals included in all studies comprises both those who were definitely exposed to Brassica plants and those who were presumptively exposed as the degree of exposure could not be ascertained in all cases. This might cause the frequency of occurrence of adverse events to appear lower than the actual value. On the other hand, our study also included case reports of individuals, all of whom were exposed, and reported adverse events. In this regard, the frequency of adverse events tends to be an overestimation, since individuals who are exposed, but do not experience an adverse event, are likely not to report. Hence, the inclusion of these case report, in which essentially all individuals exposed report adverse events, may serve to balance this underestimation. We advise the reader to treat the broad pooled frequency presented in the previous paragraph (1335/101,198) as merely gross estimations of the actual frequency, as conducting a meta-analysis *per se* was deemed inappropriate given the heterogeneity of study parameters (subjects, interventions, clinical outcomes, and study designs).

We conclude that cruciferous plants are generally safe for human consumption and use. However, individuals with known allergies/hypersensitivities to a certain member of the *Brassica* genus, or those taking warfarin, should consult with their physician before consuming such vegetables. In the future, if cruciferous derivatives are to be investigated as potential therapeutic agents, we recommend that adverse events be monitored. As our findings reflect positively on the safety of Brassica plants in humans and evidence from the current experimental literature of their benefit in certain disease states, we would encourage further exploration of their potential use in the clinical setting. The use of *brassica* or similar food products may have the potential to provide a safer alternative for the treatment of disease in comparison to current pharmaceutical interventions.

Conflict of Interests

The authors declare no conflict of interests.

Author Contribution

JYY initiated the research question; OS, EGC, DA, SS, SV, and JYY designed research; OS, EGC, DA, and SS conducted research; DA and SS provided essential materials; OS and EGC, analyzed data; OS wrote the paper; OS, EGC and JYY had primary responsibility for final content. SV provided guidance on study design and interpretation, including paper revisions. All authors read and approved the final paper.

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Research Article

Cytotoxicity of Selected Medicinal and Nonmedicinal Plant Extracts to Microbial and Cervical Cancer Cells

Gary M. Booth,[1] Robert D. Malmstrom,[1] Erica Kipp,[2] and Alexandra Paul[1]

[1] *Department of Plant and Wildlife Sciences, Brigham Young University, Provo, UT 84602, USA*
[2] *The New York Botanical Garden, 200th Street and Kazimiroff Boulevard, Bronx, NY 10458-5126, USA*

Correspondence should be addressed to Gary M. Booth, gary_booth@byu.edu

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This study investigated the cytotoxicity of 55 species of plants. Each plant was rated as medicinal, or nonmedicinal based on the existing literature. About 79% of the medicinal plants showed some cytotoxicity, while 75% of the nonmedicinal plants showed bioactivity. It appears that Asteraceae, Labiatae, Pinaceae, and Chenopodiaceae were particularly active against human cervical cancer cells. Based on the literature, only three of the 55 plants have been significantly investigated for cytotoxicity. It is clear that there is much toxicological work yet to be done with both medicinal and nonmedicinal plants.

1. Introduction

There is a one-in-four chance that a drug used from any pharmacy has an active ingredient derived from a plant [1]. Indeed, the international consumer market for medicinal herbs and botanicals is estimated to be at about US $18 billion [2]. Hence, in our technological age, plants continue to play a significant role both medically and economically.

Even the most ancient written records of human civilization tell of humans using plants in everyday life. For centuries plants have been used to feed, clothe, and heal families. Examples of medicine that contains plant derivatives include aspirin, used for pain relief and inflammation reduction; physostigmine and pilocarpine, used for glaucoma control; quinidine, which has saved the lives of many heart attack victims.

The principal goal of this study was to determine if extracts from selected medicinal and nonmedicinal plants were cytotoxic; often, the difference between a therapeutic and a toxic extract or compound is simply the dose level. Our hope is that these survey data can be used as early indicators of some plants that may have therapeutic activity. Moerman has done extensive screening studies on a variety of medicinal plants [3]. From his investigation, we selected 55 plants representing 37 different species from 8 families. The four principal families, Asteraceae, Labiatae, Ranunculaceae, and Pinaceae, represent the first, third, fourth, and fifth families with the most medicinal species. It was hoped that our data might show some trends of toxicity within medicinally rich families.

The toxicity of each extract was determined in both prokaryotic and eukaryotic cells. Prokaryote cells included *Staphylococcus aureus,* a gram-positive cocci responsible for infections of the skin and respiratory tract, food poisoning, and toxic shock; *Salmonella choleraesuis,* a gram-negative facultative aerobe responsible for food poisoning; *Pseudomonas aeruginosa,* a gram-negative rod that causes infections in wounds. For the eukaryotic system, HeLa cells, an epithelial carcinoma of the cervix, were used.

2. Materials and Methods

2.1. Plant Extraction

(i) 50 g of plant tissue were collected and dried at 45°C.

(ii) The plant was ground in a Wiley Model no. 4 plant mill.

(iii) The ground material was then extracted in methanol for twenty-four hours.

(iv) The samples were filtered in glass-fiber filters fitted with coarse pore discs, and rotary evaporated down to 20 mL of extract on a Buchi RE111 Rotary Evaporator.

2.2. Microbial Bioassay

(i) Twenty-four hours before the assay, each of the three bacteria were grown in a culture tube with 5 mL of tryptic soy broth without dextrose and incubated at 35°C.

(ii) (14.5 cm) Petri dishes were previously prepared with a coat of Muller Hinton Medium (agar). The cultures were checked on a spectrophotometer to ensure the proper growth (20% transmittance at 600 nm). A lawn was then spread in the petri dish. Six 1.4 cm circles of filter paper were then coated in plant extract, three with 20 μL and three with 30 μL, and placed on the plate. A disk with 20 μL of water was added to the plate for a negative control and to S. *aureus,* S. *choleraesuis,* 10 μL of Ampicillin (BBL Sensi-Disc (Becton Dickinson)) was added as a positive control. The plates were incubated overnight at 35°C.

(iii) The plates were then collected the next day and the zones of inhibition were measured.

2.3. HeLa Assay

(i) HeLa cells were maintained and assayed in MEM with α modification (Sigma M-0894) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1x MEM-nonessential amino acids (Sigma M-7145), 2 mM L-glutamine, and gentamicin at 50 μg/mL.

(ii) Each extract was dried down and 2 mg/mL solutions were made using 10 mM Tris buffer at pH 7.4.

(iii) 150 μL of a solution of suspended HeLa cells diluted with 15 mL of α-MEM is added to each well of a 96 well plate and incubated overnight at 37°C and 5% CO_2.

(iv) The next day 75 μL, 50 μL, 25 μL with 25 μL of α-MEM, 12.5 μL with 37.5 μL of α-MEM, or 6 μL with 44 μL of α-MEM of the 2 mg/mL extracts was added to 9 wells as a control. The prepared plate was incubated overnight.

(v) The cells were arrested the next morning with 0.4 N perchloric acid. The perchloric acid is removed, and the cells were stained in 4% sulforhodamine B in 1% acetic acid and then washed in 1% acetic acid. The dye was allowed to dry and 150 μL of 10 mM Tris base unbuffered was then added to each well, and the absorbance of each well was read using a spectrophotometer at 570 nm.

(vi) The percent viability was calculated as the ratio of absorbance of the treated sample over the average of the controls. These values were then plotted and analyzed for a dose response.

3. Results

3.1. Microbial Assay. Of the 55 plants tested, only four, *Pinus monticola, Abies procera, Salvia vaseyi,* and *Salvia apiana*, inhibited the growth of S. *aureus.* The remaining microorganisms were unaffected by the extracts. However, the zones of inhibition were quite small, only about 1 cm each. The assay is rather a crude test when compared with the HeLa cell assay. This is understandable because the zone of inhibition is directly proportional to the concentration of the biologically active agent and its diffusibility, so the possibility of active compounds not showing a positive response could be expected if the active ingredients did not diffuse. Due to the screening nature of this procedure and small sample size, the quantitative analysis of the size of the rings of inhibition was quite subjective.

3.2. HeLa Cell Assay. The LC-50 s were calculated for each of the samples. Some of the extracts were so toxic to the HeLa cells that very low doses of 0.0l and 0.001 mg/mL were studied in order to establish an LC-50. The LC-50 s were calculated from least squares regression using the LINEST function on Microsoft Excel 2000 over the dose response range or the whole data set in the case of nontoxic extracts to get a rough quantitative value in order to assess cytotoxicity. Tris buffer, the control, gave an average 92% viability with no dose response. All values were adjusted up by 8% accordingly.

We experienced four general trends in the data. The first two types we labeled "A" for active. The first type was a clear dose-response over the full range of concentrations. Type two followed a steep dose-response over the initial range of concentrations while the lower concentrations did not. Type two was the most cytotoxic. Type three was labeled with an "M" for mildly active. These showed a weaker dose-response only at the higher concentrations. Type four was labeled "N" for not active. These samples showed no dose response and only marginal mortality. These trends were then evaluated over medicinal and family lines (Table 1).

4. Discussion and Conclusions

Of the 46 medicinal plant extracts, 54% were active, 26% were mildly active, and 20% were not active against HeLa cells. Thus, 80% of the medicinal plant extracts showed some type of cytotoxicity. This strongly suggests that there may be some connection between plants known from indigenous cultures to have medicinal properties compared to empirically determined cytotoxicity. Our eight non-medicinal plants also tended to be bioactive, with 50% active, 13% mildl, and 37% not active. Only four samples showed antibacterial activity, which was only in S. *aureus,* and all these extracts were from medicinal plants. Thus, only 14% of the medicinal plants showed limited antibiotic activity.

Asteraceae, the sunflower family and one with the highest medicinal activity rating in Moerman's paper [3], was the only family from which we had a fairly large sample, 15 medicinal plants. Extracts from Asteraceae tended to be quite active and followed the general trends of medicinal plant bioactivity as stated above with 54% active, 29% mildly active, and 17% not active. The mint family, Labiateae, also tended to be cytotoxic with 86% of the plants showing bioactivity. Because only seven plants were tested, more

Table 1: Cytotoxicity of selected plant extracts to bacterial cells and HeLa cancer cells.

Family	*Genus*	*Species*	Plant part	MD	BA	HA	LC 50 (mg/mL)	*m* (slope of the line)	*b* (*y*-intercept)	$\mathbf{r^2}$
Asteraceae	*Acanthospermum*	*australe*	Whole	Y	N	M	0.191	−38.817	57.414	0.112
Asteraceae	*Ambrosia*	*ambrosioides*	Areal	Y	N	M	−2.199	−13.656	19.970	0.469
Asteraceae	*Ambrosia*	*ambrosioides*	Leaf	Y	N	A	−2.446	−11.722	21.327	0.412
Asteraceae	*Ambrosia*	*ambrosioides*	Stem	Y	N	A	0.466	−152.941	121.230	0.857
Asteraceae	*Ambrosia*	*ambrosioides*	Root	Y	N	A	0.439	−124.376	104.617	0.817
Asteraceae	*Ambrosia*	*deltoidea*	Stem	Y	**	A	0.500	−83.064	91.544	0.293
Asteraceae	*Hieracium*	*caespitosum*	Whole	Y	N	M	0.669	−95.238	113.737	0.546
Asteraceae	*Anaphalis*	*margaritacea*	Whole	N	N	N	1.195	−51.360	111.373	0.530
Asteraceae	*Gutierrezia*	*microcephala*	Areal	Y	N	A	0.470	−143.776	117.641	0.969
Asteraceae	*Pyrrhopappus*	*carolinianus*	Whole	Y	N	N	0.117	−1052.510	173.100	0.623
Asteraceae	*Silphium*	*compositum*	S/L/Fl/R	Y	N	A	1.388	−49.271	118.400	0.134
Asteraceae	*Tetragonotheca*	*helianthoides*	Root	Y	N	A	0.436	−137.978	110.164	0.760
Asteraceae	*Tetragonotheca*	*helianthoides*	S/L/Fl/R	Y	N	M	0.357	−157.865	106.395	0.844
Asteraceae	*Erigeron*	*pumilus*	Whole	Y	N	A	0.366	−142.337	102.035	0.895
Asteraceae	*Liatris*	*secunda*	Whole	Y	N	M	0.531	−156.829	133.257	0.638
Asteraceae	*Cirsium*	*undulatum*	Areal	Y	N	M	0.884	−61.281	104.180	0.642
Asteraceae	*Thelesperma*	*filifolium*	Areal	Y	N	A	0.635	−99.503	113.172	0.950
Asteraceae	*Helianthus*	*nuttallii*	Stem	Y	N	A	0.539	−135.164	122.791	0.755
Asteraceae	*Helianthus*	*nuttallii*	Twig	Y	N	A	0.399	−181.986	122.686	0.944
Asteraceae	*Haplopappus*	*annuus*	Whole	Y	N	N	0.918	−77.726	121.359	0.803
Asteraceae	*Antennaria*	*parvifolia*	Whole	N	N	A	0.273	−81.056	72.160	0.497
Asteraceae	*Hymenopappus*	*filifolius*	Areal	Y	N	A	0.112	0.167	38.910	0.611
Asteraceae	*Centaurea*	*maculosa*	Twig/Fl	N	N	M	0.247	−113.161	77.895	0.863
Asteraceae	*Scorzonara*	*laciniata*	Root	N	N	N	1.838	−28.633	102.630	0.188
Boraginaceae	*Echium*	*candicans*	Stem	Y	N	M	0.394	−124.333	99.049	0.853
Chenopodiaceae	*Atriplex*	*confertifolia*	Areal	N	N	A	0.127	−1116.726	192.195	0.694
Chenopodiaceae	*Atriplex*	*confertifolia*	Rhizome	N	N	A	0.317	−142.791	95.259	0.838
Euphorbiaceae	*Bernardi*	*myicifolia*	Stem	N	N	A	0.488	−144.404	120.448	0.863
Labiatae	*Salvia*	*vaseyi*	Root	Y	N	A	0.105	−1109.581	166.468	0.981
Labiatae	*Salvia*	*vaseyi*	Stem	Y	N	A	0.399	−177.100	120.693	0.783
Labiatae	*Salvia*	*vaseyi*	Twig/L	Y	Y	A	0.218	−120.339	76.261	0.537
Labiatae	*Salvia*	*vaseyi*	Flowers	Y	N	A	0.231	−103.088	73.816	0.589
Labiatae	*Salvia*	*apiana*	Root	Y	Y	N	0.112	−1229.526	187.967	0.765
Labiatae	*Salvia*	*dorrii*	L/T/FloBu	Y	N	A	0.512	−125.827	114.434	0.852
Labiatae	*Lavandula*	*stoechas*	Root/Fl	Y	N	A	0.110	−1069.264	167.838	0.927
Labiatae	*Lavandula*	*stoechas*	Stem/L	Y	N	A	0.440	−196.726	136.532	0.804
Labiatae	*Lycopus*	*asper*	Stem	Y	N	A	1.043	−39.514	91.202	0.094
Labiatae	*Marrubium*	*vulgare*	Areal	Y	N	A	0.241	−112.266	77.093	0.768
Labiatae	*Satureja*	*douglasii*	Whole	Y	N	A	0.293	−102.001	79.891	0.665
Malvaceae	*Sphaeralcea*	*angustifolia*	Whole	N	N	A	0.304	−129.152	89.243	0.658
Pinaceae	*Pinus*	*monticola*	Bark/St	Y	Y	A	0.346	−175.851	110.896	0.764
Pinaceae	*Pinus*	*monticola*	Twig/L	Y	N	M	0.438	−78.452	84.370	0.396
Pinaceae	*Pinus*	*monticola*	Root	Y	N	A	0.573	−95.050	104.422	0.606
Pinaceae	*Picea*	*sitchensis*	Root	Y	N	N	0.496	−125.394	112.182	0.847
Pinaceae	*Picea*	*sitchensis*	Stem	Y	N	M	0.583	−118.928	119.326	0.562
Pinaceae	*Picea*	*sitchensis*	Bark	Y	N	M	0.366	−134.091	99.034	0.684
Pinaceae	*Picea*	*sitchensis*	Twig/L	Y	N	N	0.526	−121.778	114.038	0.835
Pinaceae	*Picea*	*sitchensis*	Cone	Y	N	A	0.438	−59.520	76.046	0.326
Pinaceae	*Abies*	*procera*	Root	N	Y	N	0.381	−155.741	109.290	0.978

Table 1: Continued.

Family	*Genus*	*Species*	Plant part	MD	BA	HA	LC 50 (mg/mL)	*m* (slope of the line)	*b* (*y*-intercept)	$\mathbf{r^2}$
Ranunculaceae	*Delphinium*	*geyeri*	Areal	Y	N	M	1.306	−29.140	88.057	0.173
Ranunculaceae	*Aquilegia*	*fromosa*	Root	Y	N	M	1.532	−25.856	89.605	0.222
Ranunculaceae	*Aquilegia*	*fromosa*	Flowers	Y	N	N	0.612	−82.675	100.574	0.534
Ranunculaceae	*Aquilegia*	*fromosa*	Leaf	Y	N	N	0.521	−143.711	124.894	0.784
Ranunculaceae	*Delphinium*	*glareosum*	Whole	Y	N	N	0.824	−91.704	125.548	0.428
Ranunculaceae	*Delphinium*	*nuttallianum*	Areal	Y	N	N	1.319	−35.840	97.287	0.350

MD: medicinal plant; Y: medicinal plant; N: non-medicinal plant
BA: bacterial assay; Y: inhibition; N: no inhibition; **sample lost
HA: HeLa cell assay; A: active; M: mildly active; N: not active
Plant part; S/St: stem Fl: flower; FloBu: flowering bush; A: areal R: root; L: leaf; T: twig.

data should be collected from this family before a general conclusion can be made about its cytotoxicity. Of the nine *Pinaceae* plant extracts, 67% showed some bioactivity. Additional work is needed to determine which plant parts tend to have the highest bioactivity. The least active of our five medicinal families was Ranunculaceae with two out of six plant extracts (33%) showing mild activity. Overall these data clearly suggest that non-medicinal as well as so-called medicinal plants should be used in general cytotoxicity screening evaluations. In fact, de Oliveira Maria et al. [4] also found significant bioactivity in 12 species of Amazonian plants which were non-medicinal.

Though this work proved to be insightful, future studies should be undertaken in order to get a clearer picture of the evolutionary relationship of bioactivity and medicinal ranking of plants. From the literature, it appears that only three plants from our group, *Ambrosia ambrosioides* [5, 6], *Gutierrezia microcephala* [7], and *Atriplex confertifolia* [8] have had extensive research on their cytotoxicity. Hence, there is a great deal of toxicology work yet to be done on the remainder of the plants shown to be bioactive in our investigation.

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Research Article

A Novel Antihepatitis Drug, Bicyclol, Prevents Liver Carcinogenesis in Diethylnitrosamine-Initiated and Phenobarbital-Promoted Mice Tumor Model

Hua Sun, Linghong Yu, Huailing Wei, and Gengtao Liu

Department of Pharmacology, Institute of Materia Medica, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing 100050, China

Correspondence should be addressed to Hua Sun, sunhua@imm.ac.cn

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Bicyclol, an antihepatitis drug developed by Chinese scientists, has been shown to prevent the malignant transformation induced by 3-methylcholanthrene and 12-*O*-tetradecanoylphorbol-13-acetate in WB-F344 rat liver epithelial cells. This study provides further evidence on its role as a chemopreventive agent in experimental mice with diethylnitrosamine- (DEN-) initiated and phenobarbital- (PB-) promoted liver carcinoma. Liver tissue and serum were collected. In the two-stage model of hepatocarcinogenesis in mice, oral administration of bicyclol (100, 200 mg/kg) before DEN injection showed significant reduction in the incidence of hepatocellular foci, nodules, or carcinoma. Histopathological examination revealed that there was no hepatocellular carcinoma (HCC) and hepatoma formation in the mice pretreated with bicyclol (200 mg/kg) at week 20, while the mice treated with DEN/PB developed 33.3% HCC and 55.6% hepatoma. Furthermore, the serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and α-fetal protein (AFP) in serum significantly increased in the DEN/PB model group in comparison with the control group. Pretreatment with bicyclol showed a marked reduction in the above condition. Bicyclol also decreased the expression of AFP and proliferating cell nuclear antigen level in the liver tissue and attenuated the decrease in body weight. In this study, we also found that 10 weeks after stopping the administration of PB and drugs, the control and bicyclol-treated (200 mg/kg) animals showed no HCC and hepatoma formation at the time of termination whereas DEN/PB-induced mice developed 100% hepatoma and 50% HCC. These results further indicate that bicyclol has the chemopreventive potential for liver carcinogenesis induced by carcinogens.

1. Introduction

Hepatocellular carcinoma (HCC) comprises 90% of the liver tumors occurring worldwide in humans, with more than half a million new cases reported every year. Diagnosis of this cancer, once restricted mostly to Asia, is now rising in Europe as well as in North America [1]. Environmental factors, such as hepatitis virus infections and chemical carcinogen exposure, are causally implicated in HCC occurrence. About 80% of human HCC cases are attributable to infection [2]. Chronic hepatitis B virus (HBV) carriers are 100–400 times more likely to develop HCC than noncarriers [3]. Hepatitis C virus (HCV) is the second most common cause of HCC after HBV [4]. Currently, HCC represents more than 4% of all cancer cases in the world and causes at least 315,000 deaths every year [5]. Although early HCC can be cured by surgical resection, many HCC cases are asymptomatic; thus, most HCC patients are not diagnosed in time.

An effective approach to cancer control is chemoprevention, which is expected to interfere with the initiation, promotion, or progression of carcinogenesis. Generally, long-term therapy is required for both chronic HBV and HCV. An antihepatitis drug can be considered to be of great clinical value if it inhibits or suppresses the development of hepatocarcinogenesis besides its improvement of abnormal liver function and inhibition of hepatitis virus replication.

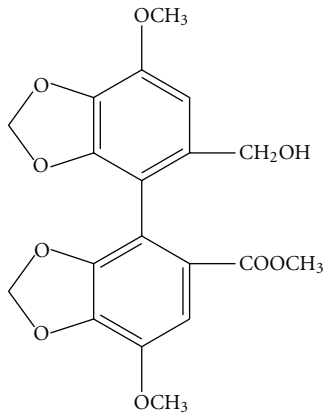

Figure 1: The chemical structure of bicyclol.

Bicyclol (4,4′-dimethoxy-2,3,2′,3′-dimethylene-dioxy-6-hydroxymethyl-6′-carbonyl-biphenyl) is a novel antihepatitis drug developed by Chinese scientists (Figure 1) [6]. Clinical trials found that bicyclol is effective in improving abnormal liver function and in inhibiting the replication of HBV in chronic hepatitis B patients [7]. Pharmacologically, bicyclol exhibits a protective action against liver injury induced by hepatotoxins in mice and rats, an antifibrotic effect on CCl_4-induced liver fibrosis in rats and mice, and an antihepatitis virus action in duck viral hepatitis and the 2.2.15 cell line [6, 8, 9]. Furthermore, bicyclol induced differentiation of human hepatocarcinoma cells ($HepG_2$ and Bel-7402 cells) and reduced AFB1 hepatotoxicity in rats by increasing the detoxifying metabolism of AFB1 in rat liver [10]. In the latest study, bicyclol showed a significant inhibitory effect on the malignant transformation of WB-F344 rat liver epithelial cells induced by 3-methylcholanthrene (3MC) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [10]. These results implicate the possibility that bicyclol has a chemopreventive effect on liver carcinogenesis.

The present study aimed to further examine the effect of bicyclol on hepatocarcinogenesis induced by the chemical carcinogens diethylnitrosamine (DEN) as the carcinogenesis initiator and phenobarbital (PB) as the carcinogenesis promoter in mice.

2. Materials and Methods

2.1. Chemicals and Drugs. Bicyclol, a white crystalline powder with 99% purity, was kindly provided by the Beijing Union Pharmaceutical Plant and was suspended in 0.5% sodium carboxymethylcellulose (Na CMC) for *in vivo* use. DEN, PB, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), aprotinin, leupeptin, *N*,*N*′-methylene-bisacrylamide, and acrylamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) determinations were purchased from Beihuakangtai Chemical Reagent Co., Ltd (Beijing, China). The ELISA kit for α-fetal protein (AFP) determinations was offered by Shanghai Shenxiong Biotech Company (Shanghai, China). All the other chemicals used were of analytical grade and were supplied by the Beijing Chemical Agents Company (Beijing, China).

2.2. Animals. Male ICR mice weighing 25–27 g (5 weeks old) were purchased from the Beijing Weitonglihua Experimental Animal Co., Ltd (Beijing, China). All mice were bred and maintained under constant conditions at a temperature of 24°C ± 1°C and humidity of 55% ± 5% with 12 h light and 12 h dark cycles. Water and feed were accessible to the mice ad libitum. Animal care and all experimental procedures were conducted in accordance with the health criteria for care of laboratory animals enacted by the Beijing municipal government.

2.3. Experimental Protocol. The experimental mice were randomly divided into 4 groups, and each group contained 15 animals housed at 5 per cage. Liver cancers were induced in all the groups with a single i.p. injection of DEN at a dose of 100 mg/kg body weight in normal saline, except in the control group. After a treatment-free interval of 1 week, the mice were kept on 0.05% PB-containing water whereas the control group was kept on PB-free water for 19 weeks. To examine the prophylactic activities of bicyclol, the animals were pretreated with bicyclol (100, 200 mg/kg) administered orally 2 days before DEN injection. After DEN injection, bicyclol was administered once daily for 6 days/week for a period of 20 weeks.

The mice were weighed periodically and their body weights were recorded. At week 20, 8–10 surviving mice of each group were sacrificed and 5 mice of each group were kept on fresh water and feed for another 10 weeks. Serum was obtained from the blood by centrifugation at 3000 rpm for 10 min. Liver specimens were removed carefully after the mice were sacrificed. Macroscopically visible liver tumors and nodules greater than approximately 1 mm in diameter on the liver surface were recorded. The right lobe of each liver and the nodules were fixed in paraformaldehyde solution for histopathological and immunohistochemical examinations. The remaining liver tissues were stored at −80°C for performing biochemical, ELISA, and Western blot assays.

2.4. Histopathology. The fixed liver samples were processed and embedded in paraffin blocks. Tissue block sections were mounted on slides, deparaffinized in xylene, dehydrated in alcohol, and sections with a thickness of 5 μm were prepared. Then, the sections were stained with hematoxylin and eosin (H&E). Liver pathological changes and the extent of liver tumors were observed and diagnosed under a microscope by a histopathologist.

2.5. Biochemical Assays of AST, ALT, and ALP. The serum levels of AST, ALT, and ALP were measured using commercial kits (Beihuakangtai Chemical Reagent Co., Ltd, Beijing, China).

2.6. Enzyme-Linked Immunosorbent Assay (ELISA) of AFP. Quantitative estimation of the tumor marker AFP in liver

and serum followed the corresponding protocols of the kits (Shanghai Shenxiong Biotech Company, Shanghai, China). Serum was obtained from blood by centrifugation at 3000 rpm for 10 min. Liver tissue was homogenized in an electron/grass homogenizer (DY89-I, Ningbo., China) using cold normal saline at 4°C. Then, the homogenates were centrifuged. Aliquots of the supernatants and the serum were used to quantify AFP. The total proteins in liver homogenates were determined by the Lowry methods using bovine serum albumin as the standard. Optical density was measured using scanning full wavelength spectrophotometer (MQX200, BIO-TEK, USA).

2.7. Western Blot Analysis of PCNA. The liver tissue was homogenized in an electron/grass homogenizer (Dounce, Ningbo, China) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA, 0.3% Triton X-100, 0.03% SDS, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM PMSF) at 4°C. After incubation on ice for 30 min, the homogenates were centrifuged at 12000 g for 20 min and the supernatants were boiled in an SDS sample loading buffer for 4 min before electrophoresis on SDS-polyacrylamide gel. After electrophoresis for 2 ~ 3 h, proteins in the SDS-PAGE gel were transferred to nitrocellulose membranes at 27 mA for 12 h at 4°C. The membranes were blocked in 5% milk-Tris buffered saline with Tween 20 (TBST) (20 mM Tris-HCl, pH 7.5, 500 Mm NaCl, 0.1% Tween 20) at 4°C overnight. The blot was probed with a mouse anti-PCNA monoclonal antibody (Santa Cruz Biotechnology Inc., USA) at a dilution of 1 : 200 in 5% milk-TBST for 2 h at room temperature and then incubated with an alkaline-phosphatase-conjugated secondary antibody in skim milk-TBST for 1.5 h at room temperature. The blot was developed using the 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP/NBT) colorimetric method, and the densities of the bands were determined using the Gel-Pro Analyzer 4.0 software.

2.8. Immunohistochemistry. The paraffin-embedded liver tissue was cut into 5 μm thick sections, deparaffinized, and incubated in 3% H_2O_2 for 10 min to quench endogenous peroxidase activity. After blocking with normal goat serum for 20 min, the sections were stained with a mouse anti-AFP monoclonal antibody (Sigma Chemical Co., USA) and mouse anti-PCNA monoclonal antibody (Santa Cruz Biotechnology Inc., USA) at 4°C overnight, followed by incubation with a horseradish-peroxidase-conjugated goat anti-mouse antibody at 37°C for 30 min. The antibody binding sites were visualized by incubation with diaminobenzidine (DAB)-H_2O_2 at room temperature for 10 min. Digital images of AFP and PCNA immunoreactive liver cells were acquired at 100x magnification on an Olympus microscope (Olympus, Tokyo, Japan) using an attached Polaroid digital microscope camera (Polaroid, Cambridge, MA, USA) and IBM PC.

2.9. Statistical Analysis. The data were presented as the mean ± SD. ANOVA was used for multiple comparisons of groups. The statistical significance between groups was assessed by the paired Student's t-test. A value of $P < 0.05$ was considered to be significant.

3. Results

The chemopreventive effect of bicyclol on DEN/PB-induced hepatocellular cancer was elucidated in male ICR mice. At week 20, DEN/PB-induced tumors or nodules could be readily observed by unaided eyes (Figure 2(a)). All macroscopically visible liver nodules greater than approximately 1 mm in diameter were counted. Oral administration of bicyclol before tumor initiation resulted in a marked inhibition of tumor development in the animals. The average number of nodules per liver in the bicyclol-treated groups was significantly lower than those in the DEN/PB model group (Figure 2(b)). In addition, the body weight of the animals decreased on DEN/PB administration when compared to the control group. Pretreatment with bicyclol prevented the decrease in body weight (Figure 3).

In the histopathological examination of liver tissue at week 20, the control group animals revealed normal architecture. DEN/PB-induced animals showed loss of architecture and presence of tumor cells. Animals pretreated with bicyclol (100, 200 mg/kg) showed fewer neoplastic cells, near-normal architecture, and significant improvement in liver histopathology (Figure 4). The tumor extent was diagnosed and evaluated by a histopathologist. As shown in Table 1, DEN initiation followed by PB promotion resulted in 33.3% hepatocellular cancer, 55.6% hepatoma, and 88.9% multiple bile canaliculi hyperplasia formations in the liver. Preadministration of bicyclol showed a significant reduction in the incidence of liver tumors. Bicyclol administered at a dose of 200 mg/kg exhibited a stronger preventive effect on liver carcinogenesis as hepatocellular cancer and hepatoma were not observed in the mice at week 20, but 50.0% low-grade multiple bile canaliculi hyperplasia formations were observed.

Table 2 shows the levels of serum marker enzymes: ALT, AST, and ALP. DEN initiation and PB promotion resulted in a marked increase in serum ALT and ALP levels as compared to those in the control group whereas no marked changes were observed in the serum AST levels. Pretreatment with 100 mg/kg bicyclol reduced serum ALT levels significantly. However, bicyclol administered at a dose of 200 mg/kg failed to considerably change the abovementioned levels of ALT, AST, and ALP.

Figure 5(b) depicts the levels of the tumor marker AFP in serum and liver by using the ELISA assay. DEN initiation followed by PB promotion resulted in a marked increase in AFP levels in liver and serum as compared with that of the control group. Pretreatment with bicyclol significantly decreased the AFP level. Bicyclol also reduced the serum AFP level, while there was no statistical significance when compared with the model group because of the large standard deviation. In the immunohistochemistry assay (Figure 5(a)), the AFP

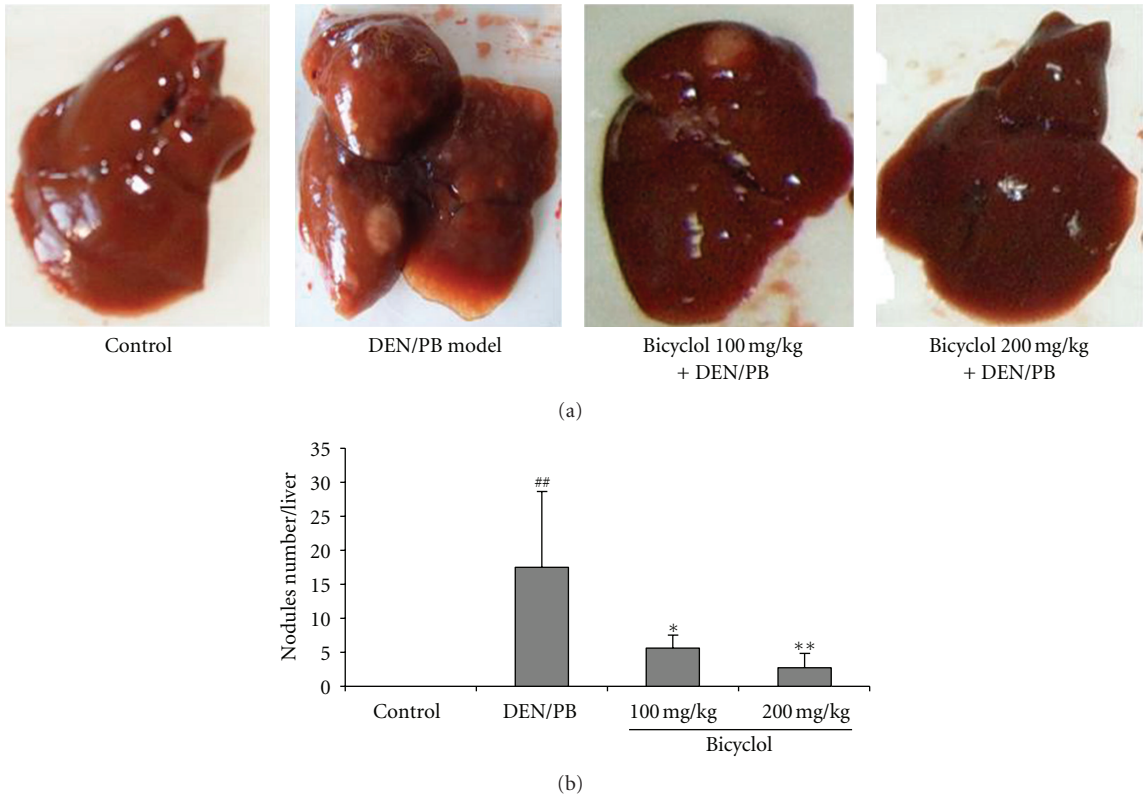

Figure 2: Gross liver tumor and nodule phenotypes in DEN/PB-induced ICR mice and the preventive effect of bicyclol at week 20. (a) Photographs of representative livers. (b) Determination of liver tumor and number of nodules per liver greater than approximately 1 mm in diameter. Average values with standard deviations are shown. $n = 8$–10. $^{\#\#}P < 0.01$ compared with the control group; $^{*}P < 0.01$, $^{**}P < 0.01$ compared with the DEN/PB model group.

Table 1: The incidence of liver tumors induced by DEN/PB in mice and the effect of bicyclol determined by H&E staining and histological evaluation.

Group	Dosage (mg/kg)	HCC	Hepatoma	Cholangioadenoma	Capillary hemangioma	MBCA
Control	—	0.0 (0/6)	0.0 (0/6)	0.0 (0/6)	0.0 (0/6)	0.0 (0/6)
DEN/PB	—	33.3 (3/9)	55.6 (5/9)	22.2(2/9)	11.1 (1/9)	88.9 (8/9)
Bicyclol	100	11.1 (1/9)	22.2 (2/9)	11.1(1/9)	0.0 (0/9)	33.3 (3/9)
	200	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	50.0 (4/8)

HCC: hepatocellular carcinoma; MBCA: multiple bile canaliculi hyperplasia.

immunoreactivity in Page: 9 DEN/PB-induced liver cells was more intense than that in the control liver cells. Pretreatment with bicyclol markedly decreased the expression of AFP. Using the same ELISA kit, we also found that bicyclol did not exhibit any lowering effect on the physiological elevation of serum AFP in pregnant mice and fetal mice livers treated with bicyclol 200 mg/kg for 10 days (Figure 5(c)).

Figure 6 reveals the immunohistochemical and Western blot analysis of PCNA. In immunohistochemical analysis (Figure 6(a)), the liver sections of the control mice stained for PCNA showed few nuclear-positive cells. However, DEN/PB treatment remarkably increased not only the intensity of immunostaining but also the number of PCNA-positive hepatocytes, which were both significantly reduced with bicyclol pretreatment. In the Western blot assay, preadministered bicyclol also decreased the increased expression of PCNA induced by DEN/PB (Figure 6(b)).

In this study, we also found that 10 weeks after stopping the administration of PB and drugs, the control and bicyclol-treated (200 mg/kg) animals showed no HCC and hepatoma formation at the time of termination whereas DEN/PB-induced mice developed 100% hepatoma and 50% HCC (data not shown).

4. Discussion

DEN is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meats,

Table 2: Levels of serum marker enzymes ALT, AST, and ALP in all groups.

Group	Dosage (mg/kg)	AST (u/L)	ALT (u/L)	ALP (u/L)
Control	—	332.0 ± 32.8	182.9 ± 25.7	5.3 ± 1.2
DEN/PB	—	334.1 ± 32.1	283.5 ± 55.9$^{\#\#}$	10.8 ± 6.4$^{\#}$
Bicyclol	100	324.3 ± 19.1	224.4 ± 23.3**	8.4 ± 4.3
	200	350.5 ± 45.6	222.2 ± 53.8	7.8 ± 2.2

Data are means ± SD values ($n = 8 \sim 10$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the control group. $^{**}P < 0.01$ compared with the DEN/PB model group.

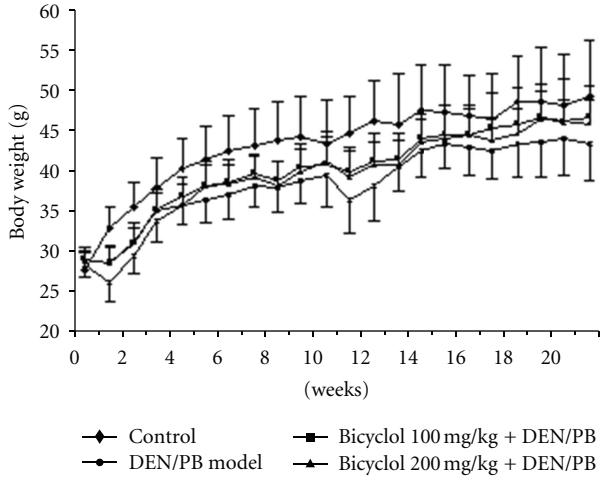

Figure 3: Body weight profile in the control and experimental groups. Average values with standard deviations are shown. $n = 14$-15.

occupational settings, cosmetics, agricultural chemicals, and pharmaceutical agents. DEN-induced HCC is one of the most accepted and widely used experimental models to study hepatocarcinogenesis [11]. There are 2 liver tumorigenesis protocols for DEN. DEN treatment at the neonatal period (2-week-old mice administered with DEN at a dose of 5 mg/kg body weight) causes severe hepatocyte toxicity. DEN treatment of young adult mice (4-5-week-old mice administered with DEN at a dose of 100 mg/kg body weight) is less toxic and requires tumor promotion by long-term treatment with PB, a procedure referred to as the two-stage protocol [12–14].

In this study, we used the DEN/PB two-stage protocol to determine whether bicyclol has a chemopreventive effect on hepatocarcinogenesis *in vivo*. As female mice are known to be resistant to hepatocarcinogenesis in experimental mouse models, including those employing chemical carcinogenesis, only male mice were used and analyzed in our experiments [15]. In this study, we found that pretreatment with bicyclol (100, 200 mg/kg orally) markedly decreased the number of liver tumor nodules per liver. According to the histopathological diagnosis, the model control group developed 33.3% HCC and 55.6% hepatoma at week 20. However, the mice pretreated with 200 mg/kg bicyclol showed no HCC and hepatoma formation in their liver. Ten weeks after stopping administration of the promoter and bicyclol, there was still no tumor formation in the 200 mg/kg bicyclol group whereas 100% hepatoma and 50% HCC was observed in the DEN/PB model group. These results further revealed that bicyclol had a preventive effect on hepatocarcinogenesis induced by carcinogens and this preventive activity is persistent.

Liver damage caused by DEN and PB could result in the deterioration or destruction of the cell membrane which leads to the leakage of transaminases and ALP from the liver tissue. The serum levels of transaminases and ALP are representative of the liver function; their increased levels are indicators of liver damage. Cancer chemoprevention and therapy depends on the investigation of these marker enzymes [16]. The elevation of ALT levels is repeatedly credited to hepatocellular damage and is usually accompanied by a rise in AST levels. Increase in ALP levels reflects the pathological alteration in biliary flow. In the present study, pretreatment with bicyclol attenuated the increased activities of ALT and ALP induced by DEN/PB, but only bicyclol-(100 mg/kg) treated mice showed statistically significant results when compared with the DEN/PB group. There were no changes in serum AST levels in the model and bicyclol-treated groups. It is shown that bicyclol helps regenerate the parenchymal cells in liver, protecting membrane integrity and thereby decreasing enzyme leakage (results in [6]). These results shown in this paper also suggest that the protective effect on cell membranes is not the major mechanism by which the chemopreventive effect of bicyclol is exerted.

AFP, an oncofetal serum protein, is progressively lost during development, such that it is virtually absent from a healthy adult [17, 18]. It has been widely used as a clinical marker in the diagnosis and monitoring of HCC. As we previously reported in human hepatocellular carcinoma MHCC97-H cells with high metastatic potential [19], bicyclol pretreatment significantly decreased the production and secretion of AFP. Since AFP overexpression has been associated with uncontrolled growth of HCC [20], our data suggests that bicyclol prevented the progression of HCC that is associated with the downregulation of cell proliferation. Another finding in this study is that bicyclol does not significantly affect the physiological AFP levels in pregnant mice and fetal mice livers. These findings indicate that the lowering effect of bicyclol on AFP is selective. This is an interesting finding, and further studies need to be carried out.

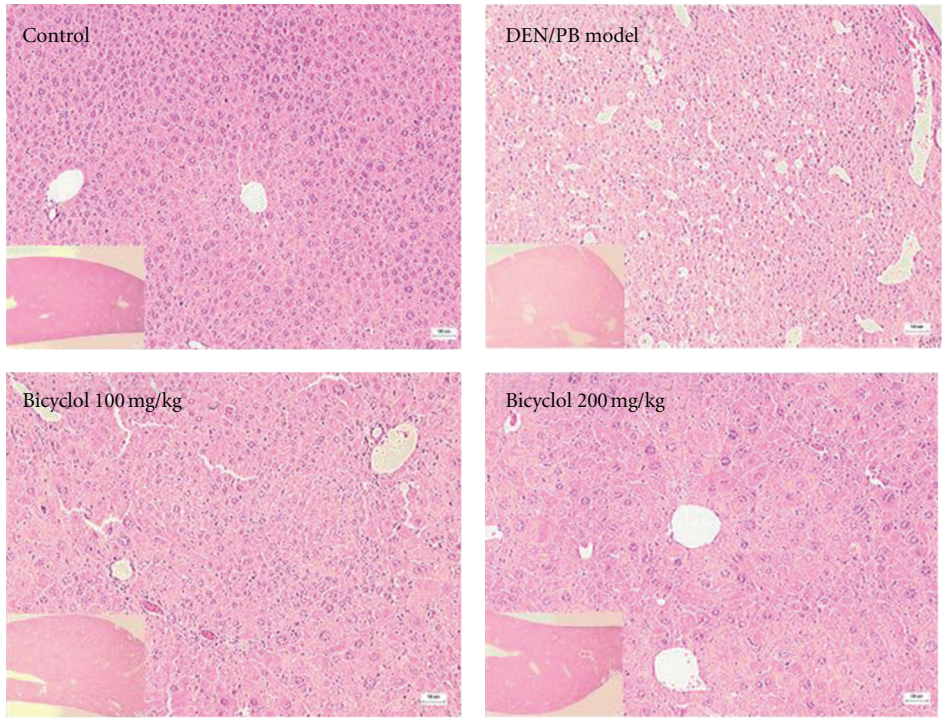

Figure 4: Histological study of the liver tissue obtained from the control and experimental groups at week 20. H&E-stained liver sections were photographed at 100x magnification. Insets in the bottom-left corners are images at 20x magnification of the liver tissue from which the 100x photographs were taken. Control group animals revealed normal architecture. DEN/PB-induced animals showed loss of architecture and presence of cancer cells. Animals pretreated with bicyclol (100, 200 mg/kg) showed fewer neoplastic cells and near-normal architecture.

PCNA is another marker of cell proliferation. It plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery [21]. This toroidal-shaped protein encircles the DNA and can slide bidirectionally along the duplex. PCNA has been identified as the DNA polymerase delta accessory protein. In our studies, the expression of PCNA significantly increased in DEN/PB-induced mice in terms of the number of PCNA-positive hepatocytes examined by immunohistochemistry, and the expression of PCNA was quantified by Western blot assay. Pretreatment with bicyclol decreased the expression of PCNA. This data suggests that bicyclol prevents the development of liver tumors along with the inhibition of cell proliferation.

In addition, cytochrome P450 (CYP) monooxygenase also plays a critical role in the activation of various chemical toxicants and precarcinogens [22–24]. Metabolic activation of DEN by CYP enzymes to form reactive electrophiles is required for its toxicity, and PB induces a large spectrum of drug-metabolizing enzymes [25]. According to the results of previous studies [26–28], bicyclol had the modulating activity on CYP isozymes, including the mild inhibition to CYP2C and CYP2D, the mild induction to CYP2E1, and reducing the metabolic rates of the selective substrates of CYP3A, 1A2, 2E1, and 2C. Bicyclol could enhance particularly the denitrosation of DEN, a low toxic pathway of metabolism. Based on these preliminary results, the modulating activity on CPY isozymes of bicyclol may also partly contribute to the prevention efficacy on hepatocarcinogenesis induced by DEN and PB found in this paper.

5. Conclusions

In summary, the findings of the present *in vivo* study are consistent with the results of our *in vitro* studies where we found that bicyclol prevents the malignant transformation of WB-F344 cells induced by 3MC and TPA [10]. Taken together, bicyclol has chemopreventive potential to the liver carcinogenesis induced by carcinogens. This is an interesting finding, and further studies on whether bicyclol can prevent liver carcinogenesis induced by chronic viral hepatitis need to be carried out. Additional studies to understand other molecular mechanisms responsible for its chemopreventive efficacy are warranted to claim bicyclol as a potential chemopreventive agent against liver cancer induced by carcinogens.

Abbreviations

AFP: α-fetal protein
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
ELISA: Enzyme-linked immunosorbent assay
DEN: Diethylnitrosamine
HBV: Hepatitis B virus
HCC: Hepatocellular carcinoma

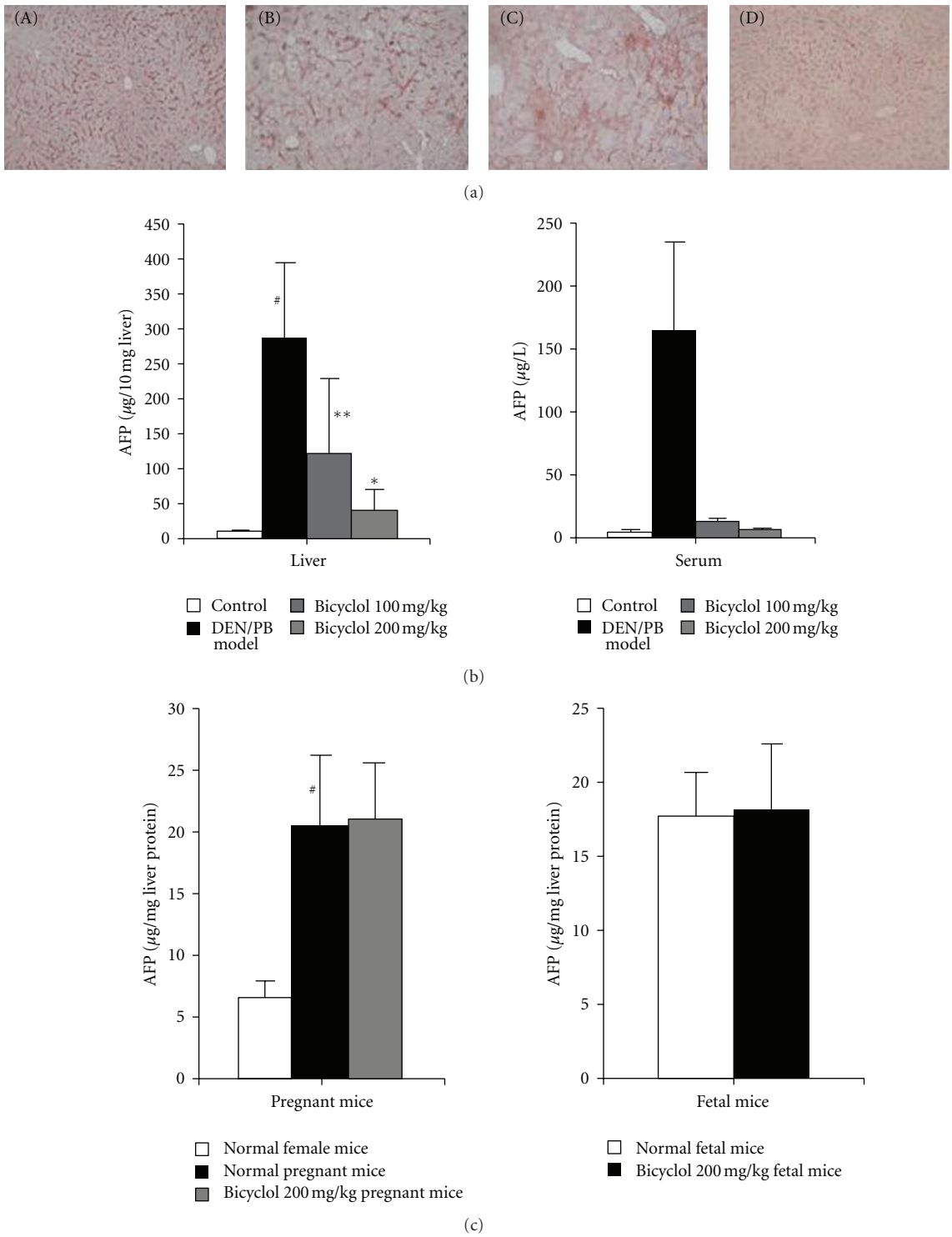

Figure 5: Expression and quantification of α-fetal protein (AFP). (a) AFP-immunoreactivity assay in the control and experimental groups at week 20 using immunohistochemistry assay. (A) control; (B) DEN/PB model; (C) treatment with bicyclol (100 mg/kg) before DEN injection; (D) treatment with bicyclol (200 mg/kg) before DEN injection. (b) Quantification of serum and tissue AFP levels in the control and experimental groups at week 20 using an enzyme immunoassay kit. Average values with standard deviations are shown. $n = 8$–10. $^{\#}P < 0.05$ compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the DEN/PB model group. (c) Quantification of liver AFP levels in pregnant mice and fetal mice using an enzyme immunoassay kit. Average values with standard deviations are shown. $n = 4$. $^{\#}P < 0.05$ compared with the control group.

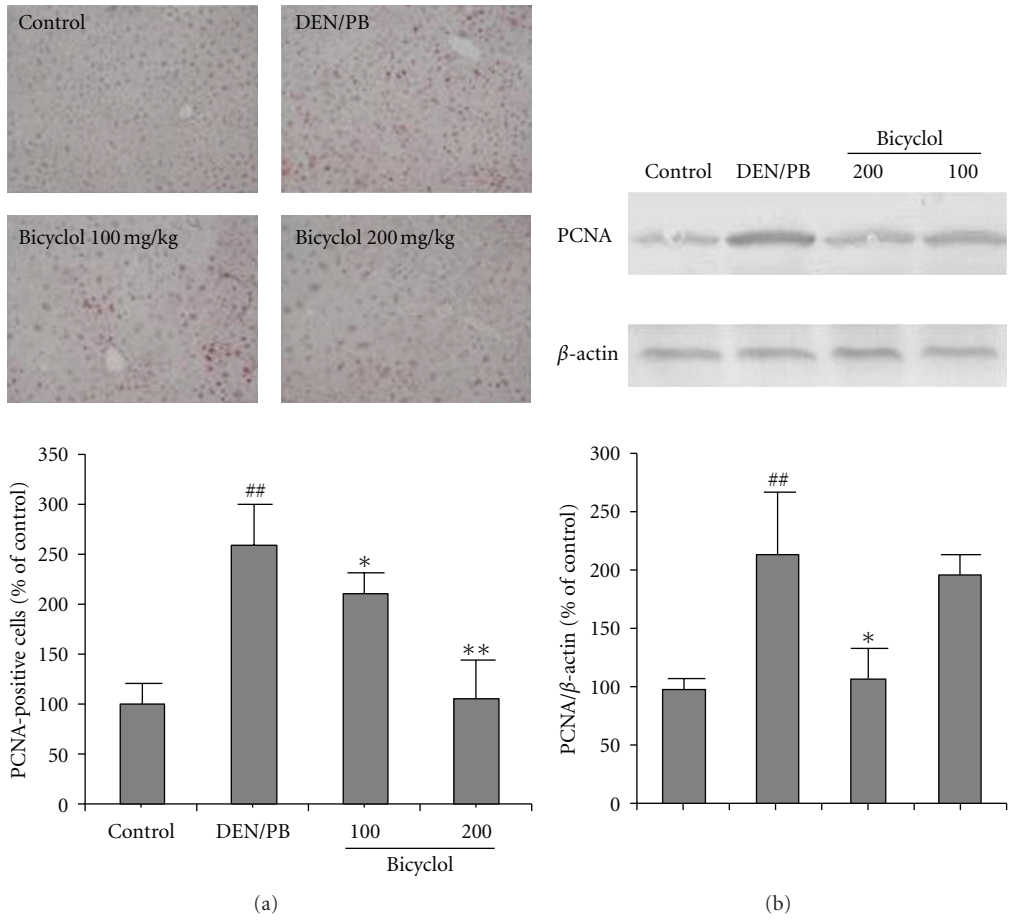

Figure 6: Expression of PCNA in the control and experimental groups at week 20. (a) Representative microscopic pictures of PCNA-stained liver sections by immunohistochemical analysis (100×) and quantification of the immunostained section of PCNA. $^{\#\#}P < 0.01$ compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the DEN/PB model group. (b) Western blot assay of PCNA and the quantitative results obtained by measuring the optical density of each band and expressed as the ratio of each targeted protein and beta-actin. Average values of 3 separate experiments along with the standard deviations are shown. $^{\#\#}P < 0.01$ compared with the control group; $^{*}P < 0.05$ compared with the DEN/PB model group.

HCV: Hepatitis C virus
3MC: 3-Methylcholanthrene
PB: Phenobarbital
TPA: 12-*O*-tetradecanoylphorbol-13-acetate.

Acknowledgments

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Review Article

Effects of Ginsenoside Rb_1 on Skin Changes

Yoshiyuki Kimura,[1] Maho Sumiyoshi,[2] and Masahiro Sakanaka[2]

[1] *Division of Biochemical Pharmacology, Department of Basic Medical Research, Ehime University Graduate School of Medicine, Shitsukawa, Toon City, Ehime 791-0295, Japan*
[2] *Division of Functional Histology, Department of Functional Biomedicine, Ehime University Graduate School of Medicine, Shitsukawa, Toon City, Ehime 791-0295, Japan*

Correspondence should be addressed to Yoshiyuki Kimura, yokim@m.ehime-u.ac.jp

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Ginseng roots (*Panax ginseng* CA Meyer) have been used traditionally for the treatment, especially prevention, of various diseases in China, Korea, and Japan. Both experimental and clinical studies suggest ginseng roots to have pharmacological effects in patients with life-style-related diseases such as non-insulin-dependent diabetic mellitus, atherosclerosis, hyperlipidemia, and hypertension. The topical use of ginseng roots to treat skin complaints including atopic suppurative dermatitis, wounds, and inflammation is also described in ancient Chinese texts; however, there have been relatively few studies in this area. In the present paper, we describe introduce the biological and pharmacological effects of ginsenoside Rb_1 isolated from Red ginseng roots on skin damage caused by burn-wounds using male Balb/c mice (*in vivo*) and by ultraviolet B irradiation using male C57BL/6J and albino hairless (HR-1) mice (*in vivo*). Furthermore, to clarify the mechanisms behind these pharmacological actions, human primary keratinocytes and the human keratinocyte cell line HaCaT were used in experiments *in vitro*.

1. Introduction

The oral administration of red ginseng root (*P. ginseng*) extracts has long been used to treat various diseases, including liver and kidney dysfunction, hypertension, non-insulin-dependent diabetes mellitus, and postmenopausal disorders, in China, Korea, and Japan. Topical applications have also been used for atopic suppurative dermatitis, wounds, and skin inflammation. The materials for Korean red ginseng products are selected from among ginseng roots (*Panax ginseng* CA Meyer) carefully cultivated in well-fertilized field for 6 years and then steamed and dried in the sun six times. The red ginseng extract produced by Korea Ginseng Corporation (Taejon, Korea) is dried and powdered by freezing prior to use. In this paper, we introduce the biological and pharmacological effects of ginsenoside Rb_1 isolated from red ginseng roots on skin damage in mice.

2. Effects of Ginsenoside Rb_1 on Burn Wound Healing in Mice

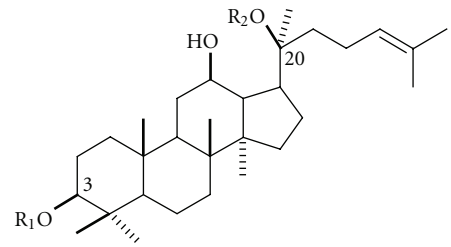
Burns and wounds initially induce coagulative necrosis and cause the formation of a scar. Macrophages migrate to an injured area to kill invading organisms and produce cytokines that recruit other inflammatory cells responsible for the diverse effects of inflammation [1, 2]. Angiogenesis in the injured area is closely associated with the process of wound healing [3]. Moreover, growth factors and cytokines are central to the wound-healing process [4–6]. Thus, the burn wound-healing process is complex, involving inflammatory factors, including monocyte migration and cytokine production, and growth factors and angiogenesis during reepithelialization. Vascular endothelial growth factor (VEGF) plays an important role in skin tissue repair through angiogenesis during the healing of burn wounds

Table 1: Effects of various ginseng saponins on angiogenesis.

Effects of ginsenoside Rb_2 (100 μg/mouse, *iv*) on tumor-induced angiogenesis in B16-BL6 melanoma-inoculated mice (*in vivo*) Ginsenoside Rb_2 showed antiangiogenesis [25].
Effects of ginsenoside Rb_2 (100 μg/mouse, *iv* and 300 μg/mouse, *po*), 20(*R*)Rg_3 (100 μg/mouse, *iv* and 300 μg/mouse, *po*), and 20(*S*)Rg_3 (100 μg/mouse, *iv* and 300 μg/mouse, *po*) on tumor-induced angiogenesis in B16-BL6 melanoma-inoculated mice (*in vivo*). Ginsenoside Rb_2, 20(*R*)Rg_3 and 20(*S*)Rg_3, showed antiangiogenesis [40].
Effects of the total saponin fraction (10–100 μg/mL) on tube formation by HUVECs (*in vitro*). The total saponin fraction enhanced angiogenesis [22].
Effects of ginsenoside Rg_1 (10 μM) and Rb_1 (10 μM) on angiogenesis in scaffold implants in mice (*in vivo*). Effects of Rg_1 (125 nM) and Rb_1 (125 nM) on chemoinvasion in HUVEC (*in vitro*). Ginsenoside Rg_1 enhanced angiogenesis, and ginsenoside Rb_1 showed antiangiogenesis in the earliest stage [23].
Effects of ginsenoside Re (10–100 μg/mL) on angiogenesis in HUVECs (*in vitro*). Effects of Re (70 μg/extracellular matrix) on angiogenesis in extracellular matrix-implanted rats (*in vivo*). Ginsenoside Re showed angiogenesis [41].
Effects of ginsenoside Rg_1 (150–600 nM) on angiogenesis in HUVECs (*in vitro*). Effects of Rg_1 (600 nM/Matrigel) on angiogenesis in Matrigel-implanted mice (*in vivo*). Ginsenoside Rg_1 promoted angiogenesis [42].
Effects of 20(*R*)-ginsenoside Rg_3(1-1000 nM) on angiogenesis in HUVECs (*in vivo*). 20(*R*)-ginsenoside Rg_3 showed antiangiogenesis [43].
Effects of Ginsenoside Rg_1 (150 nM) on angiogenesis in HUVECs (*in vitro*). Ginsenoside Rg_1 promoted angiogenesis [44].
Effects of ginsenoside Rg_1 (30 μg/mL) and Re (30 μg/mL) on angiogenesis in HUVECs (*in vitro*). Effects of Rg_1 (50 μg/mL) and Re (50 μg/mL) on angiogenesis in Matrigel-implated mice (*in vivo*). Ginsenoside $Rg_1$and Re promoted angiogenesis [45].
Effects of ginsenoside Rg_3 (3 mg/kg, ip) on growth and angiogenesis of ovarian cancer (*in vivo*). Ginsenoside Rg_3 showed antiangiogenesis [46].
Effects of ginsenoside Rb_1 (250 nM) on angiogenesis in HUVECs (*in vitro*). Ginsenoside Rb_1 showed antiangiogenesis [47].
Effects of ginsenoside Rg_1 (500 μg/Matrigel) on angiogenesis in Matrigel-implanted mice (*in vivo*) Ginsenoside Rg_1 promoted angiogenesis [48].
Effects of saponins (0.1–100 μg/mL) isolated from *Panax notoginseng* and Rg_1(10 μg/mL) on angiogenesis in HUVECs (*in vitro*). Total saponin and ginsenoside Rg_1 promoted angiogenesis [49].
Effects of ginsenoside Rg_3 (20 mg/kg, *po*) on angiogenesis and growth in lung carcinoma-implanted mice. Ginsenoside Rg_3 showed antiangiogenesis [50].

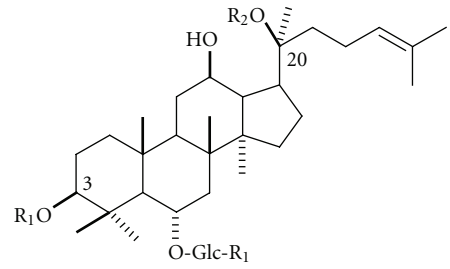
[4, 7, 8]. Furthermore, it has been demonstrated that chemokines including macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1) are expressed at high levels in murine full-thickness dermal wounds at times preceding and coinciding with maximal macrophage infiltration [9–12]. Interleukin 1-β (IL-1β) is also known to be released from monocyte-derived macrophages during inflammation and stimulates VEGF expression in endothelial cells, keratinocytes, synovial fibroblasts, and colorectal carcinoma cells [13–16]. IL-1β gene expression was reported to be upregulated in MCP-1-treated human monocytes [17]. Trautmann et al. [18] found that the expression of MCP-1 of macrophage and keratinocyte origin correlated with the accumulation of mast cells during wound healing. Weller et al. [19] reported that mast cell activation and histamine release were required for wound healing. Numata et al. [20] showed that the accelerated wound-repair activity of histamine was mediated by the activity of basic fibroblast growth factor (bFGF), which leads to angiogenesis, and macrophage recruitment in the wound-healing process. Thus, the process of wound repair is thought to be closely associated with the network systems among various cells such as keratinocytes, fibroblasts, macrophages and mast cells, and might be modulated by interactions among chemokines, cytokines, growth factors, and related biofactors secreted from these cells.

The genus *Panax* derives its name from the Greek words *pan* (all) and *akos* (healing). In 1988, Kanzaki et al. [21] reported that an orally administered red ginseng root extract stimulated the repair of intractable skin ulcers in patients with diabetes mellitus and Werner's syndrome in clinical trials. Morisaki et al. [22] showed that the local administration of ginseng saponins markedly improved wound healing in diabetic and aging rats. Sengupta et al. [23] reported that ginsenoside Rg_1 promoted functional angiogenesis into a polymer scaffold (*in vivo*) and the proliferation and chemoinvasion of tube-like capillary formation by human umbilical vein endothelial cells (HUVECs) through enhanced expression of nitric oxide synthetase, phosphatidylinositol-3 kinase, and the Akt pathway (*in vitro*). Conversely ginsenoside Rb_1 inhibited the earliest step in angiogenesis, the chemoinvasion of HUVECs [23]. Furthermore, Choi [24] reported that ginsenoside Rb_2 improved wound healing through its facilitating effects on



Ginsenoside Rb_1: R_1 = -Glc^2-Glc, R_2 = -Glc^6-Glc
Ginsenoside Rb_2: R_1 = -Glc^2-Glc, R_2 = -Glc^6-Ara (p)
Ginsenoside Rc: R_1 = -Glc^2-Glc, R_2 = -Glc^6-Ara (f)
Ginsenoside Rd: R_1 = -Glc^2-Glc, R_2 = -Glc
(p): pyranosyl, (f): furanosyl

20 (*S*) protopanaxadiol-type



Ginsenoside Re: R_1 = -Rham, R_2 = -Glc
Ginsenoside Rg_1: R_1 = -H, R_2 = -Glc

20 (*S*) protopanaxatriol-type

Figure 1: The structure of various ginsenosides.

Table 2: Effects of ginsenoside Rb_1 on angiogenesis from the area surrounding burn wounds in mice [26].

Treatment	Blood vessel length (mm/field)	Blood vessel area (mm^2/field)
Untreated burn wounds (control)	75.6 ± 24.9	10.5 ± 3.8
+Ginsenoside Rb_1		
(100 fg/wound)	228.8 ± 38.6*	46.6 ± 15.0*
(10 pg/wound)	203.0 ± 17.0*	37.6 ± 5.5*
(1 ng/wound)	274.1 ± 37.4*	49.9 ± 4.7*
+bFGF	241.5 ± 28.3*	35.8 ± 5.9*

The burn wounds were created on the backs of male Balb/c mice (6 weeks old) under anesthesia with pentobarbital. A polyethylene filter pellet (about 8 mm in diameter, 3 mm thick) containing the indicated amount of basic fibroblast growth factor (bFGF) or ginsenoside Rb_1 was applied to the burn wound surface. On day 9, any angiogenesis in the site surrounding the burn wound was photographed using a stereoscopic microscope, and the area and length of blood vessels were measured using a Coordinating Area and Curvimeter Machine (X-PLAN 360 dII, Ushitaka, Tokyo, Japan).
Values are the mean ± SE for six mice. *Significantly different from untreated burn wounds (control), $P < 0.05$.

epidermal cell proliferation, by upregulating the expression of proliferation-related factors. However, Sato et al. [25] found that the intravenous administration of ginsenoside Rb_2 inhibited metastasis to the lung by inhibiting tumor-induced angiogenesis in B16-BL6 melanoma-bearing mice. Thus, there are perplexing contradictions in the reported effects of various ginseng saponins on angiogenic activity as shown in Table 1.

To clarify these differing effects, we first attempted to examine the effects of various ginseng saponins on wound healing. Among six ginseng saponins (ginsenoside Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) (Figure 1), we found that ginsenoside Rb_1 enhanced burn-wound healing most strongly.

In summary, we reported the promotion of burn-wound healing by the topical application of ginsenoside Rb_1 at low doses (100 fg, 10 pg, and 1 ng per wound) to be due to the promotion of angiogenesis during skin wound repair through stimulation of VEGF production and an increase in hypoxia-inducible factor (HIF-) 1α expression in keratinocytes and the elevation of interleukin (IL-) 1β from macrophage accumulation in the burn wound area [26]. Furthermore, we found the facilitating effects of ginsenoside Rb_1 at low doses (100 fg, 10 pg, and 1 ng per wound) to be due to the promotion of angiogenesis via the activation of basic fibroblast growth factor (bFGF) through an increase in histamine released from mast cells recruited by the stimulation of monocyte chemoattractant protein-1 (MCP-1) as another mechanism [27]. We will explain our experiments regarding the facilitating effects of ginsenoside Rb_1 on burn-wound healing in detail. The burn area in mice treated with a topical application of ginsenoside Rb_1 in the range of 10^{-8}% to 10^{-12}% was significantly reduced on days 8–20 compared to that in vehicle-treated burn-wound control mice (Figure 2).

To clarify the mechanism behind the facilitating effect of ginsenoside Rb_1 on wound healing, we examined levels of IL-1β and VEGF in exudates of the burn. The levels increased with time over 9 days. At 1 ng of ginsenoside Rb_1 per wound, the level of IL-1β was increased on days 1, 3, and 5 but significantly decreased on day 9 compared to that in vehicle-treated control mice (Figure 3). The topical application of bFGF (2.5 μg per wound) also increased IL-1β production on day 3. The VEGF level in the exudates from the wound

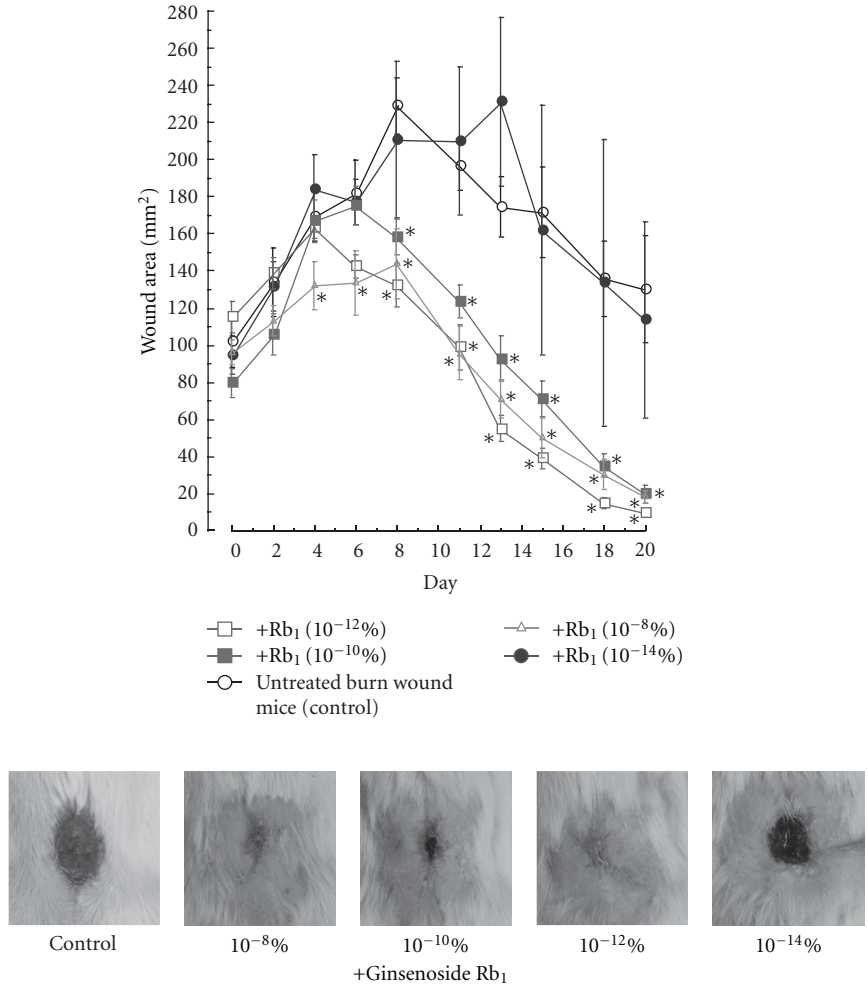


Figure 2: Effects of ginsenoside Rb_1 on wound healing in mice [26]. After the burn wound was made by applying a customized soldering iron to the skin on the backs of male Balb/c mice (6 weeks old) for 10 s at 250°C, a sterile biopsy punch (8 mm diameter) was used to excise the burnt skin, leaving the underlying fasciae intact. All surgical treatments were performed under anesthesia with pentobarbital. Indicated amounts of ginsenoside Rb_1 were applied to the burn wounds surface and then covered with a film dressing for 19 consecutive days. The burn wound site was photographed every other day and burn wound area was measured using a Coordinate Area and Curvimeter Machine (X-PLAN 360 dII). Values are the mean ± SE for 6–12 mice. *Significantly different from vehicle-treated mice, $P < 0.05$.

Table 3: Effects of *Panax ginseng* extract on skin aging.

Effects of the total ginseng saponin fraction (100 to 500 μg/mL) on luciferase reporter gene assays in human dermal fibroblast (*in vitro).* Effects of the total ginseng saponin fraction (100 to 500 μg/mL) on type I collagen in human dermal fibroblast (*in vitro*). The total saponin fraction (100 to 500 μg/mL) increased type I procollagen synthesis [51].
Effects of red ginseng extract (20 and 60 mg/kg, *po*) on acute UVB-induced skin aging in mice The extract inhibited the increases in epidermis and dermis thickness induced by UVB [52].
Effects of red ginseng extract (20 mg/kg, *ip* or topical application of 0.2% cream) on chronic UVB-irradiated skin damage in hairless mice. The extract reduced wrinkling and tumor incidence [53].
Effects of ginsenoside Rb_1 (100 fg, 10 pg, or 1 ng/mouse, topical application) on chronic UVB-irradiated skin aging in hairless mice. Ginsenoside Rb_1 inhibited the increase in skin thickness, wrinkling, and epidermis in UVB-irradiated hairless mice [31].
Effects of red ginseng extract (a diet containing 0.5 and 2.5% red ginseng extract) on UVB-irradiated skin aging in hairless mice. The extract reduced wrinkling, the mRNA level of procollagen type I, and the MMP-1 level [54].
Healthy female volunteers over 40 years of age were randomized in a double-blind fashion to receive either red ginseng extract (3 g/day) or placebo for 24 weeks. (*Clinical study*). Red ginseng extract caused an improvement in facial wrinkling and increase in type I procollagen synthesis [55].

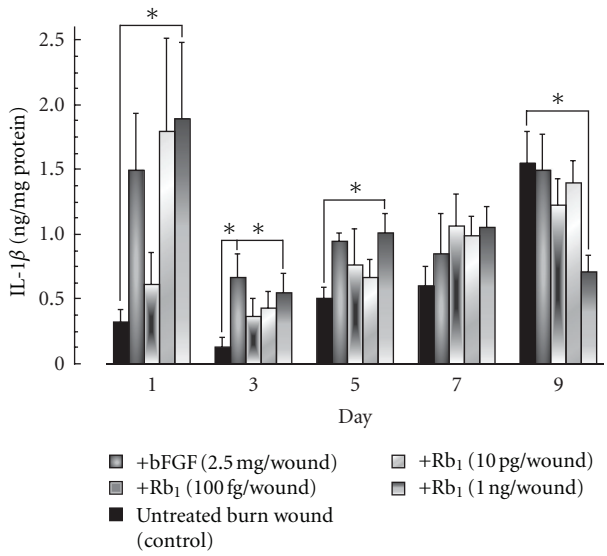


Figure 3: Effects of ginsenoside Rb_1 and bFGF on IL-1β production in the exudates of burns in male Balb/c mice [26]. The burn wounds were created on the backs of male Balb/c mice (6 weeks old) under anesthesia with pentobarbital. A polyethylene filter pellet (about 8 in mm diameter, 3 mm thick) containing the indicated amount of basic fibroblast growth factor (bFGF) or ginsenoside Rb_1 was applied to the burn wounds surface. On days 1, 3, 5, 7, and 9, the filter pellets were removed and replaced with fresh filter pellets. For control mice, filter pellets containing saline alone were applied according to the same schedule. Immediately after removal, phosphate-buffered saline (PBS, pH 7.0) (200 μl) was added to each filter pellet and mixed for 10 min. The IL-1β levels in the filter pellets were measured using a mouse IL-1β ELISA kit. Values are the mean ± SE for 6 mice. *Significantly different from control, $P < 0.05$.

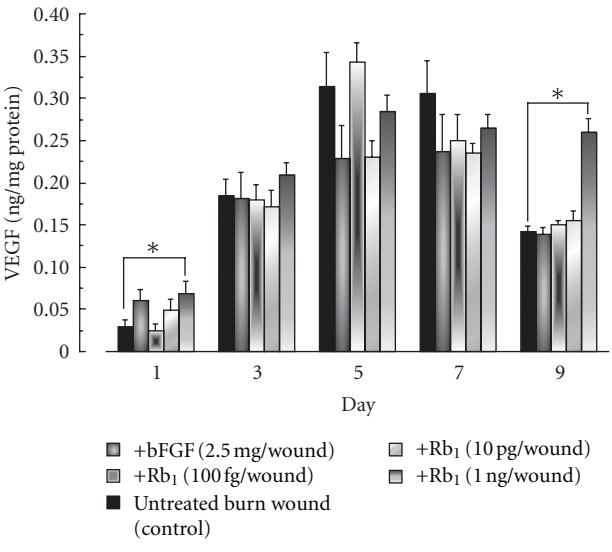


Figure 4: Effects of ginsenoside Rb_1 and bFGF on VEGF production in the exudates of burns in male Balb/c mice [26]. The experiments were performed as described in Figure 3, and the VEGF levels in the filter pellets were measured using a mouse IL-1β ELISA kit. Values are the mean ± SE for 6 mice. *Significantly different from control, $P < 0.05$.

Table 4: Effects of ginsenoside Rb_1 on the thickness of the epidermis and extracellular matrix (ECM) of the dermis at week 12 in UVB-irradiated hairless mice [31].

	Epidermis (μm)	ECM (μm) in dermis
Normal mice	14.74 ± 1.11*	332.51 ± 23.18*
Vehicle-treated UVB-irradiated mice (control)	142.59 ± 25.37	632.32 ± 31.96
+Ginsenoside Rb_1		
(100 fg/mouse)	46.00 ± 6.26*	561.86 ± 45.22
(10 pg/mouse)	49.24 ± 4.73*	560.67 ± 44.81
(1 ng/mouse)	39.84 ± 6.26*	585.63 ± 31.35

The initial dose of UVB was set at 36 mJ/cm^2, which was subsequently increased to 54 mJ/cm^2 at weeks 1–4, 72 mJ/cm^2 at weeks 4–7, 108 mJ/cm^2 at weeks 7–10, and finally to 122 mJ/cm^2 at weeks 10–12 in male albino hairless HOS: HR-1 mice. The frequency of UVB irradiation was set at three times per week. Ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/mouse) was applied topically to the dorsal region of each mouse every day for 12 weeks. The dorsal skin samples (about 3 cm^2) removed at week 12 were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm thickness, deparaffinized, and stained with hematoxylin-eosin (HE) and Azan. Four different microscopic fields (×200 magnification) per plate were photographed. The thickness of the epidermis and dermis thickness were measured from the samples stained by HE and Azan, using a Digimatic Caliper.

Values are the mean ± SE for 6 mice. *Significantly different from UVB-irradiated hairless mice (control), $P < 0.05$.

Table 5: Effects of ginsenoside Rb_1 on the numbers of apoptotic and 8-OHdG-positive cells at week 12 in the skin of UVB-irradiated hairless mice [31].

	Apoptotic cells (number/field)	8-OHdG-positive cells (number/field)
Normal mice	0 ± 0*	106 ± 7*
Vehicle-treated UVB-irradiated mice (control)	102 ± 10	286 ± 32
+Ginsenoside Rb_1		
(100 fg/mouse)	19 ± 11*	150 ± 24*
(10 pg/mouse)	11 ± 11*	183 ± 27*
(1 ng/mouse)	9 ± 9*	109 ± 26*

Ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/mouse) was applied topically to the dorsal region of each mouse every day for 12 weeks. The expression levels of apoptotic cells and 8-hydroxy-2′-deoxyguanosine (8-OHdG) (marker of oxidative DNA damage) in the dorsal skin of UVB-irradiated hairless mice were examined by the TUNEL method using an apoptosis in situ detection kit and an immunoperoxidase technique using anti-8-OHdG antibody.

Values are the mean ± SE for 6 mice. *Significantly different from UVB-irradiated mice (control), $P < 0.05$.

increased until day 5 and then decreased. The application of ginsenoside Rb_1 increased VEGF levels on days 1 and 9 (Figure 4). However, the application of bFGF did not affect VEGF production.

The application of bFGF (2.5 μg per wound) or ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng per wound) for 9 days increased the length of blood vessels by 3- to 3.5-fold and

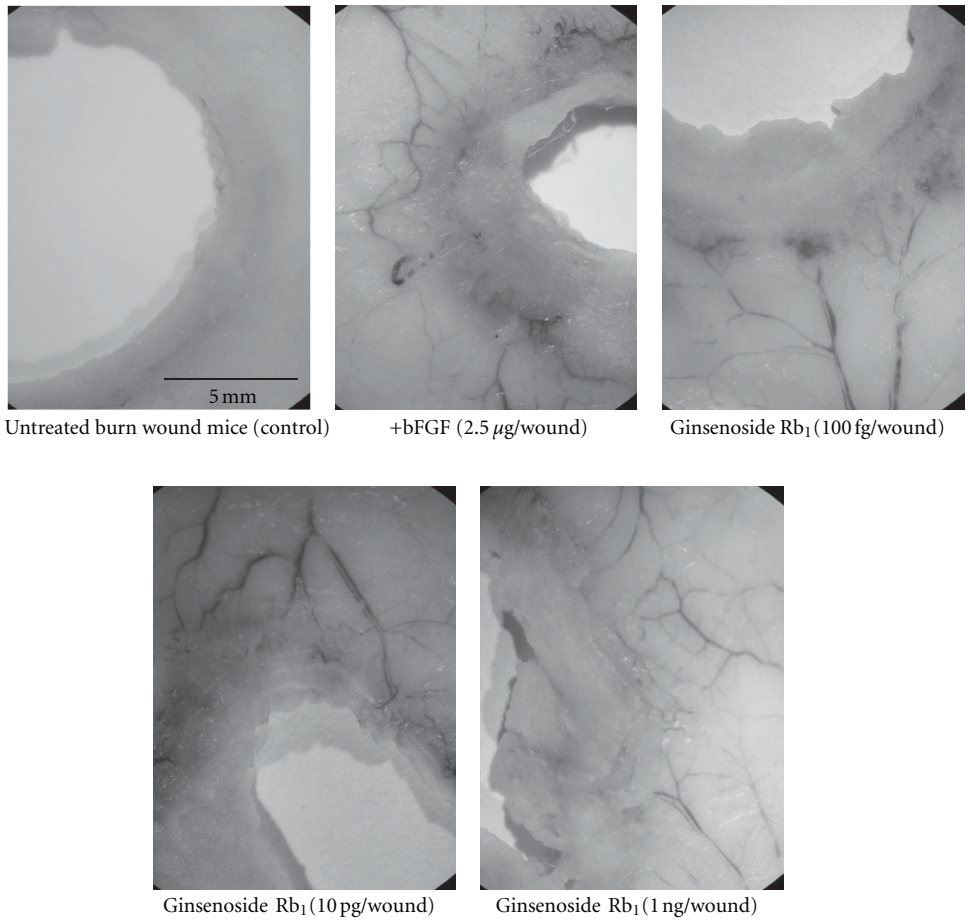


Figure 5: Photographs showing neovascularization from the tissue surrounding the burn and the effects of the topical application of ginsenoside Rb_1 [26]. The experiments were performed as described in Figure 3. On day 9, any angiogenesis in the site surrounding the burn wound was photographed using a stereoscopic microscope.

the corresponding area by 3.5- to 5.0-fold, compared to the control (Figure 5 and Table 2).

Ginsenoside Rb_1 at concentrations from 100 fg/mL to 1 ng/mL enhanced the VEGF production and HIF-1α expression induced by IL-1β in the human keratinocyte cell line HaCaT (Figure 6).

These findings suggest the enhancement of wound healing by ginsenoside Rb_1 to be due to the promotion of angiogenesis during the repair process as a result of the stimulation of VEGF production caused by the increase in HIF-1α expression in keratinocytes. Furthermore, the MCP-1 level in the exudates of vehicle-treated (control) mice reached a maximum 1 day after the burn treatment and declined rapidly from day 3. Ginsenoside Rb_1 (1 ng per wound) and bFGF (2.5 μg per wound) significantly increased the level of MCP-1 on day 1 compared to that in control mice (Figure 7). Histamine levels in the exudates of the burn wound area increased until day 7. Ginsenoside Rb_1 (1 ng per wound) significantly increased the histamine level on day 5 compared to that in control mice (Figure 7). Furthermore, ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng per wound) and bFGF (2.5 μg per wound) significantly increased histamine production on day 7 (Figure 7). The facilitating effects of ginsenoside Rb_1 may be due to the promotion of angiogenesis *via* the activation of bFGF through the increase in histamine released from mast cells recruited by the stimulation of MCP-1 production.

Based on these experimental results, the enhancing effects of ginsenoside Rb_1 on burn wound healing are summarized in Figure 8.

It has been reported that ginsenoside Rb_2 as well as ginsenoside Rb_1 promotes wound healing [24].

3. Effects of Ginsenoside Rb_1 on Ultraviolet B (UVB-) Irradiated Skin Damage in Mice

The symptoms of cutaneous aging, such as wrinkles and pigmentation, for example, develop earlier in sun-exposed skin than in unexposed skin, a phenomenon referred to as photoaging. Ultraviolet B (UVB) radiation is one of the most important environmental factors because of its hazardous effects, which include the generation of skin cancer [28], suppression of the immune system [29], and premature skin aging [30].

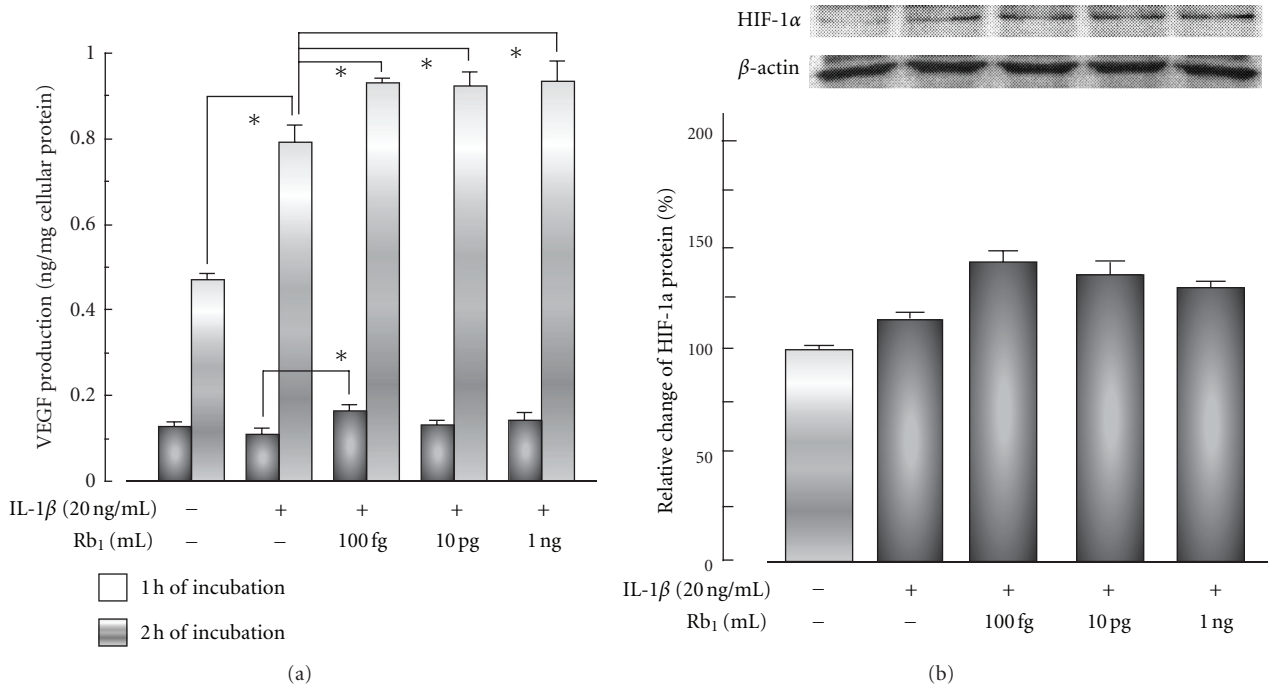


FIGURE 6: Effects of ginsenoside Rb_1 on VEGF production (a) and HIF-1α expression (b) with or without IL-1β in HaCaT cells [26]. The human keratinocyte cell line HaCaT was treated with the indicated amounts of ginsenoside Rb_1 in the presence or absence of IL-1β (20 ng/mL) for 1 or 2 h. VEGF levels in the medium were measured using a human VEGF kit. The expression of hypoxia-inducible factor (HIF-) 1α in the nuclear fraction of HaCaT cells was measured by western blot analysis with mouse anti-HIF-1α and anti-β-actin antibodies. Values are the mean ± SE for six experiments. *Significantly different from control, $P < 0.05$.

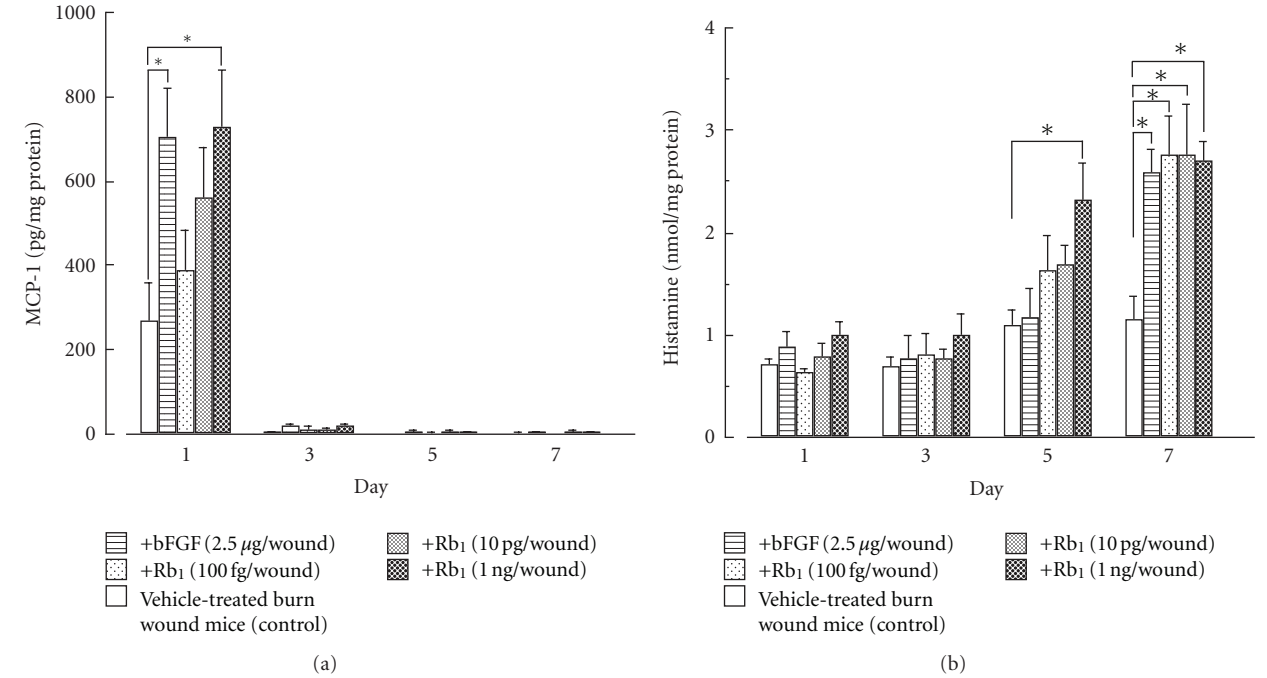


FIGURE 7: Effects of ginsenoside Rb_1 and bFGF on MCP-1 (a) and histamine (b) production in the exudates of burns in male Balb/c mice [27]. The experiments were performed as described in Figure 3, and then MCP-1 and histamine levels in the filter pellets were measured using mouse MCP-1 and histamine ELISA kits, respectively. Values are the mean ± SE for 6 mice. *Significantly different from control, $P < 0.05$.

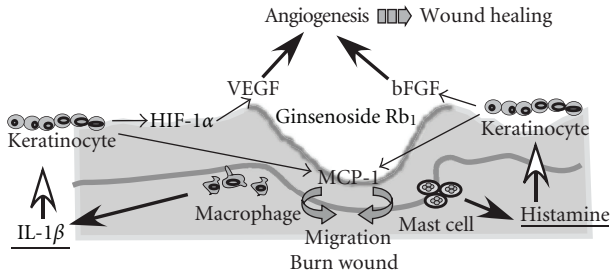


Figure 8: The proposed mechanisms of the enhancing effects of ginsenoside Rb_1 on burn wound healing.

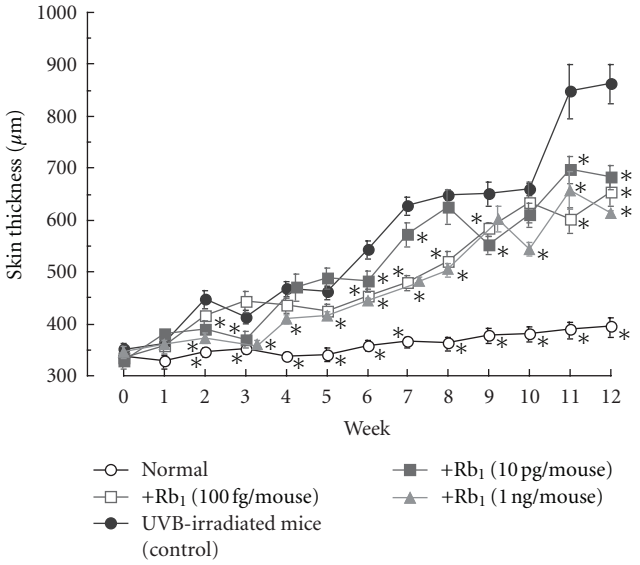


Figure 9: Effects of ginsenoside Rb_1 on skin thickness in chronic UVB-irradiated male hairless (HRM-1) mice [31]. The initial dose of UVB was set at 36 mJ/cm^2, which was subsequently increased to 54 mJ/cm^2 at weeks 1–4, 72 mJ/cm^2 at weeks 4–7, 108 mJ/cm^2 at weeks 7–10, and finally 122 mJ/cm^2 at weeks 10–12 in male albino hairless HOS: HR-1 mice. The frequency of UVB irradiation was set at three times per week. Ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/mouse) was applied topically to the dorsal region of each mouse every day for 12 weeks. The dorsal skin of the hairless mice was lifted up by pinching gently under anesthetization with pentobarbital, and skin-fold thickness was measured using a Quick Mini caliper. Skin thickness after UVB irradiation was measured every week. Values are the mean ± SE for 6 mice. *Significantly different from vehicle-treated mice, $P < 0.05$.

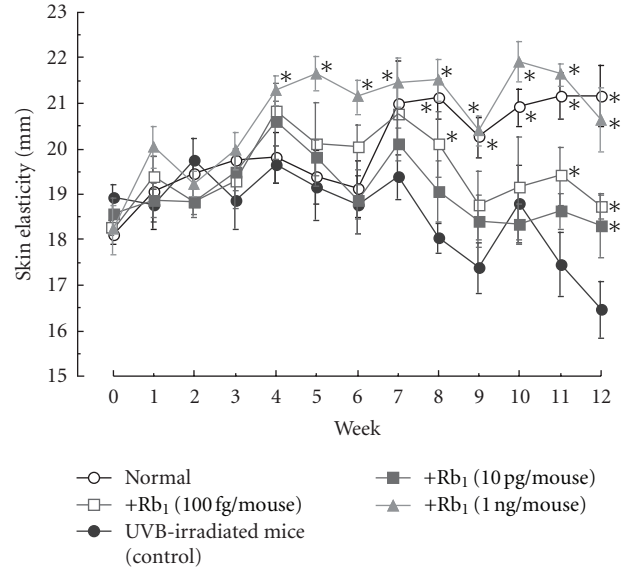


Figure 10: Effects of ginsenoside Rb_1 on skin elasticity in chronic UVB-irradiated male hairless (HRM-1) mice [31]. The experiments were performed as described in Figure 9. The dorsal skin was lifted up and skin stretch was measured using a digimatic caliper. Skin elasticity after UVB irradiation was measured every week. Values are the mean ± SE for 6 mice. *Significantly different from vehicle-treated mice, $P < 0.05$.

As shown in Table 3, it has been reported that red ginseng extract prevents skin aging induced by UVB irradiation. However, the active substance(s) has yet to be identified. We found that ginsenoside Rb_1 isolated from red ginseng roots inhibited the increases in skin thickness, epidermis, and wrinkle formation induced by chronic UVB irradiation [31]. In this paper, we will introduce the effects of ginsenoside Rb_1 on chronic UVB irradiation-induced cutaneous aging in hairless mice.

The topical application of ginsenoside Rb_1 at lower doses, 100 fg, 10 pg, and 1 ng/mouse, significantly inhibited the increase in skin thickness induced by UVB irradiation during weeks 2 to 12 compared to the skin thickness of vehicle-treated UVB-irradiated mice (control) (Figure 9).

The reduction in skin elasticity induced by UVB irradiation was significantly inhibited by the topical application of ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/mouse) during weeks 6 to 12 compared to that of control mice (Figure 10).

Wrinkling induced by UVB irradiation at week 9 was inhibited by the topical application of ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/mouse) (Figure 11).

The topical application of ginsenoside Rb_1 (100 fg, 10 pg, and 1 ng/mouse) inhibited the increase in epidermal thickness induced by UVB irradiation but had no effect on the increase in the extracellular matrix of the dermis (Table 4). The occurrence of apoptotic cells was localized to the stratum granulosum of the epidermis and was increased by UVB irradiation. The increase in apoptotic cell levels induced UVB irradiation was significantly inhibited by ginsenoside Rb_1 (100 fg. 10 pg, and 1 ng/mouse). Furthermore, 8-hydroxy-2′-deoxyguanosine (8-OHdG, a marker of oxidative DNA damage) [32] was also localized to the stratum basale and dermis, and its level was increased by UVB irradiation. The increase in 8-OHdG-positive cells induced by UVB irradiation was inhibited by ginsenoside Rb_1 (Table 5). UVB (20 mJ/cm^2) irradiation reduced the level of Bcl-2 expression in human primary keratinocytes. Conversely, UVB irradiation had no effect on Bak or Bax expression. Ginsenoside Rb_1 increased the Bcl-2 levels in UVB-treated human primary keratinocytes at the lower concentrations of 100 fg, 10 pg, and 1 ng/mL (Figure 12).

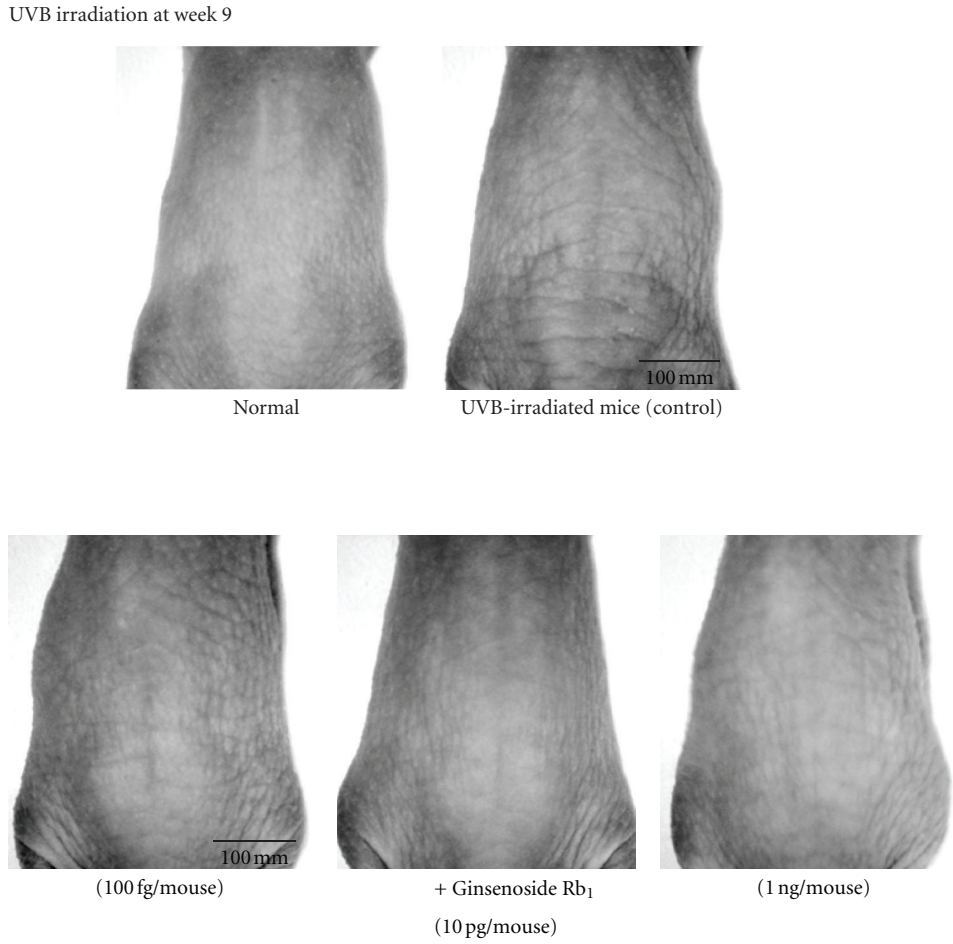


FIGURE 11: Photograph showing skin wrinkling induced by chronic UVB irradiation and the effects of topically applied ginsenoside Rb_1 [31]. The experiments were performed as described in Figure 9. To evaluate the formation of wrinkles after the UVB irradiation, the UVB-irradiated dorsal area (site of wrinkles) of each hairless mouse was photographed at 9 weeks.

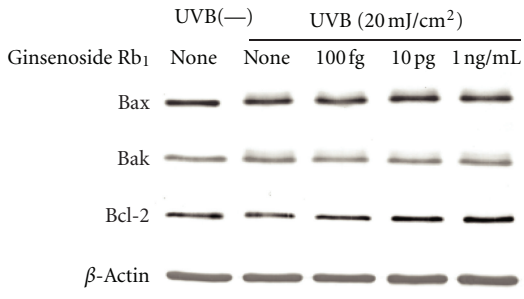


FIGURE 12: Effects of ginsenoside Rb_1 on Bax, Bak, and Bcl-2 expression levels in UVB-irradiated human primary keratinocytes [31]. Human keratinocytes (3×10^5 cells) were seeded in a 100-mm culture dish and cultured in KG-2 medium for 48 h. The cells were irradiated with UVB (20 mJ/cm^2) and treated with the indicated amounts of ginsenoside Rb_1 for 24 h in KB-2 medium. After being washed with phosphate-buffered saline (PBS, pH 7.0), the cells were treated with lysed buffer. The supernatant obtained by centrifugation was subjected to a western blot analysis with anti-Bcl-2, anti-Bax, anti-Bak, and anti-β-actin antibodies.

UVB exposure of skin cells results in several types of DNA damage such as the formation of the cyclobutane pyrimidine dimer, pyrimidine pyrimidone photodimers and 8-OHdG [33–35], and consequently DNA damage induced by long-term UV exposure leads to skin carcinogenesis. Furthermore, there are many reports that apoptotic stimuli such as UV radiation and tumor necrosis factor-α induce cell death by activating caspases [36]. Bcl-2 is a member of the large Bcl-2 family and protects cells from apoptosis. On the other hand, it has been reported that Bax and Bak appear to permeabilize the outer mitochondrial membrane, allowing the efflux of apoptogenic proteins [37–39]. The protective effect of ginsenoside Rb_1 on UVB-mediated apoptosis may be partly due to the upregulation of Bcl-2 expression in human keratinocytes. Thus, the protective effect of ginsenoside Rb_1 on skin photoaging induced by chronic UVB exposure may be due to the increase in collagen synthesis and/or the inhibition of metalloproteinases expression in dermal fibroblast and the inhibition of epidermal hyperplasia. Further research is needed to clarify the mechanism of the protective effect of

ginsenoside Rb_1 on photoaging induced by chronic UVB irradiation of the skin.

4. Conclusion

The topical application of ginsenoside Rb_1 isolated from red ginseng roots enhances burn wound healing, and ginsenoside Rb_1 prevents chronic UVB-induced skin photoaging, at very low doses. Further studies will be needed to clarify the clinical significance of these findings for skin damage induced by burn wounds or UV irradiation.

Acknowledgments

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Research Article

Antiproliferative and Anti-Invasive Effect of Piceatannol, a Polyphenol Present in Grapes and Wine, against Hepatoma AH109A Cells

Yuichiro Kita,[1] Yutaka Miura,[2] and Kazumi Yagasaki[2]

[1] *Department of Applied Biological Chemistry, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan*
[2] *Division of Applied Biological Chemistry, Institue of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan*

Correspondence should be addressed to Kazumi Yagasaki, yagasaki@cc.tuat.ac.jp

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Piceatannol is a stilbenoid, a metabolite of resveratrol found in red wine. Piceatannol and sera from rats orally given piceatannol were found to dose-dependently suppress both the proliferation and invasion of AH109A hepatoma cells in culture. Its antiproliferative effect was based on cell cycle arrest at lower concentration (25~50 μM) and on apoptosis induction at higher concentration (100 μM). Piceatannol suppressed reactive oxygen species-potentiated invasive capacity by scavenging the intracellular reactive oxygen species. These results suggest that piceatannol, unlike resveratrol, has a potential to suppress the hepatoma proliferation by inducing cell cycle arrest and apoptosis induction. They also suggest that the antioxidative property of piceatannol, like resveratrol, may be involved in its anti-invasive action. Subsequently, piceatannol was found to suppress the growth of solid tumor and metastasis in hepatoma-bearing rats. Thus, piceatannol may be a useful anticancer natural product.

1. Introduction

Cancer cells have two biological properties, that is, endless proliferation and metastasis. Invasion is a most important and characteristic step in the complicated process of cancer cells [1]. The blockage of these biological events by drugs or factors from food and natural resources will prolong the life span of an affected host. Our previous research showed that green, oolong, and black tea extracts, the sera from rats orally given these teas, and related components inhibited the proliferation and invasion of a rat ascites hepatoma cell line of AH109A [2] through induction of apoptosis and cell cycle arrest [3] and antioxidative activity [4]. Lignans also suppressed both the proliferation and invasion of AH109A hepatoma cells *in vitro*, and hence solid tumor growth and metastasis of AH109A cells *in vivo* [5]. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is known to possess cancer chemopreventive activity and strong antioxidative activity [6]. Hsieh et al. reported that resveratrol showed a greater antiproliferative effect on highly invasive breast carcinoma cells (MDA-MB-435) than on minimally invasive cells (MCF-7) [7]. However, our previous study showed that, in the hepatoma cells, resveratrol as such showed a weaker suppressive effect on the proliferation than on the invasion, and the sera from rats orally given resveratrol completely lost the antiproliferative activity in culture, although its anti-invasive activity was maintained [8, 9]. These findings suggest that the effect of resveratrol on the proliferation may be dependent on cancer cell properties. Piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene), an analogue of resveratrol, has been reported to induce apoptosis and cell cycle arrest in human melanoma cell (SK-Mel-28) [10].

In the present study, we examined whether piceatannol or its *in vivo* metabolite(s) would have antiproliferative and anti-invasive effects against AH109A and their modes of actions.

2. Materials and Methods

2.1. Materials. Piceatannol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). It was resolved in absolute ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan)

and the piceatannol solution was added to the medium at a final ethanol concentration of 0.2%. Control medium contained 0.2% ethanol alone. All other reagents were of the best grade commercially available.

2.2. Culture of AH109A Hepatoma Cells. The animal experiments in this article were conducted in accordance with guidelines established by the Animal Care and Use Committee of Tokyo University of Agriculture and Technology and were approved by this committee. Male Donryu rats (4 weeks of age) were purchased from NRC Haruna (Gunma, Japan). AH109A cells were generously provided by the Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan. AH109A cells were maintained in peritoneal cavity of male Donryu rats and isolated from accumulated ascites and then cultured in Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% calf serum (CS, JRH Biosciences, Lenaxa, KS, USA) (10% CS/MEM). These cells were cultured for at least 2 weeks after isolation to eliminate contaminated macrophages and neutrophils and used for the assays described hereinafter.

2.3. In Vitro Proliferation and Invasion Assays. Effect of piceatannol on AH109A proliferation was examined by measuring the incorporation of [methyl-^{3}H]thymidine (0.15 μCi/well, specific radioactivity; 20 Ci/mmoL, PerkinElmer Life and Analytical Sciences, Boston, MA, USA) into DNA as described previously [8, 11]. Effect of piceatannol on AH109A invasion was estimated by coculturing AH109A cells with rat mesentery-derived mesothelial cell (M-cell) monolayers, which were isolated and cultured from Donryu rats as described previously [12] with slight modifications [13]. Briefly, mesothelial cells (M-cells) were isolated from mesentery of male Donryu rats (6–8 weeks of age). After digestion by trypsin, 1.3–2.0 $\times$ 10^5 M-cells were plated in a 60 mm culture dish with 2 mm grids (Corning Incorporated, Corning, NY, USA) and cultured for 5–7 days to attain a confluent state in 10% CS/MEM. Then, AH109A cells (2.4 $\times$ 10^5 cells per dish) were applied on the monolayer of M-cells in 10% CS/MEM with piceatannol for 24 h. Invaded cells and colonies underneath M-cells were counted with a phase-contrast microscope. The invasive activity of AH109A cells was expressed as the number of invaded cells and colonies/cm^2.

2.4. Ex Vivo Assay. *Ex vivo* assay was performed with piceatannol-loaded rat sera (RS). Piceatannol was suspended in a 0.3% carboxymethyl cellulose sodium salt (CMC, Wako Pure Chemical Industries, Osaka, Japan) aqueous solution at concentrations of 0.25, 0.5, and 1 mg/mL, unless otherwise noted. Piceatannol suspension or 0.3% CMC alone (vehicle control) (1 mL/100 g body weight) was intubated to male Donryu rats (5 weeks old) which had been fasted overnight, and blood was collected 2 h after oral administration as described previously [8, 13]. Blood from rats given vehicle alone was also collected. The sera were prepared by centrifugation, sterilized by filtration, and added to the culture medium at a concentration of 10% instead of CS.

2.5. Flowcytometric Analyses of Cell Cycle Phases and Annexin-V-FITC Staining of Apoptotic Cells. For cell cycle analysis, 3.0 $\times$ 10^5 cells of AH109A per well were seeded in a 6-well plate in the medium containing various concentrations of piceatannol and cultured for 24 h. Cells were collected and washed twice with sterilized, Ca^{2+}- and Mg^{2+}-free, phosphate-buffered saline [PBS(−)]. Thereafter, 500 μL of propidium iodide (PI) solution containing 1 mg of PI (Sigma Chemical Co., St. Louis, MO, USA) in 20 mL of 1% Triton X-100 (Sigma) and 0.1% of sodium citrate (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added and cells were incubated for 30 min on ice. Cells at different cell cycle phases were then analyzed with a flow cytometer (EPICS ELITE EPS, Beckman-Coulter, Hialeah, FL, USA) as previously described [3]. The effect of piceatannol on apoptosis in AH109A cells was assessed using Annexin-V-FITC kit (IMMUNOTECH, Marseille, France) according to the manufacturer's instructions. Phosphatidylserine (PS), which can specifically bind to Annexin-V, is one of the phospholipids in the cell membrane and exists predominantly in inner leaflet of the cell membrane of normal cells. When apoptosis occurs, PS in the cell membrane immediately appears on the outer leaflet of the cell membrane. The cells with PS on their surface can thus be thought to be early apoptotic cells. Briefly, 5×10^5 cells of AH109A per well were seeded in a 6-well plate and cultured in the medium containing 0, 50, and 100 μM piceatannol for 3 h. At the end of culture, cells were labeled with Annexin-V-FITC and analyzed with a flow cytometer as described previously [14].

2.6. Pretreatment of AH109A Cells with Hydrogen Peroxide. AH109A cells were cultured for 4 h in the absence or presence of 10 μM piceatannol with or without 25 μM hydrogen peroxide (H_2O_2, Wako Pure Chemical Industries). Hydrogen peroxide was employed as exogenous reactive oxygen species (ROS). AH109A cells were then washed once with 10% CS/MEM and seeded on the M-cell monolayer in 10% CS/MEM without piceatannol and ROS. After cultured for 24 h, invaded cells and colonies underneath M-cells were counted with a phase-contrast microscope as described previously.

2.7. Flowcytometric Analysis of Intracellular Peroxide in AH109A Cells. Intracellular peroxide levels in AH109A cells were assessed by flow cytometric analysis using a fluorometric probe (2′,7′-dichlorofluorescin diacetate; DCFH-DA, Molecular Probes, Eugene, OR) [15] with a flow cytometer as described previously [16].

2.8. In Vivo Effect of Piceatannol on Soild Tumor Growth and Metastasis. Male Donryu rats (3 weeks of age) were maintained on a stock pellet diet (CE-2, CLEA Japan, Tokyo, Japan) for 10 days. Starting 4 days prior to AH109A cell injections, the rats were fed AIN-93G basal diet containing 20% casein (Oriental Yeast Co., Ltd., Tokyo, Japan), 39.75% β-corn starch (Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan), 13.2% α-corn starch (Nihon Nosan Kogyo Co. Ltd.), 10% sucrose (Nihon Nosan Kogyo Co. Ltd.), 7% corn oil (Miyazawa Yakuhin Co. Ltd., Tokyo, Japan), 5% cellulose powder (Oriental Yeast Co., Ltd.), 3.5% mineral mixture

(AIN-93G composition, Nihon Nosan Kogyo Co. Ltd.), 1% vitamin mixture (AIN-93 composition, Nihon Nosan Kogyo Co. Ltd.), 0.3% L-cystine (Wako Pure Chemical Industries, Ltd.), and 0.25% choline bitartrate (Wako Pure Chemical Industries, Ltd.). Piceatannnol was given to rats by adding 0.001% and 0.005% to the basal diet at the expense of β-corn starch. Each rat was implanted subcutaneously on one site in the dorsal region with 1×10^5 AH109A cells suspended in PBS(−) (0.5 mL/rat) to produce a solid tumor, as described previously [5]. The size of each solid tumor was measured every day. The tumor size is expressed as the sum of three measured dimensions, that is, height, length, and width. On day 20, the rats were deprived of their diets at 9:00 but allowed free access to water until they were sacrificed at 13:00. Solid tumors were dissected and weighed. Metastatic tumor foci in lung and inguinal and axillary lymphatic nodes were macroscopically examined and excised, and the number of metastatic foci was recorded.

2.9. Statistical Analysis. Data were expressed as means ± SEM. Multiple comparison was performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Piceatannol on the Proliferation of Hepatoma Cells In Vitro and Ex Vivo. When added to medium, piceatannol dose-dependently and significantly reduced the proliferation of AH109A hepatoma cells. It commenced to decrease at 12.5 μM and continued to decrease up to 200 μM (Figure 1(A(a))). Of sera obtained from rats 1, 2, 3, and 6 hours after oral administration of piceatannol at a dose of 10 mg/kg body weight, serum obtained 2 hours after administration was found to show most prominent inhibitory effect against both the proliferation and invasion of AH109A hepatoma cells (data not shown). Thus, we prepared sera obtained from rats 2 hours after oral administration of piceatannol at doses 0, 2.5, 5, and 10 mg/kg body weight. These sera also reduced dose-dependently and significantly the proliferation of AH109A cells as seen in Figure 1(A(b)).

3.2. Effect of Piceatannol on the Cell Cycle in Hepatoma Cells. When added to medium, piceatannol was demonstrated to induce cell cycle arrest at G_2/M phase in AH109A cells at lower concentrations of 25 and 50 μM (Figures 1(B(b)) and 1(B(c))) as compared with the control (0 μM) (Figure 1(B(a))).

3.3. Effect of Piceatannol on the Apoptosis Induction in Hepatoma Cells. In contrast to the effect on cell cycle, piceatannol failed to induce apoptosis at lower concentration of 50 μM as compared with the control (Figures 1(C(a)) and 1(C(b))). However, it induced strong apoptosis at higher concentration of 100 μM (Figure 1(C(c))).

3.4. Effect of Piceatannol on the Invasion of Hepatoma Cells In Vitro and Ex Vivo. When added to medium, piceatannol dose-dependently and significantly reduced the invasion of AH109A hepatoma cells. It commenced to decrease at 12.5 μM and continued to decrease up to 200 μM (Figure 2(A(a))). Sera obtained from rats 2 hours after oral administration of piceatannol at doses 0, 2.5, 5, and 10 mg/kg body weight also reduced dose-dependently and significantly the invasion of AH109A cells as shown in Figure 2(A(b)).

3.5. Effect of Piceatannol on the Invasion of AH109A Cells Pretreated with Hydrogen Peroxide. To examine whether or not piceatannol would inhibit the invasion of tumor cells by its antioxidative activity, the invasion assay was performed with AH109A cells precultured in hydrogen peroxide- (ROS-) containing medium for 4 hours. As shown in Figure 2(B(a)), the invasive activity of AH109A cells precultured in ROS (25 μM hydrogen peroxide) was significantly higher than that of AH109A cells with no treatment. Piceatannol suppressed the ROS-potentiated invasive activity of the precultured cells when added to the medium at a concentration of 10 μM on preculturing the hepatoma cells with ROS. In our preliminary experiment, 12.5 μM of piceatannol exposure for 4 hours to AH109A cells exerted no influence on the following AH109A proliferation during 24 hours (data not shown). Thus, the concentration of 10 μM of piceatannol was adopted in this experiment to avoid an influence of the piceatannol-mediated proliferation inhibition on the AH109A invasion.

3.6. Intracellular Peroxide Levels of AH109A Cells Pretreated with Hydrogen Peroxide. As illustrated in Figure 2(B(b)), AH109A cells treated with 100 μM hydrogen peroxide for 4 hours contained more intracellular peroxides than did control cells when analyzed with a flow cytometer using DCFH-DA as an indicator (control versus H_2O_2). Piceatannol (10 μM) suppressed the rise in the intracellular peroxide levels of AH109A cells (H_2O_2 versus H_2O_2 and piceatannol).

3.7. Effect of Piceatannol on Sold Tumor Growth and Metastasis in Hepatoma-Bearing Rats. Dietary piceatannol (0.001% and 0.005%) tended to suppress the AH109A tumor size dose-dependently, although significant differences were not seen (Figure 3(A)). Accordingly, at the end of the 20-day treatment period, the weights of solid tumors were lower in the piceatannol-treated groups than in the control group (Figure 3(B)). The solid tumor weight of the 0.005% piceatannol group was significantly reduced from 20.5 ± 4.4 (control) to 9.4 ± 2.5 (0.005% piceatannol group) g/rat, indicating that *ca*. 54% reduction was attained by 0.005% piceatannol. Numbers of metastatic foci were 0.22, 0.2, and 0 (number/rat) in the control, 0.001%, and 0.005% piceatannol groups, respectively.

4. Discussion

In the present study, piceatannol was demonstrated to inhibit both the proliferation and invasion of AH109A hepatoma cells *in vitro*. Sera obtained from Donryu rats orally given piceatannol also inhibited both the proliferation and invasion of AH109A hepatoma cells *ex vivo*. In our previous

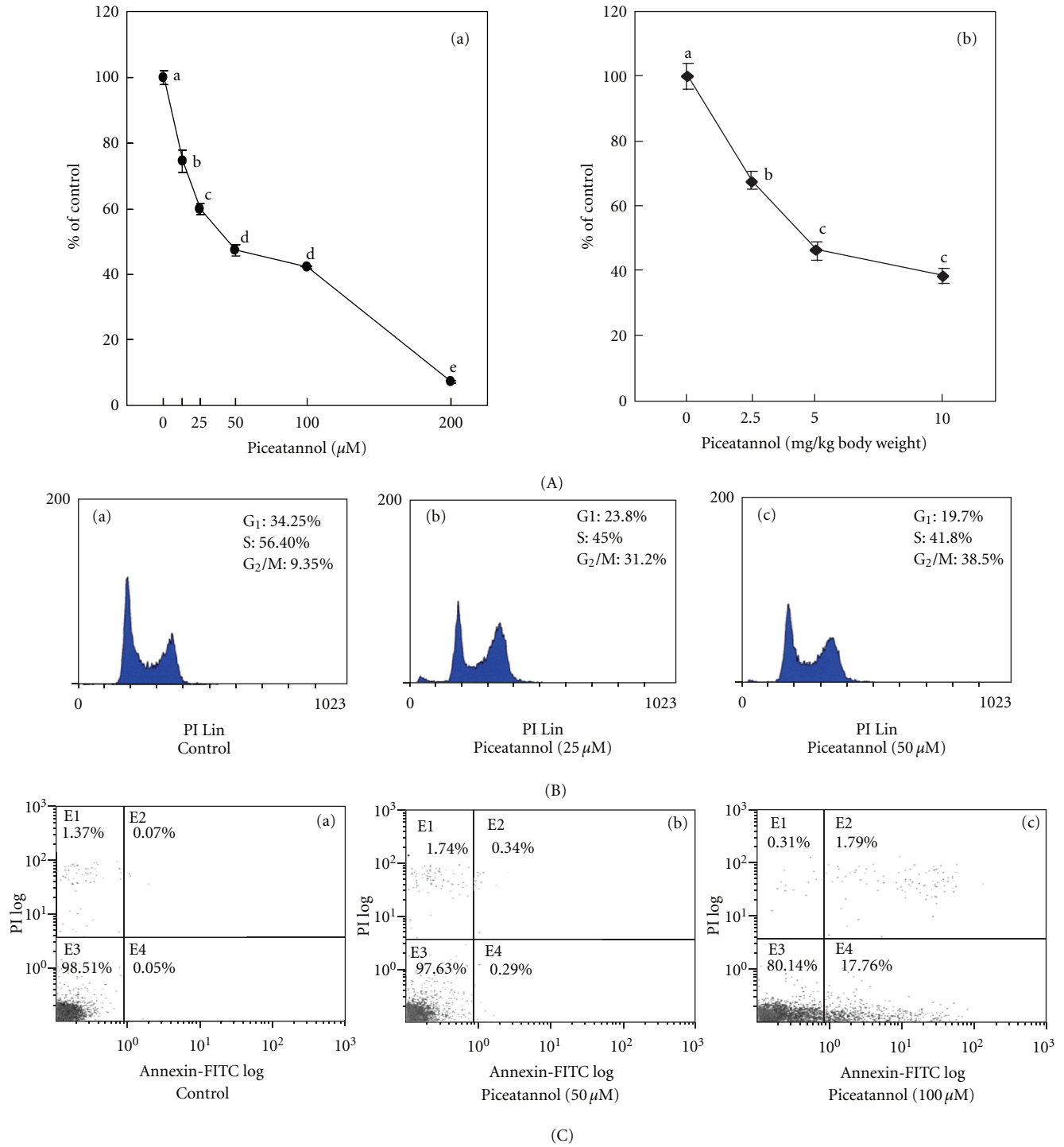

Figure 1: Effect of piceatannol on the proliferation (A), cell cycle (B), and apoptosis of AH109A hepatoma cells (C). (A) Piceatannol was dissolved in ethanol. The piceatannol solution was added to the culture medium at a final ethanol concentration of 0.2%. Piceatannol was suspended in a 0.3% carboxymethyl cellulose sodium salt (CMC) aqueous solution at concentrations of 0.25, 0.5, and 1 mg/mL. Piceatannol suspension or 0.3% CMC alone (vehicle control) (1 mL/100 g body weight) was intubated to male Donryu rats which had been fasted overnight, and blood was collected 2 h after oral administration. The proliferative activity was determined by [methyl-^{3}H]thymidine incorporation method *in vitro* (A(a)) and *ex vivo* (A(b)). Each value represents the mean ± SEM of six wells. Values not sharing a common letter are significantly different at $P < 0.05$. (B) For cell cycle analysis, 3.0×10^5 cells of AH109A per well were seeded in the medium containing 0, 25, and 50 μM piceatannol and cultured for 24 h. Cells were then collected and washed twice with PBS(−). Thereafter, propidium iodide (PI) solution was added and cells were incubated for 30 min on ice. Cells at different cell cycle phases were then analyzed with a flow cytometer. Data are from representative experiment repeated three times with similar results (B(a)–(c)). (C) The effect of piceatannol on apoptosis in AH109A cells was assessed using Annexin V-FITC kit according to the manufacturer's instructions. Briefly, 5×10^5 cells of AH109A per well were seeded in a 6-well plate and cultured in the medium containing 0, 50, and 100 μM piceatannol for 3 h. At the end of culture, cells were labeled with Annexin-V-FITC and analyzed with a flow cytometer. Data are from representative experiment repeated three times with similar results (C(a)–(c)).

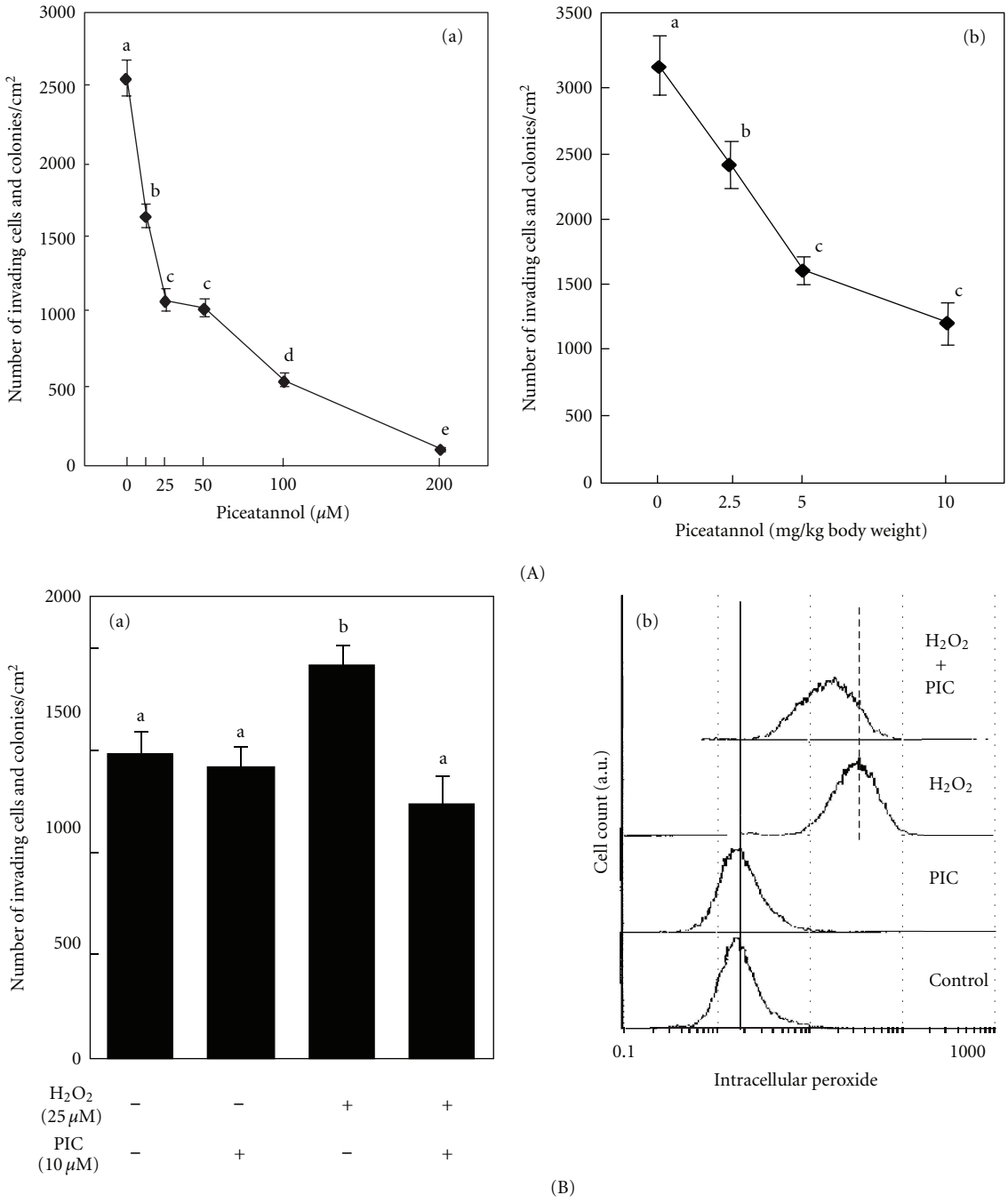

Figure 2: Effect of piceatannol on the invasion of AH109A hepatoma cells (A), reactive oxygen species- (ROS-) potentiated invasive activity and intracellular peroxide levels (B). (A) Piceatannol was dissolved in ethanol. The piceatannol solution was added to the culture medium at a final ethanol concentration of 0.2%. Piceatannol was suspended in a 0.3% carboxymethyl cellulose sodium salt (CMC) aqueous solution at concentrations of 0.25, 0.5, and 1 mg/mL. Piceatannol suspension or 0.3% CMC alone (vehicle control) (1 mL/100 g body weight) was intubated to male Donryu rats which had been fasted overnight, and blood was collected 2 h after oral administration. The invasive activity was determined by AH109A-M cell coculture method *in vitro* (A(a)) and *ex vivo* (A(b)). Each value represents the mean ± SEM of ten areas. Values not sharing a common letter are significantly different at $P < 0.05$. (B) AH109A cells were cultured for 4 h in the absence or presence of 10 μM piceatannol with or without 25 μM hydrogen peroxide (H_2O_2). AH109A cells were then washed once with 10% CS/MEM and seeded on the M-cell monolayer in 10% CS/MEM without piceatannol and ROS. After cultured for 24 h, invaded cells and colonies underneath M-cells were counted with a phase-contrast microscope. Each value represents the mean ± SEM of ten areas. Values not sharing a common letter are significantly different at $P < 0.05$ (B(a)). To evaluate the effect of piceatannol on intracellular peroxide levels, AH109A cells were treated with 100 μM H_2O_2 as ROS source. After ROS treatment for 1 hour, DCFH-DA was added, incubated for 20 minutes, and analyzed with a flow cytometer. Basal and ROS-potentiated intracellular peroxide levels are indicated as solid and dotted lines, respectively. Data are from representative experiment repeated three times with similar results (B(b)).

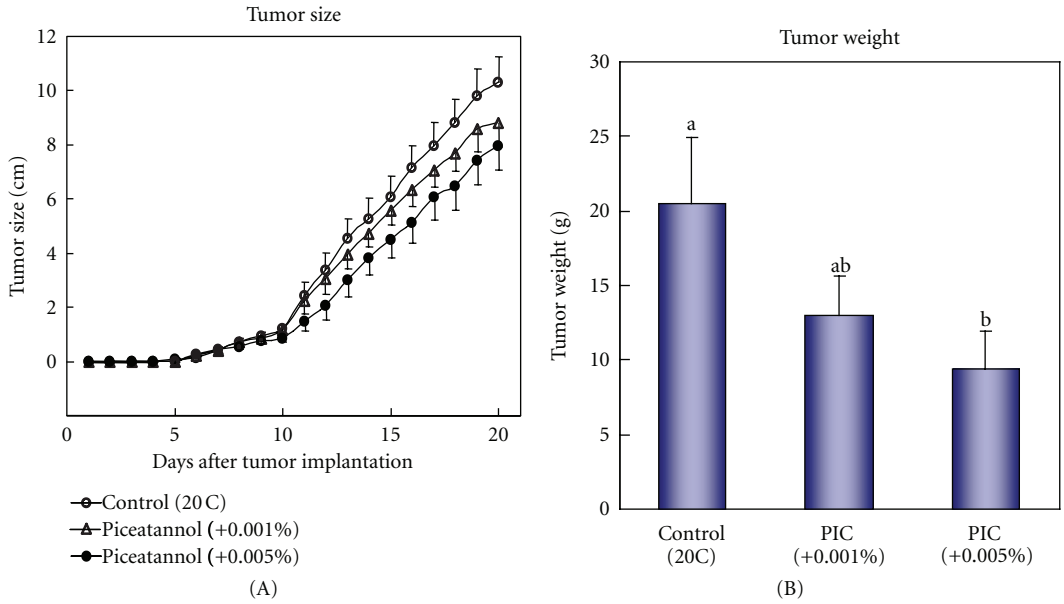

Figure 3: Effect of piceatannol on tumor size (A) and weight (B) of solid tumors in hepatoma-bearing rats. (A) Tumor size was determined in live animals by measuring the three-dimension sizes (height, length, and width). Each value represents the mean ± SEM of nine (control and +0.005% groups) and ten (+0.001% group) rats.

experiment, the sera obtained from Donryu rats orally given resveratrol completely lost the antiproliferative activity *ex vivo*, although its anti-invasive activity was preserved [8, 9]. These previous and present findings suggest that metabolism of resveratrol by intestinal flora, intestine itself, or in the liver may be different from that of piceatannol. Precise reasons for the difference between two stilbenoids, however, are unclear at present.

To elucidate the mechanisms for the inhibition of hepatoma cell proliferation by piceatannol, the effect of piceatannol treatment on cell cycle and on the rate of apoptosis was examined by using flow cytometry. Piceatannol was found to suppress the proliferation from lower concentrations (~ 25 μM) by arresting cell cycle of AH109A cells at G_2/M phase (Figure 1(B)). Furthermore, piceatannol increased the rate of apoptosis at higher concentrations (~100 μM) (Figure 1(C)). Dependencies of cell cycle arrest and apoptosis induction on piceatannol concentrations are similar to those on [6]-gingerol [11]. Although the doses of piceatannol which induced apoptosis were higher than those which induced cell cycle arrest in AH109A hepatoma cells, these results suggest that piceatannol affects both cell proliferation and cell death which at least in part account for the inhibitory effect of piceatannol on the proliferation of AH109A cells.

Our previous studies have demonstrated that the invasion of AH109A cells is accelerated by ROS [16, 17]. In the present study, we therefore examined the effect of piceatannol on the ROS-potentiated invasive activity using H_2O_2. Piceatannol was demonstrated to suppress the ROS-induced elevation of the AH109A invasion (Figure 2(B(a))). Piceatannol was also found to scavenge intracellular peroxide (Figure 2(B(b))). We have found that ROS can induce gene expression of hepatocyte growth factor (HGF), which is known as a cell motility factor [18], in M-cells as well as AH109A cells [9, 16]. Thus, HGF produced by AH109A and M-cells may potentiate the motility of AH109A cells, leading to acceleration of the invasion of AH109A cells. If piceatannol, like resveratrol [9], suppresses the production of HGF through antioxidative activity, piceatannol may reduce the motility of AH109A invasion, this leading to the reduction of the AH109A invasion. Although preliminarily, we have measured HGF secretion from AH109A cells by ELISA. Piceatannol was found to dose-dependently suppress the HGF secretion after exposure of AH109A to piceatannol for 1 hour or 24 hours at the concentration ranges of 25–100 μM (data not shown), suggesting an involvement of decreased HGF secretion in anti-invasive activity of piceatannol against AH-109A hepatoma cells.

Although not significant, piceatannol dose-dependently suppressed solid tumor size in hepatoma-bearing rats (Figure 3(A)). However, solid tumor weights, a most simple but reliable index for tumor growth, were significantly reduced by feeding the 0.005% diet (Figure 3(B)). In addition to *in vitro* efficacy, piceatannol is demonstrated to be effective *in vivo* in suppressing tumor growth. Antimetastatic effect of piceatannol was not clear *in vivo* in the present study, this being probably due to weak metastatic activity in the control group. Selection and use of highly metastatic AH109A cell clone will clarify the *in vivo* antimetastatic effect of piceatannol.

In summary, piceatannol was clearly demonstrated to suppress both the proliferation and invasion of AH109A hepatoma cells *in vitro* and *ex vivo*. Antiproliferative effect might be due to induction of cell cycle arrest and apoptosis in AH109A, while anti-invasive effect might be due to antioxidative property of piceatannol. As was expected, piceatannol

is shown to significantly suppress the solid tumor growth *in vivo*. These functions of piceatannol may be of significance from the aspects of both nutritional and pharmacological control of cancers.

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Research Article

Sesquiterpene Lactones Isolated from *Elephantopus scaber* L. Inhibits Human Lymphocyte Proliferation and the Growth of Tumour Cell Lines and Induces Apoptosis *In Vitro*

B. S. Geetha,[1] Mangalam S. Nair,[2] P. G. Latha,[3] and P. Remani[4]

[1] *Phytochemistry and Phytopharmacology Division, Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695 562, India*
[2] *Organic Chemistry Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, Thiruvananthapuram 695 019, India*
[3] *Ethnopharmacology Division, Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695 562, India*
[4] *Cancer Research Division, Regional Cancer Centre, Thiruvananthapuram 695 011, India*

Correspondence should be addressed to B. S. Geetha, dayal.geetha@gmail.com

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This study was designed to isolate the compounds responsible for the cytotoxic properties of South Indian *Elephantopus scaber* L. and further investigate their effects on quiescent and proliferating cells. Bioassay-guided isolation of the whole plant of chloroform extract of South Indian *Elephantopus scaber* afforded the known sesquiterpene lactone, deoxyelephantopin, and isodeoxyelephantopin whose structures were determined by spectroscopic methods. These compounds caused a dose dependent reduction in the viability of L-929 tumour cells in 72 h culture (IC_{50} value of 2.7 μg/mL and 3.3 μg/mL) by the cell viability assay. Both the compounds act selectively on quiescent and PHA-stimulated proliferating human lymphocytes and inhibited tritiated thymidine incorporation into cellular DNA of DLA tumour cells. The compound deoxyelephantopin at a concentration of 3 μg/mL caused maximum apoptotic cells. It also exhibited significant *in vivo* antitumour efficacy against DLA tumour cells. The results, therefore, indicate that the antiproliferative property of deoxyelephantopin and isodeoxyelephantopin could be used in regimens for treating tumors with extensive proliferative potencies.

1. Introduction

Plants have served as the major source of medication, for the treatment of human ailments since time immemorial. Plants have a long history of use in the treatment of cancer. The goal for the search for new anticancer drugs is to find drugs that act via a specific mode of action. In this manner, it is hoped that cancer cells can be targeted with little or no damage to noncancerous cells. Natural products are perfectly suited to the current molecular approach of drug development as they produce few adverse side effects, each inhibits multiple aspects of cancer cell proliferation and as a group show propensity for synergistic interaction [1].

Elephantopus scaber L. (Asteraceae) is a genus of herb, distributed all over the world, especially in America. Of the 32 species, only one species, namely, *Elephantopus scaber* is known to grow in India. Studies have shown that several species of the genus *Elephantopus* contain a large number of cytotoxic and antitumoural sesquiterpene lactones [2–4] and there is an interest in this plant over the world. Sesquiterpene lactones are characteristic constituents of the family Asteraceae and their principal characteristics are the presence of α, β-unsaturated γ-lactone [5].

Previous studies from our laboratory have shown that chloroform extract and the partially purified fraction of South Indian *E. scaber* exhibited cytotoxic as well as antitumour properties [6]. Inspired by the antitumour properties exhibited by *E. scaber*, the c hloroform extract was chosen to isolate the compounds responsible for the cytotoxic properties and further investigate their effects on quiescent and proliferating cells.

2. Methods

2.1. Experimental. All melting points are uncorrected and were determined on a Meltemp II hot stage melting point apparatus. The IR spectra were recorded on Bomem MB Series FT IR spectrometer. NMR spectra were recorded on Bruker 300 MHz FT NMR spectrometer, using $CDCl_3$ or $CDCl_3$-CCl_4 mixture (7 : 3) as solvent. TMS was used as the internal standard and chemical shifts are in δ-scale. Abbreviations used in ^{1}H NMR are s-singlet, d-doublet, dd-doublet of a doublet, and m-multiple. Analytical thin layer chromatography was performed on glass plates coated with silica gel (Merck) containing 13% calcium sulphate as binder. Column chromatography was done using 100–200 mesh silica gel. The solvents were removed from the fractions under reduced pressure using a Buchi rotary evaporator.

2.2. Animals. Swiss albino mice (males) in the age group of 8–10 weeks, weighing 20–30 g, were used for the experiments. The animals were housed under conventional laboratory conditions and fed with standard pellet diet (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. All experiments involving animals were done, strictly adhering to the guidelines for animal experimentation and handling, issued by the Government of India.

2.3. Chemicals. RPMI-1640 medium was obtained from Gibco BRL, USA, and the antibiotics streptomycin, penicillin, and gentamycin sulphate from Hi-Media Laboratories, Mumbai. Foetal calf serum, Trypsin, Ethidium bromide, Acridine Orange, Histopaque, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and vincristine were obtained from Sigma Chemical Company, USA. Phytohaemagglutinin (PHA-M) and Heparin were obtained from DIFCO, USA. The solvents used for the phytochemical analyses were LR grade, but except for final purification and spectroscopic studies, AR or spectroscopic grade solvents were used. Silica gel was obtained from Sisco Research Laboratories and Merck Laboratories, Mumbai. Tritiated thymidine was obtained from the Board of Radiation and Isotopes Technology (BRIT), Mumbai.

2.4. Tumour Cells. Murine fibroblast cell line, L-929 and Human colon carcinoma cell line, HCT 116 were grown in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamycin sulphate, in a humidified CO_2 incubator at 37°C. Daltons Lymphoma Ascitis (DLA) tumours were maintained as ascites by serial transplantation in mice, by intraperitoneal (i.p.) injection of 1×10^6 cells/mouse. The tumor cells were aspirated from the tumor-bearing mice aseptically and washed thrice in phosphate-buffered saline before transplantation.

2.5. Extraction. Fresh *E. scaber* plants, collected from TBGRI campus, Thiruvananthapuram in October 2002, after authentication by the plant taxonomist of the Institute was deposited in the Herbarium of the institute. The plants were cleaned and dried under shade for two days and 400 g of the powdered plant material was extracted with hexane (bp.60°C) using Soxhlet extractor for 12 h. The plant material was dried and again extracted with chloroform for 12 h. The extracts were concentrated under reduced pressure using rotary evaporator and afforded 9.1 g of hexane extract and 5.8 g of chloroform extract, respectively.

2.6. Preliminary Phytochemical Analysis. Several reactions for the identification of the chemical constituents present in the chloroform extract were carried out by the standard methods of Harborne [7].

2.7. Fractionation of $CHCl_3$ Extract. The $CHCl_3$ extract (5.8 g) was subjected to column chromatography on silica gel (60–120 mesh; 200 g). The column was eluted with hexane and gradient of hexane-EtOAc mixture and 50 fractions each of 100 mL were collected. The fractions were pooled according to similarities in thin-layer Chromatography (TLC).

2.8. Isolation of Isodeoxyelephantopin. Fraction 16–19 (473 mg), eluted with 15% EtOAc in hexane from the abovementioned column, was further purified by column chromatography using silica gel (60–120 mesh, 1.5 g). The column was eluted with hexane and hexane-EtOAc gradient mixture and 50 mL fractions were collected. The fractions eluted with 10% EtOAc in hexane afforded the pure compound.

2.9. Isolation of Deoxyelephantopin. Fraction 23–26 (425 mg) was purified by column chromatography using silica gel (60–120 mesh, 1.5 g). Low polar impurities were removed by eluting the column with 25% EtOAc in hexane and further elution with 40% EtOAc in hexane afforded a UV active compound.

2.10. Cytotoxicity Assay Using MTT Method. The *in vitro* response of deoxyelephantopin and isodeoxyelephantopin of *E. scaber* against L-929 cell line was studied using MTT assay [8]. L-929 cell line was maintained in RPMI-1640 medium, supplemented with 10% FCS. Briefly, cells harvested in the log phase of growth were harvested, counted, and seeded (4×10^4 cells/well in 500 μL volume) in 24-well titre plates. After 24 h of incubation at 37°C in 5% CO_2, to allow cell attachment, cultures were treated with varying concentrations of drugs (2–10 μg/mL) diluted with medium. Vehicle controls were also kept, that is, DMSO at concentrations not exceeding 1%. The plates were further incubated for 48 h. On completion of incubation, with the drugs, the plates were removed and to each well 20 μL of a 5 mg/mL stock solution of MTT were added and the plates were further incubated for 4 h. Then, the supernatant from each well was carefully removed and the MTT-formazan crystals were dissolved in 100 μL 100% DMSO and the absorbance at 570 nm was measured, using a spectrophotometer. Three replicate wells were set up for each experiment. For each drug, the concentration required to reduce absorbance by 50% (IC_{50}) in comparison to control cells was determined. IC_{50} values were derived by substituting the percentage inhibition values of

the drugs against the doses used. % growth inhibition = 100 − [(absorbance of the drug treated cells/absorbance of the untreated control cells) × 100%].

2.11. Effect of Deoxyelephantopin and Isodeoxyelephantopin on Quiescent and PHA-Stimulated Proliferating Human Lymphocytes

2.11.1. Lymphocyte Isolation. For culturing human peripheral blood lymphocytes, venous blood from healthy human volunteers was collected in heparinized tubes. Lymphocytes were separated from polymorphonuclear leukocytes and erythrocytes by Histopaque gradient method. The blood was diluted with 1 : 3 volume of PBS and layered onto 1 mL of Histopaque and centrifuged at 2,000 rpm for 20 min at room temperature. Lymphocytes were aspirated from the gradient plasma interfaces and washed twice in PBS and the final cell pellets were resuspended in RPMI-1640 medium containing 10% FCS, 100 μL penicillin, 100 μL streptomycin, and 100 μL fungicide (pH 7.4). Viability of the cells was checked by staining with 0.1% trypan blue.

2.11.2. Lymphocyte Proliferation Assay. Briefly, 200 μL of isolated lymphocytes (1×10^6/well) were seeded on 24-well titre plates and grown in 10% FCS containing RPMI-1640 medium at 37°C in 5% CO_2 in a humidified chamber. The cells were stimulated with 10 μL PHA (5 μg/mL) and wells without PHA served as positive control. To study the lymphocyte proliferation, in the absence of mitogen PHA, 10 μL of PHA was replaced by medium and served as negative control. Both PHA-stimulated and unstimulated lymphocytes were treated with varying concentrations of deoxyelephantopin and isodeoxyelephantopin (2–10 μg/mL) for 72 h. 18 h prior to termination, 2 μCi/mL [^{3}H] thymidine was added. The cells were then harvested using cell harvester and the radioactivity was measured using liquid scintillation counter. From the counts obtained (CPM), the percentage inhibition in thymidine incorporation was calculated. The percentage of incorporation was calculated as follows: % incorporation = [(CPM of treated group/CPM of control group)] × 100 % inhibition incorporation = 100 − % incorporation.

2.12. Effect of Deoxyelephantopin and Isodeoxyelephantopin on DNA Synthesis.

Thymidine labelling technique was used to evaluate DNA synthesis of Dalton's ascitic tumour cells (DLA) following drug treatment. Incorporation of tritiated thymidine into DNA was done by incubating DLA cells (1×10^6) with 2 μCi/mL of [^{3}H] labelled thymidine in the presence of various concentrations of drugs (2–10 μg/mL) in 2 mL RPMI-1640 medium containing 10% FCS at 37°C and 5% CO_2 atmosphere for 4 h. Control tubes were maintained without drugs. After incubation, the DNA was precipitated with 0.8 N perchloric acid and redissolved in 0.5 mL of NaOH (0.5 M) and cells harvested onto glass fiber discs, using a PHD cell harvester (Cambridge technology, Cambridge, MA). The glass fiber discs were counted in vials containing 3 mL scintillation-counting mixture, in a Wallac1410 β-liquid scintillation counter. From the counts obtained (CPM), the percentage inhibition in thymidine incorporation was calculated.

2.13. Morphological Analysis of Apoptotic Cells

2.13.1. Analysis of DLA Cell Death (Apoptosis) Using Fluorescence Microscopy. Cells were cultured in 24-well titre plates. 1×10^6 DLA cells were incubated in RPMI 1640 medium with 5% FCS containing various concentrations (1–10 μg/mL) of deoxyelephantopin in a CO_2 incubator at 37°C for 6 h and 18 h. For assessment of apoptosis, 6 h and 18 h after exposure to different concentrations of drug treatment, acridine orange-ethidium bromide dual staining of unfixed DLA cells was used. These dyes stain the DNA and allow visualization of the condensed chromatin of apoptotic cells. The medium was removed, cells pelleted gently, and 1 μL of acridine orange (100 μg/mL) + ethidium bromide (100 μg/mL) in 1 mL PBS was added to cells and immediately washed once with phosphate buffer saline (PBS) and resuspended in 10 μL of 10% glycerol in PBS and the slides analyzed by fluorescence microscopy (Nikon Diaphot, UV 410). The number of cells manifesting morphologic features of apoptosis such as chromatin condensation and loss of nuclear envelope [9] was counted as a function of the total number of cells present in the field. For cell death evaluation, three samples were prepared for each experimental point and the experiment was repeated three times.

2.13.2. Analysis of HCT 116 Cell Death (Apoptosis) Using Fluorescence Microscopy. 2×10^4/well HCT 116 human colon cancer cell line was seeded into each well of 24 titre-well plastic culture plate containing 12 mm cover slip. After 24 h, the cells were incubated with varying concentrations of deoxyelephantopin (1–10 μg/mL). After 48 h, the cover slip was carefully removed from the medium and washed twice with PBS and 1 μL of acridine orange (100 μg/mL) + ethidium bromide (100 μg/mL) in 1 mL PBS was added to cells. The cells were immediately washed once with PBS and suspended in 10 μL of 10% glycerol in PBS and viewed under fluorescence microscopy (Nikon Diaphot, UV 410) and apoptosis evaluated.

2.14. Effect of Deoxyelephantopin on the Ascitic Tumour Reduction in Mice.

The antitumour activity of deoxyelephantopin was determined, as described previously [10]. Briefly, Swiss albino mice (20–25 mg) were challenged with DLA cells (1×10^6 cells) and divided into 5 groups of 10 each. After 24 h, animals of group I serving as control were given 0.5 mL of 10% DMSO. The group II, III, and IV received the deoxyelephantopin 25 mg, 50 mg, and 100 mg/kg, b.w., i.p, respectively, and group V received vincristine (1 mg/kg b.w, i.p). Vincristine was used as a standard anticancer agent for comparison. The administration of deoxyelephantopin and vincristine was continued intraperitoneally for 15 days. Growth inhibitory effect of DLA tumour cells implanted into the peritoneal cavity was evaluated by determination of animal survival, recorded and expressed as mean survival time (MST)

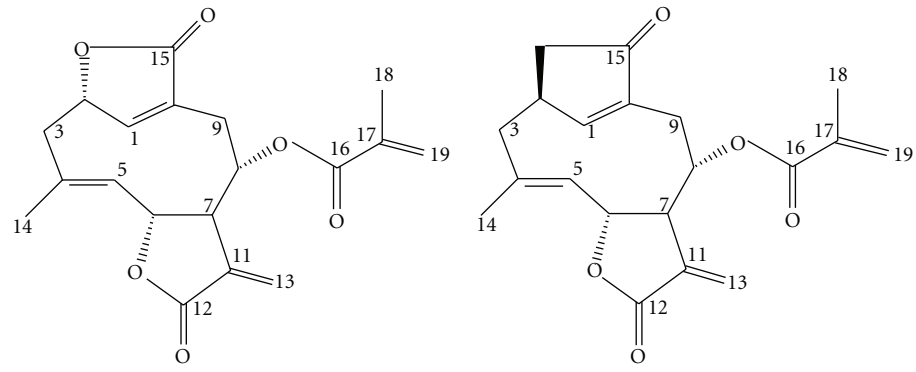

Figure 1: (a) Structure of isodeoxyelephantopin, (b) structure of deoxyelephantopin.

in days, and the percentage increase in life span % ILS) of drug treated group was determined.

2.15. Statistical Analysis. Experimental data were expressed as mean ± S.D. Student's "*t*" test was applied for expressing the significance and *P* value less than 0.05 was considered as significant.

3. Result

The preliminary phytochemical analysis of the chloroform extract revealed the presence of terpenes, flavonoids, coumarins, and alkaloids.

The extract upon fractionation using column chromatography and further purification by rechromatography yielded two UV active sesquiterpene dilactones viz: isodeoxyelephantopin and deoxyelephantopin**.** Isodeoxyelephantopin (90 mg) was crystallized from petroleum ether- EtOAc mixture as colourless needles (mp. 151–153°C, Lit.150–153°C).

3.1. Isodeoxyelephantopin. IR (cm^{-1}): 3075, 2968, 2935, 1765, 1750, 1714, 1638, 1444, 1320, 1155, 983, 951, 886, 815.

^{1}H NMR: 7.16 (s, 1H), 6.20 (d, 1H, J = 3.7 Hz), 6.15 (s, 1H), 5.68 (s, 1H), 5.65 (d, 1H, J = 3.3 Hz), 5.38 (d, 1H, J = 3.9 Hz), 5.21–5.10 (m, 2H), 4.55–4.49 (m, 1H), 3.17–2. (m, 3H), 2.75 (dd, 1H, J = 3.6 Hz, 12.6 Hz), 1.93 (s, 3H), 1.79 (s, 3H).

^{13}C NMR: 174.4, 169.5, 166.6, 149.5, 135.5, 135.5, 134.1, 131.5, 126.9, 125.4, 123.2, 79.5, 78.8, 74.1, 49.9, 40.1, 30.1, 21.6, 18.2.

From the data, the structure of the compound was assigned as isodeoxyelephantopin (Figure 1(a)). The structure was confirmed by the spectral data and melting point of the compound [11, 12].

Purification of another pool of chloroform fractions by repeated chromatography afforded another UV active sesquiterpene dilactone, deoxyelephantopin (80 mg). This compound was crystallized from petroleum ether-EtOAc mixture as colourless needles (mp. 199–200°C, Lit. 196–199°C).

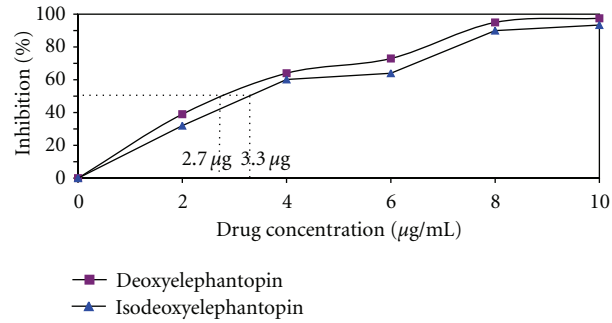

Figure 2: Percentage inhibition of deoxyelephantopin and isodeoxyelephantopin against L-929 tumour cell line using MTT assay.

3.2. Deoxyelephantopin. IR (cm^{-1}): 3100, 2962, 1751, 1770, 1707, 1632, 1432, 1295, 1201 1170, 927.

^{1}H NMR: 7.11 (s, 1H), 6.22 (d, 1H, J = 3.5 Hz), 6.14 (s, 1H), 5.65 (m, 2H), 5.46 (d, 1H, J =1.7 Hz), 5.14 (dd, 1H, J = 8.2 Hz, 10.2 Hz), 4.78 (d, 1H, J = 10.3 Hz), 4.65 (m, 1H), 3.03–2.68 (m, 5H), 1.93 (s, 3H), 1.84 (s, 3H).

^{13}CNMR: 172.4, 169.3, 166.3, 153.3, 135.9, 135.6, 134.1, 133.7, 128.5, 81.3, 77.9, 71.5, 52.3, 41.3, 33.5, 20.1, 18.2.

From the data, the structure was assigned as deoxyelephantopin (Figure 1(b)). The structure was confirmed by comparing the spectral values and melting point of the compound (13, 12).

3.3. Cytotoxicity Using L-929 Tumour Cell Line. The deoxyelephantopin and isodeoxyelephantopin showed a dose-dependent cytotoxicity to L-929 cell line in culture. The sesquiterpene lactones, deoxyelephantopin, and isodeoxyelephantopin exhibited maximum cytotoxicity, having an IC_{50} value of 2.7 μg/mL and 3.3 μg/mL, respectively (Figure 2).

3.4. Lymphocyte Proliferation. To ascertain whether deoxyelephantopin and isodeoxyelephantopin specifically inhibit the proliferation of dividing cells, their effect was assessed on unstimulated and PHA-stimulated human lymphocytes

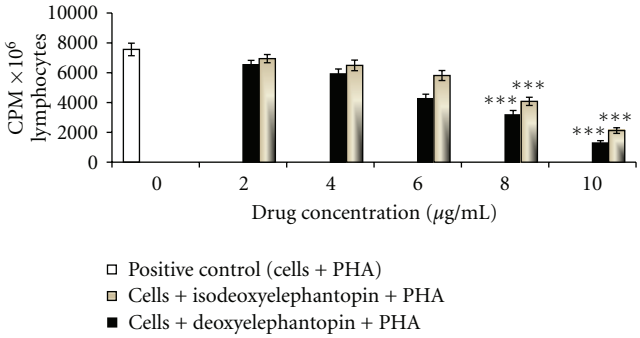

Figure 3: Influence of deoxyelephantopin and isodeoxyelephantopin on proliferative responses to the PHA-stimulation of normal lymphocytes. Values are ± SD of three replicates of three independent experiments, $^{***}P < 0.001$.

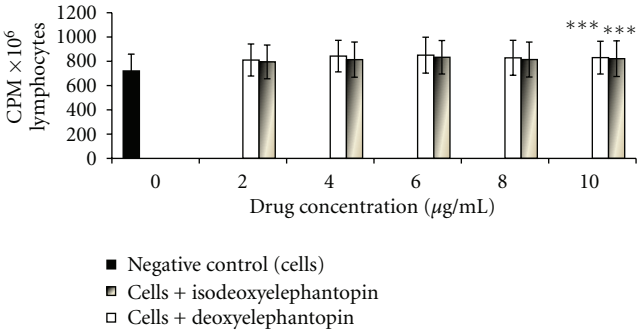

Figure 4: Influence of the deoxyelephantopin and isodeoxyelephantopin on proliferative responses in absence of mitogen PHA. Values are ± SD of three replicates of three independent experiments, $^{***}P < 0.001$.

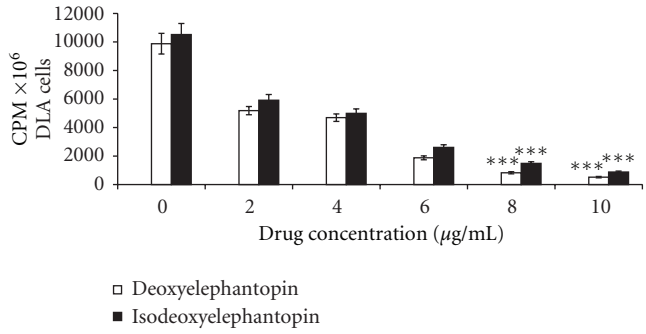

Figure 5: Effect of deoxyelephantopin and isodeoxyelephantopin on DNA synthesis of DLA tumour cells. Values are ± SD of three replicates of three independent experiments, $^{***}P < 0.001$.

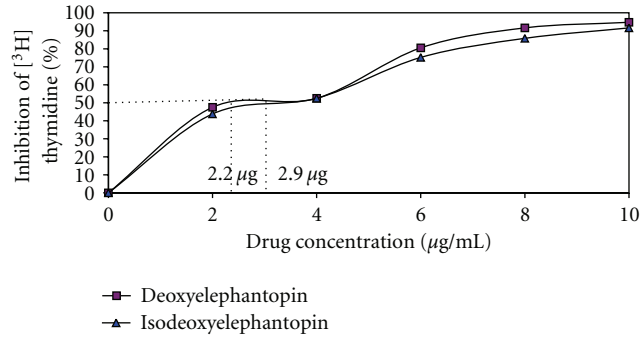

Figure 6: Effect of deoxyelephantopin and isodeoxyelephantopin on percentage inhibition of DNA synthesis of DLA tumour cells.

in culture. Deoxyelephantopin and isodeoxyelephantopin significantly decreased the [^{3}H] thymidine incorporation of proliferating (PHA-stimulated) lymphocytes in a dose-dependent manner, when compared to the positive control (Figure 3). However, there was no significant change in the proliferation of unstimulated lymphocytes exposed to deoxyelephantopin and isodeoxyelephantopin (Figure 4).

3.5. Inhibition of DNA Synthesis. DLA cells treated both with deoxyelephantopin and isodeoxyelephantopin showed decreased [^{3}H] thymidine incorporation into the nuclear DNA, suggesting that they inhibited DNA synthesis. The results on DNA synthesis also indicated a concentration-dependent decrease in [^{3}H] thymidine incorporation (Figure 5). The concentration of deoxyelephantopin and isodeoxyelephantopin required to produce 50% in-incorporation was 2.2 μg/mL and 2.9 μg/mL, respectively (Figure 6).

3.6. Morphological Analysis of Apoptotic Cells. The percentages of viable and nonviable (apoptotic) DLA cells after *in vitro* exposure to various concentrations of deoxyelephantopin with acridine orange and ethidium bromide staining during 6 h and 18 h treatment period are presented in Table 1. DLA cells exposed to 2 μg/mL deoxyelephantopin showed reduction in the percentage of viable cells, but the cells exposed to 3 μg/mL of deoxyelephantopin exhibited a significant reduction in the percentage of viable cells (52.63% and 70.65%), compared to the control group during 6 h and 18 h treatment period, respectively. The higher concentrations of drug such as 8 μg/mL and 10 μg/mL showed necrosis, which is characterized by swelling of cytoplasm and organelles, which are associated with membrane lysis, instead of apoptosis. The proportion of apoptotic cells was higher during 18 h treatment period.

During 48 h treatment period, HCT-116 colon cancer cell line stained with ethidium bromide and acridine orange exhibited a significant reduction in the percentage of viable cells (77.08%) and increase in percentage of apoptotic cells when exposed to 3 μg/mL deoxyelephantopin compared to the control group (Table 2). Deoxyelephantopin caused shrinkage after 24 h in HCT-116 cells and the shrinkage was prominent after 48 h duration of exposure due to cytoplasmic condensation, where most of the cells rounded up (dead cells).

3.7. Effect of Deoxyelephantopin on the Ascitic Tumour Reduction in Mice. The deoxyelephantopin exhibited remarkable antitumour activity. Administration of deoxyelephantopin (50 mg/kg, b.w and 100 mg/kg b.w) significantly reduced murine ascitic tumour growth and increased the life span of DLA tumour-bearing mice by 67.64% and 72.51%,

Table 1: Apoptosis (percentage of cells) of DLA cells treated with deoxyelephantopin as determined by microscopic morphological observation, stained with acridine orange and ethidium bromide.

Con. of deoxyelephantopin (μg/mL)	Treatment time (6 h) Percentage of cells[x] (%)			Treatment time (18 h) Percentage of cells[x] (%)		
	Viable cells	Non-viable cells		Viable cells	Non-viable cells	
		Apoptotic	Necrotic		Apoptotic	Necrotic
0 (control)	95 ± 4.2	—	5 ± 0.25	92 ± 4.5	2 ± 0.28	6 ± 0.49
1	68 ± 3.8	26 ± 1.71	6 ± 0.40	61 ± 4.02	30 ± 2.15	9 ± 0.56
3	45 ± 2.15	39 ± 2.79***	16 ± 1.72	27 ± 1.93	46 ± 3.29***	27 ± 1.85
6	29 ± 1.78	30 ± 1.68	41 ± 2.56	20 ± 1.37	35 ± 2.4	45 ± 4.04
8	15 ± 0.87	9 ± 0.52	76 ± 5.22	13 ± 1.17	5 ± 0.45	82 ± 5.12
10	12 ± 0.67	9 ± 0.55	79 ± 4.86	10 ± 0.90	6 ± 0.37	84 ± 7.55

[x] cells (viable + nonviable) = 100%, *** $P < 0.001$ compared to control.

Table 2: Apoptosis (percentage of cells) of HCT-116 colon cancer cells treated with deoxyelephantopin as determined by microscopic morphological observation, stained with acridine orange and ethidium bromide.

Con.of deoxy elephantopin (μg/mL)	Treatment time (24 h) Percentage of cells[x] (%)			Treatment time (48 h) Percentage of cells[x] (%)		
	Viable cells	Non-viable cells		Viable cells	Non-viable cells	
		Apoptotic	Necrotic		Apoptotic	Necrotic
0 (control)	97 ± 4.4	—	3 ± 0.25	96 ± 4.6	2 ± 0.15	1 ± 0.10
1	59 ± 4.33	28 ± 2.11	13 ± 0.87	55 ± 2.79	31 ± 2.1	14 ± 0.97
3	30 ± 1.6	39 ± 2.6***	31 ± 2.79	22 ± 1.52	42 ± 2.5***	36 ± 1.98
6	20 ± 1.51	32 ± 2.41	48 ± 3.25	18 ± 1.24	30 ± 1.9	52 ± 3.59
8	13 ± 0.35	6 ± 0.44	81 ± 4.11	12 ± 0.81	4 ± 0.28	84 ± 4.27
10	9 ± 0.60	7 ± 0.47	84 ± 5.8***	9 ± 0.46	4 ± 0.26	87 ± 5.5

[x] cells (viable + nonviable) = 100%, *** $P < 0.001$ compared to control.

respectively (Table 3). The higher dose of deoxyelephantopin (100 mg/kg, b.w, i.p) was found to be very effective when compared to the other two doses and afforded the protection of 6 animals out of 10 DLA-challenged mice. In the case of control animals treated with DMSO, the tumour grew progressively and the survival time was significantly reduced. The results were comparable with those of standard anticancer drug, vincristine.

4. Discussion

The MTT cell viability assay is widely used in determining drug sensitivity in primary screening of potential chemotherapeutic drugs. The isolated pure compounds belonging to the class of sesquiterpene lactones, deoxyelephantopin, and isodeoxyelephantopin caused a significant dose-dependent reduction in the viability of L-929 tumour cells in 72 h culture. The structural difference between deoxyelephantopin and isodeoxyelephantopin is that γ-lactone ring oxygen atom at C-2 is β-oriented in deoxyelephantopin whereas is α-oriented in deoxyelephantopin. Kupchan et al. [13] have shown that the presence of C_{11}-C_{13} exocyclic double bond conjugated to the γ-lactone function which can undergo a Michael addition is essential for cytotoxicity. Sesquiterpene which incorporated a cyclopentenone or a methylene lactone (in addition to the α, β methylene-γ lactone) appeared to produce enhanced cytotoxicity. The success of deoxyelephantopin and isodeoxyelephantopin that possess C_{11}-C_{13} exocyclic methylene conjugated to γ-lactone, to exhibit significant cytotoxicity to tumour cell line, is in agreement with these suggestions. These findings are in agreement with the studies of sesquiterpene lactones, helenain possessing both the reactive α-methylene-γ-lactone moiety, and a reactive α, β-unsubstituted cyclopentenone ring, displaying the strongest cytotoxicity against tumour cell lines using MTT assay [14].

Lymphocyte proliferation *in vitro* is usually determined by measuring [^{3}H] thymidine uptake by nuclear DNA after 72 h of culture. Mitogen stimulated cells commence DNA synthesis approximately 24 h after mitogen addition. Maximum thymidine incorporation occurs during 60–72 h time period and thus 72 h culture has been selected in the present study. The compounds, deoxyelephantopin and isodeoxyelephantopin, failed to augment human T-lymphocyte proliferation, induced by T-mitogen PHA as evidenced by decreased [^{3}H] thymidine uptake by nuclear DNA. Quiescent lymphocytes were refractory to the action of deoxyelephantopin and isodeoxyelephantopin.

It has been reported that cleistanthin A, a diphyllin glycoside from *Cleistanthus collinus* possessing cytotoxic and tumour regressing properties, was cytotoxic to PHA-stimulated proliferating human lymphocytes [15]. The effects of

Table 3: Effect of intraperitoneal administration of deoxyelephantopin on the life span of DLA-tumour-bearing mice.

Treatments	Number of mice survived after tumour inoculation (days)			Mean survival time (days)	Life span (%)	Increase in life span (%)
	15	25	35			
Group I DLA (Control)	10/10	5/10	0/10	22.56 ± 2.57	100	—
Group II DLA + Deoxy-elephantopin (25 mg/kg, i.p)	10/10	6/10	3/10	30.90 ± 3.36	136.96	36.96
Group III DLA + Deoxy-elephantopin (50 mg/kg, i.p)	10/10	8/10	5/10	37.82 ± 3.70	167.64	67.64
Group IV DLA + Deoxy-elephantopin (100 mg/kg, i.p)	10/10	8/10	6/10	38.92 ± 3.5***	172.51	72.51
Group V DLA + Vincristine (1 mg/kg, i.p)	10/10	10/10	6/10	43.54 ± 3.9	192.99	92.99

Values are mean ± SD of three experiments, $n = 10$, $^{***}P < 0.001$ compared to control (the time period required for the death of all the animals in the control group was 22).

resveratrol on the G_0-G_1 transition and cell cycle progression of mitogenically stimulated human lymphocytes have cancer-preventive properties [16]. A compound is judged as a good anticancer drug, when it is more specific towards tumour cells in comparison to normal cells. The results of the present study indicate that the sesquiterpene lactones, deoxyelephantopin, and isodeoxyelephantopin are not cytotoxic to normal human lymphocytes and only the proliferating cells were affected.

The site of action of a drug can be evaluated by [^{3}H] thymidine incorporation studies. The [^{3}H] thymidine incorporation studies revealed that the deoxyelephantopin and isodeoxyelephantopin inhibited DNA synthesis as seen by decreased incorporation of thymidine. The incorporation of [^{3}H] thymidine into DNA was high in the control than the drug-treated cultures. The growth of DLA tumour cells is due to uncontrolled cell division and the basic fact underlying this phenomenon should be enhanced DNA synthesis. Anticancer drugs exercise their effects through a variety of mechanism of action. It may inhibit stages/stages in the multiplication of DNA and/or in the synthesis of proteins. Since DNA synthesis was inhibited, it meant the compounds exerted cytotoxic effect on tumour cells. The radioactivity incorporated is inversely proportional to the concentration of the drug used. The rate of incorporation depends on the type of plant drug, the concentration of radioactive compound, and the type of cells used. Thus, [^{3}H] thymidine incorporation studies indicate the possible mechanism of action of deoxyelephantopin and isodeoxyelephantopin used at the DNA level. These findings are in agreement with the studies of sesquiterpene lactones with the exocyclic methylene groups from *Helianthus annuus* that play an important role in triggering the inhibitory effect of DNA and RNA synthesis as evident by measuring ^{14}C-labelled thymidine, uridine, and leucine incorporated into murine cells of the ascitic Ehrlich carcinoma (EAC) [17].

Analysis of cell death (apoptosis) using fluorescence microscopy indicates that maximum apoptotic cells were obtained at a concentration of 3 μg/mL of deoxyelephantopin that can be considered as optimum dose for inducing apoptosis both in DLA cells and HCT-116 cancer cells. The cytotoxic sesquiterpene lactones mediate their death-inducing effect in leukaemia T cells by triggering apoptosis [18]. The involvement of apoptosis is one of the mechanisms of cell death, induced by clinically relevant chemotherapeutic drugs in human leukemic T-lymphocytes [19]. Hence, in the present study, it is possible to confirm apoptosis as one of the methods of antitumour activity, since results of the study indicate that deoxyelephantopin, a sesquiterpene lactone isolated from *E. scaber* induces apoptosis in DLA tumour cells and HCT-116 colon cancer cells.

The deoxyelephantopin administered after DLA transplantation reduced the ascitic tumour growth as evidenced from percentage increase of life span of treated mice. This indicated the cytotoxic effect of deoxyelephantopin on tumour cells and its *in vivo* efficacy against DLA tumour cells is equal to vincristine (1 mg/kg, b.w). Although deoxyelephantopin (25 mg/kg, i.p.) evoked antitumour response *in vivo*, significant antitumoureffect was noted with higher doses of deoxyelepantopin (50 mg/kg, i.p and 100 mg/kg, i.p) exhibiting no toxic effects. Increase in life span is a reliable criterion for judging the value of any anticancer drug. Hence, the increase in life span of DLA tumour-bearing mice by treatment with deoxyelephantopin is a positive finding and supports the antitumour effect of the drug.

Our results, therefore, indicate that the antiproliferative property of deoxyelephantopin and isodeoxyelephantopin could be used in regimens for treating tumors with extensive proliferative potencies.

Declaration of Interest

The authors declare no conflict of interest.

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Research Article

The Isolation of a New *S*-Methyl Benzothioate Compound from a Marine-Derived *Streptomyces* sp.

Nor Ainy Mahyudin,[1,2,3] John W. Blunt,[4] Anthony L. J. Cole,[2] and Murray H. G. Munro[4]

[1] *Faculty of Food Science and Technology, Universiti Putra Malaysia, Selangor, 43400 Serdang, Malaysia*
[2] *School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand*
[3] *Institute of Bioscience, Universiti Putra Malaysia, Selangor, 43400 Serdang, Malaysia*
[4] *Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand*

Correspondence should be addressed to Nor Ainy Mahyudin, norainy@food.upm.edu.my

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The application of an HPLC bioactivity profiling/microtiter plate technique in conjunction with microprobe NMR instrumentation and access to the AntiMarin database has led to the isolation of a new **1**. In this example, **1** was isolated from a cytotoxic fraction of an extract obtained from marine-derived *Streptomyces* sp. cultured on Starch Casein Agar (SCA) medium. The 1D and 2D ^{1}H NMR and ESIMS data obtained from 20 μg of compound **1** fully defined the structure. The known **2** was also isolated and readily dereplicated using this approach.

1. Introduction

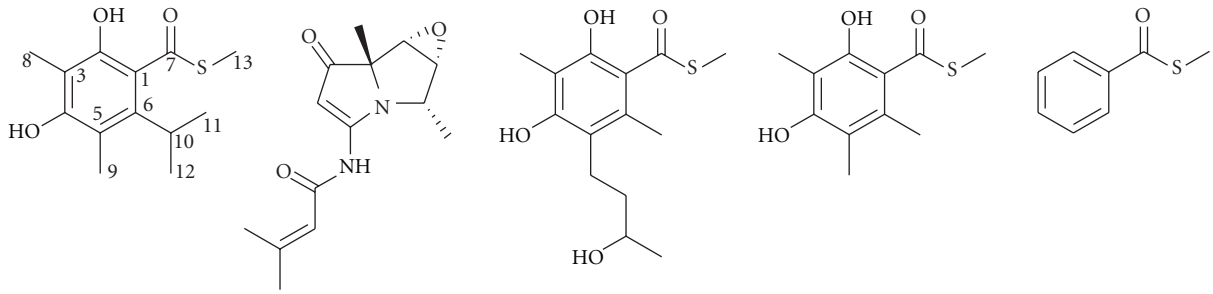
The HPLC bioactivity profiling/microtiter plate technique in conjunction with microprobe NMR instrumentation and access to the AntiMarin database [1] has been utilized by our group as a tool to enhance dereplication as well as to obtain a rapid NMR data acquisition for characterization of new metabolites by using less than 50 μg of purified material. The sensitivity of the technique to enhance structural elucidation by using only small amounts of a natural product has been described [2–10]. In our continuing efforts to rapidly characterize new bioactive metabolites, a marine-derived *Streptomyces* sp. was investigated for its bioactivity and chemical properties. Herein we report the structure of a new **1** by using 20 μg of material.

The first *S*-methyl benzothioate group of metabolites, **3**, was produced in a broth culture of *S. collinus* [11, 12]. *S. collinus* remained the only reported producer of this unusual structural type until a comparable structure, **4**, was identified from a sclerotium-colonizing isolate of the fungus, *Mortierella vinacea* [13]. The production of **5** was also reported from a marine *Streptomyces* sp. [14] and, recently, from *Phaeobacter gallaeciensis* and *Oceanibulbus indolifex* [15]. To date, **3**, **4**, and **5** are the only secondary metabolites reported for the *S*-methyl benzothioate group of metabolites (see Scheme 1).

2. Materials and Methods

2.1. General Experimental Procedures. NMR spectra were recorded on a Varian INOVA AS-500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, resp.), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 3.3 and δ_C 49.3 ppm for CD_3OD). A Protasis CapNMR microprobe was used for the microplate dereplication studies. HRESIMS were acquired using a Micromass LCT TOF mass spectrometer. MS/MS experiments were performed on a Bruker Daltonics Esquire 4000 system. Solvents used for extraction and isolation were distilled prior to use. Bioactivity assays were made using standard protocols [16, 17].

2.2. Isolation and Cultivation of Isolate. Streptomyces sp. was isolated from an unidentified tunicate collected from Lyttelton Harbour, New Zealand, in May 2004, using adapted



1 **2** **3** **4** **5**

Scheme 1

isolation techniques [18] on SCA medium. Fermentations were carried out on 60 plates of SCA for 30 days at 28°C. The isolate (LA3L2, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand) was identified by its cultural and microscopic characteristics. For the chemical study, the isolate was grown on SCA medium for 30 days at 28°C (60 plates; 20 mL). The combined agar was macerated with EtOAc and the EtOAc removed and concentrated under reduced pressure to give the crude extract (45.7 mg).

2.3. Evaluation of Extracts. The crude extract was fractionated to isolate the compounds. The extract was initially defatted with petroleum ether (Pet. Ether) yielding 21.6 mg of Pet. Ether layer (Fraction 1), and further partitioning with H_2O and EtOAc (1 : 1) resulted in 1.4 mg H_2O layer (Fraction 2) and 22.7 mg EtOAc layer (Fraction 3). Fraction 3 was further chromatographed on HPLC to obtain pure **2** and a subfraction 3a. Aliquots of subfraction 3a ($1 \times 750\,\mu$g; $2 \times 500\,\mu$g) were analyzed by HPLC (RP-18, solvents: (A) H_2O + 0.05% TFA, (B) MeCN; gradient: 0 min 10% B, 2 min 10% B, 14 min 75% B, 24 min 75% B, and 26 min 100% B; flow: 1 mL/min; 40°C. The eluent from the DAD was split in a 1 : 10 ratio between the ELSD and the fraction collector configured to collect into a 96-well microtiter plate (15 s/well). A total of 88 wells were collected (2.5– 24.5 min). A daughter plate was prepared by transferring an aliquot (5 μL) from each well of the master plate. After complete evaporation of the solvent, the wells in the daughter plate were analyzed for activity against P388 murine leukemia cells as described previously [16, 17].

The assay established that cytotoxicity was correlated with the peak observed by HPLC/ELSD/UV. The well F10 of the dried master plate, containing the bioactive **1** was analyzed using capillary probe NMR spectroscopy. The content of well F10 was dissolved in CD_3OD (7 μL) and transferred into the Protasis CapNMR microprobe. Calibrations have shown that this effectively transfers 6 μL of sample into the probe. Standard operating conditions were used to acquire 1D and 2D NMR spectra. The quantity of the compound was estimated according to the formula:

$$\left(\frac{\mathrm{MW}}{{}^{\#}\mathrm{H}}\right) \times \frac{(\text{total integral for } {}^{\#}\mathrm{H})}{(\text{integral for } \mathrm{CHD_2OD})} \times \mathrm{CF}, \qquad (1)$$

where MW is the actual molecular weight of the compound (ESMS), or an estimated value, #H is the number of protons included in the integration of the ^{1}H NMR spectrum, and CF is the calibration factor that had previously been determined from a standard solution containing quinine (30 μg in 6 μL) in the same CD_3OD solvent.

*2.4. Isolation of **1**.* The crude extract (45.7 mg) was subjected to a reversed phase semipreparative column chromatography (Phenomenex Luna C18, 10 × 250 mm, 5 μm, 1 mL/min, 40°C, gradient: 20–40% acetonitrile in 0.05% TFA in H_2O over 40 min, monitored by UV absorption at 215 nm) to yield 1.0 mg of **2** and 2.3 mg of the cytotoxic fraction, containing **1**. **2** was readily dereplicated as bohemamine using the described technique [2].

An aliquot (750 μg) of the cytotoxic fraction was injected on to the HPLC, and the fractions were collected into a microtitre plate to yield **1** (4 μg; R_t15.5 min). In the second attempt, an aliquot (up to 1000 μg) of the cytotoxic fraction was injected on to the HPLC and the fractions were collected into a microtitre plate to yield reasonably pure **1** (20 μg; R_t15.5 min).

S-methyl 2,4-*dihydroxy*-6-*isopropyl*-3,5-*dimethylbenzothioate,* **1**: light brown solid; UV (MeOH) λ_{max} 207, 227, 283; for ^{1}H NMR data and 1D and 2D spectra, see Table 1 and supporting information; HREIMS obsd, $[M+H]^+$ at *m/z* 255.1043 (calcd for $C_{13}H_{19}O_3S$, 255.1055).

3. Results and Discussion

Streptomyces sp. was obtained from liquid portions of a New Zealand marine tunicate and grown on Starch Casein Agar medium (60 plates) for 30 days at 28°C. Extraction with EtOAc yielded 45.7 mg of crude extract. This extract showed cytotoxic activity in a P388 assay (IC_{50} 383 μg/mL). Analysis by reverse-phase C_{18} analytical HPLC revealed three main peaks, one major and two minor. The result from the HPLC MTT plate assay indicated that cytotoxicity was correlated with one of the minor peaks eluted over R_t 15.0–18.5 min. In the first attempt, an aliquot of 750 μg of the cytotoxic fraction from the crude extract was chromatographed with collection of fractions into a microtitre plate. Well F10 of the microtitre plate, containing 4 μg of **1**, was analysed using the CapNMR microprobe technique and ESIMS. The ESIMS

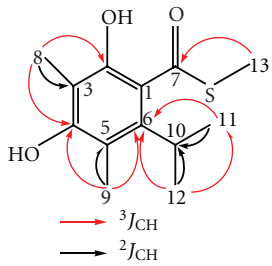


Figure 1: HMBC correlations of **1**.

spectrum indicated the mass of **1** to be 255 Da ($[M+H]^+$), and the presence of an ion at *m/z* 207, which corresponded to the loss of 47 mass units ($[M\text{-}SCH_3]^+$), suggested a thiomethyl group. Confirmation of the presence of S was supported by the HRESIMS$^+$ spectrum yielding the formula $C_{13}H_{19}O_3S$ ($M+H^+$ 255.1043 Da, calc. 255.1055 Da). The ^{1}H NMR spectrum of **1** indicated the presence of three singlet methyl signals at δ_H 2.07, 2.18, and 2.41 and an isopropyl group, represented by one doublet signal at δ_H 1.29 and a multiplet methine signal at δ_H 3.1. As the data from the ^{1}H NMR spectrum and ESIMS of **1** showed no match with those of any reported compound in the AntiMarin database [1], an additional 20 μg of **1** from the cytotoxic fraction of the crude extract was obtained for further spectroscopic analysis. Although some minor impurities contributed to the ^{1}H NMR spectroscopic data, HSQC-DEPT, HMBC, and NOE spectroscopic data were sufficient to elucidate the structure of **1**.

From the HSQC-DEPT spectrum, the chemical shifts of the protons at δ_H 1.29, 2.07, 2.18, and 2.41 were correlated with the chemical shifts of their directly bonded carbons ($^1J_{CH}$ couplings). The HMBC spectrum clearly illustrated the presence of a hexasubstituted benzenoid system. The position of one aryl methyl group at δ_H 2.07 was established by strong HMBC correlations with two oxygen-bearing carbons (C-2, δ_C 149.2 and C-4, δ_C 155.5) and with one higher field carbon (C-3, δ_C 110.2), thus, placing this group between two oxygenated aromatic carbons. The position of the other aryl methyl group was further established by strong HMBC connections of the signal at δ_H 2.18 with one oxygen-bearing carbon (C-4, δ_C 155.5) and two carbons (C5, δ_C 115.5 and C-6, δ_C 139.9). One of the two remaining aromatic carbons was substituted by an isopropyl group, proven by a long-range correlation of two methyl groups (δ_H 1.29) with the carbon (C-6, δ_C 139.9), leaving the C-1 position to be substituted by the carbonyl group (C-7, δ_C 198.4), which had a long-range HMBC correlation to the methyl group (δ_H 2.41). The long range couplings of this compound are shown schematically in Figure 1.

The positions of the isopropyl and the carbonyl group were further confirmed by an NOE experiment. When the methine proton (δ_H 3.1, m) and methyl proton (δ_H 2.18, s) signals were irradiated, the signal for C$\underline{H}_3$ (δ_H 1.29, d) was enhanced. Irradiation of the methyl protons (δ_H 1.29, d) enhanced the methine proton (δ_H 3.1, m) and methyl proton (δ_H 2.18, s) signals. No signals were enhanced when the two methyl groups C$\underline{H}_3$ (δ_H 2.07, s) and C$\underline{H}_3$ (δ_H 2.41, s) were irradiated. These data from the NOE experiment confirmed the relationship between the methine group (C-10, δ_C 31.6) and the two methyls (C-11, δ_C 20.7; C-12, δ_C 20.7) and the attachment overall of the isopropyl group at C-6 (δ_C 139.9). The ^{1}H and ^{13}C chemical shifts for the thiomethyl group of **1** were comparable to those reported for **3** [12] and **4** [13].

Table 1: NMR data of **1**.

Position	δ^{13}C, ppm		δ^1H, ppm, multiplicity (J_{HH} Hz)
1	122.2	(C)	
2	149.2	(C)	
3	110.2	(C)	
4	155.5	(C)	
5	115.5	(C)	
6	139.9	(C)	
7	198.4	(C)	
8	7.2	(CH_3)	2.07, s
9	12.1	(CH_3)	2.18, s
10	31.6	(CH)	3.10, m
11	20.7	(CH_3)	1.29, d (7.2)
12	20.7	(CH_3)	1.29, d (7.2)
13	11.4	(CH_3)	2.41, s

^{1}H: chemical shifts recorded at 500 MHz in CD_3OD. ^{13}C: chemical shifts obtained from HSQC and HMBC spectra.

A complete list of the ^{1}H and ^{13}C chemical shifts for **1** is presented in Table 1. As there were no absolute matches for this structure found, the designated **1** was therefore considered a new structure and named *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate.

The major peak identified in the HPLC analysis of the crude extract was characterized as **2** as the ^{1}H NMR, UV, and MS data obtained matched those previously reported for bohemamine [19, 20].

4. Conclusions

A new metabolite, *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate, **1**, and a known metabolite bohemamine, **2**, were isolated and identified from *Streptomyces* sp. using the CapNMR technique. The structure of **1** was fully characterized by ^{1}H, HSQC, HMBC, and NOE NMR experiments. This new compound is only the fourth natural product reported to contain the *S*-methyl benzothioate group.

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Research Article

Screening of α-Glucosidase Inhibitory Activity from Some Plants of Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae

Berna Elya, Katrin Basah, Abdul Mun'im, Wulan Yuliastuti, Anastasia Bangun, and Eva Kurnia Septiana

Department of Pharmacy, Faculty of Mathematics and Sciences, University of Indonesia, Depok 16424, Indonesia

Correspondence should be addressed to Berna Elya, elya64@yahoo.com

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Diabetes mellitus (DM) is recognized as a serious global health problem that is characterized by high blood sugar levels. Type 2 DM is more common in diabetic populations. In this type of DM, inhibition of α-glucosidase is a useful treatment to delay the absorption of glucose after meals. As a megabiodiversity country, Indonesia still has a lot of potential unexploited forests to be developed as a medicine source, including as the α-glucosidase inhibitor. In this study, we determine the α-glucosidase inhibitory activity of 80% ethanol extracts of leaves and twigs of some plants from the Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae. Inhibitory activity test of the α-glucosidase was performed *in vitro* using spectrophotometric methods. Compared with the control acarbose (IC_{50} 117.20 μg/mL), thirty-seven samples of forty-five were shown to be more potent α-glucosidase inhibitors with IC_{50} values in the range 2.33–112.02 μg/mL.

1. Introduction

Diabetes mellitus (DM) is the most common endocrine disease worldwide. About 173 million people suffer from diabetes mellitus. The number of people with diabetes mellitus will more than double over the next 25 years to reach a total of 366 million by 2030 [1]. In 2000, Indonesia is ranked the fourth largest number of people with DM, after India, China, and the United States, which is about 8.4 million people. The amount is expected to rise to 21.3 million in 2030 [2].

DM consists of several types, one of which is noninsulin-dependent diabetes mellitus (type 2 DM). This type of DM is more common, reaching 90–95% of the population with DM [3]. This increasing trend in type 2 DM has become a serious medical concern worldwide that prompts every effort in exploring for new therapeutic agents to stem its progress.

In type 2 DM, inhibition of α-glucosidase therapy is beneficial to delay absorption of glucose after a meal [4]. α-glucosidase plays a role in the conversion of carbohydrates into glucose. By inhibiting α-glucosidase, glucose levels in the blood can be returned within normal limits [5].

Natural resources provide a huge and highly diversified chemical bank from which we can explore for potential therapeutic agents by bioactivity-targeted screenings [6]. As a megabiodiversity country, Indonesia still has a lot of potential unexploited forests to be developed as a source phytopharmaca or modern medicine [7]. Opportunity exploration of medicinal plants is still very wide open in line with the development of herbal industry, herbal medicine, and phytopharmaca. Therefore, researchers try to explore the potential antidiabetic agents with the mechanism of action of α-glucosidase inhibition in several plant species from four families: Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae. The four families were chosen because members of some species have been scientifically proven to have antidiabetic activity. Based on the theory of kinship through a systematic approach to plant (chemotaxonomy), plants with the same family generally have similar chemical content,

Table 1: Phytochemical screening of 80% ethanol extracts from some plants of Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae.

Simplicia	Chemical contents						
	Alkaloid	Flavonoid	Terpenoid	Tannin	Glycoside	Saponin	Anthraquinone
				Apocynaceae			
Beaumontia multiflora Teijsm. & Binn. Folium	+	+	−	+	+	+	+
Beaumontia multiflora Teijsm. & Binn. Cortex	−	−	−	−	+	+	−
Carissa carandas L. Folium	−	+	+	+	+	+	+
Carissa carandas L. Cortex	−	−	−	+	+	+	+
Ochrosia citrodora Lauterb. & K. Schum. Folium	+	−	+	+	+	+	+
Rauvolfia sumatrana Jack Folium	+	−	+	−	+	+	−
Strophanthus caudatus (Blume.f.) Kurz Folium	−	−	+	−	+	+	+
Strophanthus caudatus (Blume.f.) Kurz Cortex	+	−	+	+	+	+	−
Strophanthus gratus Baill. Folium	−	−	+	−	−	+	−
Strophanthus gratus Baill. Cortex	+	−	+	−	+	+	−
Tabernaemontana sphaerocarpa Blume Folium	+	−	+	−	+	+	−
Willughbeia tenuiflora Dyer ex Hook.f Folium	−	−	+	+	+	+	−
Willughbeia tenuiflora Dyer ex Hook.f Cortex	+	−	+	+	+	+	−
				Clusiaceae			
Calophyllum tomentosum Wight. Folium	+	+	+	+	+	+	−
Garcinia bancana Miq. Folium	+	−	+	+	+	+	−
Garcinia daedalanthera Pierre. Folium	−	+	+	+	+	+	+
Garcinia daedalanthera Pierre. Cortex	−	−	+	+	+	+	+
Garcinia hombroniana Pierre. Folium	+	−	+	+	+	+	+
Garcinia kydia Roxb. Folium	−	+	+	+	+	+	+
Garcinia rigida Miq. Folium	+	+	+	+	+	+	+
				Euphorbiaceae			
Antidesma bunius (L.) Spreng Folium	−	−	+	+	+	+	+
Antidesma bunius (L.) Spreng Cortex	+	−	+	+	+	+	−
Antidesma celebicum Cortex	−	−	−	+	+	+	−
Antidesma celebicum Folium	−	+	−	+	+	+	+
Antidesma montanum (Blume) Folium	+	−	+	+	+	+	−
Antidesma neurocarpum Miq. Folium	+	+	−	+	+	−	+
Blumeodendron toksbrai (Blume.) Kurz. Cortex	+	−	−	−	+	+	−
Blumeodendron toksbrai (Blume.) Kurz. Folium	+	−	+	−	+	−	−
Croton argyratus Blume. Folium	−	−	+	−	+	−	−

Table 1: Continued.

Simplicia	Chemical contents						
	Alkaloid	Flavonoid	Terpenoid	Tannin	Glycoside	Saponin	Anthraquinone
Cephalomappa malloticarpa J.J.Sm. Cortex	−	−	+	+	+	+	+
Cephalomappa malloticarpa J.J.Sm. Folium	−	−	+	+	+	−	+
Galearia filiformis Blume. Folium	+	−	+	+	+	−	+
Sumbaviopsis albicans (Blume) J.J.Sm. Cortex	−	−	+	−	+	+	−
Sumbaviopsis albicans (Blume) J.J.Sm. Folium	−	−	+	−	+	+	−
Suregada glomerulata (Blume) Baill. Folium	+	−	+	−	+	−	−
			Rubiaceae				
Adina trichotoma Zoll. & Moritzi. Folium	+	−	+	−	+	−	+
Amaracarpus pubescens Blume. Folium	+	+	+	−	+	−	+
Canthium glabrum Blume. Folium	+	+	+	+	+	−	+
Chiococca javanica Blume. Folium	+	+	+	−	+	−	+
Hydnophytum formicarum Folium	+	−	+	+	+	+	−
Hydnophytum formicarum Cortex	+	+	+	−	+	−	−
Nauclea calycina (Batrl.ex DC.) Merr. Folium	+	−	−	+	+	−	−
Nauclea calycina (Batrl.ex DC.) Merr. Cortex	−	+	+	+	+	−	+
Posoqueria latifolia (Lam.) Roem. & Schult. Folium	−	−	+	+	+	−	−

Key: +: present; −: absent.

so it may just have the same potential for the treatment of a disease [8].

2. Method and Material

2.1. Plant Material. The stem bark and leaves of plants material were collected in November 2010 and identified by Center for Plant Conservation-Bogor Botanical Garden.

2.2. Extraction. Each dried powdered of wood bark, twig and leaves (20 g) were extracted by reflux with ethanol 80% then evaporated.

2.3. Inhibition Assay for α-Glucosidase Activity. The inhibition of α-glucosidase activity was determined using the modified published method [9]. One mg of α-glucosidase (*Saccharomyces cerevisiae*, Sigma-Aldrich, USA) was dissolved in 100 mL of phosphate buffer (pH 6.8) containing 200 mg of bovine serum albumin (Merck, German). The reaction mixture consisting 10 μL of sample at varying concentrations (0.52 to 33 μg/mL) was premixed with 490 μL phosphate buffer pH 6.8 and 250 μL of 5 mM *p*-nitrophenyl α-D-glucopyranoside (Sigma-Aldrich, Switzerland). After preincubating at 37°C for 5 min, 250 μL α-glucosidase (0.15 unit/mL) was added and incubated at 37°C for 15 min. The reaction was terminated by the addition of 2000 μL Na_2CO_3 200 mM. α-glucosidase activity was determined spectrophotometrically at 400 nm on spectrophotometer UV-Vis (Shimadzu 265, Jepang) by measuring the quantity of *p*-nitrophenol released from p-NPG. Acarbose was used as positive control of α-glucosidase inhibitor. The concentration of the extract required to inhibit 50% of α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

2.4. Kinetics of Inhibition against α-Glucosidase. Inhibition modes of sample that had the best α-glucosidase inhibiting activity in Clusiaceae, Euphorbiaceae, and Rubiaceae were measured with increasing concentration of *p*-nitrophenyl α-D-glucopyranoside as a substrate in the absence or presence of ethanolic extract at different concentrations. Inhibition type was determined by the Lineweaver-Burk plots analysis of the data, which were calculated from the result according to the Michaelis-Menten kinetics.

2.5. Phytochemistry Test. In this research we performed phytochemistry test which consists of alkaloid test with Mayer, Dragendorff, and Bouchardat reagents; Flavonoid test with Shinoda and Wilson Töubock reaction; tannin test with

Table 2: IC_{50} values of rude extracts against α-glucosidase.

Number	Sample	IC_{50} (μg/mL)
(1)	Acarbose	117.20
	Apocynaceae	
(2)	*Beaumontia multiflora Teijsm. & Binn.* Folium	79.80
(3)	*Beaumontia multiflora Teijsm. & Binn.* Cortex	130.20
(4)	*Carissa carandas* L.Folium	21.14
(5)	*Carissa carandas L.*Cortex	20.44
(6)	*Ochrosia citrodora Lauterb. & K. Schum.* Folium	112.02
(7)	*Rauvolfia sumatrana* Jack Folium	174.27
(8)	*Strophanthus caudatus* (Blume.f.) Kurz Folium	706.81
(9)	*Strophanthus caudatus* (Blume.f.) Kurz Cortex	13.93
(10)	*Strophanthus gratus* Baill.Folium	50.61
(11)	*Strophanthus gratus* Baill. Cortex	202.17
(12)	*Tabernaemontana sphaerocarpa* Blume Folium	554.32
(13)	*Willughbeia tenuiflora* Dyer ex Hook.f Folium	8.16
(14)	*Willughbeia tenuiflora* Dyer ex Hook.f Cortex	42.11
	Clusiaceae	
(15)	*Calophyllum tomentosum* Wight. Folium	15.83
(16)	*Garcinia bancana* Miq. Folium	22.41
(17)	*Garcinia daedalanthera* Pierre. Folium	2.33
(18)	*Garcinia daedalanthera* Pierre. Cortex	3.71
(19)	*Garcinia hombroniana* Pierre. Folium	11.30
(20)	*Garcinia kydia* Roxb. Folium	3.88
(21)	*Garcinia rigida* Miq. Folium	24.48
	Euphorbiaceae	
(22)	*Antidesma bunius* (L.) Spreng Folium	7.94
(23)	*Antidesma bunius* (L.) Spreng Cortex	3.90
(24)	*Antidesma celebicum* Cortex	3.93
(25)	*Antidesma celebicum* Folium	2.34
(26)	*Antidesma montanum* (Blume) Folium	2.83
(27)	*Antidesma neurocarpum* Miq. Folium	4.22
(28)	*Blumeodendron toksbrai* (Blume.) Kurz. Cortex	22.82
(29)	*Blumeodendron toksbrai* (Blume.) Kurz. Folium	64.78
(30)	*Croton argyratus* Blume. Folium	366.07
(31)	*Cephalomappa malloticarpa* J.J.Sm. Cortex	12.22
(32)	*Cephalomappa malloticarpa* J.J.Sm. Folium	2.66
(33)	*Galearia filiformis* Blume. Folium	21.54
(34)	*Sumbaviopsis albicans* (Blume) J.J.Sm. Cortex	42.66
(35)	*Sumbaviopsis albicans* (Blume) J.J.Sm. Folium	43.40
(36)	*Suregada glomerulata* (Blume) Baill. Folium	57.46
	Rubiaceae	
(37)	*Adina trichotoma Zoll. & Moritzi. Folium*	28.22
(38)	*Amaracarpus pubescens* Blume. Folium	3.64
(39)	*Canthium glabrum Blume.* Folium	117.85
(40)	*Chiococca javanica Blume.* Folium	23.86
(41)	*Hydnophytum formicarum* Folium	181.90
(42)	*Hydnophytum formicarum* Cortex	11.04
(43)	*Nauclea calycina (Batrl.ex DC.) Merr.* Folium	18.81
(44)	*Nauclea calycina (Batrl.ex DC.) Merr.* Cortex	25.99
(45)	*Posoqueria latifolia (Lam.) Roem. & Schult.* Folium	80.27

gelatin test, gelatin-salt test, and test with ferrous (III) chloride; glycoside test with Molisch reaction; saponin test with honeycomb froth test; anthraquinone test with Bornträger reaction; terpenoid test with Liebermann-Burchard reagent.

3. Results and Discussion

3.1. Phytochemistry Test. Compounds with α-glucosidase inhibitory activity were preliminary identificated by the existence of alkaloid, terpene, saponin, tannin, glycoside, flavonoid, and quinone (Table 1).

3.2. Assay for α-Glucosidase Inhibitory Activity. The α-glucosidase of *S. cerevisiae* is used to investigate the inhibitory activity of the rude extracts. α-glucosidase inhibitory activity of rude extracts compounds against α-glucosidases were determined using *p*-nitrophenyl-α-D-glucopyranoside (p-NPG) as a substrate and these were compared with acarbose (Table 2). The IC_{50} values of compounds range from 2.33 μg/mL to 706.81 μg/mL. There are thirty-seven of samples which have IC_{50} lower than acarbose. Extracts derived from leaves of *Garcinia daedalanthera* showed inhibitory activity against α-glucosidase enzyme significantly, with IC_{50} value of 2.33 μg/mL. Inhibitory activity of the enzyme α-glucosidase at forty-five extracts may be due to the glycoside content in each extract. Glycosides consist of sugars that may be structurally similar to carbohydrate which is a substrate of the enzyme α-glucosidase [10]. IC_{50} value of samples of plant extracts are lower than acarbose because their active chemical compounds have no further fractionation and may have a synergistic effect in inhibiting α-glucosidase [11].

Inhibition mode of leaves extract of *Antidesma celebicum* from Euphorbiaceae was investigated. Inhibition mode of 80% ethanol extract showed competitive inhibitory mode. This mode may have been due because the structure is similar with glucose. This result is similar with inhibition mode of Nojirimycin which has a competitive inhibition against α-glucosidase [9] (Figure 1).

Inhibition mode of leaves extract of *Garcinia kydia* from Clusiaceae was investigated. Inhibition mode of 80% ethanol extract showed noncompetitive inhibitory mode [12] (Figure 2).

Inhibition mode of 80% ethanol extract from *Amaracarpus pubescens* Blume. leaves had a combination of competitive and uncompetitive inhibition. Combination of competitive and noncompetitive may have been due to the extract having more than one compound that has α-glucosidase inhibitory activity [13] (Figure 3).

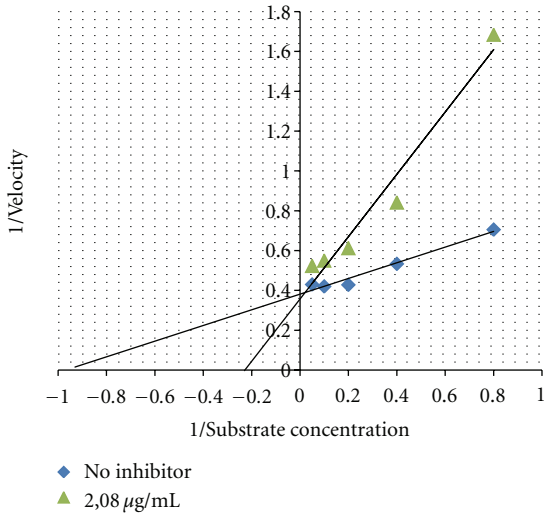

Figure 1: Lineweaver-Burk plot of 80% ethanol extract of leaves of *Antidesma celebicum* with concentration of 2.08 μg/mL with pNPG substrate concentration of 1.25, 2.5, 5, 10, and 20 mM.

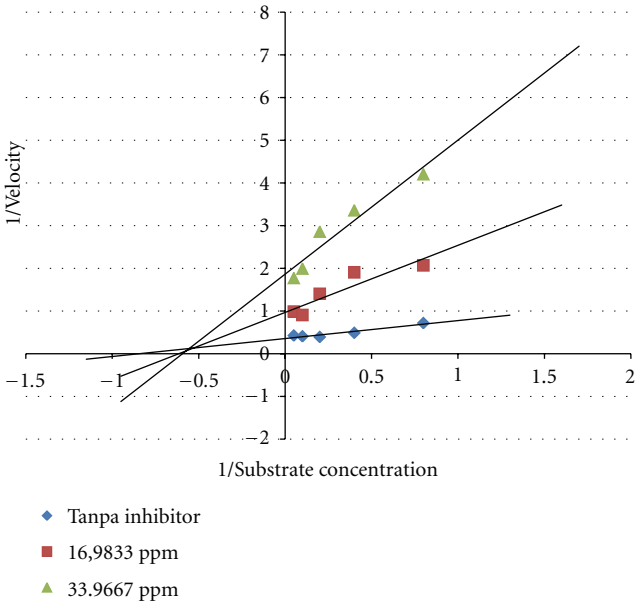

Figure 2: Lineweaver-Burke plot of the reaction α-glucosidase in the presence of 80% ethanol extract from *Garcinia kydia* Roxb. Leaves.

4. Conclusion

In vitro assays of α-glucosidase activity showed thirty-seven of forty-five samples had IC_{50} values of between 2.33 μg/mL and 112.02 μg/mL, which were lower than that of acarbose (117.20 μg/mL). Based on family, 80% ethanol extract from *Garcinia daedalanthera* Pierre. leaves (Clusiaceae), *Antidesma celebicum* leaves (Euphorbiaceae), *Amaracarpus pubescens* Blume. leaves (Rubiaceae), and *Willughbeia tenuiflora* Dyer ex Hook.f leaves (Apocynaceae) had the highest

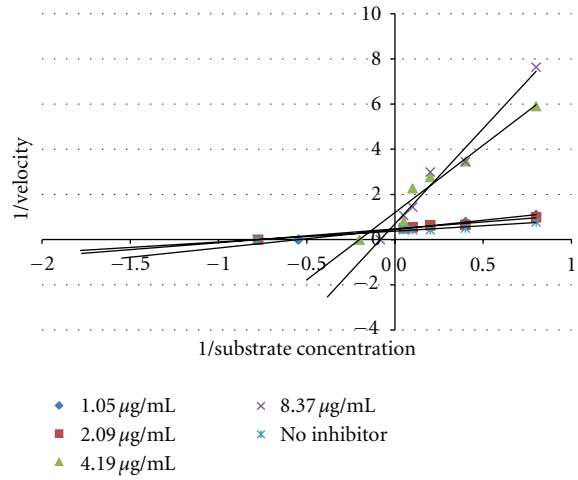

Figure 3: Lineweaver-Burke plot of the reaction α-glucosidase in the presence of 80% ethanol extract from *Amaracarpus pubescens* Blume.

α-glucosidase inhibiting activity with IC_{50} of 2.33 μg/mL, 2.34 μg/mL, 3.64 μg/mL, and 8,16 μg/mL. Meanwhile, types of enzyme inhibition mechanism from *Garcinia kydia* Roxb. leaves (Clusiaceae), *Antidesma celebicum* leaves (Euphorbiaceae), and *Amaracarpus pubescens* Blume. leaves (Rubiaceae) were noncompetitive inhibitor, competitive inhibitor, and mixed inhibitor. Currently attempts to purify the active compound from leaves extract of *Garcinia kydia* Roxb. (Clusiaceae), *Antidesma celebicum* (Euphorbiaceae), and *Amaracarpus pubescens* Blume. (Rubiaceae) are conducted to understand the inhibitory mechanisms more clearly. Moreover, further *in vivo* study is also required.

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Research Article

Structural Characterization and Antioxidative Activity of Low-Molecular-Weights Beta-1,3-Glucan from the Residue of Extracted *Ganoderma lucidum* Fruiting Bodies

Pai-Feng Kao,[1] Shwu-Huey Wang,[2] Wei-Ting Hung,[3] Yu-Han Liao,[3] Chun-Mao Lin,[4,5] and Wen-Bin Yang[3]

[1] *Division of Cardiology, Wan Fang Hospital, 111 Hsing-Long Road, Section 3, Taipei 116, Taiwan*
[2] *Core Facility Center, Office of Research and Development, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*
[3] *Genomics Research Center, Academia Sinica, 128 Academia Road, Section 2, Taipei 115, Taiwan*
[4] *Orthopedics Research Center, Taipei Medical University Hospital, Taipei 110, Taiwan*
[5] *School of Medicine, Taipei Medical University, Taipei 110, Taiwan*

Correspondence should be addressed to Wen-Bin Yang, wbyang@gate.sinica.edu.tw

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The major cell wall constituent of *Ganoderma lucidum* (*G. lucidum*) is β-1,3-glucan. This study examined the polysaccharide from the residues of alkaline-extracted fruiting bodies using high-performance anion-exchange chromatography (HPAEC), and it employed nuclear magnetic resonance (NMR) and mass spectrometry (MS) to confirm the structures. We have successfully isolated low-molecular-weight β-1,3-glucan (LMG), in high yields, from the waste residue of extracted fruiting bodies of *G. lucidum*. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay evaluated the capability of LMG to suppress H_2O_2-induced cell death in RAW264.7 cells, identifying that LMG protected cells from H_2O_2-induced damage. LMG treatment decreased H_2O_2-induced intracellular reactive oxygen species (ROS) production. LMG also influenced sphingomyelinase (SMase) activity, stimulated by cell death to induce ceramide formation, and then increase cell ROS production. Estimation of the activities of neutral and acid SMases *in vitro* showed that LMG suppressed the activities of both neutral and acid SMases in a concentration-dependent manner. These results suggest that LMG, a water-soluble β-1,3-glucan recycled from extracted residue of *G. lucidum*, possesses antioxidant capability against H_2O_2-induced cell death by attenuating intracellular ROS and inhibiting SMase activity.

1. Introduction

Beta-1,3-D-glucans are constituents of fungi, algae, and higher plants. These glucans exhibit different solubility in water-based solutions [1, 2]. *G. lucidum* is the most popular medicinal mushroom, with immunomodulating activity, applied in the treatment of a variety of diseases, including cancer, in numerous Asian countries [3–5]. Previous research has isolated several major substances with potent bioactivities, such as polysaccharides (in particular, β-1,3-glucan) or protein-conjugated β-1,3-glucan [1, 6–8]. Polysaccharides from *G. lucidum* are not linear in structure, comprising β-(1 → 6)-D-glucosyl-linked side chains on a β-1,3-D-glucan backbone. Numerous studies have described their functions [9–12].

Beta-1,3-D-glucans in *G. lucidum* have high molecular weights and low solubility in neutral water solutions. This creates the need to degrade glucan into smaller sizes for further application. Polysaccharide extraction from *G. lucidum* produces a large amount of residue as waste material. Therefore, the development of a "green" method of production, which recycles used materials and low volumes of waste, is of high importance. The present study's methods for isolating β-1,3-D-glucan are efficient and low cost when compared with other extraction methods, providing high-quality products from the residue of *G. lucidum*, which may be useful in commercial applications.

Oxidative stress is implicated in the production of superoxide radicals in biological systems. ROS released from

phagocytic cells initiates a wide range of toxic oxidative reactions and excessive formation of ROS causes cell apoptosis. Administration of antioxidants, such as medicinal herbs, can protect cells by attenuating injury caused by oxidative stress [13]. Almost all cell types express sphingomyelinase (SMase). Intracellular ceramide production increases the hydrolytic activity of SMases [14]. Ceramide is an intracellular lipid mediator that responds to various stimuli including stress. The inflammatory oxidant, H_2O_2, induces rapid increases in ceramide levels because of hydrolysis of sphingomyelin (SM) in plasma membranes, which contributes to the induction of various types of cell death. This study demonstrates that β-1,3-glucan activity is capable of interfering with SMases, and identifies β-1,3-glucan deriving from *G. lucidum* as a potential antioxidant source.

This study expanded the application of *G. lucidum*, reusing the waste materials from the extracted residue of fruiting bodies to obtain high-purity β-1,3-glucan in high yields. We also explored the biofunctions of low-molecular-weight-*G. lucidum* β-1,3-glucan; an efficient antioxidative agent that eliminates ROS in H_2O_2-treated cells by interfering with SMases. Results indicated that low-molecular-weight β-1,3-glucan has potential future use as a biomedicine. The technology used during polysaccharide degradation might also have potential application on other medicinal herbs to release various glycans for biological assay.

2. Materials and Methods

2.1. Recycling and Isolation of β-1,3-Glucans from Extracted Residue of G. lucidum. The dried fruiting bodies of *G. lucidum* were ground and extracted using dilute NaOH solution at 50°C for 12 h. The extract was centrifuged to provide water-insoluble part (waste residue, 80 to 89%) and water-soluble part (soluble heteroglycan extracts, 11 to 20%) [15]. The waste residue was further degraded by 2 N HCl at 50°C for 4 h to provide hydrolysate (45%) and insoluble residue (55%). This glucan-containing hydrolysate underwent purification by gel-filtration chromatography (Sephadex G-15) to obtain three fractions; named high-molecular-weight-glucan (HMG) in 18%, low-molecular-weight-glucan (LMG) in 60% and oligosaccharide of glucose (OSG) in 22% yield, respectively. Figure 1 displays the chromatographic diagram of recycled β-1,3-glucan from the waste residue of *G. lucidum*.

2.2. Instruments in Structural Determination of β-1,3-Glucan. High-performance anion-exchange chromatography (HPAEC) was used for the composition analysis of β-1,3-glucans. Glucose released by the acidic hydrolysis of LMG (4 N TFA 113°C 6 h) and characterized using HPAEC-PAD with known monosaccharides as standard, a Dionex BioLC system (Dionex, Sunnyvale, CA, USA) with a CarboPac PA10 analytical column (4.5 mm × 250 mm) and a CarboPac PA10 guard column (4 mm × 50 mm) was used. Gas chromatography-mass spectrometer (GC-MS) was used for analysis of saccharide composition and linkages according to the methods described by Hakomori et al. [16]. The alditol acetates and partially methylated alditol acetates were separated by GC-MS using a HP1 capillary column (dimethyl siloxane, 30 cm × 200 mm from Hewlett Packard) and a BPX-70 column (SGE Analytical Science Pty Ltd., Victoria 3134, Australia) on PolarisQ Ion Trap GC-MS/MS System (Thermo Fisher Scientific, Inc., Waltham, MA 024253, USA). The oven temperature was increased from 38°C to 150°C at 50°C per min, then to 230°C at 3°C per min and to 260°C for 5 min, with carrier gas (He) at a flow rate of 1 mL/min. NMR studies, $^1H/^{13}C$ NMR, and other 1D and 2D experiments were performed on a Bruker Fourier transform spectrometer (AV-600) equipped with a 5 mm DCI dual cryoprobe. Spectra were obtained at 298 K with solutions of polysaccharide in D_2O. The HOD signal was achieved by a presaturation for 0.5 s. NMR diffusion ordered spectroscopy (DOSY) experiments were carried out using the standard pulse sequence on the same AV-600 NB spectrometer for determination of molecular weight. The equation for DOSY experiments was as follows [17];

$$I = I_0 e^{-Dy^2 g^2 \delta^2 (\Delta - \delta/3)}, \quad D = 8.2 \times 10^{-9} M_w^{-0.49} (\mathrm{m}^2 s^{-1}). \tag{1}$$

Mass spectrometry was used for analyses of molecular weight and linkages. The molecular weights of the sodium adducts of oligosaccharides $[M + Na]^+$ were determined using MALDI-TOF MS (Applied Biosystems, Foster City, CA. USA). The glycan was mixed with 10 mg/mL of 2, 5-dihydroxybenzoic acid (2,5-DHB) and 10 mM NaCl in the ratio of 5 : 5 : 3. In MS mode, the spectra were accumulated at an average of 1,000 to 2,000 shuts at a sample concentration of 1 mg/mL.

2.3. Cell Culture. The mouse monocyte-macrophage cell line, RAW 264.7, was cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Conditions were maintained in a humidified incubator (95% air with 5% CO_2) at 37°C.

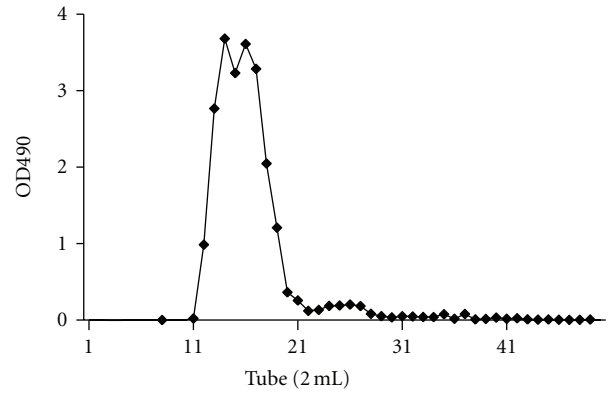

Figure 1: Size exclusion chromatography diagram of recycled β-1,3-glucan from the waste residue of *G. lucidum*. Samples were isolated using a Sephadex G-15 column (HMG: 11–13; LMG: 14–20; OSG: 23–29 tubes), and the sugar content of each tube was determined using phenol-sulfuric acid analysis (OD_{490} nm).

2.4. Cell Viability Assay. MTT assay was conducted to assay cytotoxicity and cell viability, as described previously [18], dependent on the conversion of yellow tetrazolium salt to purple formazan product. Cells (1×10^5 cells/mL) were grown on a 96-well plate. Cells were treated with LMG (0–200 μg/mL) or LMG (0–150 μg/mL) combined with 200 μM H_2O_2 for 24 h, after which an MTT solution (1 mg/mL in PBS) was added, and cells were incubated for 2 h. After the supernatant was discarded and DMSO was added to the well, the absorbance at 570 nm was measured using a spectrophotometer (Thermo Varioskan Flash, Vantaa, Finland).

2.5. Detection of Intracellular ROS Formation. Levels of cellular oxidative stress were detected using the fluorescent probe, DCFDA, as described previously [19]. After being treated with 20 μM H_2O_2 in the absence or presence of LMG (100 μg/mL), RAW264.7 cells were treated with 20 μM DCFDA for 30 min and then washed in PBS. DCFDA is mainly trapped in the cytoplasm and is oxidized to the highly fluorescent dichlorofluorescein (DCF) by intracellular ROS. DCF fluorescence intensities in cells were observed using fluorescence microscopy (Olympus IX71, Tokyo, Japan).

2.6. Suppression of Neutral and Acid SMase Activities by LMG. Neutral SMase (nSMase) activity was estimated using an Amplex Red SMase assay kit (Invitrogen), as described previously [20]. Briefly, the assay mixture contained the following components in a total volume of 200 μL. The enzyme source (0.04 U/mL SMase, 1 U/mL HRP, 0.2 U/mL choline oxidase, 8 U/mL alkaline phosphatase, and 500 μM SM at pH 7.4) in the absence or presence of LMG (0–200 μg/mL) was prepared. For the nSMase assay, assay mixtures were incubated at 37°C for 30 min. Assay of acid SMase (aSMase) activity was performed at pH 5.0. The reaction mixture contained 0.4 U/mL SMase, LMG (0–200 μg/mL), and 500 μM SM in 100 μL of 50 mM sodium acetate, pH 5.0, and cell samples were incubated at 37°C for 30 min. A 100 μL working solution (consisting of 100 μM Amplex Red, 1 U/mL HRP, 0.2 U/mL choline oxidase, and 8 U/mL alkaline phosphatase in 100 mM Tris-HCl, pH 8.0) was added, and cells were incubated at 37°C for 30 min. Fluorescence was measured using a microplate reader (Thermo Varioskan Flash) with excitation at 545 nm and emission at 590 nm.

3. Results and Discussion

3.1. Isolation of β-1,3-Glucans from Extracted Residue of G. Lucidum. This study's aim was to obtain LMG from the extracted residue of *G. lucidum* and to examine its antioxidative activities. Alkaline extracted the ground fruiting bodies of *G. lucidum* to release a water-soluble component (20%) and a water-insoluble residue (80%). The water-soluble component contained heteroglycan, glycoprotein and others, and earlier studies have reported its immunomodulating function [3, 6, 10]. The recycling of β-1,3-glucan from the residue of *G. lucidum* was of interest due to the abundance of β-1,3-glucan in the cell walls of fungi and wide application in medicinal food [1, 2, 4]. Hydrolysis of the waste material, a water-insoluble residue, by strong acidic degradation (2 M HCl, 50°C, 4 h) obtained LMG as low molecular weight glucans. This process provided smaller sized beta-glucans by narrowing down the high-molecular-weight-polyglycans, with the hydrolyzed glucans becoming water soluble. Further purification of the hydrolysate using size-exclusion chromatography (Sephadex G-15) provided three fractions; named HMG, LMG, and OSG. The isolated process is shown in Figure 1. The LMG is also obtained from the hydrolysate by ethanol precipitation (EtOH/H_2O = 5/1) or by ultrafiltration (UF) with a molecular cutoff 500 Da to remove low-molecular-weight-components for large-scale isolation. Structural analyses and biological assays used the prepared LMG following collection and lyophilization.

3.2. Structural Analysis of LMG. The isolated β-1,3-glucan from the residue of extracted *G. lucidum* fruiting bodies was a white powder and relatively pure, despite containing compounds of various sizes due to chemical degradation producing a variety of molecular weights. For composition analysis, acidic hydrolysis (4 N TFA, 110°C, 4 h) further degraded LMG and determined the monosaccharide construct in HPAEC. Procedures identified the only glucose present in the LMG fraction, suggesting that LMG is a glucan from the cell wall of *G. lucidum*. Linkages analysis used GC-MS according to the methods described by Hakomori et al. [16]. Results showed that LMG contains a high ratio of 2,4,6-trimethyl acetyl glucitol (1 → 3 linked glucose) and trace amounts of 2,3,4,6-tetramethyl acetyl glucitol (terminal glucose) and 2,4-dimethyl acetyl glucitol (1 → 6 linked glucose). NMR (Figure 2), at 298 K, elucidated the structure of LMG is β-1,3-glucan. The ^{1}H NMR spectrum of LMG protons H1–H6 appeared at 4.82, 3.59, 3.82, 3.55, 3.50, 3.97_{6a}, and 3.80_{6b} ppms, and its ^{13}C NMR spectrum appeared at 103.0 (C-1), 73.2 (C-2), 85.2 (C-3), 68.4 (C-4), 76.3 (C-5), and 60.9 (C-6) ppms, respectively. These signals are in agreement with results from previous analyses [11, 21]. As shown in Figure 2, the tiny signals of β-1,6-glucan appeared at 102.2 (C-1), 73.9 (C-2), 76.5 (C-3), 70.4 (C-4), 76.8 (C-5), and 69.7 (C-6) ppms and the protons H1–H6 at 4.70, 3.40, 4.15, 4.05, 3.90, 4.60_{6a}, and 3.78_{6b} ppms [22].

3.3. Molecular Weights of LMG as Determined Using NMR and MALDI-TOF MS. Recently, investigators developed NMR DOSY as a useful tool for measurement of molecular weights, including those of polysaccharides [17, 23]. Using the equation in the experimental section, the estimated molecular weight of LMG is 3979 Da. The signal at 4.8 ppm, $\log D = -9.0$, is H_2O with molecular weight = 18 (Figure 3). The signal of anomeric protons on LMG merged in the HOD at 4.8 ppm. The convergent dots in the same horizontal, at $\log D = -10.0$, indicated that LMG has a narrow range in terms of size. Excepting the HOD signal, no dots appeared in the parallel line in the DOSY spectrum indicating that protons in LMG are connected, and LMG is a homogeneous glucan with differences in narrow range sizes.

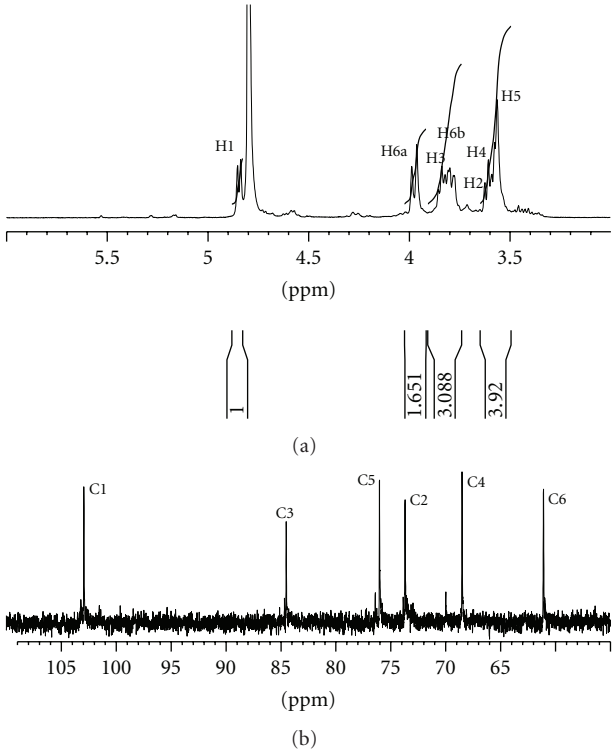

Figure 2: The ^{1}H NMR spectrum (a) and ^{13}C NMR spectrum (b) of LMG. These signals were in agreement with those of β-1,3-glucan from previously results [11].

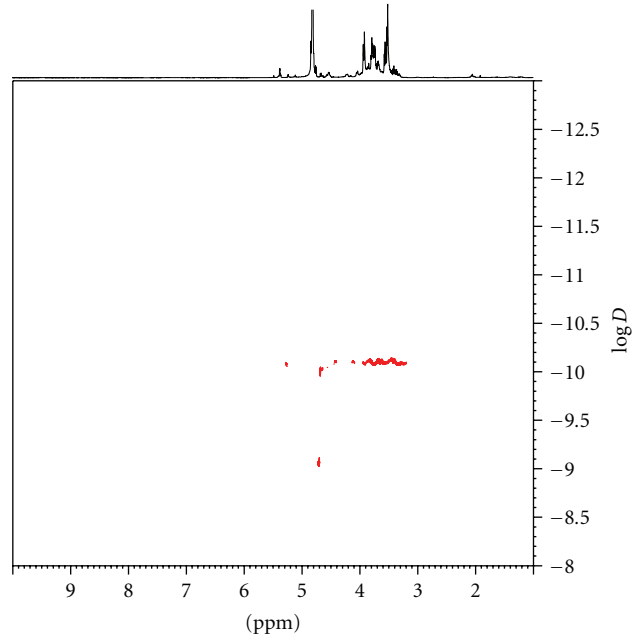

Figure 3: The 1D NMR DOSY experiment of LMG. Based on the equation, the calculated molecular weight of LMG is 3979 Da. The signal of anomeric protons merged in the HOD at 4.8 ppm and the signal, at log $D = -9.0$, is H_2O with molecular weight = 18.

MALDI-TOF MS analysis confirmed the presence of β-1,3-glucan in LMG. Figure 4 shows the molecular mass of LMG with gaps as 162 Da (one hexose unit) between peaks to peaks. Use of 2,5-DHB as a matrix substantially enhanced polysaccharide signals in the MALDI mass spectrometry [24]. This study obtained positive ions of glucan with unambiguous signals, observing LMG as sodiated ions ($[Glcn + Na]^{+}$) where n is the number of glucose units (6∼15). The mass region of ion peaks had a peak-to-peak mass difference of 162.1 Da, consistent with the repeating unit of the β-(1 → 3)-glucan. For example, when determining the degree of polymerization (DP) of LMG using MALDI-TOF, the average LMG mass appeared at DP = 9 (1499.1 Da, $[Glc_9 + Na]^{+}$) as a base peak. The molecular weights of LMG showed an average of 1,500 Da in MALDI-TOF MS, with slight differences from those obtained using the DOSY experiment. Based on these results, this study aimed to recover low molecular weight β-1,3-glucan from waste materials of *G. lucidum* residue. These recycled β-1,3-glucans might have potential commercial application as low-cost medicinal food.

Although some β-D-glucans are commercially available, they have different molecular weights, biological activities, and structures. Previous studies have identified the biological activities of β-1,3-glucan containing *G. lucidum* [5–8]. However, the antioxidative activities of low molecular weight glucans have yet to be fully understood. The structure-activity relationship (SAR) of LMG when exerting antioxidative effects was of interest. Using intracellular ceramide as a marker to determine the hydrolytic activity of SMases evaluated the antioxidative activity of isolated LMG, and also indicated if LMGs exerts protective effects on cells by attenuating injury under oxidative stress. Inflammatory oxidants cause this injury by inducing rapid increases in ceramide levels due to the hydrolysis of SM in plasma membranes. The study findings showed that LMG is capable of protecting cells against H_2O_2-induced apoptosis. LMG inhibited the activity of SMases to decrease the levels of intracellular ceramides, which may provide a useful indicator of the antioxidative activity of β-(1 → 3)-D-glucan.

3.4. Protective Effects of LMG against H_2O_2-Induced Macrophage Death. Beta-(1 → 3)-D-glucan is the one of the major components of polysaccharide from G. lucidum, and has anti-inflammatory properties [5]. This study examined the antioxidant effects of LMG. MTT assay evaluated the cytotoxicity of LMG to RAW264.7 cells (Figure 5(a)), indicating that LMG is not meaningfully cytotoxic to RAW264.7 macrophages when LMG was added at 0–200 μg/mL for 24 h. Procedures also evaluated the protective ability of LMG against H_2O_2-induced injury. After 24 h H_2O_2 (20 μM) treatment, MTT assay revealed 40% cell viability, which increased to over 80% after cotreatment of cells with LMG (100 μg/mL; Figure 5(b)). Results showed that LMG exerts dose-dependent protective effects against H_2O_2-induced cell death in RAW264.7 cells. The antioxidative activities of the other acidic hydrolyzed fractions, HMG and LMG, showed similar potency, but OSG (wich contains monosaccharide) demonstrated reduced antioxidative activity. This finding suggests that the molecular weights of beta-1,3-glucan plays important roles in determining its biological activity.

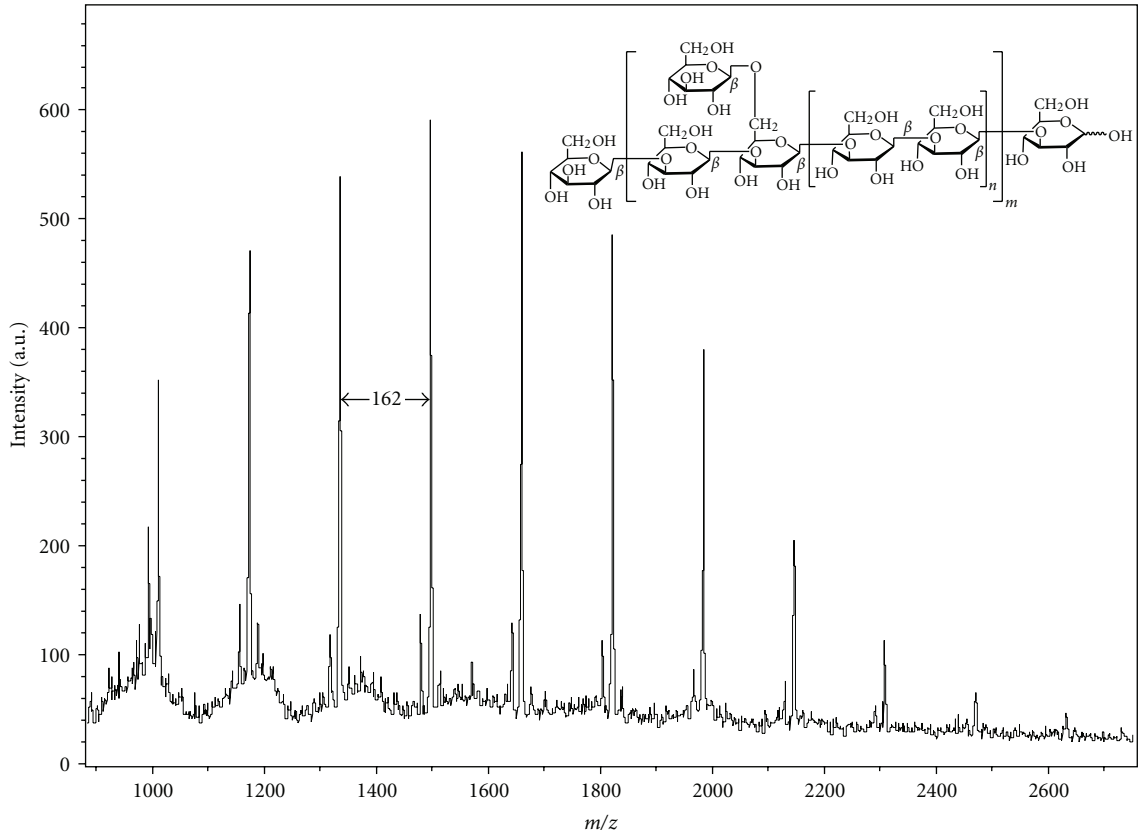

Figure 4: MALDI-TOF with 2,5-DHB as matrix, determined the molecular mass of LMG. The gap with 162 Da between peaks represents a hexose unit in LMG. Sodiated ions ($[Glcn + Na]^+$) appeared at DP = 6 to 15 with 1013.1, 1175.1, 1337.1, 1499.1, 1661.2, 1823.2, 1985.2, 2147.2, 2309.2, and 2471.3 Da, respectively.

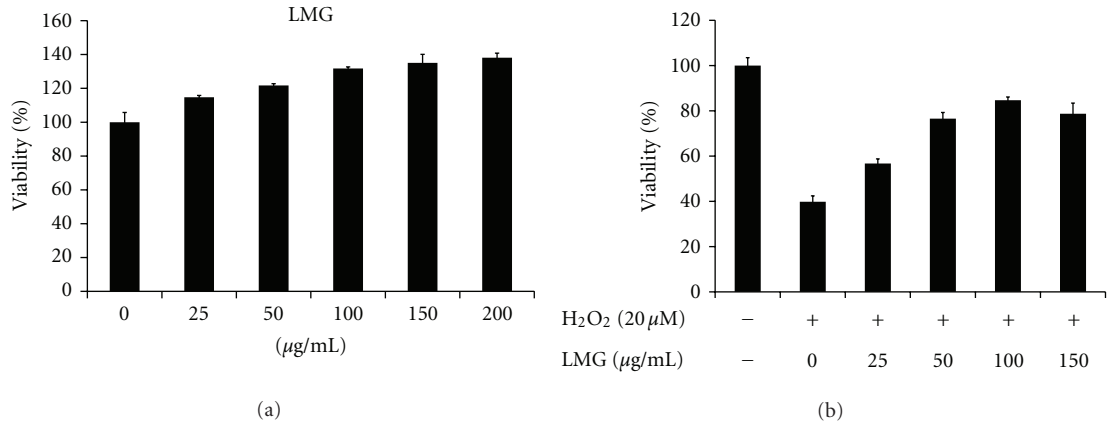

Figure 5: Protective abilities of LMG against H_2O_2-induced cytotoxicity in RAW264.7 cells. (a) MTT assay evaluated the cytotoxicity of LMG to RAW264.7 cells. Cells were treated with LMG (at 0, 25, 50, 100, 150, and 200 μg/mL) for 24 h. Results are presented as means ± SD for $n = 5$ replicates. (b) The protective ability of LMG from H_2O_2-induced cytotoxicity in RAW264.7 cells. Cells were cotreated with H_2O_2 (20 μM) and LMG (0, 25, 50, 100, and 150 μg/mL) for 24 h. Results are presented as the means ± SD for $n = 5$ replicates.

3.5. LMG Reduced H_2O_2-Induced Intracellular ROS Levels. H_2O_2 medication induces intracellular oxidative stress by increasing ROS production. This study estimated intracellular ROS in exogenous H_2O_2-injured (20 μM) macrophage cells. Fluorescence microscopy with DCFDA, a redox-sensitive fluorescent dye, visualized ROS production. In cells treated with H_2O_2 alone (Figure 6(b)), the oxidized DCF fluorescent intensity was significantly greater than in untreated control RAW264.7 cells (Figure 6(a)). Cotreatment with LMG (100 μg/mL) provided markedly reduced

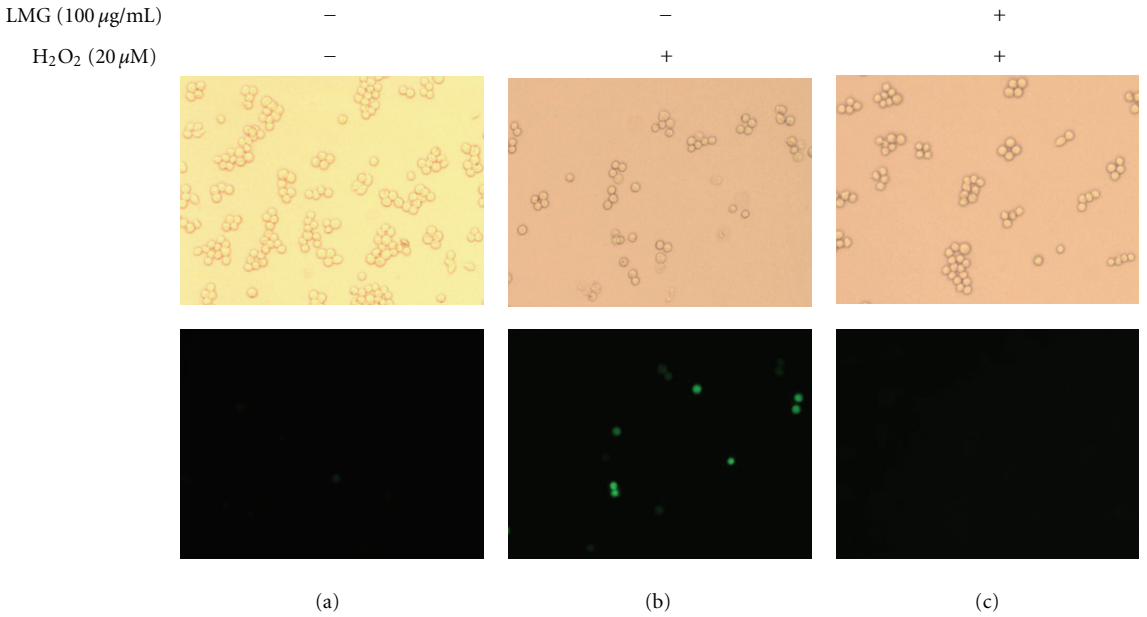

Figure 6: LMG reduced H_2O_2-induced intracellular ROS levels. RAW264.7 cells were treated with H_2O_2 (20 μM) and LMG (100 μg/mL) for 30 min, and intracellular ROS levels were measured using DCFDA fluorescent staining and fluorescence microscopy. Left panels, untreated control; middle panels, H_2O_2 added alone; right panels, co-treatment with H_2O_2 and LMG. Images in the upper panels were captured using bright-field microscopy (original magnification 200x), and those in the lower panels were captured using fluorescence microscopy.

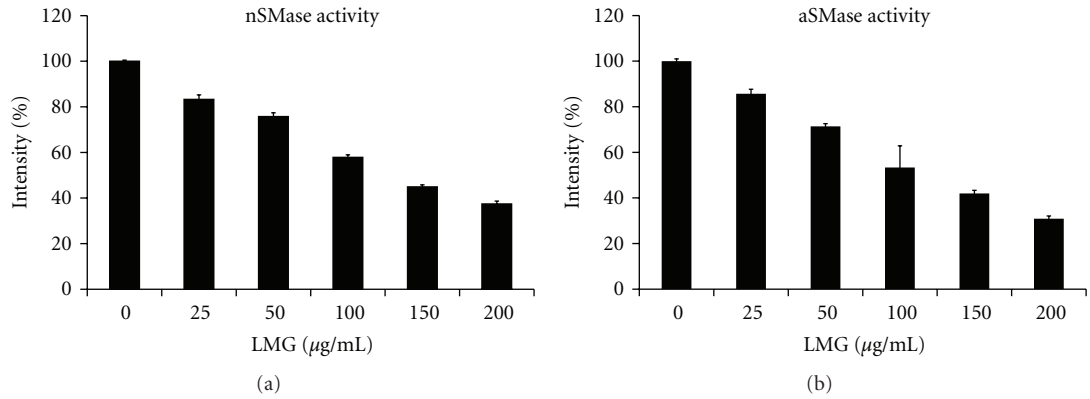

Figure 7: Amplex red assay evaluated LMG's SMase inhibitory activity. The relative activities of nSMase (a) and aSMase (b) were detected after treatment with 0, 25, 50, 100, and 200 μg/mL of LMG for 30 min. The relative activity is presented as the means ± SD for $n = 3$ replicates.

DCF fluorescence compared with fluorescence after addition of H_2O_2 only (Figure 6(c)). Increasing LMG decreased ROS production induced by addition of H_2O_2 to RAW264.7 cells.

3.6. LMG Inhibited SMase Activity. In previous studies, factors of inflammation, including TNF-α, ROS, LPS, amyloid-β, IL-1β, and LDL, stimulated SMase activation activity, which then produced ceramide and initiated ceramide signal pathways to transfer death signaling [25–27]. This study evaluated the effects of LMG on bacterial SMase activity *in vitro*. Increasing LMG (0–200 μg/mL) reduced the activity of nSMase, with an IC_{50} value of approximately 120 μg/mL (Figure 7(a)). LMG also had inhibitory effects on aSMase activity (Figure 7(b)). These data suggest that LMG has the potential to act as a SMase inhibitor. In Our previous research identified that a polysaccharide from *Cordyceps mycelia* possessed antioxidative ability and associated with SMase inhibitory effects, thus reducing levels of ceramides produced from hydrolysis of sphingomyelin [20]. Although findings did not fully elucidate the mechanism by which LMG exerts antioxidative effects, LMG's potential as a SMase inhibitor may be of importance for its future applications.

4. Conclusion

This study successfully isolated LMG, a low molecular weight β-1,3-glucan fraction, from the extracted residue of *G. lucidum* in high yields. A chemical method degraded

the high-molecular-weight insoluble cell wall glucans into smaller sized compounds. This study also investigated the antioxidant activity of isolated LMG β-1,3-glucan to indicate the potential commercial value of *G. lucidum* glucans. LMG can protect cells by attenuating injury under oxidative stress caused by inflammatory oxidants. The study finding show that LMG, a water-soluble β-1,3-glucan with low molecular weight, recycled from extracted residue of *G. lucidum*, has activity against H_2O_2-induced apoptosis. LMG, therefore, is capable of exerting antioxidant effects by inhibiting SMases. The recycling of β-1,3-glucan from the remains of fruiting bodies could potentially provide a food supplement source of antioxidants for maintenance of body health. This is the first study to report the antioxidative effects of low molecular weight β-1,3-glucan from *G. lucidum*. The methods used have potential application in the isolation of antioxidative polysaccharides from other medicinal herbs.

Acknowledgments

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Review Article

Cancer Chemoprevention by Citrus Pulp and Juices Containing High Amounts of β-Cryptoxanthin and Hesperidin

Takuji Tanaka,[1,2] Takahiro Tanaka,[3] Mayu Tanaka,[4] and Toshiya Kuno[2]

[1] *The Tohkai Institute of Cytopathology: Cancer Research and Prevention, 5-1-2 Minami-uzura, Gifu City, Gifu 500-8285, Japan*
[2] *Department of Tumor Pathology, Graduate School of Medicine, Gifu University, Gifu City, Gifu 501-1194, Japan*
[3] *Department of Physical Therapy, Kansai University of Health Sciences, 2-11-1 Wakaba, Kumatori-Machi, Sennan-Gun, Osaka 590-0482, Japan*
[4] *Department of Pharmacy, Kinjo Gakuin University, 2-1723 Ohmori, Moriyama-Ku, Nagoya City, Aichi 463-8521, Japan*

Correspondence should be addressed to Takuji Tanaka, takutt@toukaisaibou.co.jp

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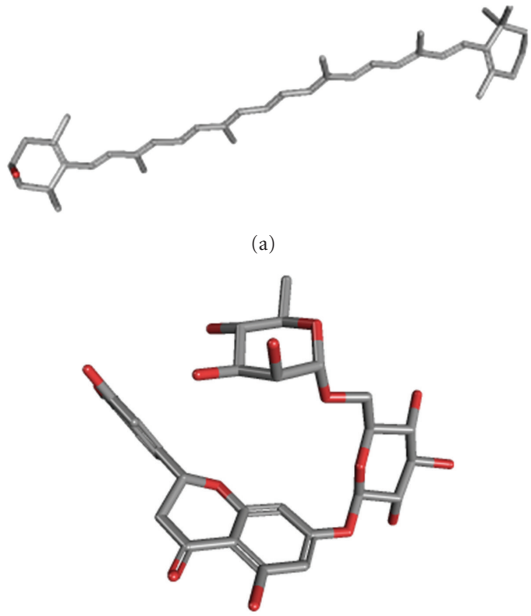
β-Cryptoxanthin, a carotenoid, and hesperidin, a flavonoid, possess inhibitory effects on carcinogenesis in several tissues. We recently have prepared a pulp (CHRP) and citrus juices (MJ2 and MJ5) from a satsuma mandarin (*Citrus unshiu* Mar.) juice (MJ). They contain high amounts of β-cryptoxanthin and hesperidin. We have demonstrated that CHRP and/or MJs inhibit chemically induced rat colon, rat tongue, and mouse lung tumorigenesis. Gavage with CHRP resulted in an increase of activities of detoxifying enzymes in the liver, colon, and tongue rats'. CHRP and MJs were also able to suppress the expression of proinflammatory cytokines and inflammatory enzymes in the target tissues. This paper describes the findings of our in vivo preclinical experiments to develop a strategy for cancer chemoprevention of colon, tongue, and lung neoplasms by use of CHRP and MJs.

1. Introduction

Epithelial malignant neoplasms remain a major health challenge in the world. Despite improvements in staging and the application and integration of therapies, including surgery, radiotherapy, and chemotherapy, the 5-year survival rate for individuals with malignancies is still low. Even if strategies for early detection are successful and malignancies are detected at a stage where local tumor resection and treatment is curative, the patients will still have significant risk for developing second primary malignancy associated with the problem, including "field cancerization" [1–6]. For this reason, it is important to focus on chemopreventive strategies to prevent the development of epithelial malignancies [5, 6].

Cancer prevention is a rapidly expanding discipline that focuses on the discovery and identification of dietary agents and drugs that prevent or inhibit epithelial malignant tumor development [5–8]. Since approximately one-third of the overall risk of cancer is attributable to diet, a large number of dietary compounds have been tested to determine their chemopreventive ability using animal carcinogenesis models [4, 9–12]. It is known that certain carotenoids and flavonoids can inhibit cancer development in animal carcinogenesis models [4, 10–12]. β-Cryptoxanthin, (Figure 1(a)), a carotenoid, and hesperidin (Figure 1(b)), a flavonoid, are such compounds. β-Cryptoxanthin with nonsubstituted β-ionone cycles and provitamin A property possesses several biological activities including scavenging of free radicals, enhancement of gap junctions, immunomodulation, and regulation of enzyme activity involved in carcinogenesis [13, 14]. β-Cryptoxanthin is reported to inhibit mouse skin tumorigenesis [15] and rat colon carcinogenesis [16]. Hesperidin, present in several vegetables and fruits, has antioxidant property, anti-inflammatory effect, and inhibiting effect on prostaglandin biosynthesis. This flavonoid inhibits chemically induced carcinogenesis in several organs [4, 10–12]. β-Cryptoxanthin and hesperidin are thus considered to be potential cancer chemopreventive compounds. However, edible plants contain only small amounts of these chemicals. To obtain higher contents of these compounds in foods, therefore, we prepared a pulp (CHRP) containing high amounts of β-cryptoxanthin and hesperidin during the process of making satsuma



(a)

(b)

Figure 1: 3D chemical structures of (a) β-cryptoxanthin and (b) hesperidin.

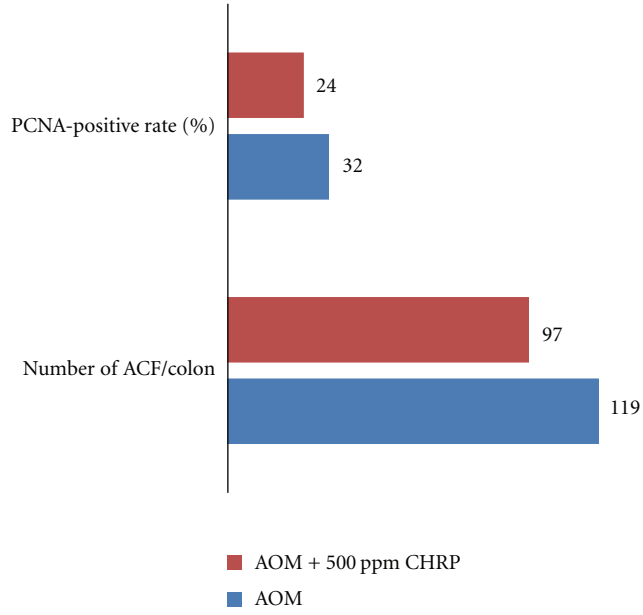


Figure 2: Dietary CHRP suppresses AOM-induced colonic aberrant crypt foci (ACF) in rats.

mandarin (*Citrus unshiu* Marc.) juice (MJ). CHRP (100 g) contained 0.67 g β-cryptoxanthin and 3.58 g hesperidin: the contents of β-cryptoxanthin and hesperidin are 583 times and 38 times greater than those in edible parts of satsuma mandarin (*Citrus unshiu* Marc.), respectively. In addition, we prepared Satsuma mandarin (*Citrus unshiu* Marc.) juices, called MJ2 (1.7 mg β-cryptoxanthin and 84 mg hesperidin/100 g) and MJ5 (84 mg β-cryptoxanthin and 100 mg hesperidin/100 g), by adding CHRP to usual mandarin orange juice (MJ: 0.8 mg β-cryptoxanthin and 79 mg hesperidin/100 g).

We describe the chemopreventive effects of CHRP and MJs on chemically induced oncogenesis in the colon and tongue of rats and mouse lung [17–19].

2. Dietary CHRP Inhibits Chemically Induced Rat Colon Carcinogenesis

To predict possible inhibitory action of CHRP in colon carcinogenesis, the effects of CHRP on the development of aberrant crypt foci (ACF), which are putative precursor lesions of colonic adenocarcinoma (ADC) of rodents and human [20–22], were examined in rats initiated with a colon carcinogen, azoxymethane (AOM) [17]. A total of 32 male F344 rats were used in the study. Animals were divided into the AOM alone, AOM and 500 ppm HCRP, 500 ppm HCRP, and untreated groups. Rats were initiated with AOM by two weekly subcutaneous injections (20 mg/kg bw) and were fed the diets containing CHRP at 500 ppm for 4 weeks (initiation stage), starting one week before the first dose of AOM. At week 11 of the experimental week, the frequency of ACF was determined. As illustrated in Figure 2, all rats in the AOM-alone group developed ACF with a frequency of 119 ± 5 ACF per rat, while rats in the AOM + 500 ppm CHRP group had a frequency of 97 ± 11 ($P < 0.05$). The CHRP-alone and untreated groups did not develop ACF. Immunohistochemical analysis of proliferation activity, proliferating-cell-nuclear-antigen- (PCNA-) positive index, was done, since promising chemopreventive compounds act through modulation of cell proliferative activity in the target organs [11, 23]. As presented in Figure 2, the PCNA-labeling index in ACF was significantly decreased by feeding with the CHIRP diet ($P < 0.05$).

Based on the findings from this preliminary experiment, a long-term animal experiment was conducted to determine the suppressing effects of CHRP on the development of colonic ADC induced by AOM. A total of 69 rats were randomly divided into 5 groups, namely, the AOM alone, AOM + 500 ppm CHRP for 4 weeks of the initiation stage, AOM → 500 ppm CHRP, for 28 weeks of the promotion stage, 500 ppm CHRP for the entire experimental period (32 weeks), and untreated groups. Animals were initiated with three weekly subcutaneous injections of AOM (15 mg/kg bw) to induce colonic neoplasms. As given in Figure 3, the frequencies of colonic ADC in the AOM + 500 ppm CHRP group (47%) and the AOM → 500 ppm CHRP group (21%, $P < 0.05$) were smaller than in group 1 (60%). In Figure 3, the PCNA-labeling indices of large bowel ADC developed in the the AOM + 500 ppm CHRP group and the AOM → 500 ppm CHRP group were significantly smaller than the AOM alone group ($P < 0.05$).

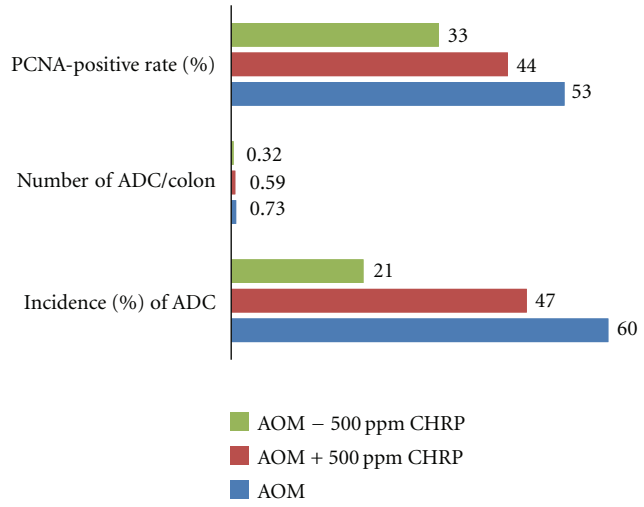


Figure 3: Dietary CHRP inhibits AOM-induced colonic adenocarcinoma (ADC) in rats.

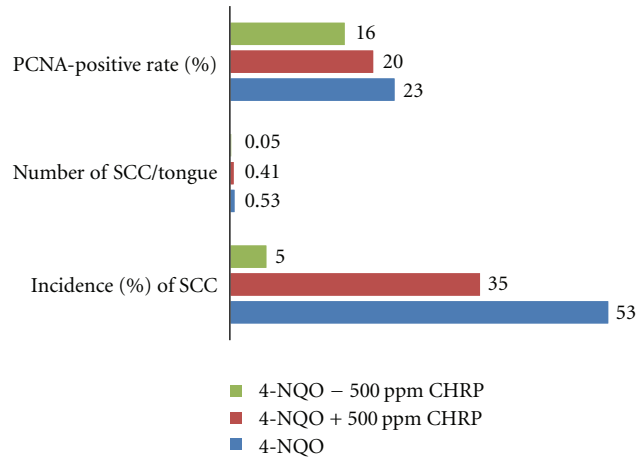


Figure 4: CHRP in diet suppresses 4-NQO-induced rat tongue squamous cell carcinoma (SCC).

3. Supplementation with CHRP in Diet Inhibits 4-Nitroquinoline-1-Oxide- (4-NQO-) Induced Rat Tongue Carcinogenesis

Oral cancer is an important public health issue because its occurrence is strongly associated with cigarette smoking and alcohol drinking [2, 3, 24]. Oral cancer is one of the ten most frequent cancers worldwide, with three-quarter of all cases occurring in the developing countries [2, 3]. It varies in the frequency greatly among different countries and geographic regions [2, 3]. The incidence and mortality of oral cancer have increased over the past decades in Europe [25] and in the United States [26]. In particular, the incidence and mortality rate of tongue cancer, as compared to other types of oral cancer, have increased in younger adults [27]. Chemoprevention against oral/tongue cancer development is thus important. To determine whether CHRP can inhibit chemically induced rat tongue carcinogenesis, a total of 67 male F344 rats were divided into 5 groups, including the 4-NQO alone group (8-week treatment with 20 ppm 4-NQO in drinking water), 4-NQO + 500 ppm CHRP group (10-week treatment during the initiation stage), 4-NQO → 500 ppm CHRP group (22-week treatment during the promotion stage), CHRP alone group (32-week of the entire experiment), and untreated group. At week 32, tongue squamous cell tumors (papilloma and carcinoma) and dysplasia developed in the posterior tongue (dorsal region) of rats that received 4-NQO. As shown in Figure 4 the incidences of tongue squamous cell carcinoma (SCC) were 53% in the 4-NQO alone group, 35% in the 4-NQO + 500 ppm CHRP group, and 5% in the 4-NQO → 500 ppm CHRP group. The incidence of tongue carcinoma in the 4-NQO → 500 ppm CHRP group was significantly smaller than the 4-NQOO alone group ($P < 0.05$). The multiplicity of tongue carcinoma of this group was also lower than the 4-NQO alone group ($P < 0.05$). The PCNA-labeling index of tongue SCC in the 4-NQO → 500 ppm CHRP group was significantly smaller than the 4-NQO alone group ($P < 0.05$).

4. MJs, Citrus Juices Rich in β-Cryptoxanthin and Hesperidin, Inhibit AOM-Induced Rat Colon Carcinogenesis

Colorectal cancer (CRC) is the fourth most common malignant neoplasm in the world [21]. Since an inverse relationship between the intake of fruits/vegetables and human CRC has been suggested [5, 6, 28–32], primary prevention, including chemoprevention utilizing the active compounds in edible plants, is important for reducing this malignancy [5, 6]. This experiment was designed to determine the modulatory effects of MJ, MJ2, and MJ5 on the occurrence of colonic neoplasms induced by AOM in rats [19]. Also, PCNA-labeling index in colonic neoplasms was analyzed immunohistochemically. A total of 113 male F344 rats were divided into 6 groups: the AOM alone group, the AOM → MJ group, the AOM → MJ2 group, the AOM → MJ5 group, the MJ5 alone group, and the untreated group. AOM was given to rat by twice weekly subcutaneous injections at a dose level of 20 mg/kg bw. MJ, MJ2, and MJ5 in black bottles were given to rats for 12 h (from 8:00 p.m. to 8:00 a.m.). Figure 5 shows the incidence and multiplicity of colonic ADC at week 38. AOM administration induced large intestinal ADC with an incidence of 69% and a multiplicity of 0.76 ± 0.57. The incidences and multiplicities of the AOM → MJ group, the AOM → MJ2 group, and the AOM → MJ5 group were significantly smaller than the AOM alone group ($P < 0.05$). The mean PCNA-labeling index of adenocarcinoma in rats of group 1 was 55 ± 7% ($n = 20$). The mean PCNA-labeling indices of adenocarcinomas present in the AOM → MJ2 and the AOM → MJ5 groups ($P < 0.05$) were significantly lower than the AOM alone group (55 ± 7%, Figure 5).

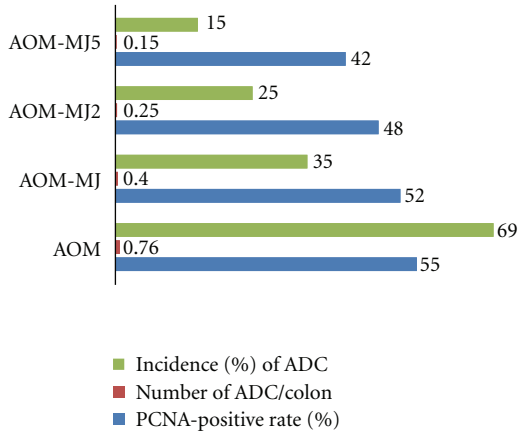


Figure 5: MJs suppress AOM-induced colonic adenocarcinoma (ADCs) in rats.

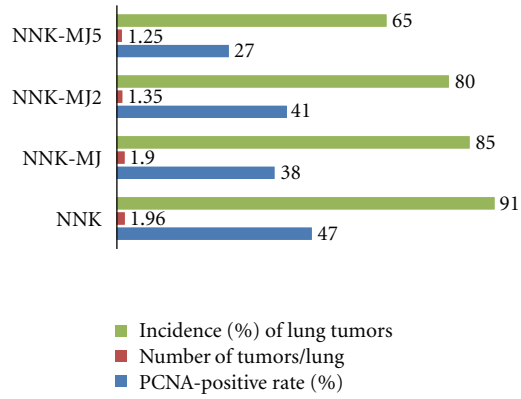


Figure 6: MJs inhibit NNK-induced lung tumors in mice.

5. MJs, Citrus Juices Rich in β-Cryptoxanthin and Hesperidin, Inhibit Chemically Induced Mouse Lung Tumorigenesis

Lung cancer is the largest cause of cancer deaths in industrial countries, and cigarette smoking is regarded as the overwhelming cause of lung cancer. Chemoprevention using naturally occurring or synthetic compounds to arrest or reverse the carcinogenic process is extremely important as a lung cancer prevention strategy. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most important carcinogenic nitrosamine, because of its strong potential in inducing lung neoplasms in rodents and its exposure to humans through smoking [33, 34]. Using the animal model for lung tumorigenesis with NNK, several promising agents for prevention of lung cancers have been reported [33–38]. This experiment was aimed to determine possible modulatory effects of MJs on the development of lung tumors induced by a pulmonary carcinogen, NNK, in mice [18]. The PCNA-labeling index in lung tumors was also determined. A total of 103 male A/J mice were divided into 6 groups: the NNK alone group, the NNK → MJ group, the NNK → MJ2 group, the NNK → MJ5 group, the MJ5 alone group, and the untreated group. NNK was given to mice by a single intraperitoneal injection (10 μmol in saline/mouse). MJ, MJ2, and MJ5 were administered to mice as a drinking water for 21 weeks, starting one week after the NNK injection. MJs in the black bottles were given to mice for 12 hr (from 8:00 p.m. to 8:00 a.m.) At week 22, lung proliferative lesions were diagnosed as hyperplasia and tumors, and we did not subclassify the tumors into adenoma and adenocarcinoma, because of the difficulty in evaluating malignancy [39, 40]. Pulmonary tumors (adenoma or adenocarcinoma) were developed in all mice treated with NNK. As illustrated in Figure 6, the incidences and the multiplicities of lung tumor of the NNK → MJ group, the NNK → MJ2 group, and the NNK → MJ5 group were smaller than NNK alone group. Statistically, the incidence of lung tumors in mice that received NNK and MJ5 was significantly lower than the NNK alone group ($P < 0.05$). The multiplicities of lung tumors of mice in the NNK → MJ group, the NNK → MJ2 group, and the NNK → MJ5 group were also low, but the differences were insignificant. The mean PCNA-labeling indices of pulmonary tumors of the NNK → MJ group and the NNK → MJ5 group were significantly lower than the NNK alone group ($P < 0.05$, Figure 6).

6. CHRP Increases Detoxifying Enzymes in the Liver, Tongue, and Colon

Induction of enzymes that enhance the detoxification of chemical carcinogens has been a broadly effective strategy for chemoprevention of experimental carcinogenesis in rodent models. Several inducing agents are now in clinical trials to evaluate utility for prevention of cancers associated with unavoidable high exposures to environmental carcinogens. Thus, certain phase II detoxifying enzyme inducers, including phenolic antioxidants, dithiolethiones, isothiocyanates, and triterpenoids, are considered to be promising chemopreventive in preclinical and clinical interventions [41–46].

To determine whether CHRP modifies glutathione *S*-tansferase (GST) and quinone reductase (QR) activities in the liver, colon, and tongue, male F344 rats were gavaged with CHRP at four dose levels (0, 40, 200 or 400 mg/kg body wt in 0.5 mL of 5% gum Arabic) of CHRP for 5 consecutive days [17]. Thirty min after the last gavage, the liver, colon, and tongue were excised immediately to measure GST and QR activities. Dosing of 40, 200, and 400 mg/kg bw of CHRP significantly elevated liver GST (1.27-fold, 1.25-fold, and 1.50-fold increases, resp., $P < 0.05$) and QR activities (1.24-fold, 1.22-fold, and 1.33-fold increases, resp., $P < 0.05$) when compared to rats that received 0 mg/kg bw. Similarly, gavage with CHRP significantly increased GST activity in the colonic (1.11-fold, 1.12-fold, and 1.15-fold increases, resp., $P < 0.05$) and tongue mucosa (1.33-fold, 1.29-fold, and 1.23-fold increases, resp., $P < 0.05$). CHRP treatment significantly increased the colonic QR activity at a dose of 400 mg/kg bw (1.13-fold increase, $P < 0.05$) and the tongue QR activity at doses of 200 and 400 mg/kg bw (1.25-fold and 1.21-fold increases, resp., $P < 0.05$).

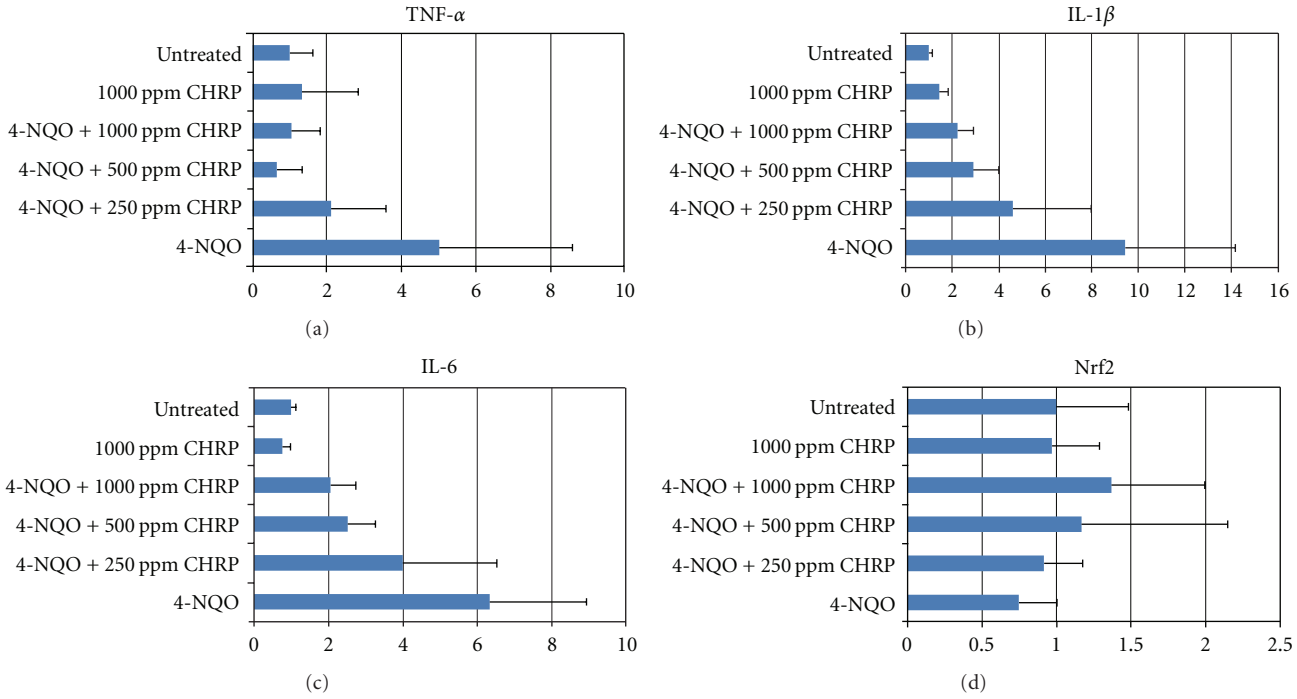


Figure 7: Dietary CHRP lowers mRNA expression of proinflammatory cytokines in the tongue of rats that received 4-NQO at week 8 of the study.

7. Modulation of Cytokine Expression by CHRP and MJs in the Tongue and Colon of Rats Initiated with Carcinogens

Chronic inflammation is closely associated with cancer development [47]. Cytokines are an important group of proteins that regulate and mediate inflammation and angiogenesis. Deregulation in their production results in tumor growth, invasion, and metastasis being facilitated. In addition to proinflammatory cytokines, inflammatory enzymes, such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), are involved in carcinogenesis [48–50], and they are good targets for cancer chemoprevention [51–54]. The NF-E2-related factor 2 (Nrf2) is a key regulator of the inducible expression of enzymes such as GST and QR in catalyzing the detoxification of reactive electrophiles and oxidants that contribute to the formation of mutations and ultimately cancers. Nrf2 is now recognized to regulate a broad cytoprotective, transcriptional response leading to prevention of damage to DNA. Nrf2 is also a good target for cancer chemoprevention in certain tissues [55–59].

Based on these reports, we assayed mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)1-β, IL-6, COX-2, iNOS, and Nrf2 in the tongue and colonic mucosa of rats to determine whether CHRP and MJs can affect this mRNA expression. Male F344 rats were initiated with a tongue carcinogen 4-NQO (20 ppm) by giving drinking water for 8 weeks, and they were also fed diets containing CHRP at doses of 250, 500, and 1000 ppm for 8 weeks, starting 4-NQO administration. At week 8, tongue mucosa was scraped. In a different experiment, male F344 rats were initiated with a colonic carcinogen AOM by twice weekly subcutaneous injections (20 mg/kg bw), and they also received MJ, MJ2, and MJ5 for 4 weeks from starting the AOM injection. Colonic mucosa was scraped at week 4 of the experimental period. Total RNA was extracted from tongue and colonic mucosa using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd, Tokyo, Japan). Quantitative real-time PCR analysis of individual cDNA was performed with ABI Prism 7500 (Applied Biosystems Japan Ltd, Tokyo, Japan) with rat gene specific primers for TNF-α, IL-1β, IL-6, COX-2, and iNOS (Applied Biosystems) on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Fold change was calculated using the $\Delta\Delta$Ct method relative to untreated with β-actin as the endogenous control. In addition, the specific primer (forward primer 5′-tgcccctggaagtgtcaaa-3′, reverse 5′-ggctgtactgtatccccagaaga-3′) was used to measure the expression of Nrf2. Data are illustrated in Figures 7, 8, 9, and 10. Administration with CHRP and MJs decreased mRNA expression of TNF-α, IL1-β, IL-6, COX-2, and iNOS in the tongue and colonic mucosa, while the treatments increased mRNA expression of Nrf2 in both mucosal tissues.

8. Discussion

We have described cancer chemopreventive ability of CHRP and MJs in experimental animal carcinogenesis models of

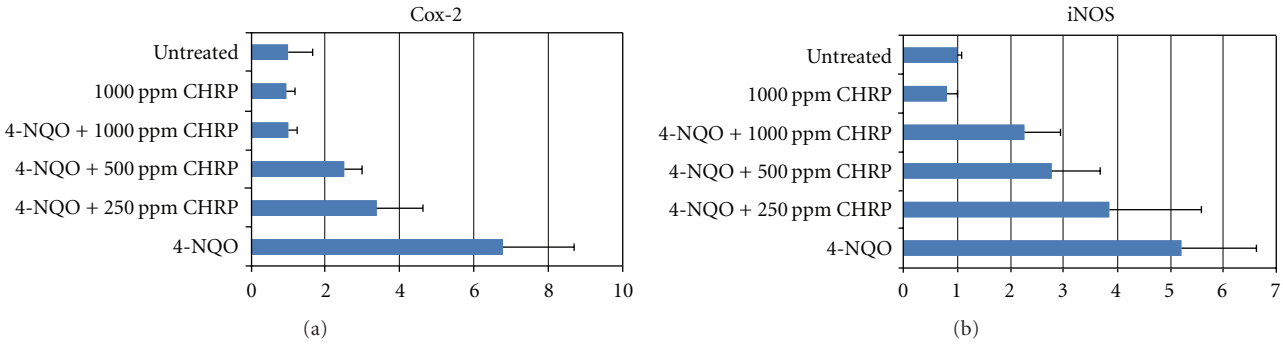


Figure 8: Dietary CHRP suppresses mRNA expression of inflammatory enzymes, Cox-2 and iNOS, in the tongue of rats that received 4-NQO at week 8 of the study.

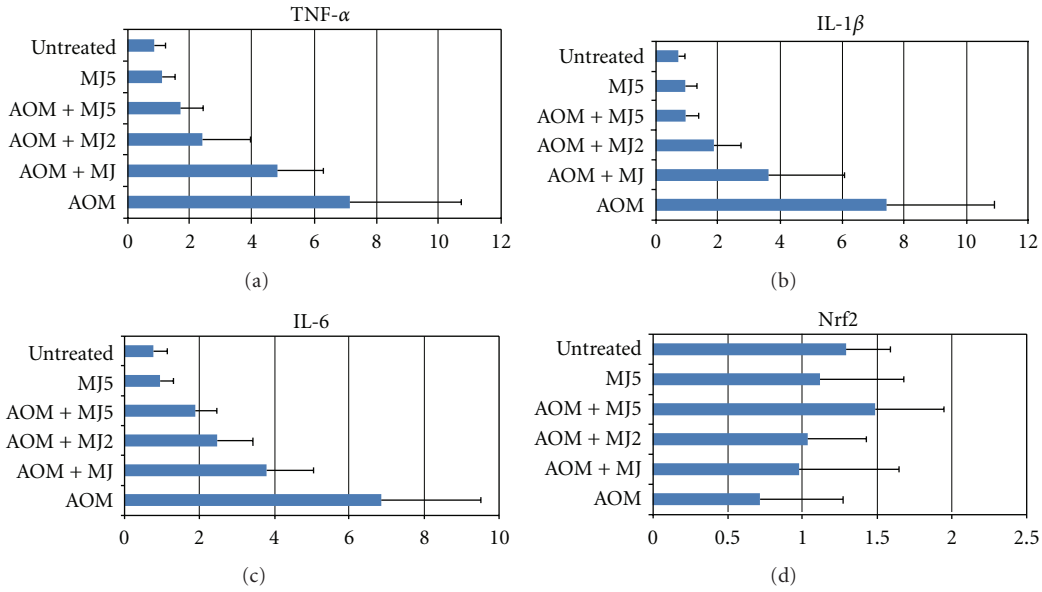


Figure 9: MJs lower mRNA expression of proinflammatory cytokines in the colonic mucosa of rats that received AOM at week 4 of the study.

colon, tongue, and lung cancers. Administration of CHRP or MJs after the carcinogen exposure clearly inhibits the development of malignant epithelial neoplasms. Miyagi et al. [60] also reported a protective effect of orange juice on AOM-induced rat colon carcinogenesis. Our data on MJs support their findings. The exact mechanism(s) involved in the suppressing effects of chemically induced tumorigenesis by CHRP and MJs is not fully known. In our studies, gavage with CHRP to rats elevated GST and QR activities in liver, colon, and tongue. Investigations by other researchers also demonstrated that limonin increased GST activities in several organs including liver, colon, and lung [61]. Therefore, it is possible that such elevation in activity of detoxifying enzyme by CHRP treatment may contribute its chemopreventive ability. Also, the modulatory effects of CHRP and MJs on specific species of liver CYPs, which are pertinent to the carcinogen metabolism [38, 62, 63], should be considered, although we did not examine their expression in our studies.

In addition, suppressing effects of CHRP and MJs on hyper-cell-proliferation activity induced by carcinogens in target organs account for their inhibition of carcinogenesis, since control of cell proliferation is one of the important effects of promising chemopreventive agents [23]. In our studies, gavage with CHRP to rats elevated GST and QR activities in liver, colon, and tongue, but strong inhibition in cancer incidence was not found when CHRP was fed to rats during the carcinogen treatment. This indicates that modification of cell proliferation of carcinoma cells rather than modification of activity of detoxifying enzymes may contribute to its chemopreventive ability when the substances were given after carcinogen exposure in long-term experiments.

Interesting findings in our studies are that CHRP and MJs were able to suppress mRNA expression of several cytokines (TNF-α, IL-1β, IL-6) and inflammatory enzymes (COX-2 and iNOS) and enhance mRNA expression of Nrf2 in the tongue and colon of rats that received a carcinogen,

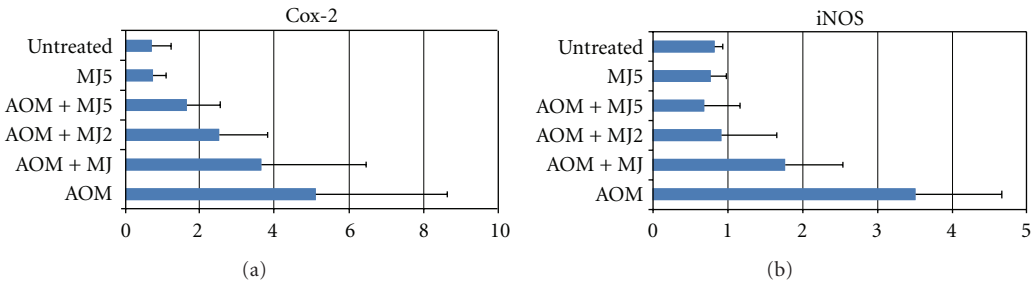


Figure 10: MJs suppress mRNA expression of inflammatory enzymes, Cox-2 and iNOS, in the colonic mucosa of rats that received AOM at week 4 of the study.

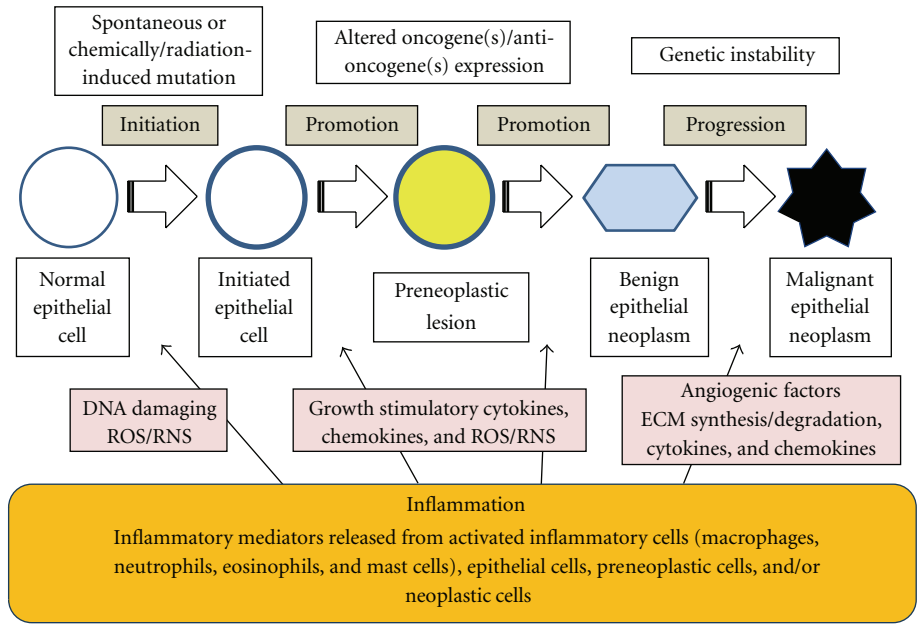


Figure 11: Working hypothesis of the involvement of inflammation in multistage carcinogenesis.

4-NQO or AOM. Chronic inflammation is known to be closely associated with cancer development in certain tissues [47], including oral cavity, colon, and lung. Cytokines regulate and mediate inflammation and angiogenesis. Tumor growth, invasion, and metastasis are facilitated when there is a deregulation in their production. Functional polymorphisms affecting gene expression of interleukin IL-1β, 4, -6, -8, and -10 as well as TNF-α are strongly associated with increased risk for oral cancer [64]. Recent findings also indicated that interactions between cytokines and sympathetic neurotransmitters and their respective receptors expressed by the nerve, immune, and tumor cells appear to influence tumor growth [65]. In the colon, inflammatory bowel disease (IBD) is an important risk factor for the development of CRC [21, 66, 67]. Inflammation is also likely to be involved with other forms of sporadic as well as heritable CRC [21]. Relationships between lung inflammation/injury and lung cancer in humans are also suggested [39]. Genetic predisposition to chronic obstructive pulmonary disease was associated with increased risk of developing lung cancer [68]. In addition to proinflammatory cytokines, inflammatory enzymes, such as COX-2 and iNOS, are involved in carcinogenesis [48–50], and they are good targets for cancer chemoprevention [51–54]. Nrf2 transcription factor was identified in the mid-1990s as a key regulator of the inducible expression of enzymes such as GST and QR in catalyzing the detoxification of reactive electrophiles and oxidants that contribute to the formation of mutations and ultimately cancers. Nrf2 is now recognized to regulate a broad cytoprotective, transcriptional response leading to prevention of damage to DNA, proteins, and lipids; recognition, repair, and removal of macromolecular damage; and tissue renewal following toxic assaults. The importance of this pathway as a determinant of susceptibility to carcinogenesis was indicated in multiple studies that demonstrated enhanced incidence, multiplicity, and/or tumor burden in Nrf2-deficient mice compared to wild-type ones in models of inflammation and CRC and lung cancer. Nrf2 is thus one of the targets for cancer chemoprevention in certain tissues [55–59]. When considering the relationship between chronic inflammation and cancer development (Figure 11), such effects of CHRP and MJs are attractive for reducing tumor occurrence.

Citrus fruit contains other possible chemopreventive agents. These include *d*-limonene [69], auraptene [70, 71], diosmin [72], limonin [73], and obacunone [73]. Some of these can modify activities and expression of the detoxifying enzymes and CYPs [74]. In addition, CHRP and MJs are able to downregulate mRNA expression of several cytokines and inflammatory enzymes and upregulate Nrf2 mRNA expression. Thus, citrus fruit is one of the rich sources of cancer chemopreventive agents.

9. Conclusion

Our studies demonstrate that oral administration of CHRP and MJs inhibits the development of malignant epithelial neoplasms in colon and tongue and lung tumors of rodents through their multiple biological functions. Further experiments, including preclinical efficacy and mechanistic studies including modification of expression of cancer-related genes in the target tissues, are warranted to fully evaluate these natural compounds for their cancer preventive properties and to understand their mode of action. One of the advantages of CHRP and MJs is that, unlike synthetic chemopreventive agents, they are natural substances present in human foods. On the basis of our observations and knowledge of the carcinogenic process, a strategy for cancer chemoprevention of colon, tongue, and lung can be developed. In conclusion, our findings may indicate that CHRP and MJs that are able to inhibit chemically induced carcinogenesis through modulation of proliferation, detoxifying enzymes, and mRNA expression of several cytokines and inflammatory enzymes are potential cancer chemopreventive agents against tongue, colon, and lung cancer development.

Acknowledgments

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Review Article

Antitumor Activity of Artemisinin and Its Derivatives: From a Well-Known Antimalarial Agent to a Potential Anticancer Drug

Maria P. Crespo-Ortiz[1] and Ming Q. Wei[2]

[1] *Department of Biomedical Science, Faculty of Basic and Health Science, Santiago de Cali University, Pampalinda Campus, Cali, Colombia*
[2] *Division of Molecular and Gene Therapies, Griffith Health Institute and School of Medical Science, Griffith University, Gold Coast Campus, Southport, QLD 4222, Australia*

Correspondence should be addressed to Maria P. Crespo-Ortiz, mdpcrespo@gmail.com

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Improvement of quality of life and survival of cancer patients will be greatly enhanced by the development of highly effective drugs to selectively kill malignant cells. Artemisinin and its analogs are naturally occurring antimalarials which have shown potent anticancer activity. In primary cancer cultures and cell lines, their antitumor actions were by inhibiting cancer proliferation, metastasis, and angiogenesis. In xenograft models, exposure to artemisinins substantially reduces tumor volume and progression. However, the rationale for the use of artemisinins in anticancer therapy must be addressed by a greater understanding of the underlying mechanisms involved in their cytotoxic effects. The primary targets for artemisinin and the chemical base for its preferential effects on heterologous tumor cells need yet to be elucidated. The aim of this paper is to provide an overview of the recent advances and new development of this class of drugs as potential anticancer agents.

1. Introduction

Cancer remains as a life-threatening disease and a leading cause of death as its control has been difficult. Although, a range of conventional therapies based on chemotherapy, surgery, and radiotherapy are available, these approaches are in many cases of limited efficacy [1]. Moreover, current anticancer regimens are frequently associated with significant levels of toxicity and the emergence of drug resistance. One major challenge to relieve cancer burden is to develop highly effective drugs with specificity on cancers but little or no side effects on normal mammalian cells.

Many research projects have been focused on developing new chemotherapies either by exploring the anticancer ability of novel compounds or by assessing drugs conventionally used in other clinical diseases. Natural products have been found to be a relevant source of novel and potent bioactive compounds with minimal side effects *in vivo*. Plant derivatives have been known to be effective against a range of diseases with broad antimicrobial activity, and some have also exhibited significant antitumor activity. One of the promising compounds is artemisinin, a naturally occurring antimalarial with anticancer properties [2]. Artemisinin and its derivatives, which are commonly used in malaria therapy, have also potent anticancer activity in the nano- to-micromolar range in sensitive and drug- or radiation-resistant cell lines [3–5]. Importantly, artemisinin is one of the very few drugs that have been widely used as antimalarials but has no significant side effects [6] or clinical resistance, although tolerance has been reported [7]. Recently, growing amount of research has focused on the mechanisms underlying the action and response to artemisinin-like drugs.

In this review, we will revisit some of the key issues in the development of artemisinin and its analogs as anticancer agents to better understand the mechanisms of their antitumor effects from the insights of new gained knowledge. By considering the benefits, limitations, and current and future development of artemisinins, we can then identify emerging

questions and address future research needs in this promising field of cancer drug discovery.

2. Artemisinin and Its Derivatives

Artemisinin is a sesquiterpene lactone with a 1,2,4-tiroxane ring system (Figure 1). This endoperoxide compound is extracted from the Chinese herb *qinghaosu* (*Artemisia annua* or annual wormwood) which was used for treating fevers for over two millennia [8]. Despite its efficacy, the prototype drug, artemisinin, has pharmacokinetic limitations. Naturally, artemisinin has low solubility in water or oil, poor bioavailability, and a short half-life *in vivo* (~2.5 h) [9, 10]. To overcome some of these problems, three generations of artemisinin-like endoperoxides including semisynthetic derivatives and fully synthetic compounds have been developed. So far, two generations of semisynthetic derivatives of artemisinin such as artesunate, arteeter, artemether, and artemisone have been effectively used as antimalarials with good clinical efficacy and tolerability (Figure 1).

Semisynthetic artemisinins are obtained from dihydroartemisinin (DHA), the main active metabolite of artemisinin [11, 12]. The first generation of semisynthetic artemisinins includes arteeter and artemether, the lipophilic artemisinins, whereas artesunate is the water soluble derivative [11, 12]. Artemisone, a second-generation artemisinin, has shown improved pharmacokinetic properties including longer half-life and lower toxicity [13]. So far, artesunate is the derivative that is commonly used in the antimalarial combination therapy.

Fully synthetic artemisinin derivatives have also been designed by preserving the peroxide moiety which confers potent drug activity. These compounds are easily synthesized from simple starting materials, thus being currently under intense development [14–17].

3. Antitumor Mechanism of Action of Artemisinin

In the malaria parasite, the endoperoxide moiety of artemisinin has been shown to be pharmacologically important and responsible of the antimalarial activity [18, 19]. The endoperoxide bond is thought to be activated by reduced heme (FPFeII) or ferrous iron (FeII) [20], leading to cytotoxic carbon-centered radicals which are highly potent alkylating agents [21]. Radicals may target essential parasite macromolecules causing parasite's death. However, the precise mechanism of action and primary target of artemisinin remain under study. In *Plasmodium*, it has been postulated that artemisinin may target organelles such as the mitochondrion, endoplasmic reticulum, and the digestive vacuole (reviewed in [22]). Some postulated molecular targets include heme alkylation, protein alkylation, Ca^{2+} ATPase (SERCA) inhibition, membrane damage, and loss of mitochondrial potential (reviewed in [22]). Despite the continuous debate on artemisinin activation and specific targets, supporting evidence points that heme or ferrous iron is required for potent activity [23]. This observation has been substantiated in other systems. In *Schistosomas*, artemether has an exquisite action against the tegument; this activity is also enhanced by iron [24].

Interestingly, the potent anticancer action of artemisinin can also be attributed to the endoperoxide bond (red square in Figure 2) and shares the same parasitical chemical base. Lack of the endoperoxide moiety does not completely abrogate anticancer activity [25] but significantly reduces cytotoxicity to only fiftieth compared to those compounds with the trioxane ring [26–28]. Residual anticancer activity may be associated with an alternative peroxide-independent mechanism [26]. In a general consensus, iron and heme or heme-bound proteins have been involved in the bioreductive activation of artemisinin [29–31]. In most of the systems, preloading of cancer cells with iron or iron-saturated holotransferrin (diferric transferrin) triggers artemisinin cytotoxicity [32–35] with an increase in artemisinin activity up to 100-fold in some cell lines [36]. Moreover, artemisinins tagged to iron-carrying compounds exhibit greater activity compared with that of artemisinin alone [37–39]. Recently, it was shown that chemical modulation using succinylacetone, a heme synthesis inhibitor, decreases DHA cytotoxicity in HL-60 (human promyelocytic leukemia cells) [35]. This was consistent with previous studies showing that induction of heme oxidase followed by downregulation of the heme synthesis genes may also inhibit cytotoxicity of novel artemisinin dimers in the same cancer line [40]. Similarly, treatment with desferroxamine (DFO), an iron chelator, renders compounds inactive [41]. Iron and heme metabolism may have a relevant role in the selective antitumor activity of artemisinin. Continued proliferation and growth of malignant cells require higher iron metabolism to achieve processes of cell survival [35]. Therefore, cancer cells exhibit an increase in transferrin receptors (TfR) which are responsible for the iron uptake and regulation of intracellular concentrations. Levels of expression of TfR in cancer cells may vary depending on the cell line. However, they differ substantially from normal cells leading to a high selectivity index of artemisinin and its derivatives. Efferth et al. reported that leukemia (CCRF-CEM) and astrocytoma (U373) cells express TfR in 95% and 43% of the cell population, whereas normal monocytes only account for approximately 1% [42, 43]. Blocking the TfR by pretreatment with specific monoclonal antibodies abrogates artemisinin activity [43].

It has been hypothesized that iron-activated artemisinin induces damage by release of highly alkylating carbon-centered radicals and radical oxygen species (ROS) (Figure 2) [28, 35]. Radicals may play a role in the cell alterations reported in artemisinin-treated cancer cells such as enhanced apoptosis, arrest of growth, inhibition of angiogenesis, and DNA damage (Figure 2). Several studies have also associated artemisinin toxicity with impaired cytokinesis, enhanced levels of oxidative stress, inhibition of tumor invasion, migration, and metastasis (reviewed in [44]). ROS generation may contribute with the selective action of artemisinin on cancer cells. Tumor cells have enhanced vulnerability to ROS damage as they exhibit lower expression of antioxidant enzymes such as superoxide dismutase, catalase, and gluthatione peroxidase compared to that of normal cells [45, 46]. Hence, increasing oxidative stress is a common anticancer

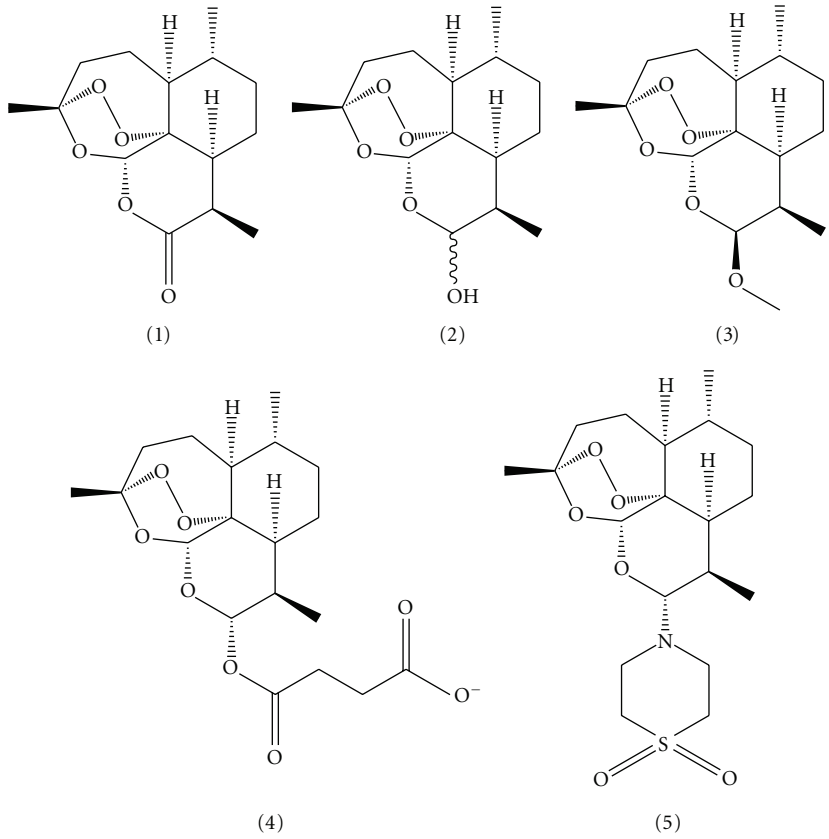

Figure 1: Chemical structure of artemisinin antimalarials (artemisinins) with anticancer activity. Artemisinin (1), dihydroartemisinin (DHA) (2), artemether (3), artesunate (4), and artemisone (5).

mechanism of antitumor agents [47]. In addition, the selectivity of artemisinin may be boosted by preferential targeting of cancer biomarkers or overexpressed cancer genes and proteins which are not detectable in normal differentiated tissues [48].

3.1. Generation of ROS as a Primary Effector of Cytotoxicity. As in *Plasmodium*, the artemisinin molecular targets in cancer cells are debatable. Although artemisinin-induced alterations in some tumor cells are consistent, it is not clear if this toxicity resides in defined molecular targets. Drug concentrations required to have an effect on cancer cells are often higher than those inducing toxicity in malaria parasites. Artemisinin, DHA, artesunate, and artemether exhibit 48 h IC_{50}s (fifty percent inhibitory concentration) up to 15 nM in malaria parasites [49, 50], whereas their anticancer activity is cell-line dependent and IC_{50}s fluctuate between 0.5 and $\geq 200\,\mu$M [5]. The exquisite sensitivity of malaria parasites to artemisinin points to the presence of specific parasitic targets. By contrast, in cancer cells, the artemisinin effect seems to be rather mediated by more general mechanisms through generation of ROS. However, it has been suggested that ROS-mediated damage may be triggered by an initiating event in the vicinity of artemisinin activation [35]. Microscopy analyses in artesunate-treated cells have shown early oncosis-like morphological changes at subcellular structures in which ROS generation may be triggered [51].

Microarray analyses found that the action of artemisinin seems to be modulated by the expression of oxidative stress enzymes including catalase, thioredoxin reductase, superoxide dismutase and the glutathione S-transferase family [5, 52]. Artemisinin-sensitive cells have downregulated oxidation enzymes whereas overexpression of these molecules renders cancer cells less sensitive [5]. Direct evidence in the HL-60 cell line has revealed that early and rapid generation (1 h) of ROS has been associated with apoptosis induction and artemisinin-induced damage. Furthermore, IC_{50} has been directly correlated with ROS levels [52]. Conversely, the action of artemisinin in several experimental systems has been reverted in presence of the antioxidant agents, N-acetyl cysteine, and 1,2-dihydroxybenzene-3,5-disulfonic acid (TIRON, an iron scavenger), which resulted in a delay in cell death [40, 52, 53]. A recent study has demonstrated that generation of ROS in artesunate-treated HeLa cells (16 h) occurring before cytotoxicity is being detected (48 h) [35], suggesting that this may be the starting event in artemisinin-induced damage. The electron transfer chain (ETC) in the mitochondrion has been proposed to play a role in the generation of ROS, however substantial cytotoxicity is still detected in HeLa cells devoid of ETC indicating that other sources of ROS may be available in the cells [35]. Indeed, emerging evidence has postulated that oxidative stress in breast cancer cells is initially generated in the lysosome as consequence of iron-activated artesunate in a process

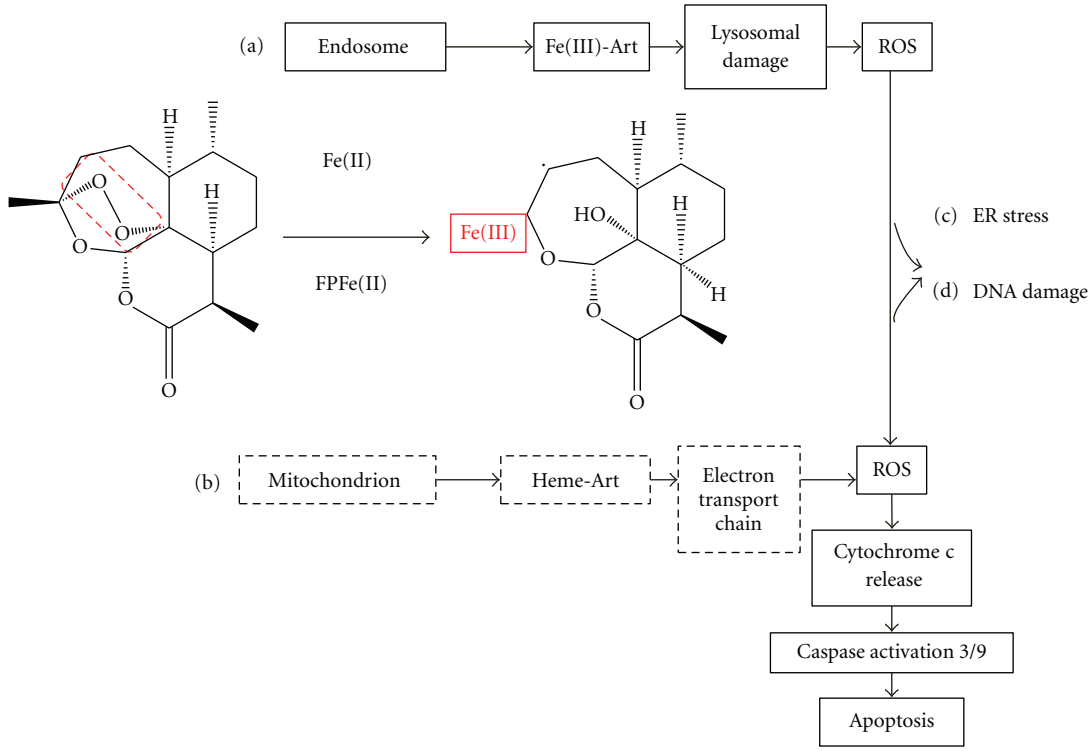

Figure 2: Postulated anticancer mechanisms of action of artemisinins. (a) It has been postulated that bioactivation of artemisinin occurs in the endosome after pH-induced release of iron from internalized transferrin. Iron activated-artemisinin generates carbon-centered radicals which may mediate lysosomal disruption and generation of ROS resulting in mitochondrial damage, activation of caspases, and cell death. (b) Alternatively, it has been suggested that only specific activation of artemisinin by heme or heme-bound protein generates cytotoxic-carbon-centered radicals. In the mitochondrion, these adducts interfere with the electron transfer chain (ETC) by interacting with heme or heme-bound proteins leading to generation of ROS and apoptosis. (c) ROS harboring may induce ER stress and (d) genotoxicity.

similar to that suggested in malaria parasites [31]. Thus, activation of the mitochondrial intrinsic apoptotic pathway is a downstream event leading to cell death [31] (Figure 2). In this model, artemisinins may be negatively controlling heme synthesis and further increase cytotoxicity [31].

Despite the growing evidence of ROS-mediated damage in many cell systems [31, 33, 35, 54], cell damage has been also independently associated with oxidative stress [35]. Particularly, novel artemisinin dimers seem to exert antitumor action with little or no ROS generation, however the underlying mechanism of cytotoxicity is still under study [26]. It also remains unclear if artemisinins-induced necrosis may be a ROS-independent mechanism of cell death [35].

The antineoplastic toxicity of artemisinins appears to be also modulated by calcium metabolism [40, 55–57], endoplasmic reticulum (ER) stress [33, 40], and the expression of the translationally controlled tumor protein, TCTP, a binding calcium protein which has been also postulated as a parasite target [5]. Although the expression of the TCTP gen, *tctp*, was initially correlated with cancer cell response to artemisinins, a functional role for TCTP in the artemisinin action has yet to be found [58].

As for malaria parasites, the role of sarcoendoplasmic Ca^{2+} ATPase (SERCA) as artemisinin target in cancer cells has also been explored [40]. Previous evidence has revealed that treatment with 10 μM artemisinin increases calcium concentrations as a result of SERCA inhibition [59]. However, studies on the mechanism of action of two artemisinin dimers have shown that potent ROS-mediated induced ER-stress after treatment was independent of SERCA inhibition [40]. Interestingly, the behavior of a highly active artemisinin dimer and thapsigargin, a well-known SERCA inhibitor, seems to be similar but mediated by different molecular events [40]. In fact, thapsigargin lacks the endoperoxide moiety and only generates discrete ROS levels. Nevertheless the ER appears to be a relevant site for artemisinin action as in HepG2 cells a fluorescent derivative has been shown to preferentially accumulate in this cell compartment [60].

Artemisinins have shown pleiotropic effects through different experimental systems. It is also possible that the underlying mechanisms mediating artemisinins cytotoxicity may vary upon specific hallmarks or shifting characteristics in cancer cell lines (Table 1). This will be only possible to elucidate if the molecular events involved in countering malignant cell proliferation are investigated in different cell lines under similar conditions.

4. Artemisinins as Anticancer Drugs

4.1. Antitumor Effects of Artemisinin and Its Derivatives. Significant antitumor activity of artemisinin and licensed semisynthetic artemisinin derivatives has been documented

Table 1: Factors that may influence artemisinins response in cancer cells.

System	Factor/characteristic
Cancer cell	Proliferating activity
	Expression of transferrin receptors
	Accumulation levels of iron
	Levels of gene expression (i.e., proapoptotic and antiapoptotic genes)
	Shifting hallmarks
	Overexpression of potential molecular targets in some tumors
	Cellular dependence on redox balance
	Expression of antioxidant enzymes
	Expression of estrogen receptors in breast cancer cells
Artemisinin compound	Dose and time of exposure
	Chemical structure: number of trioxane rings, for example, dimeric compounds can be up to >1000 fold-more potent than monomeric artemisinins [61]
	In dimeric endoperoxides: nature and stereochemistry of the linker
	In novel compounds, electrophilic substitutions in the ring or those conferring lipophilicity. Boat/chair conformation

in vitro and in animal models. Considerable research has been focused on the most active compounds, namely, DHA and artesunate. One study that tested 55 cell lines from the Developmental Therapeutics Program of the National Cancer Institute (NCI) showed that artesunate displays inhibitory activity against leukemia, colon, melanoma, breast, ovarian, prostate, central nervous system (CNS), and renal cancer cells [5]. Dihydroartemisinin has also remarkable antineoplastic activity against pancreatic, leukemic, osteosarcoma, and lung cancer cells [62]. Moreover, artemisone has shown better activity than artemisinin and considerable synergistic interactions with other anticancer agents [63].

Artemisinin has been found to act either directly by inducing DNA damage (genotoxicity) or indirectly by interfering with a range of signaling pathways involved in several hallmarks of malignancy. However, direct DNA damage is only described in specific systems, while indirect effects are more commonly refereed in the literature. In pancreatic cells (Panc-1), artesunate caused DNA fragmentation and membrane damage. Interestingly, low doses of artesunate were associated with oncosis-like cell death, whereas higher concentrations induce apoptosis [51]. Extend and type of damage seem to depend on the phenotype and the origin of cell line, and it may also vary in a time- and dose-dependent manner (Table 1). Notably, higher sensitivity to artesunate was observed in rapidly growing cell lines when compared with slow growing cancer cells [5].

Alternatively, DHA, artesunate, and artemether are likely to modulate genes and proteins coordinating growth signals, apoptosis, proliferation capacity, angiogenesis and tissue invasion, and metastasis. A complex network of interactions through different pathways may enhance the anticancer effect of these endoperoxide drugs leading to cancer control and cell death (Table 2).

4.1.1. Artemisinins Counter Cancer Proliferative Capacity. In normal cells, cyclin-dependent kinases (CDK) are the proteins translating signals in order to push cell through the cell cycle. Normal growth relies on the ability to translate signals in order to replicate and divide in an effective manner [64]. Uncontrolled proliferation in cancer cells is the result of mutations inducing amplification of growth signals, deregulation of checkpoints, and loss of sensitivity to growth inhibitors [65]. Abnormal cell growth is also triggered by deregulation of programmed cell death or apoptosis [65]. Artemisinin and its semisynthetic derivatives are able to effectively induce cell growth arrest in cancer lines either by disrupting the cell cycle kinetics or by interfering with proliferation-interacting pathways. Dihydroartemisinin and artesunate are very potent growth inhibitors with multiple studies pointing to DHA as the most potent anticancer artemisinin-like compound (DHA > artesunate > arteeter > artemether) [5, 66]. Recently, artemisone has shown impressive antitumor efficacy in 7 cells lines including melanoma and breast cancer cells [63]. Artemisinin compounds have been shown to exert cytostatic and cytotoxic action on cancer cells [63, 67]. Artemisinin-induced growth arrest has been reported at all cell cycle phases; however, arrest at G_0/G_1 to S transition seems to be more commonly affected [5] (Table 2). Arrest at all cell cycle phases at the same time has been interpreted as a cytostatic effect [63]. Disruption of the cell cycle at G_2/M was observed after DHA treatment in osteosacorma, pancreas, leukemia [68] and ovarian cancer cells [69] (Table 2). Similarly, artesunate interferes with G_2 in osteosarcoma, ovarian, and other different cancer lines (Table 2). The underlying mechanisms of artemisinins-induced growth arrest include alterations in the expression and activity of regulatory enzymes of the cell cycle, such as CDK2-4 and -6 and D type cyclins (G_1-to-S-phase transition) or CDK1, and A-type cyclin (G_2/M) [70–72]. The antiproliferative action of artemisinin induces downregulation of CDK transcription, inhibition of CDK promoters or increase of p21, p27, and CDK inhibitor [72] (Table 2). Inhibition of proliferation may be also attributed to downregulation of interacting proteins targeting multiple pathways [72]. It has

Table 2: Antitumor effects of artemisinins.

Cmpd	Cancer/cell line	Effect	Event/mechanism	Refs
DHA/ART	Osteosarcoma	Growth arrest	G_2/M, decreased survivin	[73]
	4 cell lines with different p53 status	Apoptosis	Increased Bax, activation of caspase 3,8,9 Decreased Bc12, Cdc25B, cyclin B1, NF-κB	[44]
DHA	Hepatoma (different cell lines)	Growth arrest	G_1, decreased cyclin D, E, CDK2-4, E2F1 Increased Cip 1/p21, Kip 1/p27	[67]
		Apoptosis	Increased Bax/Bcl2 ratio, activation of caspase 3 Increased poly ADP-ribose polymerase Decreased MDM2	
DHA/ART	Neuroblastoma	Growth arrest	G_1	[52]
		Apoptosis	Activation of caspase 3	
DHA	Pancreas (BxPC3 RFP)	Growth arrest	G_1, decreased cyclin D1, increased p21	[74]
		Apoptosis	Increased Bax, decreased Bcl2	[74, 75]
		Angiogenesis	Decreased VEGF	[75, 76]
			Decreased NF-κB DNA binding	[74, 76]
			IL-8, COX2, MMP9	[76]
DHA	Human promyelocytic Leukemia (HL-60)	Growth arrest	G_1	[33, 34]
		Apoptosis	ER stress, degradation of c-MYC	[33]
	Colorectal cancer (HT116)		Increased GRP78	[34]
			DNA damage	[33]
DHA	Lung cancer (SPCA1)	Apoptosis	Decreased survivin	[56]
	(PC-14)		Increased calcium levels, increased p38 MAPK	[57]
	(ASTC-a-l)		Increased oxidation, activation caspase 3,9,8 Bax translocation	[54]
DHA/ARS	Human ovarian cancer (cell panel, A2780, OVCAR-3)	Growth arrest	G_2	[69]
		Apoptosis	Increased Bax-Bad, decreased Bclx-Bcl2	[69, 77]
			Activation caspase 3/9 pathway	[69, 77]
DHA	Lymphatic endothelial cells	Apoptosis	Increased Bax, decreased Bcl2 Decreased VEGFR-3/FL-4	[78]
DHA	Melanoma (A375, G361, LOX)	Apoptosis	Increased oxidative stress, increased NOXA Activation caspase 3	[79]
DHA	Jurkat T Lymphoma	Apoptosis	DNA damage Increased oxidation, increased NOXA Increased Bak, activation of caspase 9	[80]
DHA	Fibrosarcoma (HT 1080)	Migration/invasion	Decreased NF-κB, AP-1 Decreased activation of MMP2, MMP9 Decreased PKC α/Raf/ERK and JNK	[81]
DHA	Glioma cells (C6)	Apoptosis	Decreased HIF 1α, VEGF	[41]
DHA	Chronic myeloid leukemia (K562 cells)	Growth arrest	G_2, decreased PCNA, cyclin B1, D1, E1	[82]
			CDK2-4, E2F1, DNA-PK, DNA-topo1, JNK VEGF	[68]
ART		Angiogenesis	Decreased VECF	[82]
DHA	Lewis lung carcinoma	Angiogenesis	Decreased VEGF-C, IL-1 β-induced p38 MAPK activation	[83]
			Decreased VEGF receptor KDR/flk-1	[84]
DHA/ART	Cervix carcinoma (HeLa) Human papillomavirus immortalized/transformed cells	Apoptosis	Activation of caspase 9	[85]
ART	Leukemia, melanoma, non-small cell lung cancer, colon, renal, ovarian, prostate, CNS; prostate, breast cancer (NIC cell panel)	Growth arrest	G_0/G_1, decreased CDK2, CDC25A G_2/M, decreased cyclin B1	[5]

Table 2: Continued.

Cmpd	Cancer/cell line	Effect	Event/mechanism	Refs
ART	Endometrial carcinoma (HEC-1B)	Growth arrest	G_0/G_1	[86]
		Apoptosis	Activation of caspase 3, decreased COX-2	
		Angiogenesis	Increased E-cadherin	
ART	Pancreatic cancer (BxPC3, MiaPaCa-2)	Apoptosis	Activation of caspases 3, 7 Inhibition of topoisomerase II a	[87]
ART	Non-small cell lung cancer (SPC-A1)	Metastasis	Decreased MMP2, transactivation of AP-1	[56, 88]
			NF-κB	
			uPA promoter	[88]
			MMP7	[56]
ART	Colorectal (CLY, HT29, Lovo)	Metastasis	Increased E cadherin Decreased Wnt-signalling pathway	[89]
ART	Mouse myeloma cell line SP2/0	Growth arrest	G_0/G_1	[90]
		Apoptosis	Decreased NF-κB p65, increased IκBα	
ARS	Hepatocellular cancer cells (HepG2, SMMC-7721)	Metastasis	Increased TIMP2, Cdc42, E cadherin Decreased MMP2	[91]
	Nasopharyngeal cancer lines (CNE-1 and CNE-2)	Growth arrest	G_1	[92]
	Melanoma (A375P, A375M)	Growth arrest	—	[93]
		Migration	Decreased MMP2, $\alpha v\beta 3$ integrin	
ATM	Colorectal (HCT116, SW480)	Growth arrest	G_1, S, G_2, decreased CDK1	[63]
			All phases	
	Breast (MCF-7)		G_1, decreased CDK4, cyclin D1	

Abbreviations: Cmpd: compound; DHA: Dihydroartemisinin; ART: artesunate, ARS: artemisinin, ATM: artemisone.

been shown that DHA treatment in pancreatic cells (BxPC3, AsPC-1) inhibits viability by decreasing levels of proliferating cell nuclear antigen (PCNA) and cyclin D with parallel increase in p21 [74]. Another study in the same system shows that DHA counters NF-κB factor activation leading to inhibition of its targets in the proliferation (c-myc, cyclin D) and apoptotic pathways (Bcl2, Bcl-xl) [94]. Downregulation of survivin, a protein modulating apoptosis and G_2/M cell cycle progression [95], was observed after treatment with DHA in lung cancer cells (SPC-A1) [94]. A similar effect was described by Qiang et al. in artesunate- treated osteosarcoma cells [44]. In prostate cancer, DHA induces cell cycle arrest by disrupting the interaction of Sp1 (specificity protein 1) and the CDK4 promoter [96]. Dissociation of the Sp1-CDK4 complex promotes caspase activation and cell death. In addition one work has identified artesunate as a topoisomerase II, an inhibitor which inhibits growth by interaction with multiple pathways [87]. Overall, artemisinins seem to be interfering with several pathways that are common to different cancer entities.

4.1.2. Proapoptotic Effect of Artemisinins. Apoptosis is a widely studied mechanism in antitumor therapy as its manipulation is an effective strategy for cancer control. This cellular process is mediated by a balance between the Bcl2 family genes, the proapoptotic Bax, and the antiapoptotic Bcl2 and their effects on the mitochondria [97, 98]. An increase in the Bax/Bcl2 ratio induces the release of cytochrome c followed by sequential activation of caspases and culminating with cell death [98].

Apoptosis is a common and rapid artemisinin-induced effect observed in many cancer and cell lines. Treatment with 200 μM DHA in leukemia cells induced apoptosis after 1 hour of exposure [32]. Artemisinin sensitivity has been correlated to the level of expression of antiapoptotic (Bcl2) and proapoptotic genes (Bax) in a cancer cell line [61, 97, 99] (Table 1). In general, the apoptotic effects of artemisinin have been attributed to activation of the intrinsic pathway. Hence, mitochondrial membrane damage is thought to have a pivotal role in the cascade of cell death events. Many studies have revealed that artemisinin-like compounds induce apoptosis by modulating the Bax/Bcl2 ratio [33, 44, 54, 63, 75, 77, 78, 86, 99]. Consistent with these observations, DHA and artesunate, in a panel of osteosarcoma cells, caused cytochrome c release, Bax overexpression, increase in Bax/Bcl2 ratio [44, 73], and activation of caspases 3 and 9. DHA also activates caspase 8 and decreases the levels of CDC25B, cyclin B1, and NF-κB [73]. In the same system, artesunate exposure depletes survivin which has also been involved in the apoptotic DHA response in lung cancer cells [56]. Similar events have been described in hepatoma cancer lines treated with DHA, particularly in this system DHA and the prototype drug artemisinin seem to have similar potency [67]. A microarray analysis has correlated the expression levels of c-MYC with enhanced DHA-induced apoptosis. Leukemia (HL-60) and colon cancer cells (HCT116) expressing high levels of c-MYC are significantly more sensitive to the DHA proapoptotic action. Moreover, knockdown of *c-myc* in HCT116 depleted DHA-associated cell death [33]. Downregulation of *c-myc*

may also correlate with induced G_1 arrest in this cell line [33]. Studies in metastatic melanoma (A375, G361 cell line) and Jurkat T lymphoma cells have associated the elevated apoptotic action of DHA with upregulation of NOXA (a proapoptotic protein), caspase 3 activation, and oxidative stress [79, 80]. In lung cells, the apoptotic effect of DHA occurs with increasing calcium concentration and activation of p38 [56, 57].

In some studies alterations on molecules acting on the extrinsic apoptotic pathway have also been described [54]. DHA seems to increase the transcription of the cell death receptor 5 (DR5) promoter and induces DR5 in different prostate cancer lines. In fact, a combination treatment with TRAIL, a DR5 ligand, strongly enhances DHA proapoptotic action by up to 35% on this system [100].

Artemisinins usually promote apoptosis rather than necrosis in most of the systems, however in some cases both apoptosis and necrosis have been reported. Induction of apoptosis is a major benefit of artemisinins' antitumor action as it prevents the collateral effects of inflammation and cell damage caused by necrosis. Artemisinin-induced necrosis has been associated with low levels of ATP and defective apoptotic mechanisms in some cell lines [35].

4.1.3. Artemisinins and Metastasis/Invasion Inhibition. The ability of malignant cells to invade has been associated with high mortality and morbidity in cancer patients. The spread of cancer cells to other organs is a process in which malignant cells readily invade through the extracellular matrix, reach and survive in the bloodstream, and finally seed at distant organs [101]. To achieve invasion, the cancer cell requires the loss of expression or function of E-cadherin, a calcium-binding transmembrane molecule involved in cell-cell adhesion. A range of genes encoding extracellular matrix processing proteases, motility factors, and adhesion proteins are also acting at different steps in the metastatic process [101]. Recently, PAI-1 and TIMP-1 known as endogenous protease inhibitors have also been shown to be involved in cancer metastasis [102]. An invaluable benefit of artemisinin is its relevant antimigratory activity in highly aggressive and invasive cancer entities [56, 59, 88, 91]. Antimetastatic activity of artemisinins has been correlated with modified expression of the matrix metalloproteinases (MMP) gene family and their effects on $\alpha v\beta 3$ integrins [93]. In hepatoma cells (HepG2 and SMMC 7721), treatment with 12.5 μM artemisinin depleted migration linked to a decrease in MMP2 with concomitant increase in TIMP-2. Inhibition of metastasis is achieved as artemisinin increases cell-cell adhesion by enhancing E-cadherin activity and Cdc42 activation [91]. In addition, it has been found that some cancer cells may have specific proteins cointeracting at different pathways. For example, in non-small cell lung cancer [56] and fibrosarcoma, DHA treatment induced low levels of MMP2, MMP7, or MMP9 driven by AP-1 and NF-κB transactivation or inactivation [81]. Previous studies have shown that MMP2 is regulated by Sp-1 transcription factor activity [103], moreover DHA-induced disruption of Sp-1 molecular interactions has been postulated as a crucial event for DHA regulation effects on different pathways [72]. Other investigations have found that in mouse lung Lewis cancer, lymphoid node metastasis and lymphangiogenesis were retarded by artemisinin-mediated inhibition of vascular endothelial growth factor C (VGF-C) [83].

4.1.4. Artemisinins and Angiogenesis Inhibition. As malignant tissues grow, metastases and solid tumors require extra blood supply for thriving and survival. Thus, cancer cells induce neovascularisation by regulating proteins and pathways involved in the generation and restructure of new vasculature [101]. Angiogenesis process leads to enhanced proliferation of endothelial cells through induction of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), its receptors, and cytokines [101]. This event occurs via multiple mechanisms including hypoxia-driven activation of expression of HIF-1α and the aryl hydrocarbon receptor nuclear translocator (ARNT) [104]. Angiogenesis control is mediated by angiostatin, endostatin, thrombospondin, TIMPs, PAI-1, and others [101]. Due to their role in tumor survival, the proangiogenic factors and the molecules involved in their regulatory networks are relevant drug targets. A microarray-based study revealed that artemisinins, artesunate and other derivatives inhibit neovascularisation by modulating gene expression of angiogenic factors [105]. Artemisinins responses seem to be mediated by downregulation of growth factors (VEGF, FGF) [82, 106], HIF-1α [107], new vessel mediator angiogenin (ANG), the cysteine-rich angiogenic inducer (CYR61), some metalloproteinases (MMP9, MMP11, and BMP1), and collagens [105]. In parallel, artemisinins- induced upregulation of angiogenesis inhibitors was observed [105]. These findings have been supported by experimental investigation in different systems, unveiling other molecular interactions. Exposure of human umbilical vein endothelial cells to 50 μM DHA prevents angiogenesis by depleting the levels of the VEGF flt-1 and KDR/flk-1-receptors. Similar effects were reproduced in lymphatic endothelial cells and Lewis lung carcinoma [78, 84]. In pancreatic cells (BxPc-3) and BalB/c nude mice, DHA induced inhibition of NF-κB DNA binding and downregulation of its angiogenic-related targets such as VEGF, IL-8, COX2, and MMP9 [76]. Reduced levels of NF-κB have been previously associated with proliferation and metastasis inhibition [33, 81, 90, 94] suggesting that NF-κB regulation may be a key role in the multimodal action of DHA in this system. NF-κB is a crucial factor regulating multiple processes and it has a key role in the anticancer drug response. It is activated by DNA damage and it is a mediator of apoptosis resistance in response to drug pressure.

Other anticancer properties have also been attributed to artemisinins. Artesunate has shown its ability to revert cellular transitions allowing re-differentiation of tissues by negative control of Wnt-signaling pathway [89]. Notably, artesunate has been found to be more effective in less differentiated cell lines [89].

4.2. Antitumor Action of Artemisinins in Resistant Cancer Cells. One major obstacle for a successful anticancer therapy is the development of resistance over time. Many aggressive tumors become refractory to anticancer therapy with hardly

any chemotherapeutic alternatives. A leading cause of drug resistance is the drug efflux generated by overexpression of membrane protein pumps, which results in ineffective low drug concentrations [108]. Anticancer activity of artemisinins has shown to be unaffected in otherwise resistant and multiresistant cancer cells. One study using the 55 NCI cell lines and microarray analysis revealed that genes related with resistance to the established anticancer drugs such as *MDR1 (Pgp), MRP1,* and *BCRP* showed no impact on the activity of artemisinins [5]. This was substantiated when no effects on the artesunate growth inhibition profile were observed in multidrug resistance HL-60 cell lines overexpressing *MRP-1* and *BCRP*-overexpressing cells, suggesting that antitumor activity of artemisinin is preserved when resistance to other agents is present [5]. Artemisinins are effective in a broad range of resistant cancer lines including doxorubicin, metrotexate, and hydroxyurea-resistant lines with no cross-resistance [5]. Further investigation has shown that artesunate proapoptotic effect is not affected in a doxorubicin-resistant leukemia cell line; instead artesunate potentiates doxorubicin apoptotic effects [4]. In another study, anticancer potency of artesunate is preserved in chemoresistant and chemosensitive neuroblastoma cell lines and primary neuroblastoma cultures [52]. In this system, sensitivity to artesunate was not affected in vincristin, doxorubicin, cisplatin, topotecan, mephalan, and ectoposide-adapted cells with IC_{50}s ranging from 1.4–2.7 μM similar to that of the parent sensitive cell line [52]. Only one cell line showed low sensitivity to artesunate which was related to low ROS formation and increased expression of gluthatione cysteine ligase (GCL) [52]. Depletion of glutathione mediated by a GCL inhibitor improved artesunate sensitivity in this cell line [52]. P-glycoprotein (Pgp) or p53 attenuation did not affect sensitivity to artesunate [52]. DHA has shown the lowest IC_{50} in some cell lines such as cholangiocarcinoma (CL-6) and hepatocarcinoma (Hep G2) compared to other anticancer agents; moreover, upregulation of *MDR1*, *MRP1-2*, or *MRP3* shows no effect on potency [109]. Lack of cross resistance between anticancer agents and artemisinins might be based on different mechanisms of drug action and/or resistance. Most of the conventional anticancer agents are nucleoside analogs, whereas artemisinins action is thought to be mediated by a ROS-dependent mechanism. Furthermore, in erythromyelogenous leukemia and human small cell lung cancer, artemisinins show no significant inhibition towards Pgp or MRP1 [4], thus in principle overexpression of protein pump may not affect artemisinin's potency. In another system however, artemisinin (the prototype drug) increases doxorubicin resistance by upregulating *mdrp* through a mechanism that will be discussed later.

4.3. Interactions of Artemisinins and Standard Anticancer Chemotherapy: Artemisinin Combination Therapy (ACT) for Cancer? Existing anticancer therapies predominantly target cancer proliferation either with chemotherapeutic agents, ionizing radiation or direct toxicity on growth factor signaling pathways. In a combination therapy for cancer, the antineoplastic action of artemisinin may contribute to an independent antitumor activity with no additional side effects. The benefits of combining artemisinins with other anticancer agents have been investigated showing that multifactorial action of artemisinin in different pathways may improve overall activity (synergism).

It has been reported that resistant cancer cell lines become sensitive by adding artemisinin to the conventional treatment (chemosensitization). Interestingly, DHA and artesunate have exhibited the strongest chemosensitizing/synergistic effects [4, 110], whereas the prototype drug artemisinin shows only additive and antagonistic interactions (Table 3). DHA significantly improves the anticancer effect of gemcitabine, an anticancer drug used in pancreatic cancer which develops resistance over time. *In vitro* and *in vivo* analysis in pancreatic cells demonstrated a DHA-induced increase in growth inhibition and apoptosis by 4- and 2-fold, respectively, compared with those obtained with gemcitabine alone [94]. A dual action of DHA in potentiating gemcitabine activity and possibly counteracting resistance has been attributed to DHA inhibition of gemcitabine- induced NF-κB activation and subsequent action on its targets [94]. A similar effect has been shown in hepatoma cancer cell lines irrespective of their p53 status [67]. DHA synergistically enhances tumor growth inhibition by 45% when in combination with gemcitabine, whereas artemisinin, the prototype, only induces additive effects [94].

Consistent with this observation, a greater antitumor activity was observed when DHA was used in a combination with cyclosphosphamide in murine Lewis lung carcinoma cell line or in combination with cisplatin in non-small cell lung cancer A549 in mice [84]. In rat C6 glioma cells, addition of 1 μM DHA increased by 177% the cytotoxic effect of temozolomide, a DNA-alkylating agent used in the treatment of brain cancer. Further investigation found that DHA promotes apoptotic and necrotic activity of temozolomide through ROS generation [107]. Recently, an enhancement of artesunate anticancer activity has been observed in different combination regimens. A striking synergy was achieved in combinations of artesunate and the immunomodulator drug, lenalidomide [111].

However, the benefits of an artemisinin combination therapy need to be carefully dissected. Therapeutic effects are influenced by the mode of action of the drugs and multiple interactions in particular systems and schedules. Recently, Gravetth et al. showed that gemcitabine has only additive effects when combining gemcitabine and artemisone in colon and breast cancer cells [63]. In cancer colon cells (HT-29), it has been suggested that artemisinin may impair doxorubicin activity possibly by countering the doxorubicin effect on NF-κB inhibition [59]. The same authors have reported artemisinin-induced resistance in the same system through a different mechanism. Thus, it has been postulated that artemisinin exposure inhibits SERCA with subsequent accumulation of calcium. As a result, Pgp is upregulated and leads to generation of doxorubicin resistance cells [59]. By contrast, pretreatment with a calcium chelator reverted the cells to a sensitive phenotype [59]. Notably, DHA and artesunate have not been evaluated in this system; it remains to be elucidated whether the most potent chemosensitizers have similar effects on this cell line. So far, artesunate or DHA

Table 3: Drug interactions of artemisinins.

Drug combination	Cancer/cell line	Effect	Refs
DHA + Temozolomide	Rat C6 glioma cells	Increased apoptosis, ROS Induced necrosis	[107]
DHA + Cyclophosphamide	Lewis lung carcinoma	Increased apoptosis, decreased VEGF receptor KDR/flk-1 Apoptosis	[84]
DHA + Cisplatin	Human non-small cell lung cancer (A549)	Decreased metastasis	[84]
DHA + Gemcitabine	Pancreas (Panc-1)	Inhibition of proliferation, decreased cyclin D1 Increased apoptosis, increased Bax/Bcl2 ratio, activation of caspase 3	[94]
	Hepatoma (cell panel)	Increased growth inhibition by 45%	[67]
DHA + Butyric acid	Human lymphoblastoid leukemia (Molt-4)	Synergistic. Depletion of cancer cells	[110]
DHA + Radiation	Glioma cells U373MG	Increased cytotoxicity Inhibition of radiation-induced GST	[53]
DHA + Carboplatin	Ovarian cancer cells (A2780, OVCAR-3)	Increased growth inhibition through death receptor and mitochondrial mediated pathways	[77]
DHA + TRAIL	Prostate cancer (DU145, PC-3, LNCaP)	Increased apoptosis extrinsic and intrinsic pathways	[100]
ART/DHA + Doxorubicin + Pirarubicin	Leukemia (K562/adr) Small cell lung cancer (GLC4/adr)	Synergistic	[4]
ART + Lenalidomide	Lung (A549) and breast (MCF-7)	Decreased IC50 by 48%	[111]
ART + Oxiplatin	Colon (HT 1116)	Additive. Sensitising effect	[111]
ART + Gemcitabine	Breast (MCF-7)	Additive	
	Lung (A549)	Additive	
ATM + Oxiplatin ATM + Thalidomide ATM + Gemcitabine	Colon (HCTl16, SW480) Breast (MCF-7)	All additive	[63]
ARS + Hyperbaric oxygen (HBO_2)	Molt-4 human leukemia	22% decrease in growth	[112]
ARS + Doxorubicin	Colon cancer(HT29)	Predicted as antagonic, mediated by activation of NF-κB/overexpression of Pgp	[59]
ARS + Oxiplatin	Colon (HCTl16, SW480)	Antagonism	[63]
ARS + Thalidomide		Additive	
ARS + Gemcitabine	Breast (MCF-7)	Antagonism	

Abbreviations: HBO_2: hyperbaric oxygen.

in combination with doxorubicin and pirarubicin showed chemosensitizing effect in leukemia and human-small-cell cancer-resistant cell lines, but no further increase of sensitivity was observed in the sensitive parent cell lines [4]. The chemosensitising effect was independent of Pgp inhibition [4]. Overall, this evidence suggests that DHA and artesunate have remarkable ability to potentiate antitumor agents and to counter tumor resistance.

Artemisinins also improve ionizing-based therapies. In glioma cells U373MG, DHA treatment inhibits the radiation-induced expression of GST with concomitant ROS generation. A combination treatment with DHA has been shown to be more effective than radiation or DHA alone [53]. The adjuvant effect of artemisinin in other cancer treatments including hyperbaric oxygen has also been reported [112].

4.4. Artemisinin Resistance. A salient feature of artemisinin is that artemisinin resistance *in vitro* or in the field has yet to be confirmed after 30 years of use as an antimalarial. Clinically, tolerance has been reported in patients with therapeutic failure. However, *in vitro* tolerant strains are usually unstable and only develop after several years of continuous drug exposure [113]. The multimodal action of artemisinins at different cancer pathways might also predict a delay of induced resistance in malignant cells. Indeed, only few cell lines have shown intrinsic low sensitivity or no response to artemisinin or its derivatives. For example, artemisinin (the prototype drug) seems to be less active in breast cancer cells (MCF-7) and gastric cancer (MKN) [93]. Some studies in breast cancer cells have suggested that artemisinin response may be mediated by estrogen receptors (ERα and ERβ) which

are involved in cell proliferation (reviewed in [72]). Interestingly, it has been documented that in breast cancer cells, disruption of iron metabolism may enhance potency of doxorubicin and cisplatin [114]. The low response to artemisinin has been also associated with overexpression of BMI-1 in highly metastatic nasopharyngeal cancer cell lines (CNE-1, CNE-2) [92]. A recent study found some levels of cross resistance to artesunate and DHA in a unique cisplatin chemo-resistant cell line. This effect was partially reverted by L-buthionine-S,R-sulfoximine, an inhibitor of the antioxidant GLC [52].

However, *in vitro* resistance has already been developed under experimental conditions. Microarray and experimental studies using knockouts and transfected cells indicate that upregulation of the tumor suppressor $p16^{INK4A}$ and the antioxidant protein, catalase, may confer resistance to artesunate independent of the p53 status [115]. Recently, concerns have arisen after Baechmeier et al. showed that a 24 h preincubation with 20 μM artesunate induces resistance in highly metastatic breast cancer cells. Pretreated MDA-MB-231 metastatic cells were completely refractory to further artesunate treatment, whereas a similar treatment in MDA-MB-468, a non-metastatic cell line, renders less sensitive cells. Further investigation on the mechanism of artesunate-acquired resistance indicates that upregulated transcription of NF-κB, AP-1, and NMP-1 overcome artesunate apoptotic and antimetastatic action and allows tumor progression [116]. It is not clear, however, whether artesunate-induced resistance and loss of sensitivity are preserved after long-term cell subculturing. It also remains to elucidate if other semisynthetic endoperoxides may induce a similar effect or whether a combinational therapy may delay or revert the effect on cell lines bearing this phenotype.

4.5. Artemisinins Toxicity. Dose-dependent toxicity is a major drawback that hampers anticancer therapy. This problem may be overcome by enhancing anticancer activity and thus reducing toxic drug concentrations. DHA is the most active and neurotoxic artemisinin derivative [117]. Neurotoxicity has been reported in animal studies in a dose- and time-dependent manner ($\geq$7 days) [118–120]. The toxicity of artemisinin-like compounds has been associated with long-term availability, whereas short-term peak concentrations are not toxic [121]. Thus, rapid elimination of artemisinin in oral formulations is safer than slow-release or oil-based intramuscular formulations [6, 121]. Remarkably, although artemisinins derivatives have been widely used as antimalarials, their toxicity in humans have been shown to be negligible. In cancer therapy, artemisinin may have multiple benefits as it can be used in combination with no additional side effects, but also it enhances potency and reduces doses of more toxic anticancer partners. Clinical doses used in malaria treatment after administration of 2 mg/kg in patients rise plasma concentrations of 2640 $\pm$ 1800 μg/mL (approximately 6.9 $\pm$ 4.7 mM) which can be considered up to 3 orders of magnitude higher than those artemisinin concentrations with antitumor activity [5]. It becomes relevant to closely monitor the safety of long-term artemisinin-based therapies as severe side effects may be highly unusual but significant. So far, artemisinin treatments for as long as 12 months have been reported with no relevant side effects [30, 122, 123]. However, an extremely rare case of toxic brainstem encephalopathy was described in a patient after a 2-week herbal/artemisinin combination (400 mg) regimen for breast cancer [124]. Brainstem neurotoxicity has been reported in animal studies and associated with long-term (>28 days) and high-dose treatments [118]. Recently, a fatal case of overdosing in a child who was taking antimalarial treatment was reported [125].

4.6. Artemisinins in Clinical Trials. Antitumor activity of artemisinin has also been documented in human trials [126] and individual clinical cases [30, 122]. Artemether and artesunate have been used in cancer therapy with good tolerability and lack of significant side effects.

Artesunate was successfully used in the treatment of laryngeal squamous cell carcinoma and substantially reduced the size of the tumor (by 70%) after two months of treatment [122]. Furthermore, artesunate increased survival and substantial metastasis reduction when used in combination with standard chemotherapy in patients with malignant skin cancer [30]. Another report describes a beneficial improvement in a patient with pituitary macroadenoma who was treated with artemether for 12 months [123]. Artemether has longer half-life and easily crosses the blood-brain barrier which is crucial for brain tumor treatment.

Similarly, a clinical trial in 120 patients with advanced non-small cell lung cancer has shown that artesunate in combination with a chemotherapy regimen of vinorelbine and cisplatin elevated 1-year survival rate by 13% with a significant improvement in disease control and time to progression [126]. No additional artesunate-related side effects were reported [126]. In Germany, a trial in patients with advanced breast cancer is currently ongoing. Tolerability to a combination therapy of 4-week artesunate will be assessed in this trial. Another trial in UK in colorectal adenocarcinoma to evaluate anticancer action and tolerability of artesunate was completed last year, but the results have not been published.

5. Anticancer Action of Novel Artemisinins Derivatives

5.1. Novel Semisynthetic Derivatives with Antitumor Action. Imperative need of highly effective compounds with enhanced pharmacological properties has led to the design of novel endoperoxide compounds with selective toxicity toward cancer cells. Considerable progress has been made in the design of novel compounds with enhanced potency at the nanomolar range, increased selectivity, and low toxicity *in vitro*. It has been reported that triazolyl substituted artemisinins-induced significant growth inhibitory effect [127]. Independent of stereo- or region-chemistry, strong inhibition was influenced by the functional group attached to the triazole ring. Substituted compounds with a penthyl benzene group showed the highest antiproliferative activity ranging from 0.07 to 0.39 μM 72 h IC_{50} in 6 cancer lines [127]. Recently, Feng et al. synthesized a series of dihydroartemisinin derivatives via an aza-Michael addition reaction with high selectivity index and

IC_{50} in the nanomolar range against HeLa cells (0.37 μM) [128]. In a series of deoxoartemisinins and carboxypropyldeoxoartemisinins, antitumor effect was associated with boat/chair conformations and drug-receptor interactions [129].

Different from their antiparasitic activity, it has been found that dimeric and trimeric artemisinin derivatives display much higher antitumor activity than their monomeric counterparts. In the last decade, an increase in the number of outputs in artemisinin dimeric compounds with anticancer activity has been observed. These compounds have shown IC_{50} ranging from 0.014 to 6 μM [130, 131]. Potent anticancer toxicity has been correlated with the nature of the linker [132] and with lipophilicity or electrophilic substitutions [66]. Posner et al. developed a series of artemisinin-trioxane derivate dimers from which two phosphate esters displayed nanomolar growth inhibitory values in the NCI 60 human cell line screen. Further investigation *in vitro* showed that in HL-60 cells, these compounds are more potent than doxorubicin, whereas their strongly anti-parasitic monomeric counterparts showed no anticancer activity. As suggested by the authors, two trioxane units in addition to the nature of the linker may be relevant in conferring potent anticancer activity [132]. Homodimers of artesunic acid have also nanomolar inhibitory values when tested in chemo-resistant and sensitive leukemia cells. Notably, the artesunic dimer seems to be 6-fold more potent in the multiresistant Pgp overexpressing cells (CEM/ADR500) than in its sensitive counterparts. Anticancer activity was attributed to apoptosis induction, arrest of cell cycle at G_0/G_1, and ROS generation [131]. In prostate cancer cells (LNCaP, TRAMP CIA, and C2H), two C10 non-acetal trioxane dimers displayed a 3-fold increase in potency compared to doxorubicin (17-18 nM versus 45.3 nM resp.). The dimers induced arrest at G_0/G_1 mediated by decreasing cyclin D1, cyclin E, CDK2, and an increase in p21 and p27. They also show proapoptotic action through upregulation in Bax expression [130].

In many studies, there has been an emphasis on the nature and stereochemistry of the dimer linker which may influence anticancer activity. However, it has also been shown that the linker by its own is inactive. Morrisey et al. have described that an artemisinin dimer exhibits up to 30-fold more activity than artemisinin in prostate cancer lines [61]. This dimer selectively exerted highly antigrowth potential and apoptosis in C4-2 (a cell line derived from LNCaP) and LNCaP cells compared to artemisinin [61]. An enhanced anticancer activity seems to be given by the stereoisomery of the linker [130]. In another study, C12 non-acetal dimers and one trimer of deoxoartemisinin showed similar potency to that of the conventional anticancer drugs against many cell lines. The linker with one amide or one sulfur-centered 2 ethylene groups was essential for potent anticancer activity [133]. Diastomeric-cholic-acid-derived 1,2,4,5-tetraoxanes were also tested and found to have high anticancer activity against human melanoma (Fem X), and cervix cancer (HeLa) [134] cis stereoisomers were twofold more active. The authors further suggested that an amide terminus in the linker confers increased anticancer activity.

Interestingly, Beckman et al. showed that the stereochemistry of the ether linkage of the dimers, of dihydroartemisinin (diDHA), and dihydrodeoxyartemisinin (the respective endoperoxide lacking dimer) was as important for antitumor activity as the endoperoxide moiety. Dimers were tested against 60 cells from 9 different cancers showing that although in general the diDHA was more active than dihydrodeoxyartemisinin toward anticancer growth, asymmetrical dimers of either diDHA or dihydrodeoxyartemisinin were similarly toxic [26]. The mechanism underlying the antiproliferative action of the artemisinin-derived dimers needs further study.

Recently, a series of potent artemisinin-like derivatives of easy synthesis and anticancer activity has been identified. These endoperoxides exhibit high chemical stability and greater cytotoxicity than artesunate. These compounds also exhibit relevant antiangiogenic properties as judged by studies in a zebrafish model [135].

5.2. Fully Synthetic Artemisinins. It is important to recall that some limitations of artemisinins such as short half-life (between 1 and 5 hours [136, 137]), limited affordability, and solubility need to be further addressed. Although semisynthetic compounds have partially overcome these issues, they still rely on the availability of natural precursors. In malaria, some trioxolanes and ozonides with remarkable improved pharmacokinetics are under clinical development [138]. Recently, it has been found that synthetic trioxolanes with enhanced pharmacokinetic properties may exhibit a similar toxicity than artesunate in *Schistosomas* [139]. Given that artemisinins may be potentially used as anticancer drugs and possibly in other parasitic and viral infections, the development of novel compounds with enhanced pharmacokinetic properties and targeted anticancer actions is also paramount. Although novel semisynthetic artemisinins have shown substantial antineoplastic activity, there is still limited information regarding the cytotoxicity of fully synthetic endoperoxides. A series of tetraoxacyclohexanes have been shown to potentially exhibit anticancer properties. A triol substituted compound has displayed prominent antitumor action *in vivo* toward melanoma (LOX IMVI) and ovarian (IGROV1) cancer in nanomolar concentrations (LC_{50} 60 nM) [140]. Other authors have synthesized compounds with dual action (antimalarial/anticancer effect). These deoxycholic-acid-(DCA-)—and cholic acid (CA)—derived mixed tetraoxanes are cytotoxic at very low concentrations and particularly potent against melanoma cancer (LOX IMVI, LC_{50} up to 69 nM) [141].

6. Future Development of Artemisinins as Anticancer Drugs

Artemisinins have been recommended and widely used as antimalarials for several years [142]. This drug class has shown many biological activities, in particular, strong anticancer growth activity. Supporting evidence indicates that artemisinin-like compounds may be a therapeutic alternative in highly metastatic and aggressive cancers [44] with no long-term effective therapy [44, 61] and commonly developing

drug resistance [94]. Furthermore, antimalarial endoperoxides may act synergistically with other anticancer drugs with no additional side effects [143].

6.1. What Do We Need to Know? The ability of artemisinins to kill cancer cells through multiple and heterogeneous molecular events has been well documented. However, some questions about the molecular base of artemisinin-induced cell death need further study. Growing research has been focusing on determination of the mechanism of bioactivation and molecular events underlying the artemisinin effects. However, how the antitumor activity is exerted following artemisinin activation is still not well understood. So far, the precise molecular events involved in how, when, and where ROS production is initially triggered in cancer cells remain to be defined. In addition, the relevance of any ROS- independent mechanism should be also addressed; these might not be obvious but possibly important for artemisinin cytotoxicity in some cancer cells. Some other aspects such as the direct DNA damage induced by artemisinin-like compounds and the role of p53 status in genotoxicity need to be further analysed.

One relevant aim in anticancer therapy is cotargeting multiple pathways minimizing shifting cell hallmarks and side effects. Whereas it remains important to characterize the anticancer effects of existing and novel artemisinins derivatives, research also needs to be focused on unveiling the mechanisms of cytotoxicity by identifying their relation to a particular cancer biomarkers and molecules. Artemisinins seems to regulate key players participating in multiple pathways such as NF-κB, survivin, NOXA, HIF-1α, and BMI-1. These molecules and others are to be revealed, which in turn may be involved in drug response, drug interactions, mechanisms of resistance, and collateral effects in normal cells. A better understanding of common mechanisms under similar conditions in different cell systems will greatly aid the development of targeted artemisinin derivatives. This will improve artemisinins cytotoxicity by lowering IC_{50}, emerging of resistance, drug associated toxicity, and potentiating drug interactions.

It is important to connect the molecular interactions and the regulatory effects of artemisinin on the cancer hallmarks and particularly in those tumors with poor prognosis. Some cancer cell biomarkers may be potentially useful to predict success on an artemisinin-based treatment in specific systems. Furthermore, novel endoperoxide compounds and combinational therapies can be addressed to target or cotarget markers of carcinoma progression and prevent invasiveness and metastatic properties in highly recurrent and aggressive tumors or advanced stage cancers.

Although the benefits of artemisinins in the clinical setting have been already assessed, specific interactions with established chemotherapy need to be further dissected in different cancer cells and their phenotypes. This will be crucial to implement clinical trials and treatment of individual cases. In this regard, long-term therapy with artemisinins also requires close monitoring. It is important to note that the prototype drug, artemisinin, seems to modulate responses leading to antagonistic interactions with other anticancer drugs. However, whereas it may be useful to have the prototype drug as a control *in vitro*, its pharmacokinetic properties may differ from the semisynthetic artemisinins. Therefore, artemisinin antagonistic reactions and resistance must be cautiously validated using different semisynthetic derivatives. DHA, artesunate, and artemether are the endoperoxides currently licensed for therapeutic use. So far, artemether has been shown to share similar anticancer properties than DHA and artesunate [144].

Cancer research drives a permanent discovery of new genes and interactions. The study of how artemisinin drives tumor control may become even more complex as immunological hallmarks are also involved in the generation of tumors. Immunological hallmarks in cancer cells include the ability to induce chronic inflammatory response, evasion of tumor recognition, and ability to induce tolerance [145]. Whether artemisinin may participate in the mechanisms involved in these events has yet to be determined.

Overall, the real potential and benefits of the artemisinin drug class remain yet to be uncovered. The imminent possibility of artemisinins being included in the arsenal of anti cancer drugs has opened the door for challenging research in this area, one that seems to fulfill many expectations.

Abbreviations

ADP-ribose polymerase:	Adenosine diphosphate ribose polymerase
AP-1:	Activator protein 1
BAK:	Proapoptotic member of the BCL2 protein family
BAX:	BCL2-associated protein
BCL2:	B-cell lymphoma 2
BCRP:	Breast cancer resistant protein gen
BCLX:	Bcl-2-like protein 1
CDC25B:	Dual specific phosphatase involved in the activation of cyclin-dependent kinases
CDK:	Cyclin-dependent kinase
Cip 1/p21:	Cyclin-dependent kinase inhibitor 1
CDC25A:	Dual specific phosphatase involved in the activation of cyclin-dependent kinases
COX2:	Cyclooxygenase 2
Cdc42:	GTPase of the Rho family
c-MYC:	Transcription factor
DNA-PK:	DNA-dependent protein kinase
DNA topo 1:	DNA topoisomerase 1
E2F1:	Transcription factor
ER:	Endoplasmic reticulum
GST:	Glutathione S-transferase
GRP78:	78 kDa glucose-regulated protein
HIF 1α:	Hypoxia-inducible factor 1 alpha
$\alpha v\beta 3$ integrin:	Transmembrane heterodimeric protein expressed on sprouting endothelial cells
IκBα:	Inhibitor of NF-κB
IL-1β:	Interleukin 1 beta

IL8: Interleukin 8
JNK: Jun N-terminal kinase
Kip1/p27: Cyclin-dependent kinase inhibitor 1B
KDR: Kinase insert domain protein receptor
MMP: Matrix metalloproteinase
MRP1: Multidrug resistance-associated protein gene
MDM2: Murine doble minute oncogene protein
NK-κB: Nuclear factor of kappa light polypeptide gene enhancer in B cells
NOXA: Proapoptotic protein, a member of the BH3-only Bcl-2 protein family
p38-MAPK: Mitogen-activated protein kinase
PAI-1: Plasminogen activator inhibitor 1
PCNA: Proliferating cell nuclear antigen
PKCa: Serine/threonine kinase
ROS: Radical oxygen species
Raf/ERK: Signaling pathway
uPA: Urokinase plasminogen
TIMP2: Tissue inhibitor of metalloproteinases
TRAIL: The tumor necrosis factor-related apoptosis-inducing ligand
VEGF: Vascular endothelial growth factor
VEGFR-3/FL-4: Vascular endothelial growth factor receptor
Wnt: Wingless-type signaling pathway.

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Review Article

Rhus verniciflua Stokes against Advanced Cancer: A Perspective from the Korean Integrative Cancer Center

Woncheol Choi,[1] Hyunsik Jung,[1] Kyungsuk Kim,[1] Sookyung Lee,[1] Seongwoo Yoon,[1] Jaehyun Park,[1] Sehyun Kim,[2] Seongha Cheon,[3] Wankyo Eo,[3] and Sanghun Lee[1]

[1] *Integrative Cancer Center, East-West Neo Medical Center, Kyung Hee University, 149 Sangil-Dong, Gangdong-Ku Seoul 134-727, Republic of Korea*
[2] *Graduate School of East-West Medical Science, Kyung Hee University, Yongin 449-701, Republic of Korea*
[3] *Department of Hematology and Oncology, East-West Neo Medical Center, Kyung Hee University, Seoul 134-727, Republic of Korea*

Correspondence should be addressed to Sanghun Lee, integrative@korea.com

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Active anticancer molecules have been searched from natural products; many drugs were developed from either natural products or their derivatives following the conventional pharmaceutical paradigm of drug discovery. However, the advances in the knowledge of cancer biology have led to personalized medicine using molecular-targeted agents which create new paradigm. Clinical benefit is dependent on individual biomarker and overall survival is prolonged through cytostatic rather than cytotoxic effects to cancer cell. Therefore, a different approach is needed from the single lead compound screening model based on cytotoxicity. In our experience, the *Rhus verniciflua* stoke (RVS) extract traditionally used for cancer treatment is beneficial to some advanced cancer patients though it is herbal extract not single compound, and low cytotoxic in vitro. The standardized RVS extract's action mechanisms as well as clinical outcomes are reviewed here. We hope that these preliminary results would stimulate different investigation in natural products from conventional chemicals.

1. Introduction

On September 6, 2008, the article of the title "We fought cancer… and cancer won" was in the Newsweek. The subtitle was that it is time to rethink the war on cancer, after billions spent on research and decades of hit or miss treatments. The improved understanding of molecular biology in cancer research has led to a number of new, effective treatments for cancer. However, the age-standardized death rate from all types of cancers combined first increased from 1970 to 1990 and then decreased through 2002, yielding a net decline of 2.7% [1]. Therefore, it is necessary to understand the cancer from a different point of view, which has been helped by the oriental medicine.

In view of the oriental medicine, mass or nodule is resulted from Qi and Blood stagnation. The development of a tumor could be interpreted as Qi Stagnation induced by stress, emotional disturbances, or external factors which could progress into Blood stagnation or stasis [2]. Traditionally *Rhus verniciflua* stoke (RVS) was considered to have the function of breaking up blood stasis and purging hardness [3]. Therefore, it could be applied for cancer treatment. RVS of the Anacardiaceae family, commonly known as the lacquer tree, was documented to be used for treating various stomach diseases, including tumor in East Asia including Korea at the 15th century [4, 5]. Recently several experimental studies showed that flavonoids from RVS have effective antiproliferative and apoptotic activities on various tumor cell lines including human lymphoma, breast cancer, osteosarcoma, and transformed hepatoma cells [4–7].

However, the clinical application of RVS has been limited because an allergenic component, urushiol, causes severe contact dermatitis in sensitive individuals [8, 9]. Therefore urushiol, a mixture of several derivatives of catechol, should be removed from RVS for pharmaceutical use. A standardized extract of allergen-removed RVS was manufactured

based on traditional method. Our integrative cancer center composed of oncologists and oriental medicine doctors was founded in June, 2006, and the standardized RVS extract was prescribed for the purpose of prolonging survival and improving the quality of life in patients with only palliative therapy available. Here, we review on the action mechanism and best outcomes of the standardized RVS extract with or without conventional treatment in patients with advanced or metastatic cancer.

2. Preparations of Standardized Extract from RVS

RVS stalk, which was 10 years old and grown in Wonju, Republic of Korea, was dried without exposure to direct sunlight and chopped up. The pieces were roasted in an iron pot at 240°C for 50 minutes to remove allergens and extracted two times with a 10-fold volume of water at 90°C to 95°C for 6 hours (Korean patent no. 0504160). The extract was filtered with Whatman GF/B filter paper and concentrated under vacuum to remove water. The concentrate was lyophilized to a brownish powder. The extraction yield from 100 g of chopped material was 3.3 g. A component analysis method using high-performance liquid chromatography showed that the RVS extract contained fustin, fisetin, sulfuretin, and butein, among others. The quality of the RVS extract was tested and controlled according to the quality standards of the Korea Food & Drug Administration and our hospital's standards (fustin > 13.0%, fisetin > 2.0%, urushiol not detected). Daily oral administration is 1350 mg (1 capsule containing 450 mg three times a day) of RVS extract.

3. Mechanism Studies

3.1. Anti-Angiogenesis. The RVS was experimentally shown to have antitumor properties in carcinoma of breast and uterine cervix in vitro and suppress tumor volume in a xenograft mouse model system using A549 nonsmall cell lung cancer and Lewis lung cancer cells in vivo through inhibiting the proliferation and migratory activity of vascular endothelial growth factor [10, 11]. It also significantly inhibited proliferation of human umbilical vein endothelial cells (HUVECs) induced by vascular epithelial growth factor (VEGF), despite its weak cytotoxicity against HUVECs.

Migration of endothelial cells toward a chemoattractant is an important step in angiogenesis [12]. When VEGF alone was present, the wound after scratching the cell monolayer was closed by cell migration. However, the addition of RVS significantly inhibited VEGF-induced migration of HUVECs similar to control cells (Figure 1). Maturation of migrated endothelial cells into a tube-like structure is a critical step for formation of functional vessels. HUVECs were seeded onto Matrigel and stimulated to form capillary networks with VEGF. As shown in Figure 2(b), robust and complete tube network formation was observed in VEGF-stimulated HUVECs. However, this effect of VEGF was significantly inhibited by RVS showing incomplete sprouting or branching or broken network between tubes of HUVECs (Figure 2). These results indicate that RVS blocks migration and maturation of endothelial cells induced by VEGF.

3.2. Anti-Invasion. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that remodel and degrade the extracellular matrix (ECM). Cancer cell migration and invasion of surrounding tissues are mediated in part by MMPs, especially MMP-2 and MMP-9 [13]. The effect of RVS on the invasiveness of human fibrosarcoma HT1080 cells was investigated by Boyden chamber assay [14]. The invasiveness of HT1080 cells was reduced in a dose-dependent manner following 24-hour treatment of up to RVS 200 μg/mL (Figure 3). The ability of RVS extract to inhibit the secretion of MMP-2 and MMP-9 from HT1080 cells was studied. The levels of MMP-2 and MMP-9 were reduced by RVS, suggesting that the decrease in HT1080 cell invasion is a consequence, at least in part, of reduced activities of both MMP-2 and MMP-9. RVS inhibited MMP-2 and MMP-9 activities with IC_{50} value of 1.01 ± 0.07 μg/mL and 1.94 ± 0.11 μg/mL, respectively, by spectrofluorometric method which is very low concentration compared to other various herbs [15] (Figure 4).

4. Clinical Studies

4.1. Non-Small Cell Lung Cancer (NSCLC) [16]. The RVS was investigated to prolong survival in NSCLC after the failure of first-line or second-line chemotherapy. Forty patients treated with RVS for previously treated, advanced NSCLC between June 2006 and June 2009 were eligible for the final analysis. The median RVS administration period was 75 days. The median survival time was 8.4 months with a 1-year survival of 40%, and the disease control rate was 63.6%, which is compatible with external controls in second-line chemotherapy. RVS showed more favorable outcomes in patients with better performance status or adenocarcinoma in terms of overall survival. The common RVS-related adverse events were mild epigastric pain and pruritis.

4.2. Colorectal Cancer (CRC) [17]. From July 2006 to November 2007, patients with conventional chemotherapy refractory metastatic CRC were checked. After fulfilling inclusion/exclusion criteria, 36 patients were eligible for the final analysis. Overall survival and adverse events of patients treated with RVS in the aftercare period were determined. The median RVS administration period was 2.7 months. The median overall survival for the entire population was 10.9 months (95% confidence interval, 5.6–16.1) and 1-year survival rate was 44.4%, which is compatible with external controls. By survival analysis using Cox proportional hazards model, the performance status and the prior chemotherapy regimen number significantly affected overall survival.

4.3. Pancreatic Cancer. Palliative chemotherapy-naïve patients with advanced or metastatic pancreatic adenocarcinoma were checked from July 2006 to June 2010. After applying inclusion/exclusion criteria, 42 patients were eligible for the final analysis. Overall survival, clinical benefit, and

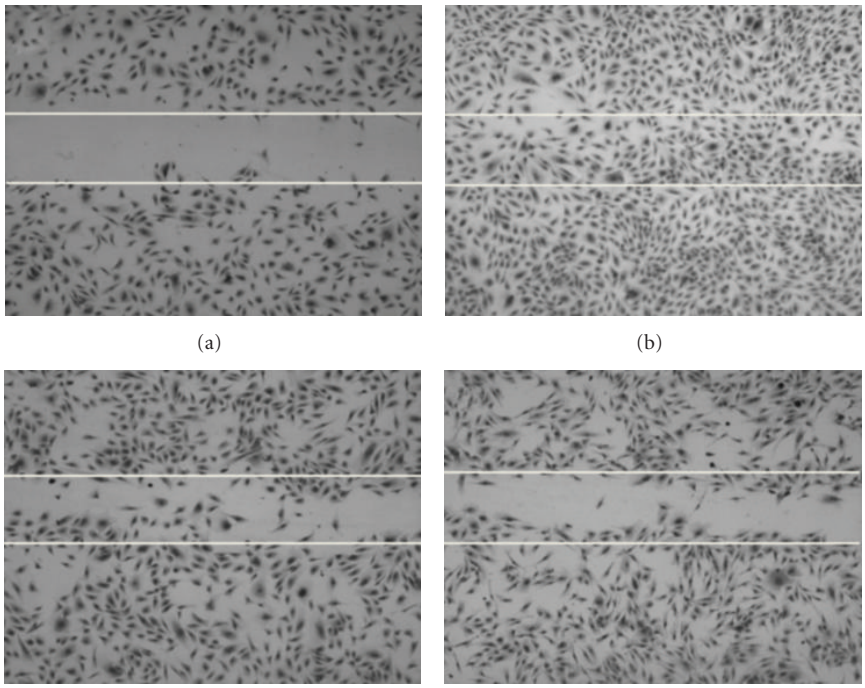

Figure 1: Effects of the standardized RVS extract on the migratory activity of VEGF-stimulated HUVEC (Wound-healing test). (a) The migrated cells in window-scraped field after scratching the cell monolayer; Control. (b) The wounds closed by cell migration from the wound edge after VEGF alone. (c) VEGF plus RVS (50 μg/mL). (d) VEGF plus RVS (100 μg/mL) showing inhibitory effect on migration compared to only VEGF-treated control.

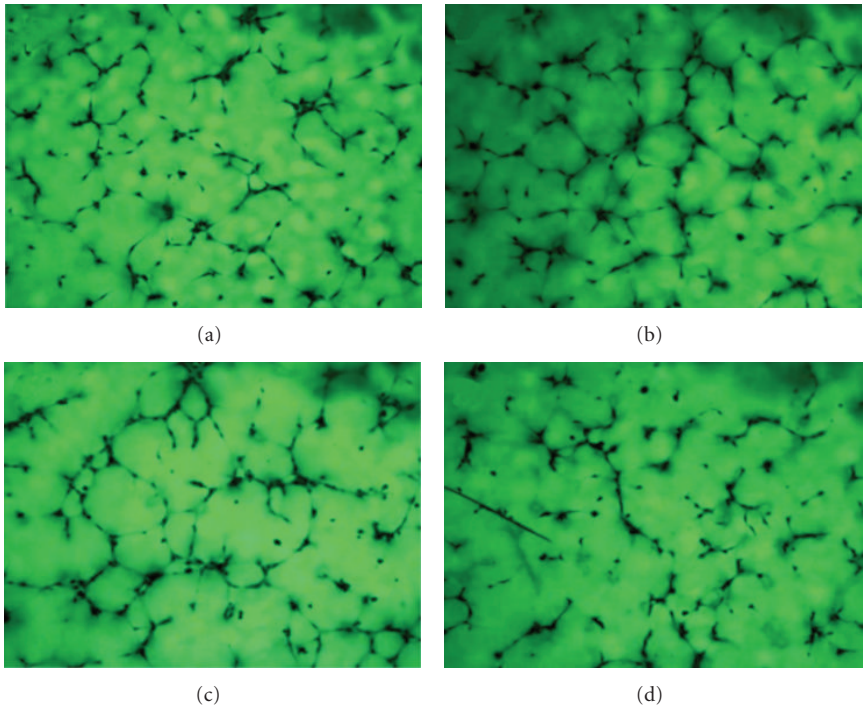

Figure 2: Effects of the standardized RVS extract on the tube formation in VEGF-stimulated HUVECs. (a) HUVECs seeding in Matrigel form capillary networks; Control. (b) The robust and complete tube network formation after VEGF alone. (c) VEGF plus RVS (50 μg/mL). (d) VEGF plus RVS (100 μg/mL) showing inhibitory effect on tube network formation compared to only VEGF-treated control.

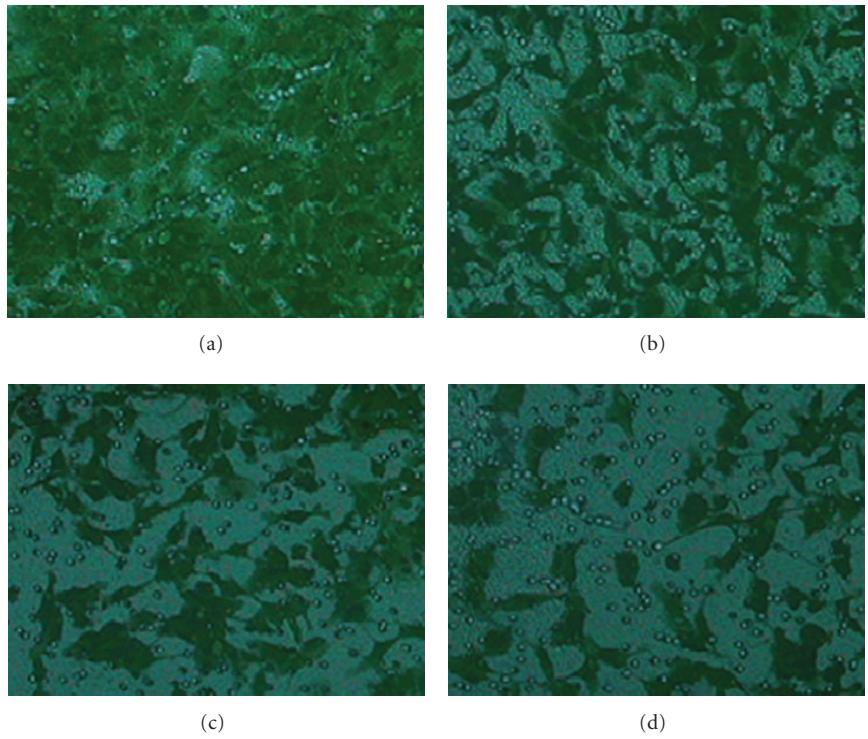

Figure 3: The effect of the standardized RVS extract on the invasiveness of human fibrosarcoma HT1080 cells using Boyden chamber assay. (a) Control. (b) RVS 50 μg/mL. (c) RVS 100 μg/mL. (d) RVS 200 μg/mL showing inhibitory effect on invasion compared to the control.

adverse events of these patients treated with RVS in the aftercare period were determined. The mean RVS administration period was 3.86 months (95% confidence interval: 2.52–5.20). The median overall survival for the entire population was 7.87 months, and 1-year survival rate was 26.2%, which is compatible with external controls. By using univariate and multivariate analyses, RVS treatment including the performance status and prognostic index significantly affected overall survival. A clinical benefit response was also shown by RVS treatment which was not dependent on concurrent chemotherapy (Data not published).

5. Case Studies

5.1. Hepatic Cellular Carcinoma (HCC) [18]. A 62-year-old man underwent living donor liver transplantation for both hepatocellular carcinoma and end stage liver disease in March 2005. He had been diagnosed with hepatitis C infection in 1977. Unfortunately, the HCC recurred with multiple metastases in both lungs in September 2005, 6 months after liver transplantation. After systemic doxorubicin chemotherapy showed progression of lung metastases, he began receiving only RVS treatment in June 2006. CT scans 5 months later showed marked shrinkage of the lung metastases. Moreover, the patient tolerated the RVS well, without hematologic and nonhematologic toxicity. Four months later, metastatic mass in the right lower lobe of lung had increased in April 2007. The patient discontinued the RVS treatment and underwent radiation therapy. After finishing radiation therapy, he developed new liver metastases and died of progressive disease in November 2007.

5.2. Gastric Carcinoma [19]. An 82-year-old female was diagnosed with gastric cancer with a polypoid gastric mass approximately 25 mm in diameter at the middle body portion of the lesser curvature, a flat elevated lesion 50 mm in diameter at the prepyloric antrum, and the small gastrohepatic lymph nodes in October 2006. The endoscopic biopsy confirmed well-differentiated adenocarcinoma with a mutation in p53 that showed high nuclear activity of more than 80%. Gastrectomy was not done because of old age and the concerns about the quality of life after surgery. RVS treatment was exclusively initiated on September 25, 2006. Five months later, the polypoid mass had markedly decreased and the flat elevated lesion had shrunk slightly, although the gastrohepatic lymph nodes were not changed. The biochemical parameters associated with liver and renal function were within the normal range, and no significant adverse effects from her RVS treatment have been observed. She maintains a good performance status up to now (July 2011).

5.3. Renal Cell Carcinoma (RCC) [20]. A 47-year-old man presented a 12 cm right renal mass and received a right radical nephrectomy which was pathologically the clear cell type of RCC in July 2006. Four months later, follow-up CT and PET scans revealed metastatic multiple pulmonary nodules. Palliative chemotherapy was recommended, but he refused it.

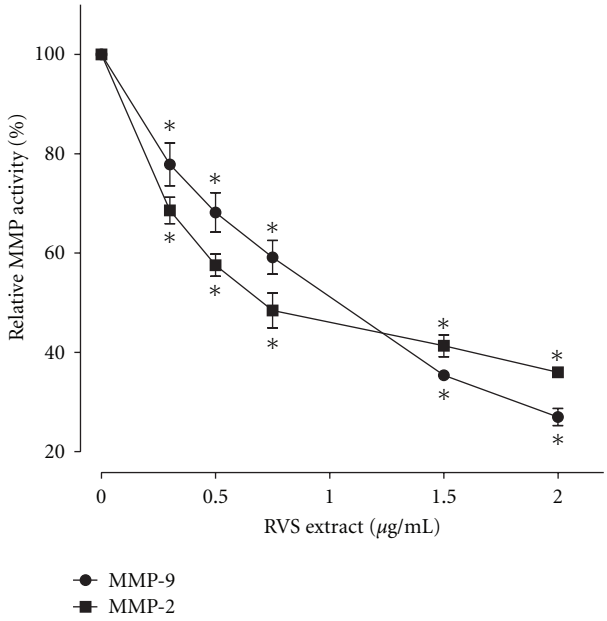

Figure 4: The effect of the standardized RVS extract on matrix metalloproteinase (MMP) activities by spectrofluorometric method. The IC_{50} of the standardized RVS extract for MMP-2 and MMP-9 was 1.01 ± 0.07 μg/mL and 1.94 ± 0.11 μg/mL respectively. $^*P < 0.05$ versus respective controls.

Instead, only RVS treatment was initiated in December 2006. After 4 months, CT scans showed a complete response in all pulmonary metastases including resolution of right pulmonary artery thrombosis. Follow-up CT scans continued to demonstrate a complete response. He maintains a good performance status up to now (July 2011).

A 47-year-old man was diagnosed with the clear cell type of RCC accompanying multiple pulmonary nodules and underwent a radical nephrectomy for a 6.3 cm mass in September 2006. After cytoreductive surgery, the metastases in both lungs aggravated and a right adrenal mass was newly developed. After palliative sunitinib 2 cycles showed progression of lung metastases and both adrenal masses, he began receiving only RVS treatment in July 2007. After 9 months of RVS administration, chest CT scans showed the resolution of the masses, noted previously in the left upper lung. After 13 months of RVS therapy, CT scans showed significant reduction in the size of the metastatic masses in both adrenal glands. No evidence of disease continues in CT scans obtained up to now (July 2011).

5.4. NSCLC [21]. A 52-year-old female was diagnosed with pulmonary adenocarcinoma accompanying malignant pleural effusion confirmed by histologic examination in August 2006. Immunochemical staining pattern was a cytokeratin 7 (strong positive), cytokeratin 20 (negative), and thyroid transcription factor-1 (positive). One month later, CT scans showed an aggravation in malignant pleural effusion. She strongly refused recommended chemotherapy because of concerns about adverse effects. Instead, only RVS treatment was initiated in October 2006. After 1-month RVS treatment, CT scans showed marked decrease in pleural effusion and no interval change in mass. There was no significant change in tumor and pleural nodularity in a chest CT scans until January 2009. After progression of her disease, she was enrolled in a clinical trial (erlotinib) at other hospital in July 2009 and was lost to follow-up. Adverse effects from 34-month RVS treatment were not observed.

6. Discussion and Conclusion

As several plants were used for cancer in traditional practice, rigorous in vitro and in vivo studies are essential and necessary to evaluate these extract's efficacy and safety before clinical trials, where the efficacy is dependent on the cancer-cell toxicity. Throughout this drug screening model such as the US National Cancer Institute (NCI) 60 human tumor cell line anti-cancer drug screen (NCI60) based on cytotoxicity, many drug developments could be possible from natural products [22]. In fact, half of all anti-cancer drugs approved internationally were either natural products or their derivatives and were developed on the basis of knowledge gained from small molecules or macromolecules that exist in nature [23]. However, it is questionable that the cell death is a critical end point for anti-cancer treatments. These days, the advances in the knowledge of cancer biology have developed some molecular-targeted agents for cancer treatment. Multiple tyrosine kinase inhibitors targeted on angiogenesis clinically prolong overall survival through cytostatic rather than cytotoxic effect to the cancer cells [24].

Korean health care system is different from other countries where two separate medical systems, western medicine and traditional Korean medicine, exist. The natural extract administered traditionally could be legally prescribed by traditional Korean medicine doctors. Therefore, it is possible for cancer patients to receive medication which might not be approved on conventional drug screen model because of low cytotoxicity. In fact, the RVS extract gave poor outcomes in NCI60 cell lines (Data not shown). However, some cancer patients in our center survived advanced cancer and are practically proven as responder to RVS extract. Except all previously described patients to be published, there are many responders in various cancer cell types including pancreatic cancer, cholangiocarcinoma, and sarcoma refractory to conventional chemotherapy. We suggest that standardized RVS extract could be a natural anticancer candidate from our experiences.

Single active constituents such as sulfuretin or butein have been characterized from RVS extract, following the conventional pharmaceutical paradigm of drug discovery [25, 26]. Nevertheless, it is hypothesized that the active compound isolated from plants may rarely have high activity against cancer as the extract at comparable concentration without synergistic interaction or multifactorial effects between compounds present in herbal extracts [27]. Cancer is also complex disease having various biological capabilities such as sustaining proliferative signaling, evading growth

suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, genome instability, tumor-promoting inflammation, deregulating energy metabolism, and evading immune destruction [28]. In response to targeted therapy, cancer cells may also reduce their dependence on a particular capability, becoming resistant to it. For example, even if potent angiogenesis inhibitors succeed in suppressing specific molecular targets, tumors adapt and shift from a dependence upon continuing angiogenesis to invasiveness and metastasis instead [28, 29]. Therefore, multiple targeted agents with synergistic interaction are necessary to inhibit the cancer cell progression. The whole extract containing many constituents from natural products might make it possible to be more effective and less toxic in clinic than single potent compound. Based on clinical outcomes of RVS, it could be postulated to have various therapeutic targeting on cancer cell except the previously mentioned mechanism of action focused on angiogenesis.

In the era of targeted therapy, the predictive biomarkers have been identified to select patients more likely to benefit from it, which indeed makes cancer treatment personalized. In recent development of new drug, well-validated biomarker becomes more and more important from the excellent example of crizotinib targeting ALK mutation [30]. Unfortunately, there are no known clinically relevant biomarkers which could identify responders to the drugs targeted on angiogenesis [31]. Therefore, more research is needed for the predictive factors in order to find RVS extract-responders using molecular biology and traditional Korean medicine's diagnostic methodology. From our clinical results, effective outcome from RVS extract is not dependent on not only the cancer type but also the patient. Natural products are also known to affect immune function which could be monitored by immunomodulating cytokines such as IFN-γ, IL-2, and IL-10 [32]. Actually, there are reports on anti-inflammatory function of RVS or RVS flavonoids. Butein from RVS inhibits cytokine-induced nitric oxide production by suppression of inflammatory signaling in NF-κB pathways [33]. RVS hexane fraction and its major component, fisetin, significantly inhibit inflammatory cytokine production such as TNF-α, IL-6, IL-8, and monocyte chemoattractant protein-1 [34]. RVS glycoprotein (36 kDa) also has an inhibitory activity of T-helper type 2 (Th2) cytokines such as IL-4 and IL-10, which means to help immune dysfunction [35]. In aggressive pancreatic cancer, a cross talk between tumor cells and cancer-associated fibroblasts was reported to result in the induction of Th2-type inflammation which was well correlated with poor survival of patients [36]. Therefore, we try to focus cytokines-related immune system and find common genotype or phenotype backgrounds concerning drug metabolism in RVS extract-responders.

In conclusion, drug developments from natural products should be differently approached from the conventional pharmaceutical paradigm, and we hope that these preliminary results would stimulate further investigation into the effectiveness and tolerability of other natural products for the treatment of advanced cancers in clinical practice.

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Research Article

The Indolic Diet-Derivative, 3,3′-Diindolylmethane, Induced Apoptosis in Human Colon Cancer Cells through Upregulation of NDRG1

A. Lerner,[1,2] M. Grafi-Cohen,[1,2] T. Napso,[3] N. Azzam,[4] and F. Fares[4,5]

[1] *Pediatric Gastroenterology and Nutrition Unit, Carmel Medical Center, Haifa 34362, Israel*
[2] *B. Rappaport School of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel*
[3] *Department of Evolutionary and Environmental Biology, University of Haifa, Mount Carmel, Haifa 31905, Israel*
[4] *Department of Human Biology, University of Haifa, Mount Carmel, Haifa 31905, Israel*
[5] *Department of Molecular Genetics, Carmel Medical Center, Haifa 34362, Israel*

Correspondence should be addressed to F. Fares, ffares@sci.haifa.ac.il

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N-myc downstream regulated gene-1 participates in carcinogenesis, angiogenesis, metastases, and anticancer drug resistance. In the present study, we analyzed the expression pattern of N-myc downstream regulated gene-1 following treatment of human colonic cancer cell lines; HCT-116 (well differentiated with wild-type p53 gene) and Colo-320 (poorly differentiated with mutant p53 gene), with 3,3′-diindolylmethane, a well-established proapoptotic agent product derived from indole-3-carbinol. Treatment of Colo-320 and HCT-116 with 3,3′-diindolylmethane disclosed inhibition of cell viability in a dose-dependent manner, mediated through apoptosis induction. The increased expression of N-myc downstream regulated gene-1 was detected only in poorly differentiated colon cancer cells, Colo-320 cell line. Our results suggest that N-myc downstream regulated gene-1 expression is enhanced by 3,3′-diindolylmethane in poorly differentiated cells and followed by induction of apoptosis. 3,3′-diindolylmethane induced apoptosis may represent a new regulator of N-myc downstream regulated gene-1 in poorly differentiated colonic cancer cells.

1. Introduction

N-myc downstream regulated 1 gene (NDRG1) is a 43-kDa protein located mainly in the cytoplasm but occasionally translocates into the nucleus in a p53-dependent manner [1]. This protein interacts with many cell elements and consequently participates in various processes in the cells, such as differentiation, response to stress conditions, involvement in carcinogenesis, angiogenesis, and metastasis. NDRG1 is induced, among others, by stress conditions, inhibition of DNA methylation, and histone deacetylation [2–7]. Furthermore, NDRG1 is necessary for caspase-3 activation and is required for p53-dependent apoptosis in a cell type-specific manner [8]. Nevertheless, the precise function of NDRG1 and its regulation in cancer cells is far from being clear.

In colon cancer, NDRG1 protein levels appear to be decreased with the progression from normal colonic epithelium to carcinoma [9, 10]. Moreover, NDRG1 expression is associated with a more aggressive tumor phenotype and poor outcome in colorectal cancer [11].

NDRG1 mediates apoptosis in several tissues [8, 12, 13]. However, the regulation of NDRG1 during apoptosis state in cancer cells in general, and in colon cancer in particular, was not explored previously. The purpose of the current study was to investigate the NDRG1 expression pattern in human colon cancer cell lines which vary in their differentiation level and in their p53 status. For apoptosis induction, we used 3,3′-diindolylmethane (DIM), a well-established proapoptotic agent product derived from indole-3-carbinol (I3C). In a low environmental pH, I3C is converted into polymeric products, among which 3,3′-diindolylmethane (DIM) is the main one. Studies showed that DIM induced cell-cycle arrest in human prostate cancer through, the upregulation of cycline-dependent kinases [14] and apoptosis via mitochondrial pathway [15–21].

2. Materials and Methods

2.1. Cell Culture and Treatment. Two human colon cancer cell lines HCT-116 (well differentiated, p53 wild type) and Colo-320 (poorly differentiated, p53 mutant) were grown in a medium 10% fetal calf serum and 100 U/mL penicillin-streptomycin in the presence of DIM (10–100 μM). Cell viability (XTT) [22], cytotoxicity, and leakage of lactate dehydrogenase [23, 24] were tested 24, 48, and 72 hours after treatment.

DAPI staining for visualization of chromatin condensation [25] were conducted after treatment with 60 μM DIM for 72 hr [26].

2.2. RNA Isolation and Real-Time PCR. RNA from colon cancer cells was isolated using Tri-reagent (Tal-Ron). Total RNA (3 μg) was reverse-transcribed to cDNA. 1197 bp product of NDRG1 was amplified by real-time PCR (ABI Prism 7900) using probe and the following primers: 5′-AGGGACATGTCTCGGGAGATGCAGGAT-3′ and 5′-GGCCGCCTAGCAGGAGACCTCCATGG-3′. As a control gene, we amplified β-actin (838 bp) with 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ and 5′-CGTCATACTCCT GCTTGCTGATCCACATCTGC-3′.

Alkaline phosphatase activity assay [27] was conducted 4 and 8 h after treatment with 60 μM DIM.

2.3. Statistical Analysis. Data analyses were carried out using SPSS version 17. Experiments were repeated three times, each performed in triplicate, and the data are presented as means ± SD (Standard Deviation). Statistical analysis of the differences between controls and treated groups were performed using Student's t-test. $P < 0.05$ was considered significant.

3. Results

3.1. DIM Reduced Cell Viability by Apoptosis Induction. We observed that the two colon cancer cells treated with DIM (10–80 μM) for 72 hr had a significant inhibitory effect ($P < 0.001$) in a dose-dependent manner (Figure 1). The IC_{50} values were 52 μM for HCT-116 and 56 μM for Colo-320. Furthermore, no toxic effects were manifested for these concentrations, as it was determined with LDH enzyme activity (Data not shown).

Following the reduced viability, apoptosis was tested in cells treated with DIM (60 μM) for 72 hr (Figure 2(a)). Both cell lines showed DNA fragmentation, a main feature of apoptosis occurrence. In addition, cell treatment with 60 μM DIM for 48 h followed by DAPI staining (Figure 2(b)) showed apoptotic cells that exhibited typical apoptotic morphology, condensation, and fragmentation of the nucleus, in comparison to untreated cells.

3.2. Alteration in NDRG1 Expression in Colo 320. Basal expression of NDRG1 prior treatment showed low levels of mRNA and no expression of protein in Colo 320 cell lines; alternatively, high levels of both mRNA and protein were detected in HCT-116 cell line (Data not shown).

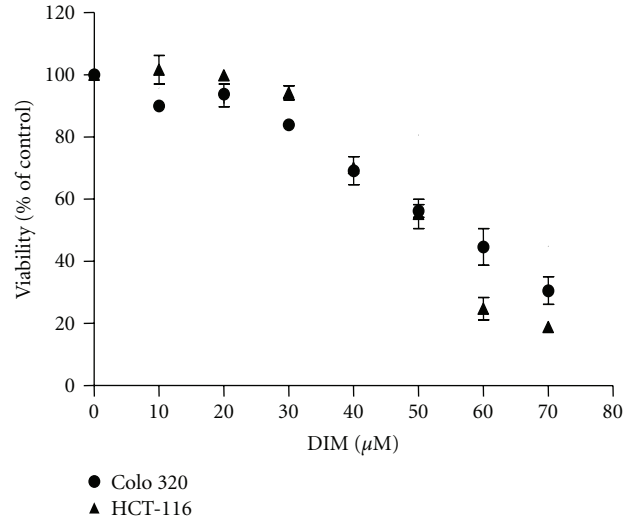

Figure 1: Effect of DIM on cell viability. Exponentially growing cells were incubated in the presence of 10 to 100 μM of DIM for 72 hours, and the viable cells were detected by XTT assay. The results are presented as percentage of control and expressed as means ± SD of three independent experiments.

Exposure of the Colo 320 to DIM for 24–72 hr resulted in a significant increase (9-fold for 24 h, 10-fold for 48 h, and 15-fold for 72 h following treatment) of the NDRG1 mRNA levels (Figure 3). However, exposure of HCT-116 to DIM resulted in uninfluenced expression of the NDRG1 mRNA compared to control cells.

3.3. Effect of DIM and NDRG1 Expression on Cell Differentiation. Alkaline phosphatase activity, a molecular marker of colon epithelial cells differentiation was used to determine whether the increase in NDRG1 expression is related to differentiation alteration in the cells. The results revealed that treated cells had a nonsignificant increase of alkaline phosphatase activity in Colo 320 cells and had no effect in HCT-116 cells (Figure 4).

4. Discussion

In the present study, we examined NDRG1 expression in human colon cancer cells during apoptosis induced upon DIM application. First, we showed that indol derivative found in crucifers (DIM) induced apoptosis in both human colon cancer cells HCT-116 and Colo 320. We have previously shown that DIM has a suppressive effect on the growth of breast cancer and prostate cell lines [15, 16, 20]. The IC_{50} values for DIM on growth inhibition of colon cancer cells (50–60 μM) were similar to those found for prostate and breast cancer cells (20–40 μM and 17–30 μM, resp.) [15, 28]. DIM affected the viability of the cancer cell in a time- and dose-dependent manner irrespective of their p53 phenotype. The effect was mediated by apoptosis as shown morphologically and by DNA fragmentation. Thus, colonic

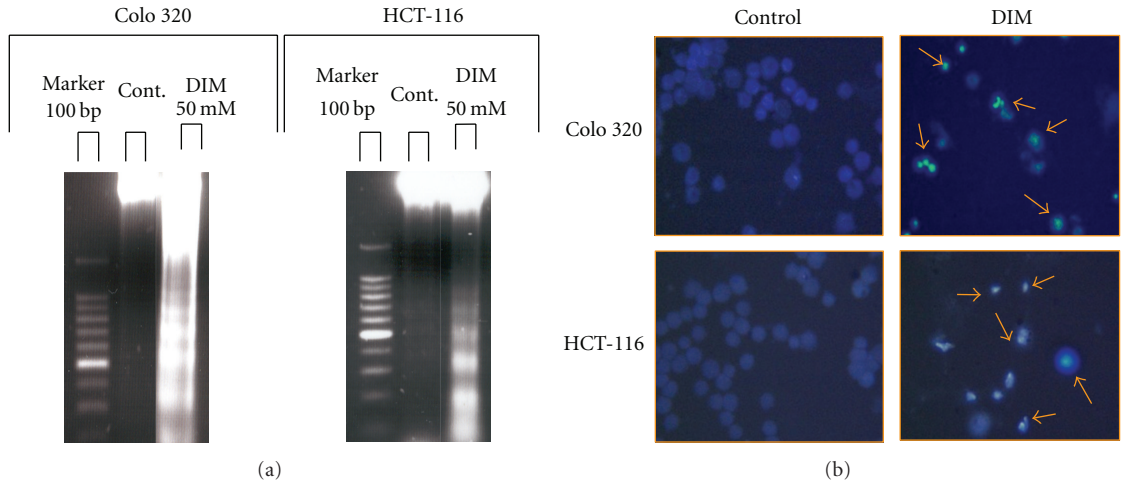

Figure 2: Apoptosis formation by DIM in Colo 320 and HGT116 cell lines. Cells were treated with DIM 60 μM for 72 hours. DNA was extracted and separated on a 1.5% Agarose gel. The data shown were repeated three times (a). Morphologic changes, denoting apoptotic cells were shown by cell morphological changes. Cells were incubated in the presence of 60 μM DIM for 48 hours and stained with DAPI. Morphologic changes are shown by arrows (b). The data shown were repeated three times.

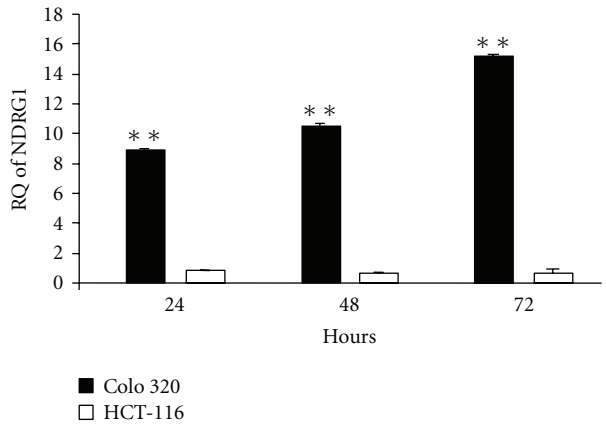

Figure 3: Expression of *NDRG1*. Cells treated with 60 μM DIM for 24, 48 and 72 h. The levels of mRNA were examined by real-time PCR and are represented as RQ (relative quantification) = 2^{-ddCt}. $^{*}P$ value <0.01, $^{**}P$ value <0.001.

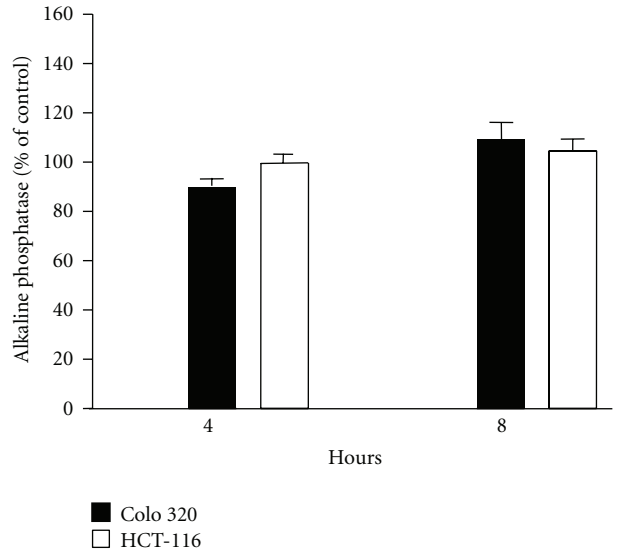

Figure 4: DIM treatment effect on alkaline phosphatase activity. Alkaline phosphatase activity (differentiation factor of colon cells) was detected after 4 and 8 hours of treatment. The results are presented as percentage of control and expressed as means ± SD of three independent experiments. $^{**}P$ value <0.001.

cell lines can be added to an increasing list of other organs' cell lines, namely, prostate and breast [16, 21, 29] that are affected by environmental factors which induce apoptosis.

The basal expression of NDRG1 reported here, high levels in well-differentiated cells (HCT-116) and low levels in poorly differentiated cells (Colo-320), was reported in colon [30], gastric cancer specimens [31], and in esophageal squamous cell carcinoma [32].

Quantization of NDRG1 expression showed that NDRG1 mRNA is increased 9–15-fold ($P < 0.001$) during treatment of the Colo-320 cells with DIM for 24–72 hr compared to control cells, indicating NDRG1 upregulation during apoptosis induced by DIM.

Up to now, it was known that NDRG1 expression is induced by DNA damage in a p53-dependent fashion, and it is necessary but not sufficient for p53-mediated caspase activation and apoptosis [8]. The present study shows for the first time that DIM increased NDRG1 expression in a p53 independent pathway followed by induction of apoptosis.

In conclusion, in this study, we provide evidence that the indole diet-derived DIM induce apoptosis in two human colon cancer cell lines, well- and poorly differentiated. However, induction of apoptosis by DIM is followed by increased NDRG1 expression only in a poorly differentiated and p53 mutant cells. On the other hand, DIM induced apoptosis in well-differentiated cells in a NDRG1-independent pathway. Our results may contribute to a better understanding of the

molecular mechanisms by which DIM exerts its effects in colon cancer cells and tumors. It is suggested that NDRG1 is not only a differentiation factor but might impact the apoptotic process. In all the NDRG1 transfection studies, apoptotic effects should be taken in account. In fact, NDRG1 is on the list of metastatic-related/cancer suicide genes drug development in the battle against cancer [33, 34]. Further studies are needed in order to examine NDRG1 role in colon cancer genesis. Results from the present research may contribute to the development of new and efficient strategies for colon cancer therapy.

Conflict of Interests

The authors declare no potential conflict of interests with respect to the authorship and/or publication of this paper.

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Research Article

The Effect of *Tinospora crispa* on Serum Glucose and Insulin Levels in Patients with Type 2 Diabetes Mellitus

Theerawut Klangjareonchai and Chulaporn Roongpisuthipong

Department of Medicine, Ramathibodi Hospital, Faculty of Medicine, Mahidol University, Bangkok 10400, Thailand

Correspondence should be addressed to Chulaporn Roongpisuthipong, racrp@mahidol.ac.th

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Objective. To determine the effects of *Tinospora crispa* on serum glucose and insulin levels in healthy subjects and patients with type 2 diabetes mellitus. *Method*. Serum from 10 healthy subjects and 10 diabetic participants, who had fasted overnight, were obtained every 30–60 minutes during the 3 hours of continued fasting and during the 3 hours after ingestion of 75 g of glucose with or without ingestion of 125 or 250 g of *Tinospora crispa* dry powder capsule. Glucose and Insulin levels were analyzed and the areas under the curve for mean serum glucose and insulin levels were calculated. *Result*. The areas under the curve of mean serum glucose and insulin levels in both healthy and diabetic participants were not significantly different between with or without *Tinospora crispa* dry powder capsule. In diabetic participants the area under the curve of glucose was slightly lesser when 250 mg of *Tinospora crispa* was ingested, but not reaching statistical significance (478 and 444 mg min/ml, resp., $P = 0.57$). *Conclusion*. The results suggest that *Tinospora crispa* ingestion cannot affect serum glucose and insulin levels in healthy subjects or patients with type 2 diabetes mellitus.

1. Introduction

Diabetes mellitus (DM), one of the major non-communicable diseases, has become a major public health problem and has increased prevalence in the modern world, even in developing countries such as Thailand [1]. As with other diseases, DM has been treated by oral administration of plant extracts based on traditional medicine since ancient times [2]. *Tinospora crispa* (*T. crispa*, Menispermaceae), a medicinal plant used to treat DM, was able to cause a reduction in serum glucose level in diabetic rats, and the hypoglycemic effect was probably due to its insulinotropic activity [3]. *T. crispa* also increased peripheral utilization of glucose and inhibited hepatic glucose release [4].

Sangsuwan et al. [5] showed that *T. crispa* had no efficacy for therapy in patients with type 2 DM who did not respond to an adequate dose of oral hypoglycemic drugs for at least 2 months and still had a glycosylated hemoglobin of greater than 8.5%. There were no significant changes in fasting serum glucose or glycosylated hemoglobin between those collected at baseline and during the study period in either group. In this study the author suggests that *T. crispa* did not stimulate insulin secretion in poor controlled diabetes because these patients might not the have ability to secrete insulin. Efficacy of *T. crispa* should be investigated in diabetic patient who responded to oral hypoglycemic drugs and did not use insulin therapy because pancreas in these patients might have ability of insulinotropic activity.

The objective of this study was to determine the effects of *T. crispa* on serum glucose and insulin levels in patients with type 2 DM and healthy subjects.

2. Material and Method

2.1. Subjects. After approval from the Ethics Committee for Human Studies of Ramathibodi Hospital, Mahidol University, 10 participants (7 women and 3 men) with type 2 DM between the ages of 32 and 64 years and 10 healthy participants (6 women and 4 men) with normal oral glucose tolerance test between the ages of 23 and 33 years were enrolled, type 2 DM participants responded to oral hypoglycemic drugs and discontinued oral hypoglycemic drugs at least 1 month. Patients with liver disease, heart disease, renal impairment, or those who previously received traditional medicine

Table 1: Baseline characteristics of the subjects in the study.

Characteristics	Diabetic participants ($n = 10$)	Healthy participants ($n = 10$)
Gender, male : female	7 : 3	6 : 4
Mean age, year (SD)	54.5 (9.5)	27.6 (2.9)
Mean FPG, mg/dL (SD)	151.0 (57.1)	83.4 (11.3)
Mean HbA1c, % (SD)	7.7 (1.1)	5.8 (0.3)
Mean creatinine, mg/dL (SD)	0.9 (0.5)	0.8 (0.2)
Mean cholesterol, mg/dL (SD)	175.5 (26.6)	204.0 (35.7)
Mean triglyceride, mg/dL (SD)	131.2 (56.7)	85.0 (26.4)
Mean AST, U/L (SD)	32.2 (13.6)	16.9 (5.7)
Mean ALT, U/L (SD)	46.6 (21.0)	29.7 (5.3)
Mean bilirubin, mg/dL (SD)	0.6 (0.3)	0.6 (0.4)

were excluded. Informed, written consent was obtained from all subjects after explanation of the nature, purpose, and potential risks of the study.

2.2. Plant Material and Fraction Preparation. The stems of *T. crispa* were collected from Supanburi and Nakhonsithammarat provinces, Thailand. Plant material was identified by Dr. Kongkathip N, Department of Chemistry, Faculty of Science, Kasetsart University. Coarse powder of *T. crispa* stem was defatted with petroleum ether (60–80°C) and dried material was extracted with 70% ethanol using soxhlet apparatus at 65–70°C for 7 days. The extract was dried under vacuum in a rotary evaporator. Powder was used microcrystalline cellulose at 7% of powder weight and using dry granulation technique. Powder of 125 and 250 mg *T. crispa* stem was prepared in form of capsule [6]. We designed to use 250 mg of *T. crispa* dry powder capsule because in previous study, 250 mg twice a day *T. crispa* can reduce fasting blood glucose significantly from baseline in metabolic syndrome patients [7].

2.3. Methods. After the screening visit, all subjects adhered to an alcohol-free, high-carbohydrate diet (>150 g/d) and discontinued all oral hypoglycemic agents at least 72 hours before the study. Exercise was discontinued 24 hours before and fasting was instituted 12 hours till the morning of the study. The study was performed in three visits, 1 to 2 weeks apart. The doses at each visit were as follows: 75 g of glucose at first visit; 75 g of glucose and 125 mg of *T. crispa* dry powder capsule at second visit; 75 g of glucose and 250 mg of *T. crispa* dry powder capsule at third visit. Venous blood was drawn and then ingestion of 75 g glucose with or without ingestion of 125 or 250 mg of *T. crispa*. Additional venous blood samples were drawn at 30, 60, 90, 120, and 180 minutes after ingestion. Serum glucose was obtained by an automated glucose oxidase method and serum insulin was measured by radioimmunoassay kit. All participants were examined and blood was taken for complete blood count, liver enzyme profile, and renal function at entry and end of the study.

2.4. Statistical Analyses. Areas under the curve (AUC) for increase in serum glucose and serum insulin were calculated by trapezoidal rule, and means were tested for significance by paired t-test. Mean glucose and insulin blood levels of all subjects were calculated for each time point. $P < 0.05$ was considered statistically significant.

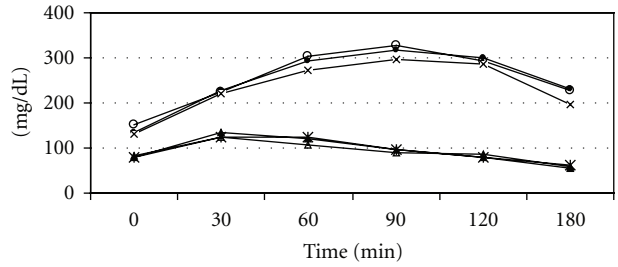

Figure 1: Mean serum glucose level at timed intervals during glucose tolerance test for 10 diabetic and 10 healthy participants. Mean serum glucose levels in mg/dL without (o) and with 125 (•) and 250 mg (×) ingestion of *Tinospora crispa* diabetic participants, and without (△) and with 125 (▲) and 250 mg (*) ingestion of *Tinospora crispa* in healthy participants.

3. Results

All of the 20 participants who had enrolled completed the study. At the dosage used there were no adverse effects reported by our subjects. During the treatment period no elevations in serum transaminase were detected. The baseline characteristics of the participants are shown in Table 1.

3.1. Glucose Levels with and without Ingestion of Tinospora crispa. Table 2 shows incremental serum glucose AUC (time 0 to 180 min) over the baseline in mg min/mL for 75 g glucose ingestion with and without 125 or 250 mg of *T. crispa* for each participant. The mean serum glucose AUC in both diabetic and healthy participants was not significantly different between with and without *T. crispa*. In diabetic participants the mean serum glucose AUC were slightly lesser when 250 mg of *T. crispa* was ingested, but not reaching statistical significance (478 and 444 mg min/mL, resp., $P = 0.57$). The mean glucose levels at each time interval showed small differences with *T. crispa* ingestion (Figure 1).

Table 2: Mean of 3 h total area under the curve (AUC) glucose and 3-h total AUC insulin for 75 g oral glucose tolerance test performed with and without 125 or 250 mg of *Tinospora crispa*.

	3 h total AUC for glucose (mg min/mL)			3 h total AUC insulin (mIU min/mL)		
	Without *T. crispa*	125 mg *T. crispa*	250 mg *T. crispa*	Without *T. crispa*	125 mg *T. crispa*	250 mg *T. crispa*
DM	478 ± 135	475 ± 102	444 ± 117	61 ± 56	50 ± 37	39 ± 21
Healthy	165 ± 25	171 ± 38	171 ± 30	73 ± 55	85 ± 34	58 ± 16

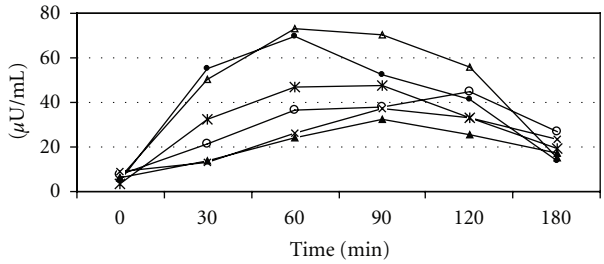

Figure 2: Mean serum insulin level at timed intervals during glucose tolerance test for 10 diabetic and 10 healthy participants. Mean serum insulin levels in μU/mL without (o) and with 125 (•) and 250 mg (×) ingestion of *Tinospora crispa* in diabetic participants, and without (△) and with 125 (▲) and 250 mg (*) ingestion of *Tinospora crispa* in healthy participants.

3.2. Insulin Levels with and without Ingestion of Tinospora crispa. Table 2 shows incremental insulin in μU min/mL AUC (time 0 to 180 min, 75 g glucose tolerance tests without and with 125 or 250 mg of *T. crispa*) for each participant. Mean insulin AUC (Table 2) and mean levels at each time interval (Figure 2) in both diabetic and healthy participants varied, little with *T. crispa* ingestion.

4. Discussion

This study was unable to demonstrate the efficacy of *T. crispa* water extract to dry powder capsule for changes in areas under the serum glucose and insulin curves in healthy or diabetic subjects.

This result was inconsistent with other studies in animal model and metabolic syndrome subjects, Noor et al. [8] showed *T. crispa* aqueous extract to the drinking water for one week. Fasting blood glucose levels were significantly lower and serum insulin levels were significantly higher in treated diabetic rats (10.4 ± 1.0 mmol/l and 12.8 ± 1.1 μU/mL, resp.) compared to untreated diabetic controls (17.4 ± 1.7 mmol/l and 8.0 ± 0.7 μU/mL, resp.). Kongkathip et al. [9] found that the water extract of *T. crispa* caused a 13.9–15.6% reduction of serum glucose levels in normal rats after 120-minute oral feeding while 70% ethanol extract decreased serum glucose levels by 8.9–13.0% after 120 minute. Acute toxicity testing showed that LD50 values of water extracts are about 20–24 g/kg. It was suggested that the water extract of *T. crispa* is highly safe. Sriyapai et al. [7] presented metabolic syndrome patients who received 250 mg twice a day *T. crispa* dry powder capsule for 2 months can reduce fasting blood glucose significantly from baseline (6.29 ± 10.47 mg/dL, $P < 0.01$).

on the other hand, Sangsuwan et al. [5] showed that *T. crispa* had no efficacy for therapy in patients with type 2 DM who did not respond to oral hypoglycemic drugs since there were no significant changes in fasting serum glucose between those collected at baseline and during the study period in either group (250 and 245 mg/dL, resp.) or glycosylated hemoglobin (10.3 and 11.2%, resp.). Our study demonstrated that *T. crispa* can not significantly reduce mean serum glucose AUC in diabetic patient but can show a trend to reduce mean glucose AUC 478 to 444 mg mL/min. 250 mg of *T. crispa* may not be adequate dosage to reduce serum glucose and stimulate insulin and another reason is that it may not last long enough to show the effect of treatment. Therefore, we need further investigation about adequate dose of *T. crispa* to reduce serum glucose and stimulate insulin secretion in type 2 diabetic patients.

5. Conclusion

The results suggest that *Tinospora crispa* ingestion cannot affect serum glucose and insulin levels in healthy subjects or patients with type 2 diabetes mellitus.

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Review Article

Applications of the Phytomedicine *Echinacea purpurea* (Purple Coneflower) in Infectious Diseases

James B. Hudson

Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada V5Z 3J5

Correspondence should be addressed to James B. Hudson, jbhudson@interchange.ubc.ca

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Extracts of *Echinacea purpurea* (EP, purple coneflower) have been used traditionally in North America for the treatment of various types of infections and wounds, and they have become very popular herbal medicines globally. Recent studies have revealed that certain standardized preparations contain potent and selective antiviral and antimicrobial activities. In addition, they display multiple immune-modulatory activities, comprising stimulation of certain immune functions such as phagocytic activity of macrophages and suppression of the proinflammatory responses of epithelial cells to viruses and bacteria, which are manifested as alterations in secretion of various cytokines and chemokines. These immune modulations result from upregulation or downregulation of the relevant genes and their transcription factors. All these bioactivities can be demonstrated at noncytotoxic concentrations of extract and appear to be due to multiple components rather than the individual chemical compounds that characterize *Echinacea extracts*. Potential applications of the bioactive extracts may go beyond their traditional uses.

1. Traditional Uses of *Echinacea*

Herbal medicines derived from several species of the indigenous *Echinacea* genus were in use throughout the plains of North America long before the introduction of European medicines, primarily as treatments for various infectious diseases and wounds. Nine discrete species were classified subsequently by botanists, as indicated in Table 1, although medical records suggest that considerable interchange between uses of designated species occurred and consequently the association of a specific species with particular treatments has to be viewed with caution [1–3]. Even in recent years there have been revisions in the taxonomy of the genus [4, 5]. Nevertheless it is generally agreed that *Echinacea purpurea*, the purple coneflower, was widely used by Native peoples and later by the Eclectic practitioners of North America, possibly because it was so widespread, and also because it was apparently effective in a number of diseases. Current herbal preparations, which have become very popular in North America, Europe, and elsewhere, have tended to favor this species over the others, and the majority of the basic scientific studies have focused on this one. Accordingly, this review is restricted primarily to discussion of *E. purpurea* (abbreviated EP), with occasional reference to alternative species.

The source material for scientific and clinical studies is usually an aqueous "pressed juice" or ethanol tincture/extract of aerial parts of the dried plant or roots. The chemical composition differs substantially between such preparations, at least in terms of the known "marker compounds", such as caffeic acid derivatives, alkylamides, and polysaccharides, all of which have been claimed to contribute to the medicinal benefits [5–7]. However, the uncertainty in the identity of the principal bioactive compounds has made interpretation of basic and clinical studies difficult, and unfortunately the problem has been exacerbated by the frequent use of uncharacterized source material.

In an attempt to validate some of the traditional uses of *Echinacea*, numerous studies have been made recently on the effects of characterized EP preparations on viruses, bacteria and other organisms, inflammatory responses, and gene expression in infected and uninfected human cell cultures, as well as animal studies. These results are discussed in following sections. However I have omitted reference to

Table 1: Traditional uses of *Echinacea* (Coneflower) extracts.

Echinacea species	Traditional applications
E. purpurea, E. angustifolia, E. pallida	Respiratory infections: colds and 'flu, bronchitis, strep throat, toothache Urinary tract infections: herpes sores, gonorrhea Skin disorders: staph infections, cold sores, ulcers, wounds, burns, insect bites, eczema, allergies Others: rheumatoid arthritis
E. atrorubens, E. laevigata *E. paradoxa, E. sanguinea* *E. simulata, E. tennesseensis*	Not frequently used

Adapted from [1].

studies that used combinations of EP with other herbs, for reasons explained below (Section 16).

2. Antiviral Activities

2.1. Respiratory Virus Infections. Acute respiratory infections in humans are usually caused by one or more of a group of well-known viruses, which includes over 100 rhinoviruses ("common cold" viruses), influenza viruses A and B, parainfluenza viruses, corona viruses, respiratory syncytial virus, and certain adenoviruses [8–10]. Influenza virus A deserves special consideration because of its unique capacity for genetic reassortment between animal and human strains and consequent production of epidemics (Section 2.3 below). The SARS virus (SARS-HCoV) also merits additional comments, as explained in Section 15. In addition the recent application of more sensitive molecular detection techniques has revealed the presence of other viruses, such as metapneumoviruses and bocaviruses, which might also be involved in the generation of respiratory symptoms [10]. However we do not know if these newly recognized viruses are really pathogenic or are simply "passengers" that eluded previous diagnostic techniques. Herpes simplex virus type 1 (HSV-1) also produces oral mucosal infections ("cold sores"), and these may be accompanied by symptoms reminiscent of respiratory viruses.

Clearly various families of viruses, with different structures and replication schemes, and consequently bearing different potential molecular targets, are involved in respiratory symptoms, and many of them are susceptible to *Echinacea* extracts, as indicated in Table 2. Among the possible viral targets are: (i) the virion itself (membrane components); (ii) cellular attachment or entry; (iii) one or more of the many stages in virus replication and development, particularly those that involve virus-specific enzymes; (iv) egress of progeny virus from infected cells. However, because of the variety of replication schemes among these viruses the chances of success for a single therapeutic drug are low, especially considering that in the majority of respiratory infections specific virus information is lacking.

Another problem with the specific antiviral target approach, especially in the case of compounds directed at specific viral genes or their products, is the inevitable emergence of virus resistant mutants [14, 15] and their subsequent spread through the community and environment. The conventional answer to this problem has been to use combinations of two or more antiviral drugs, with distinct molecular targets, notwithstanding the likely increase in undesirable side effects. However, a logical alternative approach is the use of a noncytotoxic agent that has the capacity to inhibit many different respiratory viruses simultaneously, and recent evidence indicates that certain herbal extracts could fulfill this requirement [15–17].

2.2. Causes of Respiratory Symptoms. "Colds", "flu", and "bronchitis" are terms that have been coined to describe various permutations of common symptoms, supposedly brought about by the actions of specific viral infections of the upper respiratory tract. These symptoms may include such familiar discomforts as sneezing, stuffy nose, irritation of mucous membranes, excess mucus production, sinusitis, cough, sore throat, malaise, and fever, as well as exacerbation of asthma and COPD (chronic obstructive pulmonary disease). In some cases symptoms may spread to include the lower respiratory tract and lungs and result in bronchiolitis or pneumonia [16, 18–20]. However these symptoms may not be a direct result of virus replication, which in many cases is minimal in differentiated airway tissues [21, 22], but rather an indirect consequence of virus-induced inflammatory responses [17, 23].

In respiratory infections, the invading virus initially encounters epithelial tissues, which are composed largely of epithelial cells and occasional dendritic cells and macrophages. These cells respond by means of the various antimicrobial strategies that make up the innate immune system, including defense peptides (antimicrobial peptides) and the secretion of various proinflammatory cytokines and other mediators of inflammation [24–26]. Other molecules such as kinins are released and are probably responsible for some of the early symptoms. Rhinoviruses have also been shown to induce kinin gene expression [27], although this effect is more likely a delayed effect of virus infection. In response to all these mediators phagocytic cells and various types of inflammatory cell may then be attracted to the site of infection [26]. In addition the redox balance of the cells may be adversely affected, either by the virus infection itself or as a consequence of the proinflammatory response [28].

Since most of the symptoms reflect this common nonspecific host response to infecting agents, rather than to the direct cytolytic or cytopathic effects of a specific virus [15–17], then a more rational therapeutic approach would be the application of anti-inflammatory agents, especially if the intention of the therapy is to ameliorate symptoms. If a potential safe anti-inflammatory agent also contains multiple antiviral activities, then this would provide a bonus. Several herbal extracts have been shown to possess a combination of bioactivities that could be useful in the control of respiratory infections [17, 29, 30], and these *Echinacea* extracts have

become very popular, although, because of the variation in their chemical composition (as mentioned in Section 1), not all of them are necessarily beneficial.

2.3. Influenza Virus Type A. Influenza viruses are ubiquitous and produce significant annual morbidity and mortality throughout the world, with potentially devastating consequences for human and animal health, and the global economy [31, 32]. Thereare three types of influenza virus, A, B, and C, the latter two being confined mainly to humans, in which they produce relatively mild seasonal outbreaks. However the greatest impact derives from Influenza A virus, which has been associated with several well-known human pandemics during the last century and an increasing number of epidemics (epizootics) in domestic birds [31–34]. It is believed that influenza A virus originated in wild birds, possibly waterfowl such as ducks and geese and that these birds act as reservoirs and vectors for the many known subtypes (strains) of influenza A virus [35].

The classical symptoms of human influenza include cough, malaise, and fever, often accompanied by sore throat, nasal obstruction, and sputum production, which resolve spontaneously in most healthy individuals, although immune compromised and elderly individuals tend to be more vulnerable. Complications may include bronchitis and pneumonia, and exacerbation of asthma, and chronic obstructive pulmonary disease (COPD) [16, 18, 20].

More serious disease in healthy individuals, especially during pandemics, is often accompanied by excessive overreaction of the innate immune response with the secretion of dangerous levels of cytokines ("cytokine storms") and other inflammatory mediators [32–34]. Also the importance of concurrent bacterial infection cannot be overlooked, since this may lead to more serious outcomes [36]. Thus an ideal control agent should be able to prevent or reduce the replication and spread of the virus, as well as any potentially pathogenic bacterial infection, and also counteract the overproduction of inflammatory mediators.

Vaccines are generally advocated for routine application during each influenza seasonal outbreak, based on the prevailing strain of the previous season; but because of the unpredictable nature of influenza epidemics one cannot be sure of the success of any vaccine, and several researchers have questioned the wisdom of widespread vaccination [15, 31, 37, 38].

Numerous antiviral drugs for use in infected patients have been tested experimentally, in animal models and in humans, but none has proven satisfactory [30, 39]. The most recent synthetic compounds are the neuraminidase inhibitors oseltamivir (Tamiflu) and zanamivir (Relenza), but drug-resistant strains of human and avian Influenza viruses have been documented with increasing frequency [40, 41].

2.4. Antiviral Activities of Echinacea Purpurea (EP). Early studies showed that only certain *Echinacea* extracts possessed significant antiviral activity. *E. purpurea* (EP) aerial parts and roots contained potent antiviral activities (virucidal) against influenza virus, herpes simplex virus, and coronavirus, and these were distributed among more than one solvent derived fraction, probably reflecting the presence of more than one antiviral compound [11, 12]. However there was no correlation between antiviral activity and composition of the recognized marker compounds, that is, caffeic acids, polysaccharides, and alkylamides, and in fact a purified polysaccharide fraction from EP possessed no significant activity, while cichoric acid and several caffeic acid derivatives showed only weak to moderate activity, insufficient to account for the potent activities of EP [42]. In addition the antiviral activity of commercial ethanol tinctures from EP can remain stable for at least several years at ambient temperatures, which would seem to rule out many potential candidate bioactive compounds. Furthermore some, but not all, of the antiviral activities were due to photosensitizers, which again limits the number of prospective candidates [12, 43].

Recent studies with the standardized preparation Echinaforce (EF, comprising ethanol extracts of *E. purpurea*, 95% aerial parts plus 5% roots) showed that this preparation was a very potent virucidal agent against several viruses with membranes, as indicated in Table 2. In addition to HSV-1 and respiratory syncytial virus, all tested human and avian strains of influenza A virus, as well as influenza B virus, were susceptible [13, 44]. In addition rhinovirus and feline calicivirus were also equally susceptible at the relatively high concentrations of EF recommended for oral consumption [45]. Thus EF at 1 : 10 dilution (equivalent to 1.6 mg/mL dry weight/volume) was capable of killing at least 10^5 infectious viruses by direct contact. Adenoviruses however were resistant.

In further studies EP was found to be much less effective against intracellular virus [13, 44]. Consequently virus already present within a cell could be refractory to the inhibitory effect of EP, whereas virus particles shed into the extracellular fluids should be vulnerable. Therefore EP can act during initial contact with the virus, that is, at the inception of infection and also during transmission of virus from infected cells.

Additional experiments showed that continuous passage of influenza A virus in cell cultures in the presence of EP did not result in the emergence of resistant strains, whereas passage of the virus through successive cultures in the presence of Tamiflu rapidly generated Tamiflu resistance. Furthermore Tamiflu-resistant virus remained fully susceptible to EP [13]. Therefore continuous usage of EP in the population would be less likely to generate resistant strains of virus than Tamiflu or other anti-influenza compounds currently in the market.

It was shown by hemagglutination assays that EP inhibited the receptor-binding activity of influenza A viruses, over a range of EP concentrations, suggesting that EP interfered with viral entry into the cells, thus effectively rendering the virus noninfectious [13]. EP also inhibited neuraminidase activity *in vitro* (unpublished results), suggesting that the active compounds could block influenza virus entry and spread by acting on at least two virion targets. However, the susceptibility of other viruses, which do not rely on HA or

Table 2: Antiviral activities of EP at noncytotoxic concentrations.

virus	Relevant properties	Susceptible to EPethanol extracts (+ or -)	Susceptible to EP aqueous extracts (+ or -)
rhinoviruses	ss RNA, no membrane	+ only at high conc.	+ only at high conc.
Influenza viruses	segmented RNA, + membrane & 2 target virion proteins (HA, NA)	+	+
Respiratory syncytial virus	ss RNA, + membrane & fusion protein	+	nt
Coronavirus	ss RNA + membrane	+ (mouse virus)	nt
Calicivirus	ss RNA, no membrane	+ high conc.	+ high conc.
Poliovirus	ss RNA, no membrane	-	-
Herpes viruses	ds DNA + membrane	+	+

Data from [11–13].

NA functions, to EP, indicates that there must be additional molecular targets.

3. Effects of EP on Virus-Infected Cells: Involvement of Cytokines and Chemokines

Rhinovirus-infected human bronchial and lung epithelial cells were used to study the effects of EP on cellular gene expression and secretion of cytokines and chemokines, principal mediators of inflammatory responses. Rhinoviruses stimulated the secretion of many different cytokines [46, 47], including the proinflammatory IL-1, IL-6, IL-8, and TNFα, which are known to be collectively involved in many of the symptoms common to respiratory infections, such as sneezing, fever, sore throat, nasal discharges, and inflammation in various respiratory tissues. Several EP preparations were able to completely or partly reverse this stimulation [48–50]. EP could be added before or after virus infection, with similar success, and also the results were not affected by virus dose or the time of exposure to EP [49].

It is noteworthy that RV14 and RV1A, which are known to use distinct cellular receptors (ICAM-1 and LDL, resp.), both stimulate cytokine secretion that is reversed by EP, suggesting the involvement of multiple signaling pathways. This concept was supported by the demonstration that many transcription factors (TF's) known to be associated with cytokines and chemokines were also stimulated by RV14, and these TF's were modulated by EP treatment [50, 51].

Other viruses, including HSV-1, influenza A virus, adenovirus type 3 and 11, and respiratory syncytial virus, stimulated the secretion of pro-inflammatory cytokines, and in each case the stimulation was reversed by EF (Table 3, and [44]). However only live infectious viruses were able to do this, for infection by equivalent doses of ultraviolet-inactivated viruses failed to elicit the responses. This suggests that the virus may have to enter the cells and undergo some degree of gene expression in order to stimulate the cytokine expression or secretion. It is also interesting that viruses such as adenoviruses, which are not vulnerable to direct attack by *Echinacea*, but could nevertheless stimulate cytokine secretion, were still susceptible to cytokine reversal.

In a more recent study with influenza A-infected mouse macrophage-like cells, the viral induced production of cytokines and chemokines was also suppressed to various degrees by EP extracts and some of their constituent alkylamides (details below).

Several conclusions can be derived from these results. Probably the most important is that we could not correlate cytokine inhibitory effects, that is, anti-inflammatory properties, with specific individual compounds or groups of marker compounds, namely, alkylamides, polysaccharides, and caffeic acid derivatives. In the case of EP, all the fractions derived from roots, leaves plus stems, and flowers, were anti-inflammatory according to IL-6 and IL-8 measurements [52]. Numerous viral and bacterial infections, as well as wounds, result in substantial stimulation in levels of proinflammatory cytokines, especially IL-6 and IL-8, which are consequently considered to represent useful markers of an inflammatory condition [23, 29]. Thus any compound or herbal extract that inhibits or reverses this elevation in IL-6/8 and so forth, could be considered as a potential anti-inflammatory agent. Consequently many preparations derived from roots or aerial parts of EP would be expected to possess anti-inflammatory properties.

4. Mucin Secretion

Secretion of excessive mucus is one of the more annoying symptoms of colds and other respiratory infections and occurs frequently in chronic pulmonary infections. Many pharmaceuticals have been designed to relieve this feature of an infection, usually with the accompaniment of undesirable side effects.

Mucins, the products of at least 18 mucin genes in humans, are highly glycosylated macromolecules that constitute part of the innate defense system against respiratory pathogens [53], But in some chronic conditions, and in response to certain airway infections such as rhinovirus [54], one or more genes may be induced to hypersecrete mucins. In studies on bronchial epithelial cells in culture, and in organotypic bronchial tissue cultures, rhinovirus induced the secretion of excess MUC5A, the dominant respiratory mucin, and EP reversed this secretion [55], suggesting that this could be an additional benefit of EP treatment. Table 4 shows a representative result.

Table 3: Cytokines/chemokines induced by viruses (+) and reversed by EP.

cytokine	RV1A	RV14	FluV	RSV	Ad 3
IL-1a	+	+	+	+	+
IL-5	+				+
IL-6	+	+	+	+	+
IL8 (CXCL-8)	+	+	+	+	+
TNFα	+	+	+	+	+
GROα		+	+	+	
CCL-3				+	+
CCL-4				+	+

Adapted from [44]. RV: rhinovirus (1A/14); FluV: influenza virus (H1N1/H3N2); RSV: respiratory syncytial virus; Ad 3: adenovirus type 3.

5. 3-D Tissues of Human Airway Epithelium

It is important that the cell culture models used to evaluate anti-infectious agents reflect conditions *in vivo* as far as possible [56]. This condition was confirmed for EP by means of a commercial source of normal human airway epithelial tissue (EpiAirway tissue, a 3-D organotypic model), which could be propagated *ex vivo* under defined conditions such that tissue architecture and differentiation patterns were preserved, according to standard histological examination [57]. Under these conditions the effects of rhinovirus infection, and EP, on various parameters of tissue integrity and cytokine induction were evaluated [55]. Individual replicate tissue samples, maintained as inserts in culture for three days or three weeks, were infected with rhinovirus type 1A (RV1A), EP alone, a combination of the two, or medium only. None of the treatments affected the histological appearance or integrity of the tissues, all of which maintained a high level of cell viability and preservation of cilia, with the exception of the conspicuous virus-induced mucopolysaccharide inclusions in the goblet cells. There was no evidence of virus replication, although the RV infected tissues secreted substantial amounts of the proinflammatory cytokines IL-6 and IL-8, and this response was reversed by EF treatment (Table 5). The goblet cells also appeared normal and free of inclusions in the EP-treated tissues. Therefore these results confirmed the findings derived from studies of bronchial and lung epithelial cell lines (above), namely, that RV infection resulted in a substantial inflammatory response and mucin secretion in the absence of virus replication.

6. Effects of EP on Gene Expression in Rhinovirus-Infected Cells

Gene expression in human bronchial cells was analyzed by means of DNA microarrays, following treatment by one of two EP preparations, an aqueous extract and an ethanol extract, with or without infection by rhinovirus type 14 [58, 59]. Both extracts influenced the expression of many genes, including cytokine genes, although the pattern of expression was different for the two extracts. In addition the virus itself induced numerous changes, mostly increases in expression, and the extracts tended to decrease (i.e., restore to normal levels) these expression levels. Further analysis of the effects revealed that some of the changes in cytokine expression were interconnected through a specific transcription factor, C/EBPb (CAAT/enhancerbinding protein b, [59]). Since numerous transcription factors were known to be affected by EP extract in this same cell-virus system [50], it is tempting to conclude that many gene expression effects of *Echinacea* extracts could be due to changes in expression or activation status of multiple transcription factors. This in turn could be brought about by interaction with surface receptors or their intracellular modulators.

Analyses of IFNα (interferon alpha) gene expression, by DNA microarray analysis and PCR assays, revealed only a few changes in cells treated by different EP extracts, in uninfected bronchial epithelial cells, or in rhinovirus-infected cells, although certain interferon stimulated genes (ISG's) were upregulated by the virus and downregulated by the EP extracts [59]. Interferon bioassays in bronchial cells failed to detect IFN, in contrast to cells stimulated by the known IFN inducer poly I : C (Vimalanathan and Hudson, unpublished data). Negative findings were also reported recently in a mouse macrophage cell line infected with HSV-1 [60], from which the authors concluded that EP induced little if any IFN alpha or beta, and consequently antiviral effects of EP are not likely to involve the interferon system.

Table 4: Mucin secretion in human epithelial cell cultures.

Treatment	Ratio, treatment/control
None (control)	1.00
Echinacea (EP) only	0.76
Rhinovirus (RV1A) infection	2.18
Echinacea (EP) + RV1A	0.64

Bronchial epithelial cell cultures (BEAS-2B) were treated with combinations of EP and rhinovirus RV1A, as indicated, and supernatants were assayed for secreted MUC5A by ELISA (details in [55]).

7. Antibacterial Activities

Several potentially pathogenic bacteria have the capacity to cause respiratory symptoms, resulting from initial interactions with epithelial cells of the oral and nasal mucosa and various parts of the lung. General features of infection by these organisms include proliferation and spread of the bacteria with resultant cellular damage, often aided by products of bacterial virulence genes, and the induction of excessive proinflammatory cytokines, which can lead to chronic inflammation. A herbal medicine with bactericidal and anti-inflammatory properties could provide benefits to individuals suffering from respiratory symptoms, and certain preparations of EP possess these activities, in addition to their antiviral activities described above. Results are summarized in Table 6.

Streptococcus pyogenes (Group A streptococcus, or GAS) is responsible for widespread infections, ranging from hundreds of millions of relatively mild cases of pharyngitis

globally, to more severe toxic shock syndromes and necrotizing fasciitis ("flesh-eating disease"), the more severe symptoms being ascribed to inflammatory cytokines ("cytokine storms"). In addition several *Streptococcal* gene products or virulence factors have been described which aid the bacteria in persistence in oral epithelia and saliva and dissemination to other tissues [63–66]. Consequently the dual actions of EP in killing the bacteria and reversing their proinflammatory activities could be a significant aid in combating such infections.

Hemophilus influenzae is part of the normal nasopharyngeal flora. Recently additional pathogenic strains have been associated with otitis media, chronic bronchitis, and pneumonia. Initial interaction with epithelial cells can result in proinflammatory cytokine secretion, via toll receptors and other mediators [66, 67]. EP can kill this organism readily and also inhibit the cytokine induction in bronchial epithelial cells (Table 6).

Legionella pneumophila, associated with Legionnaires' disease and sometimes more severe cases of pneumonia, is widely distributed in water and soil, from which the organism can be inhaled as an aerosol and once inside alveolar macrophages localizes in a relatively resistant vacuole, in which it replicates [68, 69]. The organism is however very sensitive to EP, and its induction of proinflammatory cytokines is inhibited by EP treatment (Table 6).

Staphylococcus aureus has long been recognized as part of the normal skin flora, but Methicillin-resistant (MRSA) strains have been associated in recent years with increased frequency of hospital-acquired infections, resulting in severe pneumonia [70, 71]. Preparations of EP had relatively little effect on growth of MRSA or MSSA (methicillin sensitive *S. aureus*) but were very effective in inhibiting the proinflammatory response to the bacteria, indicating at least partial benefits in counteracting the detrimental effects of MRSA infection. *Klebsiella pneumoniae*, often associated with antibiotic resistant pneumonia, was also relatively resistant to the bactericidal effects of EP, as were *Mycobacterium smegmatis* and several other bacterial opportunists [62].

Several conclusions were drawn from these results: (i) EP and other *Echinacea* extracts were selective in their antibacterial activities; (ii) different organisms showed significant differences in their patterns of sensitivity; (iii) there were no correlations between chemical composition of extracts, in terms of known marker compounds, and their corresponding antibacterial activities; (iv) different preparations of *Echinacea* showed markedly different effects on bacteria, indicating that EP has distinct mechanisms of action against each bacterium; (v) in general EP can reverse the stimulation of proinflammatory cytokines regardless of the inducing bacterium or virus.

In addition to the studies with live bacteria, several groups have examined the effects of EP on the stimulation of inflammatory mediators by bacterial lipopolysaccharide (LPS usually derived from *Escherichia coli*) in various human and mouse cell cultures (see below, Section 12). Such models can serve as useful tests for anti-inflammatory agents [23, 29], although they do not necessarily represent live bacteria. These studies together indicate that EP is effectively a general anti-inflammatory agent and should be capable of ameliorating many of the symptoms of respiratory infections.

Table 5: Effects of EP on cytokines/mucin in 3-D tissues (ratio, treatment/control).

Treatment	IL-6	IL-8	MUC5A
EP	0.7	1.5	0.82
Rhinovirus	3.1	22.0	2.0
EP + rhinovirus	0.5	2.0	0.76

Data from [55].

8. Coinfections with Viruses and Bacteria

Many authors have commented on the importance of coinfections of the airways between a respiratory virus and bacterial opportunists, and the possible enhancement of pulmonary diseases [36]. This has already been alluded to above, but a number of interesting studies have suggested possible mechanisms. Rhinoviruses, respiratory syncytial virus, and influenza virus, all altered signaling pathways in cultured epithelial cells, leading to cell membrane changes, including enhancement of attachment of certain bacteria, such as *H. influenzae* and *S. pneumoniae*, and resulting in cytopathic effects and possible colonization [72–75]. The reverse process was also demonstrated; prior infection of epithelial cells with *H. influenzae* increased the level of ICAM-1 receptors and resulted in enhanced rhinovirus infection [76]. Since EP can inactivate these viruses and bacteria on contact, as well as inhibiting their induction of proinflammatory cytokines, it is conceivable that such coinfections could be halted by EP oral administration in the form of tinctures or sprays.

9. Skin Infections

9.1. Herpes Simplex Viruses. Many skin infections are caused by viruses and microorganisms, and some of these could be amenable to topical treatment with EP. Herpes simplex virus types 1 and 2 (HSV-1/2) are common infections of oral and genital mucosa ("cold sores" and "genital sores") and may become chronic infections with painful recurrent eruptions of the skin. Keratitis, an infection of the corneal epithelium and stromal tissue, which may also be recurrent, is a major cause of blindness. Fortunately most of these infections are accessible to topical agents, and not surprisingly many antiviral drugs have been marketed for their control, with variable success; but since most of these were designed to target viral genes, then emergence of resistant mutants is always a threat. However many plant extracts have been evaluated as anti-HSV agents [77, 78], and some of these have shown promising results. Several EP preparations showed potent virucidal activity against HSV, and these results have already been summarized in Section 2.1 above, in connection with respiratory viruses. The advantages of EP are its broad spectrum of activity, which minimizes the chances of resistant mutants arising, its high potency, and its relative lack of cytotoxicity.

9.2. Acne. *Acne vulgaris* is a multifactorial chronic disorder of the pilosebaceous follicles of the skin. *Propionibacterium acnes*, the dominant microbe in the sebaceous glandular regions, and inflammation, possibly initiated by this bacterium, appear to be the two main instigators and drivers of the disease, although other factors also appear to be involved, such as hormones and host nutritional status [79]. One explanation for the chronic nature of the disease is that the *P. acnes* induces the production of proinflammatory cytokines and chemokines, as well as other inflammatory mediators, which attract leukocytes to the site of infection and thereby set up a cascade of inflammatory responses, which also involve production of reactive oxygen species and other radicals, all of which in combination lead to the development of the acne lesion [79, 80].

Conventional therapy targets the development of the lesions, by means of retinoic acid analogues and other compounds and antibiotics directed against the bacterium [80]. Needless to say that the continued application of antibiotics entails the risk of resistant bacteria emerging. As an alternative approach, several recent reports have indicated the possibility of using plant extracts to counter the growth of the bacteria and/or the inflammatory response, although these have yet to be evaluated *in vivo* (e.g., [81]).

The organism itself, including laboratory and clinical isolates, was readily inactivated by dilutions of EP well below the normal recommended dose for topical treatment or for oral consumption in the control of colds and flu symptoms. Furthermore, the bacterial induction of proinflammatory cytokines, evident in three human cell lines examined (bronchial epithelial, lung epithelial, and skin fibroblasts) was also inhibited by EP, which suggests that this extract could offer dual benefits to acne patients [82]. Results are summarized in Table 7. The bacterial-induced cytokines included IL-6 and IL-8 (CXCL8), and to a lesser extent TNFα, which are hallmarks of inflammatory responses and would be expected to lead to influx of various inflammatory leukocytes. In addition the secretion of GROα normally results in attraction of monocytes. Such a combination of cytokines could well explain the production of inflammation at the site of the infection; consequently an agent capable of safely reversing this effect should be beneficial to the patient.

9.3. Fungi. Fungi are the causative agents of numerous acute and chronic infections of the skin in many parts of the body and are often inaccessible to immunological attack. A limited number of studies have been reported on antifungal activity of various *Echinacea* extracts, including some EP extracts, against *Candida* species and some dermatophytes, but these were essentially qualitative by design and merely indicated that certain extracts could inhibit growth of some fungi [83, 84]. Since EP is formulated for oral and superficial applications, a more comprehensive evaluation of their fungistatic and fungicidal activities could support and validate some of the traditional uses and anecdotal reports of success in controlling chronic fungal infections of the skin [1–3].

10. Other Organisms

10.1. Clostridium Difficile. *C. difficile* is a Gram positive spore-forming gut anaerobe, which has been associated increasingly in recent years with epidemics of diarrhea (*Clostridium difficile*-associated diarrhea, CDAD) and pseudomembranous colitis, especially in health care institutions where patients may be subjected to chronic administration of antibiotics with resulting disruption of the normal balance of gut flora [85, 86]. Certain preparations of EP are capable of killing the organism [61], as shown in Table 8. This suggests that oral consumption of appropriate extracts, and possibly some teas made from EP, could be beneficial in infected patients. It has been reported that EP supplementation to the diet can apparently cause changes in gut bacterial flora [87], but it is not clear how significant such changes are and whether they are beneficial or not.

10.2. Parasites. Leishmaniasis and trypanosomiasis are diseases caused by protozoans *Leishmania* and *Trypanosoma* species belonging to the trypanosomatidae family. *Leishmania* species are mainly transmitted by the bite of an infected female phlebotomine sandfly [88]. Trypanosoma species are transmitted to humans by the bite of an infected tsetse fly (*Glossina* Genus), causing sleeping sickness, or an infected Assassin bug (sub-family *Triatominae*), causing Chagas disease in humans.

Both parasites cause widespread disease, with hundreds of thousands of new cases each year. Although drugs are available for the treatment of different stages of the diseases, they are frequently associated with severe side effects [88, 89]. However some recent studies have examined antiparasitic properties of several plant extracts, with promising results [89, 90].

Three different preparations of EP, one an ethanol extract and the others aqueous extracts of aerial plant parts, were evaluated for their ability to inhibit growth of the organisms *in vitro* and antiinflammatory activity [90].

All three EP preparations exhibited dose-dependent antileishmanial and trypanocidal activities after 24, 48, and 72 h incubation, although their relative potencies varied somewhat between extracts and target species. In general the ethanol extract was the most effective.

The mode of action of EP on these parasites is not known. It may differ between species, as it does for bacterial species (Section 8, above). Microscopic observations on the parasites indicated that EP slowed or eliminated their motility and caused cell rounding at high EP concentrations.

L. donovani also showed proinflammatory activity by stimulating the secretion of IL-6 and IL-8 (CXCL8) in two different human cell lines a bronchial epithelial line and a skin fibroblast line, similar to the stimulation shown in these cell lines by a variety of other viral and bacterial pathogens (as described above). In both cell types, the selected EP ethanol extract inhibited these *Leishmania*-induced responses (Table 9). Thus certain *Echinacea* preparations are capable of controlling growth of these parasites, and can inhibit the inflammatory activity induced by them.

Table 6: Antimicrobial effects of EP.

Species	Relative microbicidal activity of EP (− to ++)	Anti-inflammatory response by EP (+ or −)
Streptococcus pyogenes	++	+
Hemophilus influenzae	++	+
Legionella pneumophila	++	+
Staphylococcus aureus	+/−	+
Klebsiella pneumoniae	+/−	nt
Propionibacterium acnes	++	+
Mycobacterium smegmatis	+	+
Clostridium difficile	++	+
Candida albicans	+	nt
Leishmania donovani	+	+
Trypanosoma brucei	+	nt
Bacterial lipopolysaccharide (LPS)	n/a	+

Data from [61, 62]. nt = not tested; n/a = not applicable.

Table 7: EP reversal of *P. acnes* induced cytokines/chemokines.

Cytokine/chemokine	Ratio, *P. acnes*/control	Ratio, *P. acnes* + EP/control
IL-6	7.0	2.7
IL-8 (CXCL8)	3.0	0.35
GROα	1.9	0.56
TNFα	1.2	0.52

Data from [82].

11. Antioxidant Properties

Many plant extracts have been shown to contain antioxidant properties, according to several standard tests, and phenolic components have often been incriminated [5, 29, 84, 91]. In addition to the generally accepted nutritional and health benefits of adding antioxidants to the diet, various acute and chronic infections have been associated with negative effects on the intracellular redox balance, including decreases in reduced glutathione and other important cellular antioxidants, which could have adverse effects on cellular metabolism [28].

Several reports have demonstrated the anti-oxidant property of EP preparations [84, 92]. Therefore supplementation with EP during infections could provide additional host protection by restoring the normal redox status.

12. Effects of EP on Immune Cell Functions

Several studies have reported the effects of *Echinacea* preparations on cellular gene expression in uninfected human cells relevant to the immune system. Changes in NK cells and cell surface antigens were described for blood cells taken from humans treated with EP [93, 94]. Randolph et al. [95] described changes in levels of expression (in terms of mRNAs and proteins) of several cytokine genes in human blood samples taken at different times after treatment with a commercial *Echinacea* product, and Brovelli et al. [96] found that the expression of several cytokine genes in cultured human monocytes was influenced by the nature of the *Echinacea* preparation (stage of development and plant part used), presumably a reflection of their different chemical compositions.

Wang et al. [97] described the effects of a butanol fraction, derived from aerial parts of *E. purpurea*, on gene expression of immune-related molecules in human dendritic cells, which are part of the adaptive immune response. Many dendritic cell genes were affected, either upregulated or downregulated. Modulation of several cytokines by EP extract was also reported for cultured mouse dendritic cells [98]. None of these studies included infected cells, but they clearly confirmed that EP produces multiple gene effects in immune cells.

Many groups during the last 20 years have investigated the effects of *Echinacea* preparations on immune functions in cell cultures of mouse origin. In general, incubation of peritoneal, alveolar, or spleen monocyte/macrophage/lymphocyte preparations with EP resulted in stimulation of phagocytosis and cytokine secretion (reviewed in [5, 6]). This was in contrast to the studies described above for infected airway epithelial cells and fibroblasts, in which EP generally acted as an anti-inflammatory agent, via numerous changes in gene expression. In studies with partially purified EP polysaccharides (derived from *in vitro* EP plant cultures), stimulation of phagocytic activity in macrophages was also observed [99, 100], whereas isolated *Echinacea* alkylamides, individually or in groups, invariably displayed immune suppression in various cultured cells [101–105].

Unfortunately the earlier studies gave rise to a popular belief that *Echinacea* acted as a general "immune stimulant" or "immune booster", statements that persist today on many commercial labels and web site descriptions. More recent studies however refer more appropriately to immune modulation rather than generalized immune stimulation (e.g., [47, 101–105]). Thus the net result of interactions between EP and cells *in vivo* will likely be influenced by the composition of the extract and the nature and location of the cell type.

Table 8: Effect of EP on *Clostridium difficile*.

EP extract	*C. difficile* (cfu/2.5 μL)	$\log_{10}$decrease
None	1.5×10^4	—
EP ethanol (moderate alkylamides; no polysaccharide)	<10	>3
EP aqueous (+ polysaccharide; no alkylamides)	4.0×10^3	0.6

Data from [61].

Rininger et al. made a different series of observations [106]. They reported that several preparations of EP showed minimal effects on production of cytokines in mouse cultures, unless the extracts were first exposed to a simulated gastric and intestinal digestion protocol, whereupon they acquired the ability to stimulate cytokine secretion. This result is clearly relevant to normal consumption of *Echinacea*, but remains unexplained.

Mouse macrophage cell line RAW 274 responded to LPS (bacterial lipopolysaccharide) treatment by stimulating the production of the inflammatory mediator nitric oxide, and EP was able to inhibit this effect [92], in accordance with the anticytokine effects described above. In our preliminary studies (Sharma and Hudson, unpublished results) rhinovirus also stimulated NO production in these same cells, and EP reversed this effect. Thus although potential phagocytic cells can be stimulated by EP, similar cells that have already been stimulated by LPS (or possibly by live bacteria) are inhibited by EP, indicating the tendency of EP to restore normal immune functions.

13. Studies in Rodents

EP-treated rats were assessed for spleen and lung macrophage-related functions, including phagocytic activity and cytokine stimulation, both of which were stimulated in the treated animals [107], in agreement with the studies on mouse cells cultured *in vitro*.

In the studies with EP polysaccharides derived from plant cultures, treated normal and immunosuppressed mice could be protected from a lethal dose of either *Listeria monocytogenes* or *Candida albicans*, apparently as a result of reduced titers of the organisms in target tissues [108]. A similar beneficial effect of standard EP extract was also observed in a recent study in which mice were infected with *Listeria monocytogenes* [104]. Whether the protection in these cases was due to enhanced phagocytic activity and clearance of the organisms in various tissues, or to direct contact of EP components with the organisms, or a combination of these and other factors, is not clear from these studies.

A recent report described the successful use of a polysaccharide-enriched aerial EP extract in controlling the course of influenza virus A infection (a well characterized H1N1 strain) in mice. There was no apparent effect on lung virus titers, but there were significant effects on various cytokine levels in lungs and sera [109]. The authors concluded that the benefits of EP treatment resulted from modulation of inflammatory cytokines, rather than direct antiviral activity, and this concurs with the studies indicating anti-inflammatory properties of EP. Thus EP could still be beneficial in systemic infections in which EP components are unlikely to make direct contact with the virus.

Miller and colleagues [110–112] have made several studies of the effects of dietary EP extract on the aging and survival of mice. Several parameters of growth indicated possible benefits of the EP diet, and a noteworthy positive effect was the lifelong stimulation of NK cell activity and function, which could theoretically help the animals deal with infectious diseases.

14. Veterinary Applications

Most domestic animals, including pets, livestock, and fish, require treatment at some point in their lives for viral and microbial diseases, and the causative organisms are usually analogous to the corresponding human counterparts that have already been discussed, for example, avian influenza viruses, animal herpes viruses, various respiratory viruses and bacteria, and many fungal and parasitic infections. Consequently some of them should be responsive to *Echinacea* treatment, either as direct antivirals, antimicrobials, or as an anti-inflammatory agent. In addition some of these organisms, especially bacteria such as *Salmonella* and *Campylobacter* species, are also important sources of contaminated foods. Furthermore some commentators have pointed out the need to evaluate herbal preparations as replacements for at least part of the antibiotic onslaught that farmed animals often receive.

Certain herbs, including *Echinacea,* have a modern tradition of veterinary applications [113, 114], in North America and Europe, and although relatively few reports have described basic studies analogous to those described for human diseases, or even controlled trials in animals, invariably the treatments were concluded to be safe and free of significant side effects. This conclusion is also supported by the studies in mice and rats described in the previous section, in which toxic effects were not observed.

In regard to infections, a study in chicks infected with the protozoan parasite *Coccidia* concluded that dietary supplementation with EP root extract significantly decreased lesion scores and improved the health of the animals, in comparison with animals raised on a normal diet [115], although immune parameters were not measured; consequently it is not clear if the effect of EP was directed against the parasite itself or on the immune system. Nevertheless an effective treatment for coccidiosis would be welcome in the poultry industry.

On the other hand, in a study in young pigs [116], dietary EP was found to offer no protection against the porcine reproductive and respiratory syndrome virus (PRRS virus). Since this virus is a member of the arterivirus family (related to coronaviruses) and possesses a membrane, it would be expected to be susceptible to direct contact with EP. However the systemic nature of the infection could render it

Table 9: Effect of EP on *Leishmania*-induced secretion of cytokines.

Cells	Treatment	IL-6 (pg/mL)	IL-8 (pg/mL)
Bronchial epithelial cells	None, control	19.8	42.8
	+ EP	29.2	45.7
	+ Ld (*Leishmania donovani*)	63.1	147.5
	+ Ld + EP	25.8	18.1
Human skin fibroblasts	None, control	29.8	62.0
	+ EP	38.2	74.4
	+ Ld	207.8	320.0
	+ Ld + EP	9.6	42.8

Data from [90], (standard deviations removed for simplicity).

inaccessible to dietary *Echinacea* components. Alternatively, the treatment protocol might have been inadequate.

In addition to controlling infections in domestic animals, herbal preparations have been advocated for such things as immune stimulation, growth promotion, and performance enhancement. Studies carried out in uninfected horses [117] and fish (Tilapia, [118]) suggest possibilities for *Echinacea* preparations. Again safety was not considered a problem for the animals. Fish, like other farmed animals, are always potentially vulnerable to viral and microbial infections, especially under conditions of stress; consequently alternative treatments to synthetic antimicrobials could be useful.

15. Emerging Infections: The Example of Severe Acute Respiratory Syndrome (SARS)

In case we needed to be reminded of the unpredictable nature of microbial epidemiology, the SARS epidemic erupted in China in late 2002 and quickly spread to many other countries over the next year and a half, resulting in more than 8,000 seriously infected individuals, of whom nearly 10% died. The epidemic was officially recorded as the first pandemic of the 21st century. As a result of emergency global public health measures the disease was halted and appears to have been controlled, although continued vigilance is necessary in case the causative virus "returns" [119].

The novel coronavirus responsible (SARS CoV), believed to be of animal origin, was isolated and its genome quickly sequenced [119]. However no satisfactory antiviral therapy was or is available. A study on autopsy lung tissues revealed that viral nucleoprotein and RNA were present mainly in alveolar epithelial cells, the probable initial site of virus replication, and to a lesser extent in macrophages, which could be the mode of systemic transmission [120]. A more recent review summarized studies on SARS CoV infection in cell cultures and emphasized the role of innate immune responses to the virus infection [121]. This virus, like other coronaviruses and other respiratory viruses, stimulates inflammatory cytokine secretion, although coronaviruses generally do not induce antiviral interferon. Thus modulation of cytokines and evasion of the innate immune defences could be important contributors to SARS virus pathology. Therefore a herbal preparation like EP, with anti-inflammatory and virucidal properties, could be useful if the SARS were to return.

16. Standardization and Herb Mixtures

The need for standardization of EP has already been stressed in this paper, and a few studies have reinforced the concept of variation among different preparations from different suppliers [45, 106, 122]. This is a common problem with herbs, since the exact chemical composition can vary with different parts of a plant, the age and season of harvest, and the exact method of extraction [6, 7, 45, 96]. On the other hand we found in our studies that a standardized preparation of EP retained its full antiviral activity over several years, provided that storage conditions were appropriate (unpublished results). Similarly, preparations of EP maintained their cytokine-stimulating activity after storage for two years [123].

The presence of multiple antiviral activities among different extracts and fractions suggests that many kinds of *Echinacea* preparation, such as tinctures, sprays, tablets, teas, and so forth could be beneficial in the treatment of infections, although not all preparations are likely to be effective, and a recent study of 10 commercial preparations highlighted the variability of antiviral activity between different preparations, although lot-to-lot variation was less evident [45]. In general ethanol-based extracts had greater antiviral activities than aqueous extracts; but it was not possible to identify a specific component responsible for the activity. Furthermore there was no correlation between antiviral and anti-inflammatory activities.

Some commercial preparations of *Echinacea*, including those with EP, comprise mixtures of EP with other non-Echinacea herbs, the rationale being that two herbs are better than one, and three are even better, etc. In this respect such mixtures may be considered analogous to the complex mixtures often advocated in Chinese traditional medicine (TCM) and Ayurvedic medicines. However, this basic assumption is not necessarily valid, and in preliminary studies we examined a number of mixtures of EP with other standard herbs, for antiviral activities. In the majority of cases the mixtures were much less effective than the EP by itself and none were better than EP (unpublished data).

However since we do not know the exact mechanisms of the EP antiviral activity, or the nature of the active "compounds", then we cannot explain these phenomena. But the lesson is obvious, namely, that some EP preparations by themselves can be potent antiviral agents and should not be mixed with unknowns.

17. Relevance of Bioactivities to Normal Consumption

Echinacea extracts intended for treatment of colds and flu, and sore throats are normally marketed for oral consumption. The active ingredients therefore acquire immediate exposure to the mucosal epithelia. According to our studies with standardized preparations (as described above), the recommended applications ensure that physiologically relevant amounts, that is to say, adequate local antiviral, antibacterial, and anti-inflammatory concentrations, are achieved under normal conditions of consumption. Subsequent absorption and metabolism of the various components, however, are less relevant to this application, since the sites of infection and inflammation are at the level of airway epithelial tissues.

Nevertheless, it has been demonstrated that alkylamides (and possibly additional components) can be absorbed quickly into the blood and remain in the circulation for some time, at least following *E. angustifolia* administration [124], although EP extracts tend to have relatively few alkylamides [6, 7]. Therefore, depending on the exact chemical composition of the *Echinacea* extract, there could be benefits to the consumer in addition to the initial interactions with the oral mucosa.

18. Clinical Trials

Numerous studies have been carried out in humans, with the objective of preventing or treating common colds, and these have been critically summarized [3, 5, 124, 125]. Unfortunately some of them used inadequately characterized *Echinacea* preparations derived, with various extraction protocols, from different species and plant parts. It is likely that the chemical composition of the extracts, and consequently their bioactivities, differed substantially. Attempts to rationalize the interpretation of results by the use of meta-analyses encountered the same problem, namely, the resulting variability in test materials. Consequently it is no surprise that the issue is "controversial", and many popular scientists and pseudoscientists have jumped to conclusions about the efficacy or lack of efficacy of *Echinacea* in preventing or treating the common cold. In spite of these reservations there was an overall trend towards beneficial effects of the *Echinacea*, but equally importantly the safety of the preparations was confirmed repeatedly, including trials in children [125–127]. A more comprehensive trial that also includes influenza virus and other respiratory viruses, incorporating the use of a well-characterized bioactive *Echinacea* extract, is needed to confirm the benefits of oral *Echinacea* use.

Until that has been done, we are left with overwhelming anecdotal experience, in addition to the very encouraging results from the cell and tissue culture studies and animal studies, but no definitive conclusion regarding clinical efficacy in humans.

19. Mechanisms of Action

The results described have indicated that some *Echinacea* extracts evidently contain compounds, or combinations of compounds, with the ability to interact specifically with viral and microbial targets [13, 17, 62, 128]. In addition, these extracts can affect various signaling pathways of epithelial cells and inhibit the virus/bacterium-induced secretion of cytokines/chemokines and other inflammatory mediators that were responsible for the pulmonary symptoms. Since many signaling pathways can be affected by *Echinacea* in different cell types, including immune cells [47, 59, 97], it is conceivable that the overall beneficial effects are due to a particular combination of compounds acting synergistically. Examples of synergism in herbal medicine have been described and in some cases validated experimentally [23, 129, 130], and it is likely that certain *Echinacea* preparations also display synergism. However, in spite of our attempts to correlate bioactivities of *Echinacea* preparations with recognized chemical markers, that is, polysaccharides, caffeic acid derivatives, and alkylamides [5–7], we have not succeeded in doing so. In contrast, preliminary evidence in our laboratory has implicated other classes of compounds (unpublished data).

20. Conclusions

These studies on EP indicate multiple actions of the herbal preparation, resulting either from the individual activities of several compounds or the synergistic effect of different compounds. The resulting benefits are: (1) direct virucidal activity/activities against several viruses involved in respiratory infections, at concentrations which are not cytotoxic; (2) direct bactericidal actions against certain potentially pathogenic respiratory bacteria; (3) inactivation of other microbial pathogens relevant to humans and their domesticated animals; (4) reversal of the proinflammatory response of epithelial cells and tissues to various viruses and bacteria; (5) modulation of certain immune cell functions; (6) reversal of the excessive mucin secretion induced by rhinovirus. These bioactivities result from changes in gene expression. Thus a combination of these beneficial activities could reduce the amount of prevailing viable pathogens, and their transmission and also lead to amelioration of the symptoms of the infection.

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Research Article

Antilipogenic and Anti-Inflammatory Activities of *Codonopsis lanceolata* in Mice Hepatic Tissues after Chronic Ethanol Feeding

Areum Cha,[1] Youngshim Choi,[1] Yoojeong Jin,[1] Mi-Kyung Sung,[2] Yun-Chang Koo,[3] Kwang-Won Lee,[3] and Taesun Park[1]

[1] *Department of Food and Nutrition, Yonsei University, 262 Seongsanno, Seodaemun-gu, Seoul 120-749, Republic of Korea*
[2] *Department of Food and Nutrition, Sookmyung Women's University, 52 Hyochangwon-gil, Yongsan-gu, Seoul 140-742, Republic of Korea*
[3] *Department of Food Science, College of Life Sciences and Biotechnology, Korea University, 1, 5-ga, Anam-dong, Sungbuk-ku, Seoul 136-701, Republic of Korea*

Correspondence should be addressed to Taesun Park, tspark@yonsei.ac.kr

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This study evaluated the antilipogenic and anti-inflammatory effects of *Codonopsis lanceolata (C. lanceolata)* root extract in mice with alcohol-induced fatty liver and elucidated its underlying molecular mechanisms. Ethanol was introduced into the liquid diet by mixing it with distilled water at 5% (wt/v), providing 36% of the energy, for nine weeks. Among the three different fractions prepared from the *C. lanceolata* root, the *C. lanceolata* methanol extract (CME) exhibited the most remarkable attenuation of alcohol-induced fatty liver with respect to various parameters such as hepatic free fatty acid concentration, body weight loss, and hepatic accumulations of triglyceride and cholesterol. The hepatic gene and protein expression levels were analysed via RT-PCR and Western blotting, respectively. CME feeding significantly restored the ethanol-induced downregulation of the adiponectin receptor (adipoR) 1 and of adipoR2, along with their downstream molecules. Furthermore, the study data showed that CME feeding dramatically reversed ethanol-induced hepatic upregulation of toll-like receptor- (TLR-) mediated signaling cascade molecules. These results indicate that the beneficial effects of CME against alcoholic fatty livers of mice appear to be with adenosine- and adiponectin-mediated regulation of hepatic steatosis and TLR-mediated modulation of hepatic proinflammatory responses.

1. Introduction

Fatty liver is the most common and earliest response of the liver to heavy alcohol consumption and may develop into alcoholic hepatitis and fibrosis [1]. It has been traditionally held that, during alcohol metabolism, the increase in the cellular NADH concentration generated by alcohol and aldehyde dehydrogenases impairs β-oxidation and tricarboxylic acid cycle activity in the liver, which in turn leads to severe FFA overload and enhanced synthesis of triacylglycerol in the liver [2]. More recently, two transcription factors, the peroxisome proliferator-activated receptor (PPAR) α and PPARγ, have been discovered as new mechanisms that control hepatic fatty acid oxidation and synthesis, respectively [3, 4]. Ethanol feeding impairs fatty acid catabolism in the liver partly by blocking PPARα-mediated responses in C57BL/6J mice. In the meantime, dietary supplementation of ethanol-fed animals with a PPARα agonist induces the expression of PPARα target genes and stimulates the rate of fatty acid β-oxidation, which prevents alcoholic fatty liver [5]. In contrast to PPARα, hepatic PPARγ contributes to triglyceride homeostasis, which regulates both triglyceride clearance from the circulation and the lipogenic program [6]. Ethanol increases the mRNA expressions of PPARγ and lipid synthetic enzymes, ATP citrate lyase (ACL), and fatty acid synthase (FAS) in mice liver [1].

Ethanol is well known to stimulate increased extracellular adenosine concentration through its action on the nucleoside transporter. Ethanol ingestion increases purine release into the bloodstream and urine in normal volunteers [7–9] and into the extracellular space in liver slices obtained from mice [10, 11]. Extracellular adenosine regulates various

physical processes [12], including hepatic fibrosis [10, 11], ureagenesis [13, 14], and glycogen metabolism [15, 16], as well as peripheral lipid metabolism [17, 18]. These physiological effects of adenosine are mediated by a family of four G-protein-coupled receptors: A_1, A_{2A}, A_{2B}, and A_3 (A1R, A2AR, A2BR, and A3R), each of which has a unique pharmacological profile, tissue distribution, and effector coupling [19]. Recently, Peng et al. reported that ethanol-mediated increases in extracellular adenosine, which act via adenosine A1R and A2BR, link the ingestion and metabolism of ethanol to the development of hepatic steatosis. Thus, they suggested that targeting adenosine receptors may be effective in the prevention of alcohol-induced fatty liver [1].

Although fatty liver was thought to be relatively benign, more recent studies show that fat accumulation makes the liver more susceptible to injury from other agents such as drugs and toxins, especially endotoxins [20], which are believed to be involved in the pathogenesis of alcoholic hepatitis and fibrosis [21, 22]. Alcohol consumption induces a state of a "leaky gut" that increases plasma and liver endotoxin levels and leads to the production of the tumor necrosis factor α (TNFα) by Kupffer cells via the toll-like receptor (TLR) 4, which is known to mediate lipopolysaccharide-(LPS-) induced signal transduction and to eventually contribute to liver injury [23]. Ethanol-fed mice exhibited an oxidative stress that was dependent on the upregulation of multiple TLRs (TLR1, 2, 4, 6, 7, 8, and 9) in the liver and was sensitive to liver inflammation induced by multiple bacterial products recognized by the TLRs [24].

Codonopsis lanceolata (*C. lanceolata*), which belongs to the Campanulaceae family, is a perennial herb that grows naturally in moist places in woods, low mountains, and hills [25]. It is commonly found in East Asia, particularly in China [25]. *C. lanceolata*, which is composed of various active components including tannins, saponins, polyphenolics, alkaloids, essential oils, and steroids, has long been prescribed in traditional folk medicine in Korea, Japan, and China [26–28]. The dried roots of *C. lanceolata* have been used as a traditional remedy for lung inflammatory diseases such as asthma, tonsillitis, and pharyngitis. The total methanol extracts of the fresh leaves or roots of *C. lanceolata* significantly suppress the production of TNFα and nitric oxide, the expression of interleukin (IL)-3 and IL-6, and LPS-mediated phagocytic uptake in RAW 264.7 cells [28]. In a rat model, *C. lanceolata* water extract significantly improved the hepatic accumulation of triglyceride and cholesterol induced by a high-fat diet [29]. Although the lipid-lowering effect of *C. lanceolata* extract has been demonstrated in a rodent model with diet-induced obesity, the protective effects of this plant against alcoholic fatty liver diseases have not yet been explored. In this study, the potent bioactivities of the *C. lanceolata* methanol extract that can be used as natural compound or pharmaceutical supplements for the prevention and/or treatment of alcoholic fatty liver were demonstrated. The mechanisms by which the *C. lanceolata* methanol extract performs its antilipogenic and anti-inflammatory actions were investigated in the hepatic tissues of mice with chronic ethanol consumption.

2. Materials and Methods

2.1. Preparation of C. lanceolata Extract. *C. lanceolata*, which is cultivated in Gangwon-do, Korea, was purchased and freeze dried. Freshly dried material was ground into fine powder in an electric grinder and stored in dessicator. 200 g plant powder was refluxed with 95% methyl alcohol (MeOH) in a round bottom flask on a water bath for 3 h. Crude MeOH extract was filtered out and evaporated to dryness for the preparation of the *C. lanceolata* methanol extract (CME). The residual methanol extract was again refluxed with distilled water for 5 h and filtered. The filtrate, thus obtained, was again added with ethanol at the ratio of 1 : 4 and then stirred for 1 h at 4°C. Following their centrifugation at 5,000 ×g for 30 min at 4°C, the supernatant was concentrated in a rotary vacuum evaporator and freeze dried for the preparation of the *C. lanceolata* ethanol supernatant (CES), and the pellets were air dried and freeze dried for the preparation of the *C. lanceolata* ethanol precipitate (CEP). The yields of these three extracts were 3.84% (CME), 5.05% (CES), and 3.30% (CEP) of the wet *C. lanceolata* root, respectively.

2.2. Animals and Diets. Specific pathogen-free male C57BL/6N mice (eight weeks old) were purchased (Orient, Gyeonggi-do, Korea) and acclimatized to the authors' animal facility for one week prior to the experimentation. The mice were kept individually in sterilized animal quarters with controlled temperature (at 21 ± 2°C) and humidity (at 50 ± 5%) and with 12 h light and dark cycles and were allowed free access to standard chow and tap water during the acclimatization period. The animals were then randomly divided into five groups ($n = 8$) and fed the normal diet (ND), the ethanol diet (ED), the CME-supplemented ethanol diet (MED), the CES-supplemented ethanol diet (ESD), or the CEP-supplemented ethanol diet (EPD) for nine weeks. The mice in the ED group consumed a liquid diet wherein ethanol provided 36% of the energy, as described by Lieber et al. [30]. Ethanol was introduced into the diet by gradually mixing it with distilled water, from 0% (wt/v) to 5% (wt/v), over a one-week period for adaptation, and was provided at 5% (wt/v) for the next eight weeks. The ND mice received the same diet but with isocaloric amounts of dextrin-maltose instead of ethanol, and the MED, ESD, and EPD mice were fed the ethanol diet that contained 0.091% (wt/v) CME, CES, and CEP, respectively. The compositions of the experimental diets are given in Table 1. The ND, MED, DES, and EPD mice were pair fed so that their consumption would be isocalorically equivalent to the consumption on the previous day of the ED mice that were individually paired with them. The animals that were used in this study were treated in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996), as approved by the Institutional Animal Care and Use Committee of Yonsei University.

2.3. Histological Examination. The liver tissue specimens were fixed in 10% buffered formalin and embedded in

Table 1: Compositions of experimental diets.

Ingredient	ND	ED	MED	ESD	EPD
			g/L		
Casein	41.4	41.40	41.40	41.40	41.40
L-cystine	0.50	0.50	0.50	0.50	0.50
DL-methionine	0.30	0.30	0.30	0.30	0.30
Corn oil	8.50	8.50	8.50	8.50	8.50
Olive oil	28.40	28.40	28.40	28.40	28.40
Safflower oil	2.70	2.70	2.70	2.70	2.70
Dextrine-maltose[1]	115.20	25.60	25.60	25.60	25.60
Vitamin mix (AIN-76G)[2]	2.50	2.50	2.50	2.50	2.50
Mineral mix (AIN-76G)[3]	8.75	8.75	8.75	8.75	8.75
Choline bitartrate	0.53	0.53	0.53	0.53	0.53
Cellulose	10.00	10.00	10.00	10.00	10.00
Xanthan gum	3.00	3.00	3.00	3.00	3.00
Ethanol		50.00	50.00	50.00	50.00
CME			0.91		
CES				0.91	
CEP					0.91
Water	778.22	817.82	816.91	816.91	816.91
Total	1000	1000	1000	1000	1000

[1] Dextrin : maltose = 80 : 20.

[2] Vitamin mixture (g/kg mix); thiamin · HCl 0.6; riboflavin 0.6; nicotinamide 25; pyridoxine· HCl 0.7; nicotinic acid 3; D-calcium pantothenate 1.6; folic acid 0.2; D-biotin 0.02; cyanocobalamin (vitamin B_{12}) 0.001; retinyl palmitate (250,000 IU/gm) 1.6; DL-α- tocopherol acetate (250 IU/gm) 20; cholecalciferol (vitamin D_3) 0.25; menaquinone (vitamin K_2) 0.05; sucrose, finely powdered 972.9.

[3] AIN-76 Mineral mixture (g/kg mix); $CaHPO_4$ 500; NaCl 74; $K_2H_6O_7H_2O$ 220; $K_2SO_4$52; MgO 24; $MnCO_3$ 3.57; Fe ($C_6H_5O_7$) · $6H_2O$ 6; $ZnCO_3$ 1.6; $CuCO_3$ 0.3; KIO_3 0.01; Na_2SeO_3· $5H_2O$ 0.01; CrK $(SO_4)_2$ 0.55; sucrose, finely powdered 118.

paraffin, cut at thicknesses of 5 μm, and later stained with hematoxylin and eosin (H&E) for the histological examination of fat droplets.

2.4. Biochemical Assays. The serum concentrations of total cholesterol, HDL cholesterol, triglyceride, and free fatty acid were determined using commercial kits (BioClinical System, Gyeonggi-do, Korea). Hepatic lipids were extracted through the procedure developed by Folch et al. [31], using a chloroform-methanol mixture (2 : 1, v/v). The dried lipid residues were dissolved in 1 mL of ethanol for the cholesterol and triglyceride measurements. The hepatic cholesterol, triglyceride, and free fatty acid concentrations were analyzed using the same enzymatic kit that was used in the serum analyses. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using commercial reagents (Bayer, USA).

2.5. Isolation of Total RNA and Semiquantitative RT-PCR. The total RNA was extracted from the liver using a Trizol reagent (Invitrogen, USA) and was reverse transcribed using the Superscript II kit (Invitrogen, USA) according to the manufacturer's recommendations. The primers for the PCR analysis were synthesized by Bioneer (Korea). The forward (F) and reverse (R) primer sequences for the genes that were involved in the experiment are shown in the Supplementary Material available online at doi: 10.1155/2012/141395. The PCR for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on each sample as an internal positive-control standard. The number of cycles and the annealing temperature were optimized for each primer pair. Amplification of the GAPDH, A1R, A2AR, A2BR, A3R, PPARα, carnitine palmitoyltransferase (CPT-1), microsomal triglyceride transfer protein (MTP), long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), PPARγ, retinoid X receptor (RXR), CCAAT-enhancer-binding protein-α (C/EBPα), lipoprotein lipase (LPL), adipocyte protein 2 or fatty acid binding protein 4 (aP2), FAS, TLRs, LPS binding protein (LBP), cluster of differentiation 14 (CD14), myeloid differentiation primary response gene 88 (MyD88), myelin and lymphocyte protein (MAL), TLR adaptor molecule (TRIF), TNF-receptor-associated factor 6 (TRAF6), interferon regulatory factors (IRFs), nuclear factor kappa B-p50 (p50), p65, interferon α (IFNα), IFNβ, IL-12-p40, chemokine (C-X-C motif) ligand 2 (CXCL2), adiponectin receptor 1 (adipoR1), adipoR2, silent-mating-type information regulation 2 homolog 1 (SIRT1), peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC1α), and sterol regulatory element binding transcription factor 1 (SREBP-1c) was initiated via 5 min of denaturation at 94°C for 1 cycle, followed by 30 or 35 cycles at 94°C for 30 s, 55 or 60°C for 30 s, and 72°C for 1 min and via 10 min of incubation at 72°C. The sequences of the primer pairs and PCR conditions (annealing temperatures, cycles, and product sizes) that were used in the RT-PCR experiments are shown in the Supplementary Material. The PCR products were then separated in a 2% agarose gel and visualized in a gel documentation system (Alpha Innotech, USA). The intensity of the bands on the gels was converted into a digital image with a gel analyzer.

2.6. Western Blot Analysis. For the Western blot studies, protein extracts were obtained from the mice livers using a commercial lysis buffer (Intron, USA) that contained a protease inhibitor cocktail (Roche, USA). The protein concentrations were determined with the Bio-rad protein assay kit (Bio-rad, USA). The Western blot analysis was performed with antibodies that were specific to AMP-activated protein kinase (AMPK), phospho-AMPK (Thr 72), Acetyl-CoA carboxylase (ACC), phospho-ACC (Ser 79) (Cell Signaling, USA), and β-actin (Santa Cruz, USA). After the SDS-PAGE at 10% acrylamide concentrations, the proteins were transferred to the nitrocellulose membrane, blocked with 5% skim milk in a Tris-buffered saline/Tween buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), and incubated with appropriate antibodies overnight. The blots were washed extensively, incubated with a horseradish-peroxidase-linked

Table 2: Body weight gain and serum biochemistries of mice fed experimental diets.

	ND	ED	MED	ESD	EPD
Body weight gain (g/9 weeks)	10.9 ± 0.4^{a1}	5.3 ± 0.8^{c}	7.6 ± 0.7^{b}	7.5 ± 0.6^{b}	4.6 ± 0.6^{c}
Triglyceride (mmol/L)	0.78 ± 0.01^{b}	0.81 ± 0.02^{ab}	0.74 ± 0.03^{b}	0.86 ± 0.02^{a}	0.75 ± 0.02^{b}
Total cholesterol (mmol/L)	2.17 ± 0.19^{b}	2.15 ± 0.13^{b}	1.96 ± 0.13^{b}	2.65 ± 0.18^{a}	2.23 ± 0.10^{a}
HDL cholesterol (mmol/L)	0.88 ± 0.04^{b}	1.10 ± 0.10^{a}	1.17 ± 0.06^{a}	1.04 ± 0.08^{ab}	1.09 ± 0.06^{ab}
VLDL + LDL cholesterol (mmol/L)[2]	1.29 ± 0.19^{ab}	1.06 ± 0.15^{b}	0.79 ± 0.12^{b}	1.60 ± 0.21^{a}	1.14 ± 0.15^{ab}
Atherogenic index[3]	1.52 ± 0.25^{a}	1.07 ± 0.21^{ab}	0.70 ± 0.13^{b}	1.65 ± 0.21^{a}	1.12 ± 0.10^{ab}
Free fatty acid (μEq/L)	808 ± 31^{b}	990 ± 27^{a}	848 ± 36^{b}	1074 ± 52^{a}	883 ± 32^{b}
ALT (IU/ml)	6.87 ± 0.42^{c}	21.2 ± 0.89^{a}	17.5 ± 0.53^{b}	21.3 ± 0.78^{a}	21.2 ± 1.04^{a}
AST (IU/ml)	17.63 ± 1.63^{b}	22.3 ± 2.05^{a}	17.4 ± 1.05^{b}	24.6 ± 1.04^{a}	24.9 ± 0.98^{a}

[1] Each value is expressed as mean ± SEM ($n = 8$). Means with different letters within a row are significantly different ($P < 0.05$).
[2] VLDL + LDL cholesterol (mmol/L) = Total cholesterol − HDL cholesterol.
[3] Atherogenic index = (Total cholesterol − HDL cholesterol)/ HDL cholesterol.

anti-rabbit or -mouse Ig (Santa Cruz, USA), and washed again. The detection was carried out with the ECL Western blotting kit (Animal Genetics, Korea), after which the blots were exposed to an X-ray film (Agfa, Belgium).

2.7. Statistical Analysis. The results of the body weight gain, liver weight, and serum and hepatic biochemistries were expressed as the mean values ± SEM of the eight mice in each group. The results of the semiquantitative RT-PCR and Western blot were expressed as the mean values ± SEM of the three independent experiments in which the RNA and protein samples from the eight mice were used, respectively. The analysis of the variance (ANOVA) and the Duncan's multiple range method were used to compare significant differences among the groups. The level of significance was set at $P < 0.05$ for all the statistical tests.

3. Results

3.1. Body Weight Gain and Blood Biochemistries. The nine-week body weight gain of the mice in the ED group was 51% lower than the value for the pair fed ND mice. The animals that were fed the MED (43% greater) or the ESD (41% greater) exhibited significantly greater body weight gains than the ED mice, whereas the mice that were fed the EPD did not recover the weight they lost from ethanol consumption (Table 2).

Compared to the values for the ED mice, the mice that were fed the MED exhibited significantly lower levels of serum FFA (17% reduction) and lower activities of serum ALT (17% reduction) and AST (22% reduction). They also demonstrated a tendency towards decreasing serum triglyceride, total cholesterol, VLDL + LDL cholesterol, and atherogenic indices, albeit at statistically insignificant levels than those for the mice that were fed the ED. In contrast, the ESD or EPD failed to improve both the lipid levels and enzyme activities in the blood of the mice with chronic ethanol consumption (Table 2).

3.2. Liver Weight and Hepatic Lipid Levels. Histological analysis of the livers with H&E staining revealed prominent lipid accumulation in the livers of the ED fed mice whereas lipid droplets were rare in the livers of the MED, ESD, and EPD-fed mice (Figure 1). ED feeding of the mice for nine weeks resulted in more significant increases in the relative weight of their livers (by 8%) and in the hepatic levels of their triglycerides (43% increase), cholesterol (36% increase), and FFA (52% increase) than in the ND group (Figure 1). The MED or ESD significantly improved the ethanol-induced enlargement of the liver (the relative liver weight was 7% or 6% lower, resp.). The MED, ESD, or EPD significantly reversed the ethanol-induced hepatic accumulations of triglycerides (18%, 11%, and 18% reductions, resp.), cholesterol (22%, 12%, and 18% reductions, resp.), and FFA (29%, 14%, and 22% reductions, resp.). The triglyceride and cholesterol levels in the liver were lowest after the MED consumption among the diets that were supplemented with different fractions of the *C. lanceolata* root, but the differences between the diets were not statistically significant. The MED group exhibited the most prominent and the most significant improvement in the reduction of the hepatic FFA accumulation, better than did the ESD and EPD groups ($P < 0.05$) (Figure 1).

3.3. Regulation of Adenosine Receptor-Mediated Signaling Molecules. MED feeding significantly reversed the ethanol-induced upregulation of the A1R and A2BR genes in the livers of the mice and restored the ethanol-induced downregulation of the PPARα, CPT-1, MTP, LCAD, and MCAD genes in the livers of the mice (Figures 2(a) and 2(b)). The supplementation of the ethanol diet with CME significantly blunted the upregulation of these transcription factors and of their target genes due to ethanol.

3.4. Regulation of Adiponectin Receptor-Mediated Signaling Molecules. Chronic ethanol ingestion led to more significant decreases in the hepatic expression of the adipoR1 and adipoR2 genes than in the ND mice, and MED feeding significantly restored these ethanol-induced changes in the adiponectin receptor genes. Similarly, the MED feeding significantly reversed the marked decreases in the mRNA levels of the hepatic SIRT1 and PGC1α, which were induced

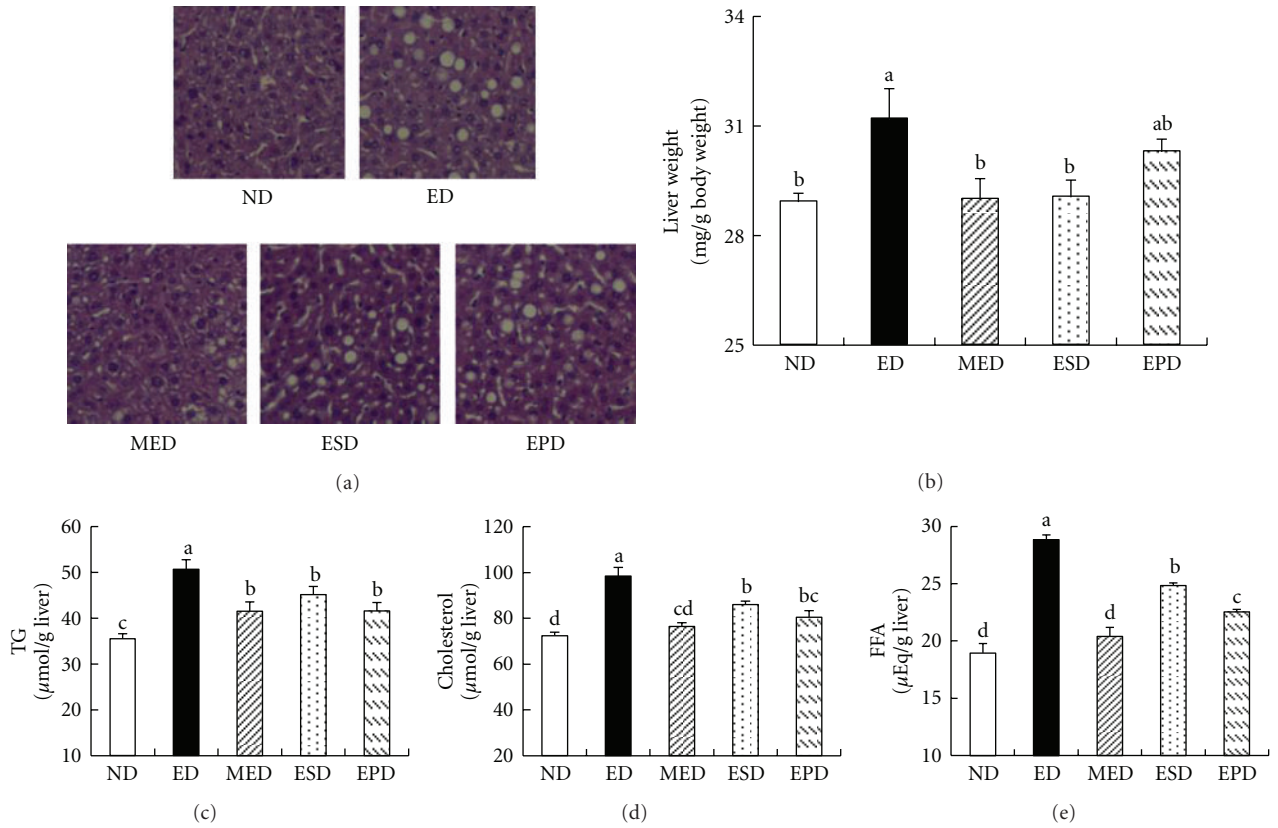

Figure 1: *Codonopsis lanceolata* extracts substantially reduces alcohol-induced liver steatosis. (a) Hematoxylin and eosin-stained sections of representative liver samples of the treated groups (100×). (b) Changes in the liver-to-body weight ratio. Hepatic triglyceride, free fatty acid, and cholesterol levels are shown in (c, d, and e). The data are expressed as means ± SEM ($n = 8$). ^{a,b,c,d}Means not sharing a common letter are significantly different ($P < 0.05$).

by ethanol feeding. In addition, the MED efficiently counteracted the ethanol-induced upregulation of the SREBP-1c gene (higher 57% reduction than in the ED mice, $P < 0.05$) (Figure 3). The MED significantly blunted the downregulation of the phospho-AMPK/total AMPK (146% increase) ratio and of the phospho-ACC/total ACC ratio (700% increase), which were caused by chronic ethanol ingestion, in the livers of the mice (Figures 3(b) and 3(c)).

3.5. Regulation of TLRs-Mediated Signaling Molecules. The hepatic expression of the TLR mRNAs was measured after nine weeks of ethanol exposure. More significant increases in the TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, TLR8, TLR9, TLR12, and TLR13 mRNA levels were observed in the livers of the ethanol-fed control mice than of the pair fed ND mice. The MED significantly alleviated the ethanol-induced upregulation of the hepatic TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR12, and TLR13 gene expressions. The hepatic TLR3, which was upregulated by the ethanol feeding, and the hepatic TLR5, which was unaffected by the ethanol feeding, did not respond to the MED feeding (Figure 4).

After endotoxin bound itself to the LPS-binding protein (LBP), it associates in the portal blood with the CD14 prior to its binding to TLR4 and all the TLRs that were recruited by MyD88, MAL, TRIF, and TRAF6 for their proinflammatory signaling. The mRNA levels of the LBP and CD14, as well as of the adaptor molecules of the TLRs (MyD88, MAL, TRIF, and TRAF6), all more significantly increased in the livers of the ED mice than of the ND mice, whereas the MED feeding led to more significant decreases in the hepatic mRNA levels of the LBP, CD14, MyD88, MAL, TRIF, and TRAF6 than in the ED mice (Figure 5(a)). Among the extremely significant recent discoveries on TLR signaling is that the MyD88-dependent pathway also activates some IRFs, such as IRF1, 3, 5, and 7. In this study, the mRNA levels of the IRF1, 3, 5, and 7 genes more significantly increased in the livers of the ED mice than of the ND mice and these ethanol-induced upregulations of the hepatic IRFs were significantly reversed by the MED feeding (Figure 5(b)). The RT-PCR analysis of the hepatic mRNAs of NFκB (p50 and p65) and of its target cytokine genes such as TNFα, IL-6, IFNα, IFNβ, IL-12-p40, and CXCL2 was performed next. The MED feeding significantly downregulated the expressions of the p50, p65, TNFα, IL-6, IFNα, IFNβ, IL-12-p40, and CXCL2 genes compared to the ED mice (Figure 5(c)).

4. Discussion

The authors' previous study showed that the supplementation of the Lieber-DeCarli ethanol diet with *C. lanceolata*

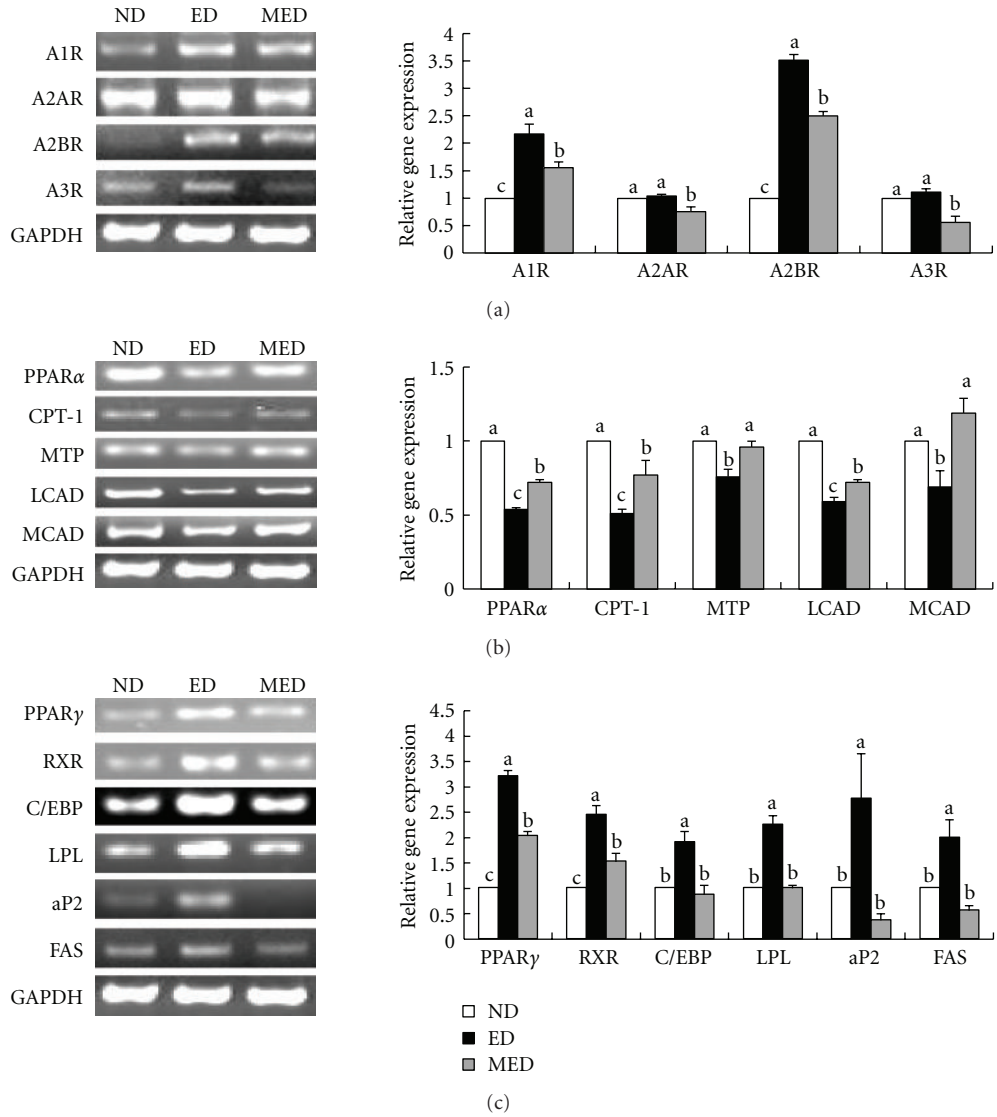

Figure 2: mRNA expression of the hepatic genes that are involved in fatty acid oxidation or adipogenesis. The left panel shows an RT-PCR analysis of (a) A1R, A2AR, A2BR, and A3R (b) PPARα, CPT-1, MTP, LCAD, and MCAD and (c) PPARγ, RXR, C/EBPα, LPL, aP2, and FAS expression. The images show the representative agarose gel electrophoresis of the PCR products. The right panel shows the relative expressions of (a) A1R, A2AR, A2BR, and A3R (b) PPARα, CPT-1, MTP, LCAD, and MCAD and (c) PPARγ, RXR, C/EBPα, LPL, aP2, and FAS levels. The data were normalized to the GAPDH levels, and all the expression levels that are displayed are relative to the ND. The results that are shown represent the means ± SEM of the three independent experiments in which RNA samples from eight mice were used. [a,b,c]Means not sharing a common letter are significantly different ($P < 0.05$).

water extract (5 mg/L of a liquid diet) showed a significant improvement in the amount of weight that was gained and in the hepatic lipid levels (in the press). In this study, the effects of three different fractions of *C. lanceolata* roots, which were prepared using different solvents, on the protection from alcoholic fatty liver were tested. Several studies have demonstrated that the protective effects of various plant extracts from alcoholic liver injury in animals, such as of fenugreek seed (*Trigonella foenum fraecum*) methanol extract [32], and hot water extracts of avaram leaves (*Cassia auriculata*) [33] and green tea (*Camellia sinensis*) [34] were manifested at dosages of between 200 and 500 mg/kg of body weight. Based on these studies, *C. lanceolata* root fractions were added to a liquid diet at 0.091% (wt/v), which is equivalent to a daily intake of 300 mg/kg of body weight, assuming that a mouse weighs 30 g and consumes 10 ml of a liquid diet per day.

Among the three different fractions that were prepared from the *C. lanceolata* root, CME exhibited the most remarkable attenuation of alcohol-induced fatty liver in terms of various parameters. For example, CME more significantly reduced the serum ALT and AST activities and the hepatic FFA concentration in the mice that were fed ethanol than did CES or CEP. Besides, alcohol-induced body weight loss and

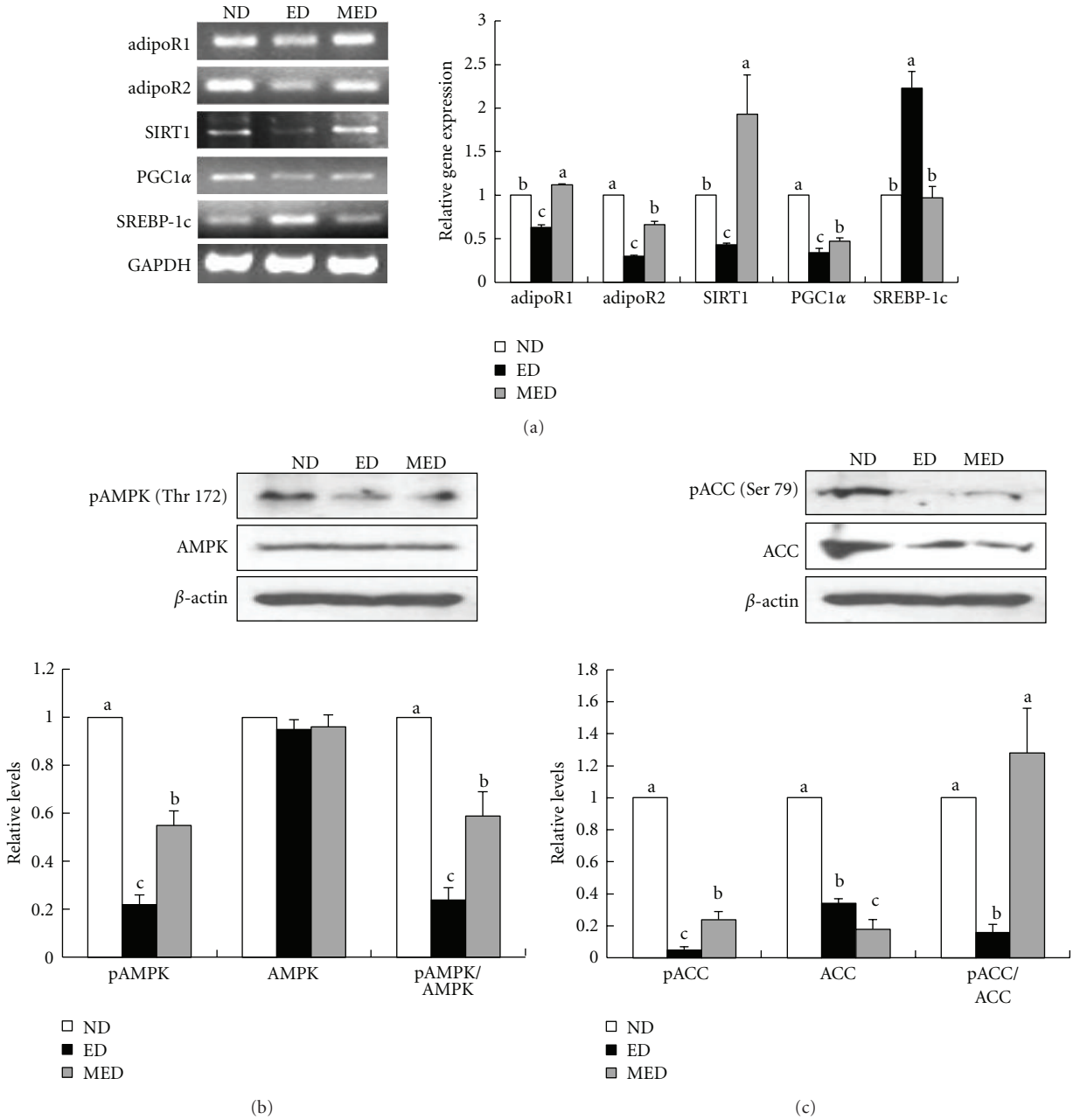

Figure 3: Alcohol-induced liver adiponectin receptors and involved genes in mRNA or protein expression. (a) The left panel shows the results of the RT-PCR analysis of adipoR1, adipoR2, SRIT1, PGC1α, and SREBP-1c expressions. The images show the representative agarose gel electrophoresis of the PCR products. The right panel shows the relative expressions of the adipoR1, adipoR2, SRIT1, PGC1α, and SREBP-1c levels. The data were normalized to the GAPDH levels, and all the expression levels that are shown are relative to the ND. (b) The upper panel shows the results of the Western blot analysis of the phosphorylated and total AMPK. The images show representative Western blotting images. The bottom panel shows the relative expressions of those levels and the ratios of the phosphorylated protein level to the total protein level. (c) The upper panel shows the results of the Western blot analysis of the phosphorylated and total ACC. The images show representative Western blotting images. The bottom panel shows the relative expressions of those levels and the ratios of the phosphorylated protein level to the total protein level. These results represent the means ± SEM of the three independent experiments in which RNA or protein samples from eight mice were used. [a, b, c]Means not sharing a common letter are significantly different ($P < 0.05$).

hepatic accumulations of triglycerides and cholesterol more significantly improved in mice that were supplemented with CME than in animals that were supplemented with ESD or EPD, although the difference was statistically insignificant. Therefore, CME appeared to be the most potent, among the fractions that were obtained from the *C. lanceolata* root, in protecting animals from alcohol-induced hepatic accumulation of lipids. Further investigated were the underlying mechanisms of CME against hepatic steatosis induced by chronic ethanol administration. The ethanol-induced

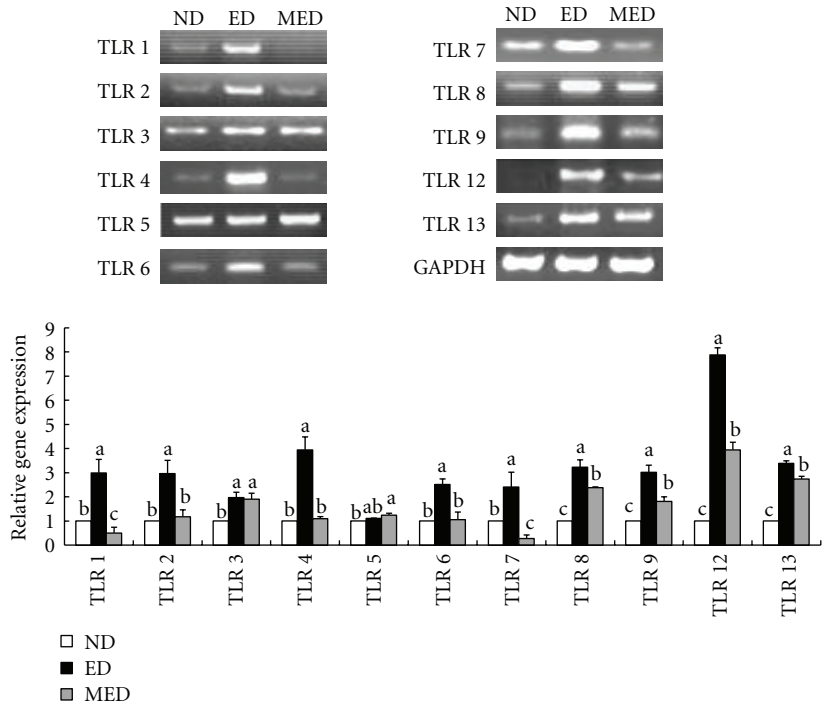

Figure 4: Alcohol-induced liver TLR mRNA expression. The upper panel shows the results of the RT-PCR analysis of the TLR1~9, 12, and 13 expressions. The images show the representative agarose gel electrophoresis of the PCR products. The bottom panel shows the relative expressions of the TLR1~9, 12, and 13 levels. The data were normalized to the GAPDH levels, and all the expression levels that are shown are relative to the ND. The results that are shown represent the means ± SEM of the three independent experiments in which RNA samples from eight mice were used. [a, b, c]Means not sharing a common letter are significantly different ($P < 0.05$).

elevation of the plasma HDL cholesterol concentration, as observed in the current study, has been suggested as a cardioprotective response in animals chronically loaded with ethanol [35]. The elevated HDL concentration and the reduced LDL concentration, which are characteristic of the lipoprotein pattern in chronic alcoholics, could well explain the reduced risk of coronary heart disease in alcoholics [36].

In this present study, the mRNA expression of the hepatic A2BR and A1R was markedly elevated by chronic ethanol feeding. Furthermore, the expression of PPARα and of its target genes, such as CPT-1, MTP, LCAD, and MCAD, all significantly decreased, whereas the expression of PPARγ and of its target genes, such as RXR, C/EBP, LPL, aP2, and FAS, all significantly increased in the livers of the ethanol-fed ED mice than of the ND mice (Figure 6). These results are in accordance with recent findings from experiments with mice that adenosine generated by ethanol metabolism plays an important role in ethanol-induced hepatic steatosis via A1R and A2BR, which leads to the upregulation of PPARγ and the downregulation of PPARα, respectively [1]. It was observed that CME supplementation reversed ethanol-induced elevation of the expressions of A2BR, A1R, PPARγ, and their target genes in the liver tissues of mice. These results suggest that CME ameliorates hepatic steatosis in mice, at least partly by modulating adenosine-receptor-mediated signaling molecules that are responsible for fatty acid oxidation and lipogenesis.

Adiponectin stimulates hepatic AMPK, which, in turn, phosphorylates ACC on Ser-79 and attenuates ACC activity. Inhibition of ACC directly reduces lipid synthesis and indirectly enhances fatty acid oxidation by blocking the production of malonyl-CoA, an allosteric inhibitor of CPT-1 [37]. Activation of AMPK by adiponectin in the liver also leads to decreased mRNA and protein expression of SREBP-1c [38–41], which results in decreased hepatic lipid synthesis. Adiponectin is also known to stimulate the activities of both PGC1α and PPARα, which mainly control the transcription of a panel of genes that encode fatty acid oxidation enzymes [42, 43]. As a result, adiponectin-mediated AMPK activation favors lipid catabolism and opposes lipid deposition in the liver [44–49]. Chronic ethanol administration is known to significantly decrease the plasma adiponectin level in mice [50]. In this study, more significant decreases in the mRNA levels of AdipoR1 and AdipoR2 were observed in the livers of the ED mice than of the pair fed ND mice. These alcohol-induced reductions in the hepatic AdipoRs expression appear to have been associated with the subsequent decreases in the phosphorylation of AMPK and ACC as their phosphorylations in the liver more significantly decreased in the ED mice than in the ND mice (Figure 6).

Besides, the hepatic mRNA levels of SIRT1, PGC1α, SREBP-1c, PPARα, and CPT1 were also more significantly reduced in the ED mice than in the ND mice (Figure 6). SIRT1 has been gaining recognition as one of the critical agents in the mediation of adiponectin signaling to AMPK [37], which is the central mechanism in the regulation of lipid metabolism [52–55]. Although PGC1α was initially identified as a coactivator of PPARγ, it has subsequently

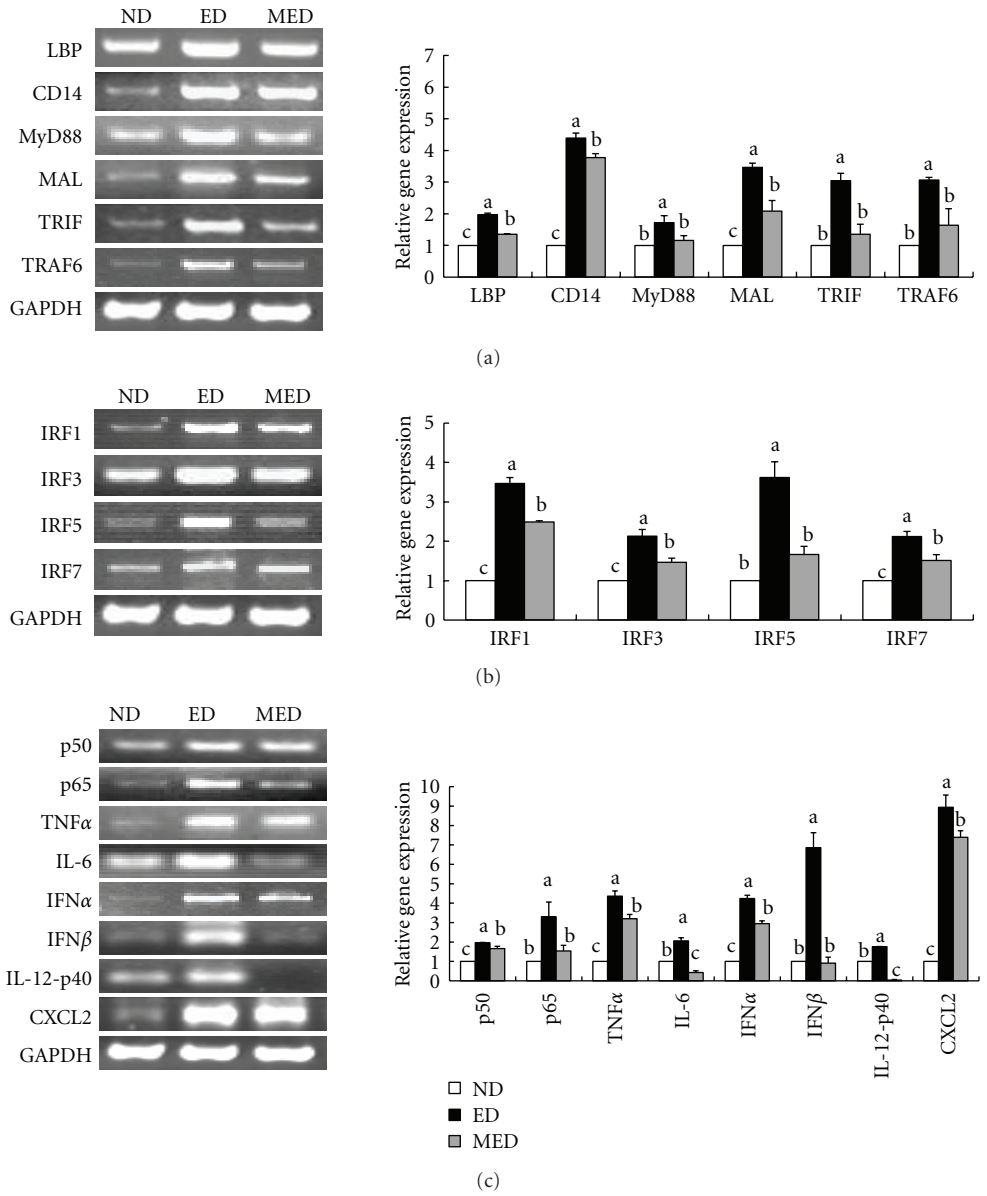

Figure 5: mRNA expressions of the hepatic genes that were involved in the inflammation. The left panel shows the results of the RT-PCR analysis of the (a) LBP, CD14, MyD88, MAL, TRIF, and TRAF6, (b) IRF1, IRF3, IRF5, and IRF7, and (c) p50, p65, TNFα, IL-6, IFNα, IFNβ, IL-12-p40, and CXCL2 expressions. The images show the representative agarose gel electrophoresis of the PCR products. The right panel shows the relative expressions of the (a) LBP, CD14, MyD88, MAL, TRIF, and TRAF6, (b) IRF1, IRF3, IRF5, and IRF7, and (c) p50, p65, TNFα, IL-6, IFNα, IFNβ, IL-12-p40, and CXCL2 levels. The data were normalized to the GAPDH levels, and all the expression levels that are shown are relative to the ND. The results that are shown represent the means ± SEM of the three independent experiments in which RNA samples from eight mice were used. [a,b,c]Means not sharing a common letter are significantly different ($P < 0.05$).

been shown to serve as a cofactor of several other transcription factors, including PPARα [56, 57]. Treatment with adiponectin restored the ethanol-inhibited PGC1α/PPARα activity in cultured hepatic cells and in animal livers, which suggests that the stimulation of adiponectin-SIRT1 signaling may serve as an effective therapeutic strategy for treating or preventing human alcoholic fatty liver [38–40]. In this study, CME significantly restored the ethanol-induced downregulation of adiponectin-mediated signaling molecules, including adipoR1, adipoR2, pAMPK/AMPK, pACC/ACC, SIRT1, and PGC1α, which led to increased fatty acid oxidation and decreased lipogenesis in the livers of the mice.

It was recently shown that innate immune cells recognize conserved pathogen-associated molecular patterns through pattern recognition receptors, among which the family of TLRs occupies an important place [24]. LBP, which is present in normal serum, recognizes and binds to LPS with high affinity through its lipid moiety [58, 59]. LPS-LBP complexes

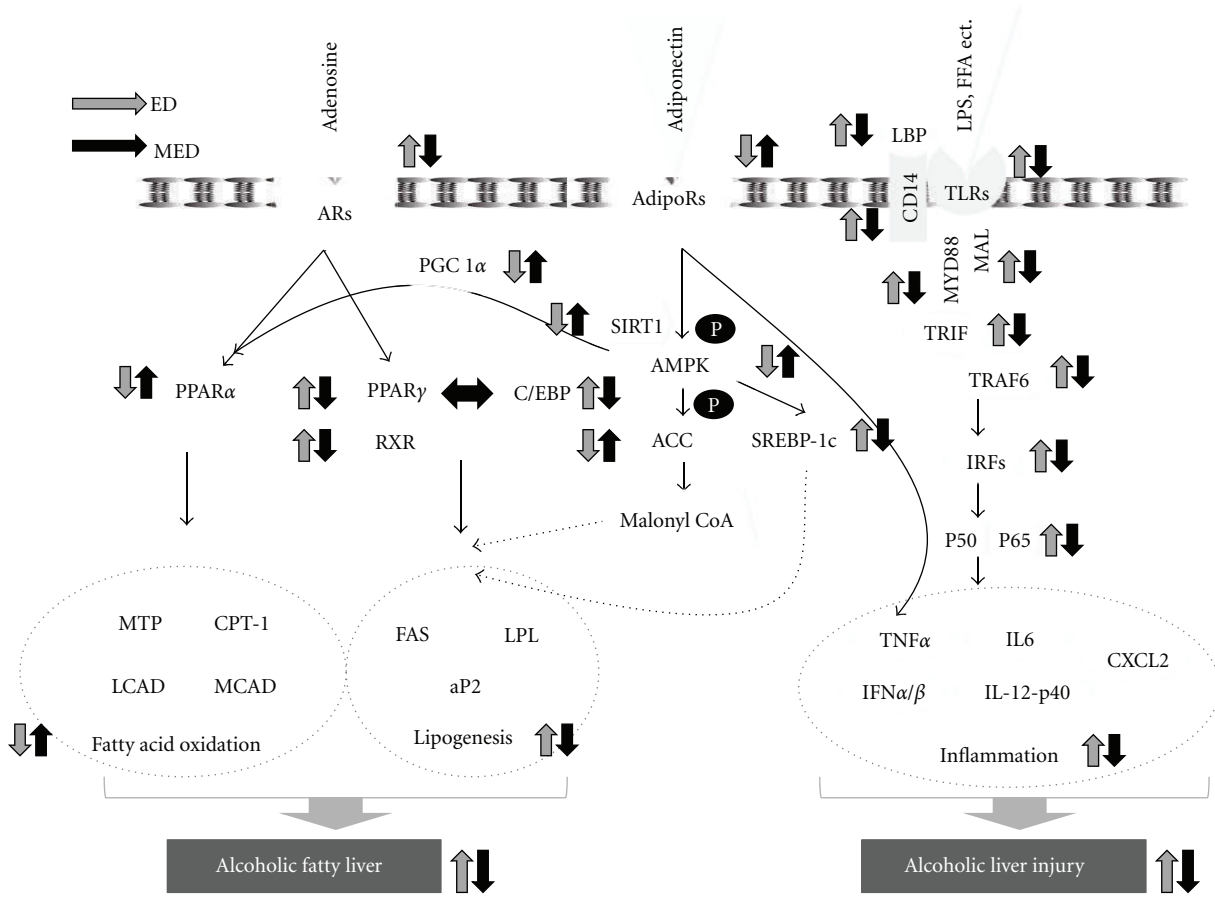

Figure 6: Schematic diagram illustrating the antilipogenic and anti-inflammatory activities of *Codonopsis lanceolata* in the liver of mice with chronic ethanol consumption. Adapted from Peng [1], You and Rogers [37, 51]. The arrows denote the direction of the responses to altered conditions with ED feeding relative to ND feeding (gray arrows), and with MED feeding relative to ED feeding (black arrows).

then activate cells through the second glycoprotein, the membrane-bound CD14, to produce inflammatory mediators [60–62] in the presence of only a functional TLR4 [63]. Although LPS was not detected in the blood, more significant increases in the mRNA levels of LBP and CD14 were observed in the livers of the ED mice than of the ND mice (Figure 6). TLRs have a TIR domain that initiates the signaling cascade through TIR adapters, such as MyD88 [64], Mal [65, 66], and TRIF, which interact with TRAF6, the downstream adaptor. TRAF6 activates the IRFs, which leads to NF-κB activation and induction of the expressions of proinflammatory cytokines [67], such as of TNFα, IL-6, IFNα, and IFNβ.

It was demonstrated that chronic ethanol feeding induces hepatic steatosis and clear upregulation of TLR1, 2, 3, 4, 6, 7, 8, 9, 12, and 13 in the liver. These findings are supported by previous reports that ethanol-fed mice exhibited hepatic inflammation that was dependent on the upregulation of multiple TLRs in the liver [24]. The expressions of the MyD88, MAL, TRIF, TRAF6, IRF1, 3, 5, and 7 genes, along with the mRNA levels of NFκB and proinflammatory cytokines, were all more significantly elevated in the livers of the ED mice than of the ND mice (Figure 6). These results are also in accordance with previous observations that MyD88 is required for the development of fibrosis [68], a predisposing condition for hepatocellular cancer development, and that IRF7 expression more significantly increased in the livers of alcohol-fed MyD88-deficient mice than of pair fed control mice [69]. These results suggest that chronic ethanol intake may activate TLR-mediated proinflammatory signaling cascades. The study data show that these hepatic inductions of TLR-mediated signaling cascade molecules, which involve both MyD88-dependent and -independent pathways, along with their target proinflammatory cytokine genes, such as TNFα, IL-6, IFNα, IFNβ, IL-12-p40, and CSCL2, due to ethanol feeding were all dramatically reversed with CME supplementation.

Taken together, these beneficial effects of CME against alcoholic fatty livers in mice appear to have occurred with adenosine- and adiponectin-mediated regulations of hepatic steatosis and TLR-mediated modulation of hepatic proinflammatory responses, since ethanol-induced changes in the expression of the molecules that are involved in these three signaling pathways were all significantly reversed with CME feeding. Although alcoholic fatty liver is a major risk factor for advanced liver injuries such as steatohepatitis,

fibrosis, and cirrhosis [51], previous studies have mostly focused on the antilipogenic or -inflammatory activity alone, but not in combination, of a compound and extracts. For example, Kumar et al. evaluated the effect of the *Cassia auriculata* leaf extract on lipid metabolism in alcohol-induced hepatic steatosis [33]. Resveratrol prevents the development of hepatic steatosis induced by alcohol, by restoring the inhibited hepatic SIRT1-AMPK signaling system [70]. This study demonstrated that both lipogenesis and inflammation are associated with the development of alcoholic fatty liver and that CME has both antilipogenic and anti-inflammatory effects through coordinated multiple signaling pathways. Although much further study is required concerning the exact identities of the chemical constituents of the *C. lanceolata* methanol extract that are responsible for the findings described herein, the results of this study provide some insights on the basic mechanism that underlies the therapeutic effect of the extract on mice hepatic tissues after chronic ethanol feeding.

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Research Article

Antiproliferative Activity of Xanthones Isolated from *Artocarpus obtusus*

Najihah Mohd Hashim,[1] Mawardi Rahmani,[1] Gwendoline Cheng Lian Ee,[1] Mohd Aspollah Sukari,[1] Maizatulakmal Yahayu,[1] Winda Oktima,[1] Abd Manaf Ali,[2] and Rusea Go[3]

[1] *Department of Chemistry, Universiti Putra Malaysia, Selangor, 43400 Serdang, Malaysia*
[2] *Faculty of Agriculture and Biotechnology, Universiti Sultan Zainal Abidin, 20400 Kuala Terengganu, Malaysia*
[3] *Department of Biology, Universiti Putra Malaysia, Selangor, 43400 Serdang, Malaysia*

Correspondence should be addressed to Mawardi Rahmani, mawardi@science.upm.edu.my

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An investigation of the chemical constituents in *Artocarpus obtusus* species led to the isolation of three new xanthones, pyranocycloartobiloxanthone A (**1**), dihydroartoindonesianin C (**2**), and pyranocycloartobiloxanthone B (**3**). The compounds were subjected to antiproliferative assay against human promyelocytic leukemia (HL60), human chronic myeloid leukemia (K562), and human estrogen receptor (ER+) positive breast cancer (MCF7) cell lines. Pyranocycloartobiloxanthone A (**1**) consistently showed strong cytotoxic activity against the three cell lines compared to the other two with IC_{50} values of 0.5, 2.0 and 5.0 μg/mL, respectively. Compound (**1**) was also observed to exert antiproliferative activity and apoptotic promoter towards HL60 and MCF7 cell lines at respective IC_{50} values. The compound (**1**) was not toxic towards normal cell lines human nontumorigenic breast cell line (MCF10A) and human peripheral blood mononuclear cells (PBMCs) with IC_{50} values of more than 30 μg/mL.

1. Introduction

Tropical rain forests contain a lot of interesting pharmacologically active constituents and many more are still waiting to be discovered as they still offer undoubtedly valuable and amazing chemical entities. The World Health Organization (WHO) reported that 11% of the 252 drugs considered as basic and essential in this century were exclusively origin from flowering plants [1]. Many impressive modern drugs available in the market were derived from plants source and most of them were based on their ethnomedicinal uses. Hence, many works on tropical plants from Malaysia are being and have been extensively studied as well as their biological activity. One of them is *Artocarpus* species. The *Artocarpus* species is one of the genus in the Moraceae family [2] and is made of about 55 species. The genus is widely distributed throughout subtropical and tropical region of the Indian subcontinent south of the Himalayas, Sri Lanka, Burma, Thailand, Indo-China, Southern China, Taiwan, Hainan, Malesia, and Melanesia [2, 3]. Some parts of the plants are also used in traditional medicine preparations for the treatment of various diseases such as diarrhea, fever, liver cirrhosis, hypertension, diabetes, inflammation, malaria, ulcers, wound, and for tapeworm infection [4–8]. Many interesting biological activities and phytochemical work have been carried out on the plants with the identification of various classes of interesting phenolic compounds [9–18]. Our previous works on the stem bark of *A. altilis* and *A. obtusus* have led to the isolation of prenylated flavonoids and xanthones [19, 20].

In preliminary screening for cytotoxic, antioxidant and antimicrobial activity of thirteen Malaysian *Artocarpus* species has resulted in the potential of a few species for further examination in particular *A. obtusus* Jarret [13]. The plant is endemic to Sarawak, and, in continuation of our investigation on Malaysian plants, we now described the antiproliferative activity of three new xanthones isolated from the stem bark of *A. obtusus*. The cytotoxic activity

was tested using MTT assay against human promyelocytic leukemia (HL60), human chronic myeloid leukemia (K562), and human estrogen receptor (ER+) positive breast cancer (MCF7). The most active xanthone was further tested against mouse embryo fibroblast (3T3), human cervical cancer (HeLa), human estrogen receptor (ER−) negative (MDA-MB 231), human colon cancer (HT29) and human hepatocarcinoma (HepG2), human nontumorigenic breast cell line (MCF10A), and human peripheral blood mononuclear cells (PBMCs). The potential compound was also analyzed for its antiproliferative activity and effect on cell morphology of HL60 and MCF7 cell lines at selected concentrations using BrdU and acridine orange and propium iodide staining, respectively. Currently, there is no recognized ethnomedicinal data and reported biological activity for *A. obtusus*; thus, this is the first report on antiproliferative activity studies of the isolated xanthones from the plant as well as the isolation and structural elucidation of compound (**3**).

2. Materials and Methods

2.1. General Experimental Procedures. All melting points (m.p.) were determined using a hot stage melting point apparatus model Leica GALEN III equipped with microscope and are uncorrected. UV and IR spectra were measured with Shimadzu UV 2100 and Perkin Elmer FTIR (model 1725X) spectrophotometers, respectively. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained with JEOL ECA-400 spectrometer operating at 400 and 100 MHz with tetramethylsilane (TMS) as an internal standard, respectively. The MS were obtained with Shimadzu GCMS-QP5050 spectrometer with Direct Induction Probe (DIP) using ionization induced by electron impact at 70 eV. Column chromatography was carried out using Merck Si gel 60 No. 1.07734, Darmstadt, Germany. Analytical thin layer chromatography (TLC) was performed on commercially available Merck TLC plastic sheet precoated Kieselgel 60 F_{254}, 0.2 mm thickness, and the chromatotron plates were coated with Kieselgel 60 F_{254} and scrapped to 1 mm thickness.

2.2. Plant Material. Sample of the air-dried stem bark of *Artocarpus obtusus* was collected from Sarawak in 2004, identified by Dr. Rusea Go, and a voucher specimen (S94402) has been deposited at the Herbarium, Department of Biology, Faculty of Science, Universiti Putra Malaysia.

2.3. Preparation of Plant Extracts and Isolation Procedure. The dried ground stem bark (640 g) of *Artocarpus obtusus* was sequentially extracted with *n*-hexane, chloroform and methanol at room temperature. The extracts were concentrated *in vacuo* to give 3 g (0.47%), 9 g (1.4%), and 6 g (0.94%) of dark viscous *n*-hexane, chloroform, and methanol extracts, respectively. The chromatographic isolation procedure to obtain pyranocycloartobiloxanthone A (**1**) and dihydroartoindonesianin C (**2**) from the chloroform extract has been described previously [20]. Similarly, the dark viscous methanol extract (6 g) was separated by gravity column chromatography and eluted with mixture of solvents of increasing polarity consisting of *n*-hexane, *n*-hexane/$CHCl_3$, $CHCl_3$, $CHCl_3$/MeOH, and MeOH to afford 45 fractions of 200 mL each. Another batch of compounds (**1**) and (**2**) were obtained from fractions 11–19 after a series of silica gel column chromatographic separation. Fractions 26–32 were combined and applied on column chromatography of Sephadex LH20 and eluted with methanol to give 45 fractions of 100 mL each. The combined fractions 18–28 were further chromatographed by a series of column chromatography using silica gel and eluted with solvent mixture of *n*-hexane, *n*-hexane/$CHCl_3$, $CHCl_3$, $CHCl_3$/MeOH, and MeOH to afford yellow solid (25.0 mg). The solid was recrystallized with chloroform as needle-shaped pyranocycloartobiloxanthone B (**3**) with R_f value of 0.5 (EtOAc : $CHCl_3$; 4 : 1) and m.p. 218–220°C.

2.4. Spectral Data. Pyranocycloartobiloxanthone A (**1**); $C_{25}H_{22}O_8$, yellow needle-shaped crystals, and m.p. 288–290°C. The spectral data of the compound are identical to literature values [20].

Dihydroartoindonesianin C (**2**); $C_{25}H_{24}O_6$, yellow needle-shaped crystals, and m.p. 210–212°C. The spectral data of the compound are identical to literature values [20].

Pyranocycloartobiloxanthone B (**3**), $C_{25}H_{22}O_8$, yellow needle-shaped crystal with m.p. 218–220°C; UV (Acetone) λ_{max} nm (log ε): 381 (0.57), 256 (4.00), and 229 (0.32); IR ν_{max} cm^{-1} (KBr): 3390 (OH), 1642 (C = O), 1474, 1270, 1160, 982; EIMS m/z (% intensity): 450 (26.50), 436 (28.00), 435 (100), 377 (60.00), 378 (18.00), 349 (6.00), 331 (4.50), 188 (14.00), and 166 (6.00); HREIMS m/z 450.1329 $[M]^+$ (calcd for $C_{25}H_{22}O_8$, 450.1314). ^{1}H-NMR (400 MHz, Acetone-d_6): 1.19 (3H, *d*, *J* = 6.4 Hz, H-15), 1.45 (6H, s, H-19, H-20), 1.91 (1H, *m*, H-11), 2.25 (1H, *dd*, *J* = 15.6, 7.7 Hz, H-13), 2.76 (1H, *m*, H-12), 3.32 (1H, *m*, H-11), 5.47 (1H, *s*, H-14), 5.65 (1H, *d*, *J* = 10.0 Hz, H-17), 6.12 (1H, *s*, H-6), 6.49 (1H, *s*, H-3′), 6.93 (1H, *d*, *J* = 10.0 Hz, H-16), 7.96 (1H, *brs*, OH-13), 8.44 (1H, *brs*, OH-2′), 8.84 (1H, *brs*, OH-4′), 13.37 (1H, *brs*, OH-5); ^{13}C-NMR (100 MHz Acetone-d_6): 16.5 (C-15), 24.2 (C-11), 29.8 (C-19), 30.0 (C-20), 33.9 (C-12), 38.8 (C-13), 80.2 (C-18), 96.5 (C-14), 101.3 (C-6), 103.6 (C-3), 105.5 (C-3′), 105.8 (C-10), 107.1 (C-8), 114.3 (C-1′), 117.6 (C-16), 127.3 (C-6′), 129.4 (C-17), 135.0 (C-5′), 153.2 (C-4′), 153.6 (C-2′), 153.8 (C-5), 161.0 (C-9), 163.1 (C-2), 164.1 (C-7), and 182.3 (C-4) (Figure 1).

2.5. In Vitro Assay for Antiproliferative Activity

2.5.1. Cell Lines. The cancerous and noncancerous cell lines (suspension and anchorage-dependent cells) were obtained from the American Type Culture Collection (ATCC) and the RIKEN Cell Bank (RCB). The cancerous cell lines used were HL-60 (human acute promyelocytic leukemia) (RCB), K562 (human chronic myeloid leukemia) (ATCC), MCF7 (human estrogen receptor (ER+) positive breast cancer) (ATCC), HeLa (human cervical cancer) (ATCC), HepG2 (human hepatocarcinoma) (ATCC), HT29 (human colon cancer) (RCI), and MDA-MB 231 (human estrogen receptor (ER−) negative) (ATCC). The noncancerous cell line and normal cells were 3T3 (Mouse embryo fibroblast) (ATCC),

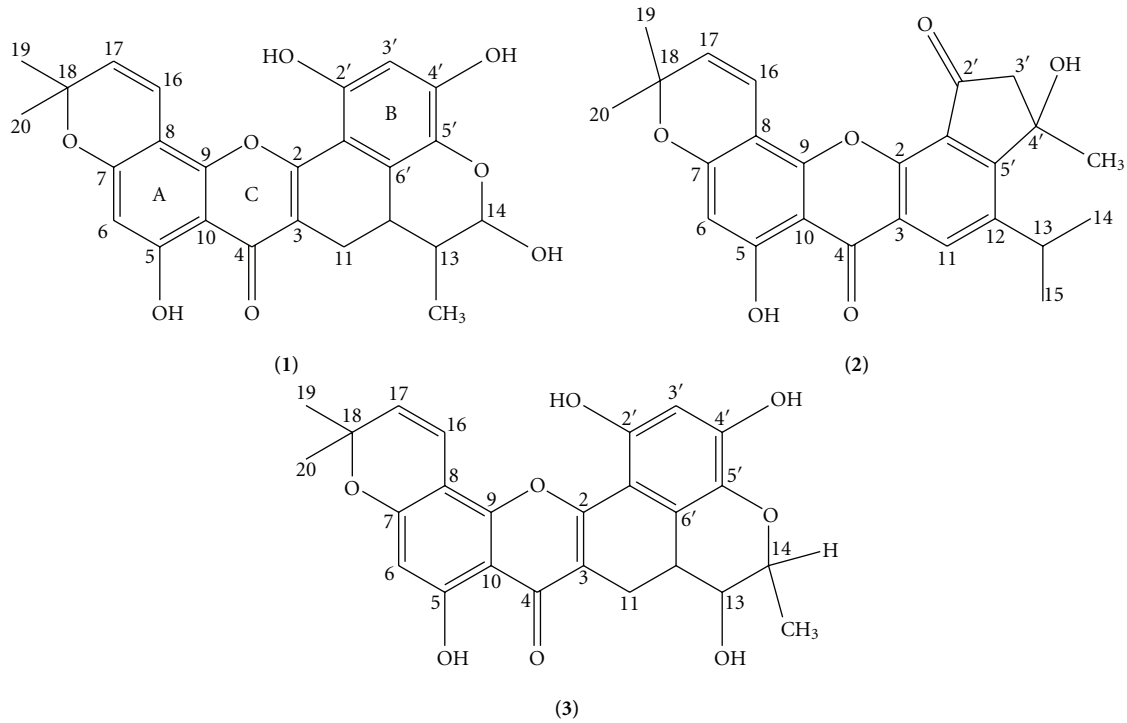


Figure 1: The chemical structure of the three isolated compounds tested for antiproliferative activity, pyranocycloartobiloxanthone A (**1**), dihydroartoindonesianin C (**2**), and pyranocycloartobiloxanthone B (**3**).

MCF10A (human nontumorigenic breast cell line) (ATCC) and PBMC (human peripheral blood mononuclear cells). PBMCs were obtained from one of the authors herself. Blood (10–12 mL) was taken from the donor by using the 25 mL syringe. The blood sample was diluted with same volume of PBS. The diluted blood sample was carefully layered on Ficoll-Paque Plus (Amersham Biosciences, USA) the ratio of 2 : 1. The mixture was centrifuged at 1500× g for 40 minutes at 18–20°C. The undisturbed lymphocyte layer was carefully transferred into centrifuge tube. The lymphocyte was washed and spun down three times by adding 10 mL sterile PBS and to get the pellet and resuspended in RPMI 1640 (Sigma, USA) with 100 IU/mL of penicillin, 100 μg/mL of streptomycin (Flowlab, Australia), and 10% v/v Fetal Bovine Serum (FBS) (PAA, Austria). Cell counting was performed to determine the PBMC number in equal volume of trypan blue.

2.5.2. Cytotoxic Analysis by the MTT Assay. The colorimetric assay was performed on the isolated compounds using a modified microculture tetrazolium salt (MTT) assay (Sigma, USA) [21]. Varying concentrations of 0.46, 0.93, 1.87, 3.75, 7.5, 15, and 30 μg/mL of pyranocycloartobiloxanthone A (**1**), dihydroartoindonesianin C (**2**), and pyranocycloartobiloxanthone B (**3**) were prepared from the substock solutions by serial dilution in RPMI 1640 to give a volume of 100 μL in each microtitre plate well (96-flat bottom microwell plates). Each well was then added with 100 μL of cell lines in complete growth media (RPMI 1640) 5×10^5 cells/mL. Untreated cells were used as control and included for each sample. The assay for each concentration of compounds was performed in triplicate. The culture plates were incubated for 72 hour at 37°C in a humidified (90%) incubator with 5% CO_2. After incubation, the fractions of surviving cells were determined relative to the untreated cell population using the colorimetric MTT assay. In this method, the viabilities of the cells were determined by measuring the amount of blue formazan crystals formed after 20 mL of freshly prepared MTT solution (5 mg in 1 mL PBS) was added to each well followed by four hours of incubation at 37°C. Then, 170 μL of the remaining supernatant was removed, 100 μL of DMSO was added to each well, and the mixture was stirred thoroughly to dissolve the blue crystal formazan. The plate was then incubated for 30 minutes to ensure that all the crystals were dissolved. Finally, the absorbance (OD) of each well at 570 nm of wavelength was read using the microplate reader (ELISA reader, EL340 Biokinetic Reader, Bio-Tek Instrumentation). Doxorubicin was used as a positive control. A graph was plotted for the percentage of cell viability against concentration of the extract, and the cytoxicity index used was IC_{50}, the concentration that yields 50% inhibition of the cell compared with untreated control. The percentage of cell viability was determined as follows:

$$\%\ \text{Viability} = \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100\%. \quad (1)$$

2.6. Cell Proliferation ELISA BrdU (5-bromo-2′-deoxyuridine) Assay (Colorimetric). This assay was conducted to determine the antiproliferative effects of the tested compound. In this assay, three different concentrations of

Table 1: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral data of pyranocycloartobiloxanthone B (3) and pyranocycloartobiloxanthone A (1).

H/C	(**3**) In acetone-d_6			(**1**) In DMSO [13]	
	δ_H (in ppm, J in Hz)	δ_C (in ppm)	HMBC	δ_H (in ppm)	δ_C (in ppm)
2	—	163.1	—	—	160.7
3	—	103.6	—	—	100.8
4	—	182.3	—	—	179.1
5	—	153.8	—	—	151.2
6	6.12 (1H, s)	101.3	C-5, 7, 8	6.17	98.7
7	—	164.1	—	—	160.7
8	—	107.1	—	—	104.3
9	—	161.0	—	—	157.9
10	—	105.8	—	—	103.6
11	1.91 (1H, *m*)	24.2	C-3, 6′	1.88	21.5
	3.32 (1H, *m*)		C-3, 4, C-12	3.32	
12	2.76 (1H, *m*)	33.9	C-13, 1′, 5′, 6′	2.55	31.2
13	2.25 (1H, *dd*, J = 15.6, 7.7)	38.8	C-12, 14, 6′	1.82	35.7
14	5.47 (1H, *s*)	96.5	C-13, 15	5.32	93.1
15	1.19 (3H, *d*, J = 6.4, CH_3)	16.5	C-12, 13, 14	1.08	14.6
16	6.93 (1H, *d*, J = 10.0)	117.6	C-7, 9, 18	6.86	114.8
17	5.65 (1H, *d*, J = 10.0)	129.4	C-16, 18, 19	5.74	127.3
18	—	80.2	—	—	78.0
19	1.45 (3H, *s*, CH_3)	29.8	C-17, 18	1.44	27.7
20	1.45 (3H, *s*, CH_3)	30.0	C-17, 18	1.42	27.9
1′	—	114.3	—	—	111.0
2′	—	153.6	—	—	151.2
3′	6.49 (1H, s)	105.5	C-2′, 4′, 5′	6.43	103.1
4′	—	153.2	—	—	151.1
5′	—	135.0	—	—	132.5
6′	—	127.3	—	—	124.6
OH-5	13.37 (1H, *brs*)	—	C-6, 7, 10	13.36	—
OH-13	7.96 (1H, *brs*)	—	—	—	—
OH-14	—	—	—	6.93	—
OH-2′	8.44 (1H, *brs*)	—	—	9.91	—
OH-4′	8.84 (1H, *brs*)	—	C-3′	9.81	—

compound or test sample were prepared together with control. The concentration chosen were 1 μg/mL, the IC_{50} concentration, and 20 μg/mL. Two types of cells were selected: HL-60 (suspension) and MCF7 (adherent) cells, since they gave the most significant results for the cytotoxic activity. The procedures are according to the instruction manual from Roche. Each sample was assayed in triplicate, and control samples include a blank (no cells) and background (without BrdU). The cells were treated by pyranocycloartobiloxanthone A (**1**) for 24, 48, and 72 hours. The cells were cultured together with various dilutions of test substance (tested compound) in 96-well MP flat bottom in a final volume of 100 μL/well, and 10 μL BrdU label (diluted by 1/2000 in media) was added to each well and incubated for 24 hour at 37°C. BrdU label was then replaced with 200 μL of fix/denaturing solution for 30 min at room temperature (25°C) and removed by inverting the plate. The anti-BrdU-POD working solution (100 μL/well) (diluted by 1/100 in dilution buffer) was added and incubated for one hour at room temperature. The cells were washed three times with wash buffer and once with dH_2O. Then, removed dH_2O and 100 μL of substrate solution was added to each well and incubated for 15 min at room temperature in the dark. The stop solution (100 μL/well) was added, and the absorbance was measured using ELISA reader at 450 nm, (reference wavelength 690). The activity was observed by the optical density (OD) of the absorbance at 450 nm, and graphs were plotted from the optical density obtained against time of exposure for each cell lines.

2.7. Acridine Orange (AO) and Propodium Iodide (PI) Staining. Cell suspensions were mixed with an equal volume of staining solution (1 : 1) containing 10 μg/mL acridine orange and 10 μg/mL propium iodide (dissolved in PBS) and observed under fluorescence microscope within 30 minutes. The viable (green intact cells), apoptotic (green

Table 2: The IC_{50} values of isolated compounds towards various cell lines.

Cell line	IC_{50} (μg/mL)			
	(**1**)	(**2**)	(**3**)	Doxorubicin
HL60	2 ± 0.7	26 ± 0.8	17 ± 0.8	0.2 ± 0.03
K562	0.5 ± 0.05	>30	>30	nt
MCF7	5 ± 1.2	27 ± 1.9	23 ± 1.3	0.2 ± 0.06
HeLa	8 ± 3.3	nt	nt	nt
MDA-MB 231	12 ± 1.1	nt	nt	nt
HT29	14 ± 1.5	nt	nt	nt
HepG2	20 ± 2.0	nt	nt	nt
3T3	7 ± 1.6	nt	nt	nt
MCF10	>30	nt	nt	nt
PBMC	>30	nt	nt	>30

Note: (nt)-not tested, (**1**)-pyranocycloartobiloxanthone A, (**2**)-dihydroartoindonesianin C, and (**3**)-pyranocycloartobiloxanthone B. Data represent mean ± SD of triplicate determinations from three independent experiments.

shrinking cells with condensed of fragmented nucleus), and necrotic (red cells) were the morphological changes that were examined under fluorescence microscope.

3. Results and Discussion

Based on the biological screening test results on 39 extracts from thirteen species of *Artocarpus* species, the three extracts of *Artocarpus obtusus* exhibited excellent cytotoxic activity against HL60 cells with IC_{50} values from 1.4 to 8.4 μg/mL [13]. However, only chloroform extract of *A. obtusus* exhibited cytotoxic activity towards MCF7 cells with IC_{50} values 22.60 μg/mL, while the other extracts were inactive towards the cell. These extracts were further phytochemically investigated with the isolation and identification of three new xanthones, pyranocycloartobiloxanthone A (**1**), dihydroartoindonesianin C (**2**), and pyranocycloartobiloxanthone B (**3**). The structural elucidation of two of xanthones, (**1**) and (**2**) isolated from the chloroform extract, has already been published recently [20], and the structure determination of the third xanthone (**3**) is included in this presentation. After chromatographic separation of the methanol extract, pyranocycloartobiloxanthone B (**3**) was obtained as yellow needle-shaped crystals with m.p. 218–220°C. The UV spectrum exhibited absorption bands at λ_{max} 381, 256, and 229 nm for a typical xanthone skeleton. Broad and strong absorptions were noted at 3390 and 1642 cm^{-1} in the IR spectrum due to the presence of hydroxyl and carbonyl functionalities. The EIMS gave molecular ion peak at m/z 450 which is consistent with molecular formula $C_{25}H_{22}O_8$. The high-resolution EIMS exhibited molecular ion peak at m/z 450.1329 (calculated $C_{25}H_{22}O_8$ for m/z 450.1314). The ^{1}H-NMR displayed characteristic signals for chelated phenolic hydroxyl group at δ 13.37 and a 2,2-dimethylchromene ring substituent with the observation of a pair of olefinic protons at δ 6.93 and 5.65 with identical coupling constant of 10.0 Hz and a sharp singlet of two overlapped methyl groups at δ 1.45. The aromatic region also revealed the occurrence of two

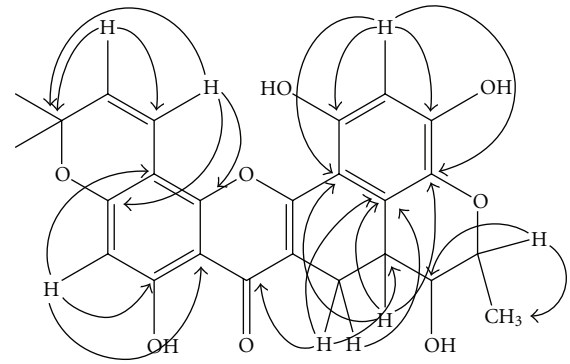


Figure 2: Selected HMBC of pyranocycloartobiloxanthone B (**3**).

singlets at δ 6.12 (H-6) and 6.49 (H-3′), and another high field resonance at δ 1.19 assigned to methyl group at C-15. The two broad chemical shifts noted at δ 8.44 and 8.84 were due to the hydroxyl groups attached to aromatic ring at C-2′ and C-4′.

The ^{13}C-NMR and DEPT spectra indicated the existence of twenty-five signals contributed by fourteen quaternary carbons including a conjugated carbonyl (δ 182.3), seven methine, one methylene (δ 24.2), and three methyl (δ 16.5, 29.8, and 30.0) carbon atoms. These ^{1}H-NMR and ^{13}C-NMR spectral data were quite similar to pyranocycloartobiloxanthone A (**1**) but with some obvious differences especially the location of one the hydroxyl groups at C-14 now shifted to C-13 which occurred at δ 38.8. The methyl group at C-13 shifted to C-14 and the chemical shift of this carbon now shifted upfield at δ 96.5 (Table 1). On the basis of HMQC and HMBC spectral analysis, the chemical shifts were fully assigned, and the positions of the substituents on the pyran and aromatic rings were determined. The ^2J and ^3J correlations of both protons at H-6 to -5, C-7, and C-8, H-3′ to C-2′, C-4′, and C-5′ confirmed the position of each respective aromatic protons. The two-bond and three-bond correlations were also used to locate the positions of both methyl and hydroxyl groups in the pyran ring (Figure 2). In (**1**), the methyl protons at C-15 exhibited correlations with C-12, -13, and -14 [20]. However, the methyl protons only showed correlations to C-13 and 14 in compound (**3**) which supported the attachment of this methyl group at C-14. Similarly, the methine proton at C-12 do not indicate any correlation to the methyl group. Based on these spectral data, the structure of this new xanthone was assigned and given trivial name pyranocycloartobiloxanthone B (**3**).

By using three tumor cell lines (HL60, K562, and MCF7), a comparison was made between the three new xanthones to establish which has greater cytotoxicity. The cytotoxic activity was assayed at various concentrations, expressed in IC_{50} values (μg/mL) under continuous exposure for 72 hours, and is summarized in Table 2. The estimated IC_{50} values were obtained from the plotted graphs of percentage cell viability (%) against various concentrations of the compounds (μg/mL) (Figures 3 and 4). The study showed pyranocycloartobiloxanthone A (**1**) exhibited potent cytotoxic activity against the three cell lines with IC_{50} ranged from

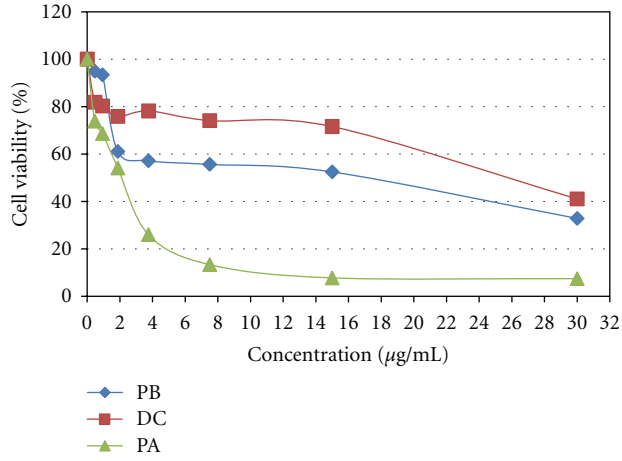


Figure 3: Percentage viability of HL60 cells treated with different concentration of pyranocycloartobiloxanthone A (**1**) (PA), dihydroartoindonesianin C (**2**) (DC), and pyranocycloartobiloxanthone B (**3**) (PB) measured after 72 hours using MTT assay.

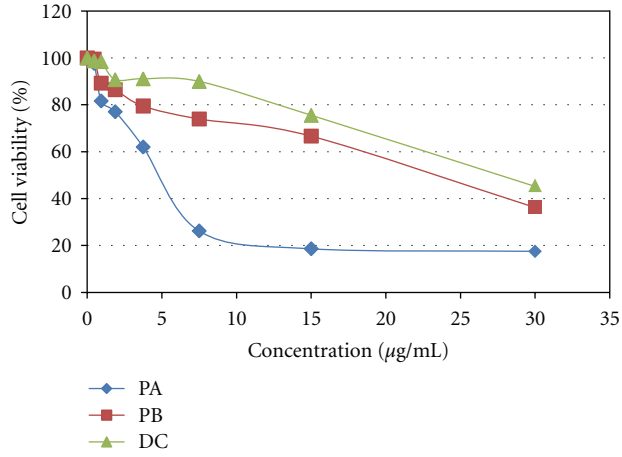


Figure 4: Percentage viability of MCF7 cells treated with different concentration of pyranocycloartobiloxanthone A (**1**) (PA), dihydroartoindonesianin C (**2**) (DC), and pyranocycloartobiloxanthone B (**3**) (PB) measured after 72 hours using MTT assay.

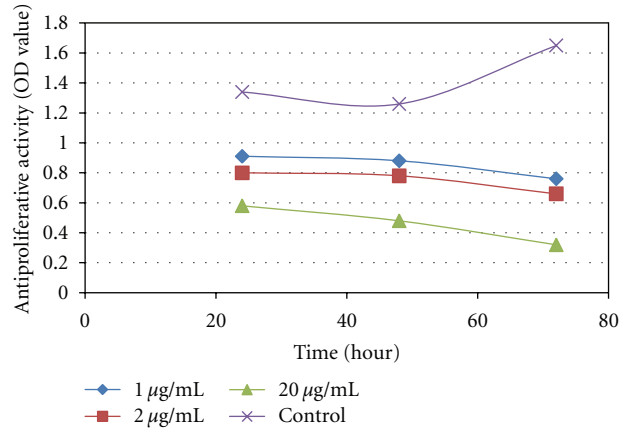


Figure 5: Effect of pyranocycloartobiloxanthone A (**1**) on the proliferation of HL60 cells *in vitro*. After treatment with 1 μg/mL, 2 μg/mL (IC_{50} based on MTT results) and 20 μg/mL for 24, 48, and 72 hours cellular proliferation of HL60 cells was assayed using BrdU incorporation ELISA.

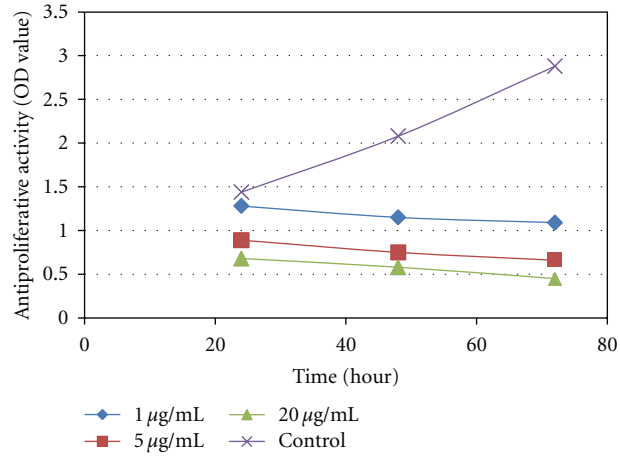
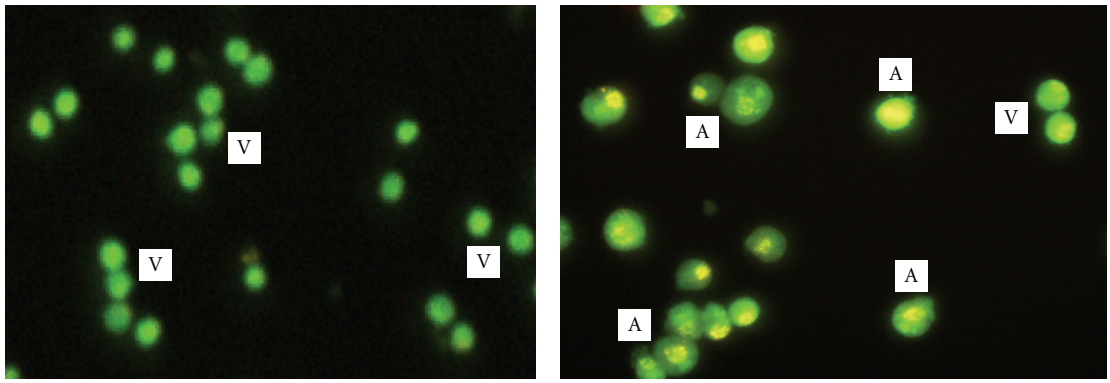


Figure 6: Effect of pyranocycloartobiloxanthone A (**1**) on the proliferation of MCF7 cells *in vitro*. After treatment with 1 μg/mL, 5 μg/mL (IC_{50} based on MTT results) and 20 μg/mL for 24, 48, and 72 hours cellular proliferation of MCF7 cells was assayed using BrdU incorporation ELISA.

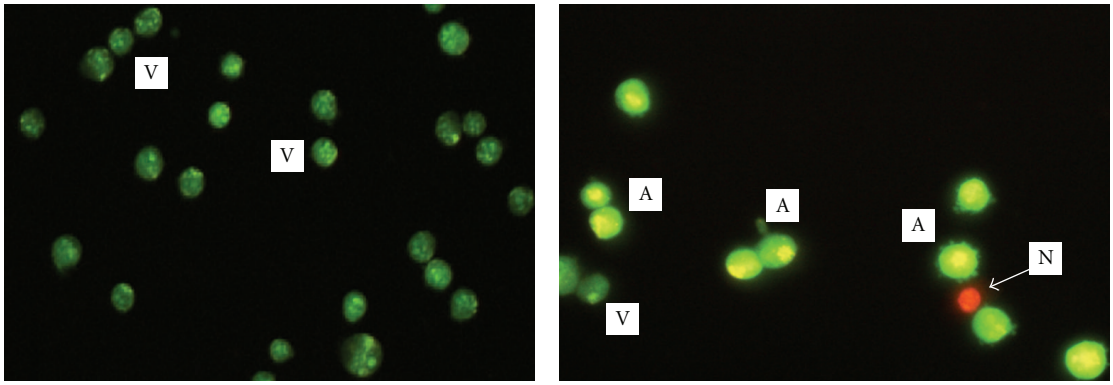
0.5–5.0 μg/mL in comparison with the other two compounds which only displayed moderate cytotoxic activities towards HL60 and MCF7 cell lines. Both compounds also failed to inhibit the growth of K562 cell line. In order to establish the potential of pyranocycloartobiloxanthone A (**1**) as a good cytotoxic agent, the compound was further tested against seven other cell lines (HeLa, MDA-MB 231, HT29, HepG2, 3T3, MCF10A, and PBMC). Due to insufficient amount of samples, compounds (**2**) and (**3**) were not further tested against these cell lines. The IC_{50} values on the sensitivity of MDA-MB 231, HT29, and HepG2 cell lines towards pyranocycloartobiloxanthone A (**1**) were obtained at 12.0, 14.0, and 20.0 μg/mL, respectively. However, the compound was not toxic towards normal cell lines, MCF10A and PBMC except for 3T3. The toxic effect of the compound displayed against 3T3 may be due to genetic changes occurred in the cell, or the compound was toxic to mouse cells. However further tests are needed to be carried out to underline the effect. The sensitivity of the compound towards the cell lines were comparable to doxorubicin used in the test.

The investigation on the potential cell proliferation inhibition activity of pyranocycloartobiloxanthone A (**1**) on HL60 and MCF7 cells was conducted due to their significant sensitivity (based from MTT assay) towards the compound. The results showed that at high and IC_{50} concentrations of pyranocycloartobiloxanthone A (**1**) on treated HL60 and MCF7 populations, the degree of cell killing or antiproliferative activity was significant, denoted by slight declined of OD value of absorbance over prolonged exposures in comparison to the untreated control population (Figures 5 and 6). The OD values for the IC_{50-} treated HL60 populations were 0.8, 0.78, and 0.66 for 24, 48, and 72 hours, respectively. On the other hand, OD values of the untreated



(a) (b)

Figure 7: Morphology features of HL60 cells incubated with pyranocycloartobiloxanthone A (**1**) after being stained with acridine orange and propidium iodide were observed under fluorescence microscope (magnification 200x) (a) untreated HL60 cells (b) treated with pyranocycloartobiloxanthone A (**1**) at 2 μg/mL (IC_{50} value), after 72 hours. Note: V: viable, A: apoptosis, and N: necrosis.



(a) (b)

Figure 8: Morphology features of MCF7 cells incubated with pyranocycloartobiloxanthone A (**1**) were observed under fluorescence microscope (magnification 200x) (a) untreated MCF7cells (b) treated with pyranocycloartobiloxanthone A (**1**) at 5 μg/mL (IC_{50} value), after 72 hours. Note: V: viable, A: apoptosis, and N: necrosis.

HL60 populations were 1.34, 1.26, and 1.65 for 24, 48, and 72 hours, respectively. Therefore, the exposure of HL60 cells to pyranocycloartobiloxanthone A (**1**) at IC_{50} concentration for 24, 48, and 72 hours resulted in a reduction from 60 to 40% of BrdU incorporation in comparison with the untreated population. A slight decline of OD values for IC_{50}–treated MCF7 populations was observed from 0.89, 0.75, and 0.66 at 24, 48, and 72 hours, respectively. In the untreated populations, the OD values were 1.44, 2.08, and 2.88 for 24, 48, and 72 hours, respectively. The exposure has resulted in reduction from 61.8 to 23% of BrdU incorporation in comparison with untreated population. As indicated above, the data exhibited the exposure time remarkably influenced on the growth of HL60 and MCF7 cells in the ELISA BrdU assay. Both cells experienced a noteworthy decline in cell proliferations compared to the control populations at any concentration as the time of exposure increased. The time course studies indicated that pyranocycloartobiloxanthone A (**1**) was sensitive to HL60 and MCF7 cells by inhibiting the cell proliferation. Hence, the inhibition of cellular proliferation for both cells treated with pyranocycloartobiloxanthone A (**1**) was consistent with the decline in cell viability from the MTT assay evaluation. Hence, the reduction of OD or viability which is observed in MTT assay may reflect cell death and/or reduced rate of proliferation.

In contrast, by utilizing AO/PI staining, we were able to determine the percentage and mode of cell death by uptake of different fluorescence dyes and morphological changes of individual cell in the treatment group. In the morphological study, pyranocycloartobiloxanthone A (**1**) was chosen for further study as it has shown significant activity against selected cancer cell lines compared to other isolated compounds. The cells, HL60 and MCF7, were treated with respective IC_{50} values, 2 μg/mL, and 5 μg/mL of pyranocycloartobiloxanthone A (**1**) after 72 hours of incubation. The cells were observed under fluorescence microscope prior AOPI staining. Both apoptotic and necrotic features were observed in HL60 and MCF7 cells population after treatment with pyranocycloartobiloxanthone A (**1**) at their respective IC_{50} dose (Figures 7 and 8). However, the compound seemed to kill both cells in apoptotic manner at IC_{50} dose, whereby upon swelling of the cells, little buds

started to appear around the cells. The buds disintegrate from the mother cell to form apoptotic bodies while the mother cell will shrunk. Cell rupture was observed after the prolonged exposure (72 hours) and resulted in the formation of cellular debris. There are several genes involved with apoptosis in mammalian cells, and the apoptotic response is determined by a balance between antiapoptotic genes (Bcl-2 and p53) and proapoptotic genes (C-myc and Bax proteins) [22]. In cancer, there is a lack of equilibrium between the rate of cell division and cell death (apoptosis), therefore, any compounds or agents that are able to promote or suppress apoptosis are potential to be developed as anticancer drugs.

The current study showed that the compounds were sensitive or toxic towards the selected cell lines, and the response of each cell lines towards the compounds was different. Differential antiproliferative activity of the compounds indicated that the cells may have different particular molecular site or receptor for selected compounds to interact, which may influence the response of the activity [22]. Pyranocycloartobiloxanthone A (**1**) exhibited poor toxicity with high IC_{50} values (>30 μg/mL) against PBMC and MCF10A cells which is a good indication that normal human cell lines may be resistant to the compound. The suggested effective doses for a 50% inhibition in cell viability for plant extracts and pure compound to be considered active according to National Cancer Institute (NCI) guidelines should be less than 20 and 4 μg/mL, respectively [23]. It is interesting to note that, pyranocycloartobiloxanthone A (**1**), at low concentration, exhibited its antiproliferative effect against most of the selected cancer cell lines. On the other hand, the other two compounds, dihydroartoindonesianin C (**2**) and pyranocycloartobiloxanthone B (**3**) require high concentrations to exert their antiproliferative effect towards HL60, K562, and MCF7 cells. This finding is possibly due to the presence of active sites in the structure of the compounds, whereby the presence of the resorcinol moiety in ring B and isoprenyl substituent at C-3 of the flavones skeleton may contribute to the interesting activity [9, 24]. Therefore, by having the above two features together in the chemical structure of pyranocycloartobiloxanthone A (**1**), they have substantiated the remarkable antiproliferative activity displayed. Surprisingly, in spite of having both active features together and also an isomer to compound (**1**), pyranocycloartobiloxanthone B (**3**) displayed lower antiproliferative activity towards the target cell lines. This may be due to the migration of the hydroxyl group from C-14 to C-13 and methyl group in the opposite direction in compound (**3**). As for dihydroartoindonesianin C (**2**), position C-3 is also substituted with isoprenyl group but without the resorcinol skeleton in the chemical structure, and this may be caused much lower antiproliferative effect than compound (**1**). These findings indicated that both resorcinol moiety in ring B and isoprenyl substituent at C-3 position must be the most important features for revealing the potent activity. Thus, the continuing phytochemical investigation of Malaysian *Artocarpus* species cannot only identify new lead compounds as anticancer agents but also provide a pool of chemicals for future biological target studies.

4. Conclusion

The extracts *of A. obtusus* were found to exhibit good cytotoxic and potential antiproliferative activity against some cell lines and further isolation work on the extracts resulted in the isolate ion of three new xanthones. The structures of the compounds were established by spectroscopic method and comparison with literature values. One of the xanthones, pyranocycloartobiloxanthone A (**1**), showed potent antiproliferative activity towards various cell lines and inactive when tested on normal cell lines. The compound (**1**) was able to induce apoptosis against HL60 and MCF7 cell lines at its respective IC_{50} values.

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Research Article

Synergistic Antibacterial Effect between Silibinin and Antibiotics in Oral Bacteria

Young-Soo Lee,[1] Kyeung-Ae Jang,[2] and Jeong-Dan Cha[2]

[1] *Department of Dental Hygiene, Sun Moon University, Asan-si 336-708, Republic of Korea*
[2] *Department of Dental Hygiene, College of Natural Sciences, Dongeui University, Busan 614-714, Republic of Korea*

Correspondence should be addressed to Jeong-Dan Cha, joungdan@deu.ac.kr

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Silibinin is a composition of the silymarin group as a hepatoprotective agent, and it exhibits various biological activities, including antibacterial activity. In this study, the antibacterial activities of silibinin were investigated in combination with two antimicrobial agents against oral bacteria. Silibinin was determined with MIC and MBC values ranging from 0.1 to 3.2 and 0.2 to 6.4 μg/mL, ampicillin from 0.125 to 64 and 0.5 to 64 μg/mL, gentamicin from 2 to 256 and 4 to 512 μg/mL, respectively. The ranges of MIC_{50} and MIC_{90} were 0.025–0.8 μg/mL and 0.1–3.2 μg/mL, respectively. The antibacterial activities of silibinin against oral bacteria were assessed using the checkerboard and time-kill methods to evaluate the synergistic effects of treatment with ampicillin or gentamicin. The results were evaluated showing that the combination effects of silibinin with antibiotics were synergistic (FIC index < 0.5) against all tested oral bacteria. Furthermore, a time-kill study showed that the growth of the tested bacteria was completely attenuated after 2–6 h of treatment with the MBC of silibinin, regardless of whether it was administered alone or with ampicillin or gentamicin. These results suggest that silibinin combined with other antibiotics may be microbiologically beneficial and not antagonistic.

1. Introduction

Dental plaque is a film of microorganisms on the tooth surface that plays an important role in the development of caries and periodontal diseases [1–3]. Corrective treatment for such infectious diseases requires the reduction and/or elimination of bacterial accumulations in the retentive sites on the top of the teeth (occlusal surfaces) and between teeth by daily toothbrushing and frequent dental cleanings or prophylaxis [4, 5]. Several antibacterial agents including fluorides, phenol derivatives, ampicillin, erythromycin, penicillin, tetracycline, and vancomycin have been used widely in dentistry to inhibit bacterial growth [6–8]. However, excessive use of these chemicals can result in derangements of the oral and intestinal flora and cause side effects such as microorganism susceptibility, vomiting, diarrhea, and tooth staining [9–11]. These problems necessitate further search for natural antibacterial agents that are safe for humans and specific for oral pathogens. Natural products have recently been investigated more thoroughly as promising agents to prevent oral diseases, especially plaque-related diseases such as dental caries [12–15].

Silymarin is a standardized extract obtained from the seeds of milk thistle (*Silybum marianum*), which contains approximately 70–80% of the silymarin flavonolignans [16–18]. Silibinin is a major bioactive component of silymarin flavonolignans [19, 20]. Both silymarin and silibinin have been used as traditional drugs for $\geq$2000 years to treat a range of liver disorders, including hepatitis and cirrhosis, and to protect the liver against poisoning from exposure to chemical and environmental toxins, including insect stings, mushroom poisoning, and alcohol [16, 20, 21]. Recently, *in vitro* and *in vivo* studies have reported that silibinin possesses antioxidant, anti-inflammatory, and antiarthritic activities, and it has chemopreventive efficacy on lung carcinoma, prostate cancer, breast carcinoma, hepatic disorder, and colon carcinoma [22–25]. In a previous study, silibinin showed antibacterial activity against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus epidermidis* [26].

In this study, we investigated the synergistic antibacterial activity of silibinin in combination with the existing antimicrobial agents against oral bacteria.

2. Materials and Methods

2.1. Bacterial Strains. The oral bacterial strains used in this study were *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Streptococcus sobrinus* ATCC 27607, *Streptococcus ratti* KCTC (Korean collection for type cultures) 3294, *Streptococcus criceti* KCTC 3292, *Streptococcus anginosus* ATCC 31412, *Streptococcus gordonii* ATCC 10558, *Actinobacillus actinomycetemcomitans* ATCC 43717, *Fusobacterium nucleatum* ATCC 10953, *Prevotella intermedia* ATCC 25611, and *Porphyromonas gingivalis* ATCC 33277. Brain-heart infusion broth supplemented with 1% yeast extract (Difco Laboratories, Detroit, Mich) was used for all bacterial strains except *P. intermedia* and *P. gingivalis*. For *P. intermedia* and *P. gingivalis*, brain-heart infusion broth containing hemin and menadione was used.

2.2. Minimum Inhibitory Concentrations/Minimum Bactericidal Concentrations Assay. The minimum inhibitory concentrations (MICs) were determined for silibinin by the broth dilution method [15] and were carried out in triplicate. The antibacterial activities were examined after incubation at 37°C for 18 h (facultative anaerobic bacteria), 24 h (microaerophilic bacteria), and 1-2 days (obligate anaerobic bacteria) under anaerobic conditions. MICs were determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. Following anaerobic incubation of MICs plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of silibinin that kills 99.9% of the test bacteria by plating out onto each appropriate agar plate. Ampicillin and gentamicin were used as standard antibiotics in order to compare the sensitivity of silibinin against test bacteria.

2.3. Checker-Board Dilution Test. The antibacterial effects of a combination of silibinin, which exhibited the highest antimicrobial activity, and antibiotics were assessed by the checkerboard test as previously described [15]. The antimicrobial combinations assayed included silibinin with ampicillin or gentamicin. Serial dilutions of two different antimicrobial agents were mixed in cation-supplemented Mueller-Hinton broth. After 24 h of incubation at 37°C, the MIC was determined to be the minimal concentration at which there was no visible growth. The fractional inhibitory concentration index (FICI) is the sum of the FICs of each of the drugs, which in turn is defined as the MIC of each drug when it is used in combination divided by the MIC of the drug when it is used alone. The interaction was defined as synergistic if the FIC index was less than or equal to 0.5, additive if the FIC index was greater than 0.5 and less than or equal to 1.0, indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC index was greater than 2.0.

2.4. Time-Kill Curves. Bactericidal activities of the drugs under study were also evaluated using time-kill curves on oral bacteria. Tubes containing Mueller-Hinton supplemented to which antibiotics had been added at concentrations of the MIC_{50} were inoculated with a suspension of the test strain, giving a final bacterial count $5\sim6 \times 10^6$ CFU/mL. The tubes were thereafter incubated at 37°C in an anaerobic chamber, and viable counts were performed at 0, 0.5, 1, 2, 3, 4, 5, 6, 12, and 24 h after addition of antimicrobial agents, on agar plates incubated for up to 48 h in anaerobic chamber at 37°C. Antibiotic carryover was minimized by washings by centrifugation and serial 10-fold dilution in sterile phosphate-buffered saline, pH 7.3. Colony counts were performed in duplicate, and means were taken. The solid media used for colony counts were brain-heart infusion (BHI) agar for streptococci and brain-heart infusion agar containing hemin and menadione for *P. intermedia* and *P. gingivalis*.

3. Results and Discussion

The antibacterial activities and synergistic effects of silibinin alone or with antibiotics were evaluated in oral bacteria. The antibacterial activities of the ATCC and KCTC strains of oral bacteria to silibinin, ampicillin, and gentamicin alone and in combination are presented in Table 1. The MICs/MBCs for silibinin were found to be either 0.1/0.2 or 3.2/6.4 μg/mL, for ampicillin either 0.125/0.5 or 64/64 μg/mL, and for gentamicin, either 2/4 or 256/512 μg/mL. Silibinin MIC_{50} and MIC_{90} values for oral cariogenic bacteria were 0.025–0.2 μg/mL and 0.1–0.8 μg/mL, respectively, while for periodontopathogenic bacteria these values were 0.1–0.4 μg/mL and 0.4–3.2 μg/mL, respectively (Table 1).

In combination with silibinin, the MIC for ampicillin was reduced to ≥4–8-fold in all tested bacteria, producing a synergistic effect as defined by FICI ≤ 0.5. The MBC for ampicillin has shown synergistic effects in all tested bacteria expect *S. sanguinis*, *S. ratti*, and *P. intermedia* (Table 2). In combination with silibinin, the MIC/MBC for gentamicin was reduced to ≥4–8-fold in all tested bacteria expect *S. sanguinis* and *S. ratti* by FICI ≥ 0.75 (Table 3). Many articles have revealed that Gram-positive bacteria was more sensitive to plant antimicrobials than Gram-negative bacteria, suggesting that the results are due to the difference between the presence and absence of the outer membrane which can limit drug diffusion in harmony with multidrug transporters [15, 27–29]. In this study, silibinin also shows susceptibility on Gram-positive bacteria as well as Gram-negative bacteria. Many attempts have been made to eliminate *S. mutans* from the oral flora [30]. Antibiotics such as ampicillin, chlorhexidine, erythromycin, penicillin, tetracycline, and vancomycin have been very effective in preventing dental caries [6, 31, 32]. Moreover, the antifungal activities have shown that neither silibinin nor silymarin II had antifungal activity against yeast [30]. The Gram-positive bacteria-specific properties of silibinin are caused

Table 1: Antibacterial activity of silibinin and antibiotics in oral bacteria.

Samples	Silibinin (μg/mL)			Ampicillin	Gentamicin
	$MIC_{50<}$	$MIC_{90<}$	MIC/MBC	MIC/MBC (μg/mL)	
S. mutans ATCC 25175[1]	0.05	0.2	0.2/0.4	0.125/0.5	8/16
S. sanguinis ATCC 10556	0.1	0.4	0.4/0.4	0.5/1	64/64
S. sobrinus ATCC 27607	0.1	0.4	0.4/0.8	0.5/1	4/8
S. ratti KCTC 3294[2]	0.2	0.8	0.8/0.8	0.5/1	16/32
S. criceti KCTC 3292	0.2	0.8	0.8/1.6	1/2	8/16
S. anginosus ATCC 31412	0.1	0.8	0.8/1.6	1/2	32/32
S. gordonii ATCC 10558	0.025	0.1	0.1/0.2	0.5/1	32/32
A. actinomycetemcomitans ATCC 43717	0.4	1.6	1.6/3.2	64/64	4/8
F. nucleatum ATCC 51190	0.4	3.2	3.2/6.4	2/4	2/4
P. intermedia ATCC 49049	0.4	1.6	1.6/3.2	4/8	16/32
P. gingivalis ATCC 33277	0.1	0.4	0.4/0.8	0.5/1	256/512

[1] American Type Culture Collection (ATCC).
[2] Korean collection for type cultures (KCTC).

Table 2: Synergistic effects of the silibinin with ampicillin against oral bacteria.

Strains	Agent	MIC/MBC (μg/mL)		FIC	FICI[2]	Outcome
		Alone	Combination[1]			
S. mutans ATCC 25175[3]	Silibinin	0.2/0.4	0.05/0.1	0.25/0.25	0.5/0.5	Synergistic/synergistic
	Ampicillin	0.125/0.5	0.0312/0.125	0.25/0.25		
S. sanguinis ATCC 10556	Silibinin	0.4/0.4	0.1/0.2	0.25/0.5	0.5/0.75	Synergistic/additive
	Ampicillin	0.5/1	0.125/0.25	0.25/0.25		
S. sobrinus ATCC 27607	Silibinin	0.4/0.8	0.1/0.2	0.25/0.25	0.5/0.5	Synergistic/synergistic
	Ampicillin	0.5/1	0.125/0.25	0.25/0.25		
S. ratti KCTC 3294[4]	Silibinin	0.8/0.8	0.1/0.2	0.125/0.25	0.375/0.75	Synergistic/additive
	Ampicillin	0.5/1	0.125/0.5	0.25/0.5		
S. criceti KCTC 3292	Silibinin	0.8/1.6	0.2/0.4	0.25/0.25	0.5/0.375	Synergistic/synergistic
	Ampicillin	1/2	0.25/0.25	0.25/0.125		
S. anginosus ATCC 31412	Silibinin	0.8/1.6	0.2/0.4	0.25/0.25	0.5/0.5	Synergistic/synergistic
	Ampicillin	1/2	0.25/0.5	0.25/0.25		
S. gordonii ATCC 10558	Silibinin	0.1/0.2	0.025/0.05	0.25/0.25	0.5/0.5	Synergistic/synergistic
	Ampicillin	0.5/1	0.125/0.25	0.25/0.25		
A. actinomycetemcomitans ATCC 43717	Silibinin	1.6/3.2	0.2/0.8	0.125/0.25	0.25/0.5	Synergistic/synergistic
	Ampicillin	64/64	8/16	0.125/0.25		
F. nucleatum ATCC 51190	Silibinin	3.2/6.4	0.4/1.6	0.125/0.25	0.375/0.5	Synergistic/synergistic
	Ampicillin	2/4	0.5/1	0.25/0.25		
P. intermedia ATCC 49049	Silibinin	1.6/3.2	0.2/0.8	0.125/0.25	0.375/0.75	Synergistic/additive
	Ampicillin	4/8	1/4	0.25/0.5		
P. gingivalis ATCC 33277	Silibinin	0.4/0.8	0.1/0.2	0.25/0.25	0.5/0.5	Synergistic/synergistic
	Ampicillin	0.5/1	0.125/0.25	0.25/0.25		

[1] The MIC and MBC of the silibinin with ampicillin.
[2] The fractional inhibitory concentration index (FIC index).
[3] American Type Culture Collection (ATCC).
[4] Korean collection for type cultures (KCTC).

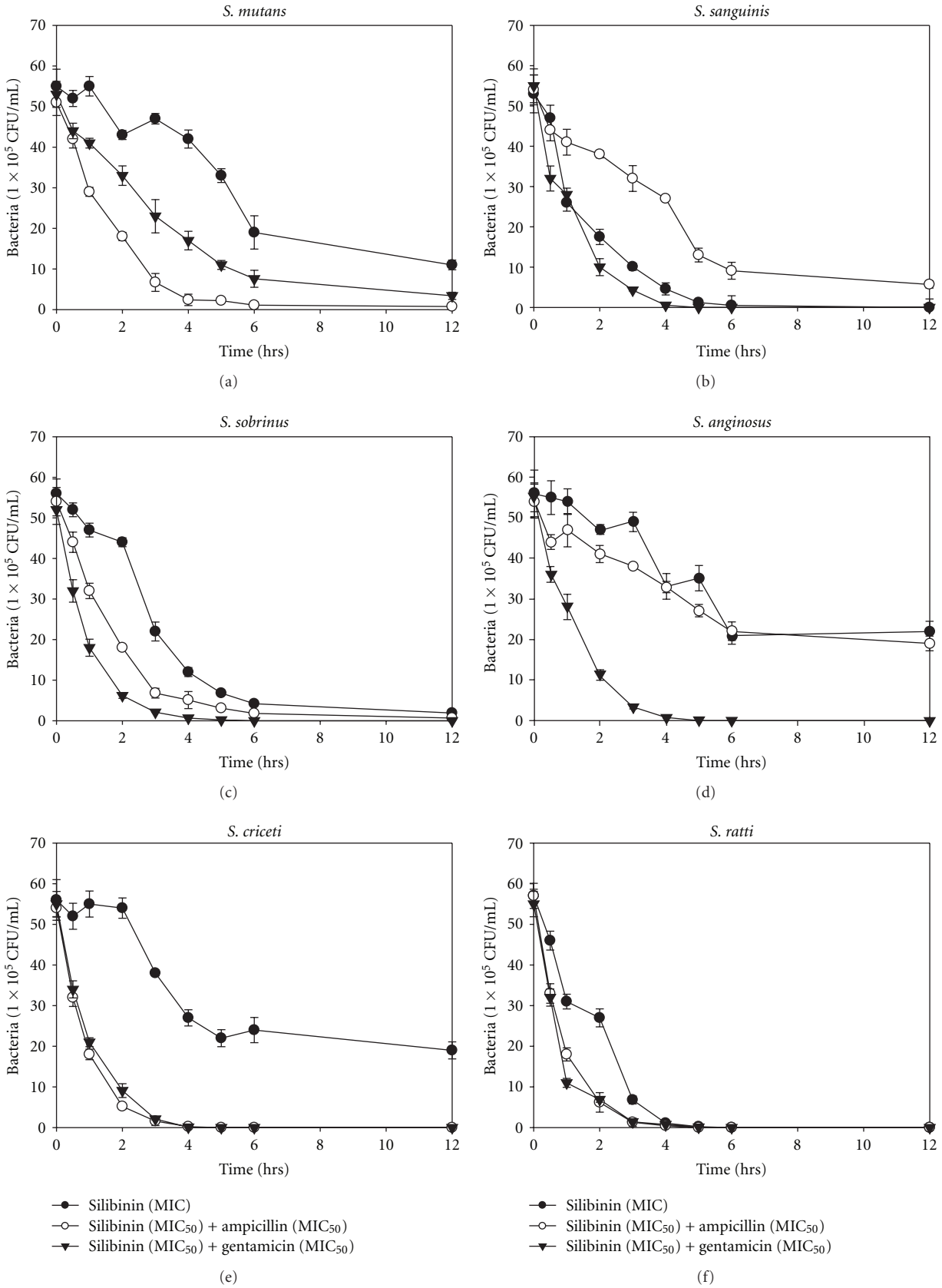

Figure 1: Time-kill curves of MIC of silibinin alone and its combination with MIC_{50} of ampicillin or gentamicin against *S. mutans*, *S. sanguinis*, *S. sobrinus*, *S. anginosus*, *S. criceti*, and *S. ratti*. Bacteria were incubated with silibinin (•), silibinin + ampicillin (◦), and silibinin + gentamicin (▼) over time. Data points are the mean values ± S.E.M. of six experiments. CFU: colony-forming units.

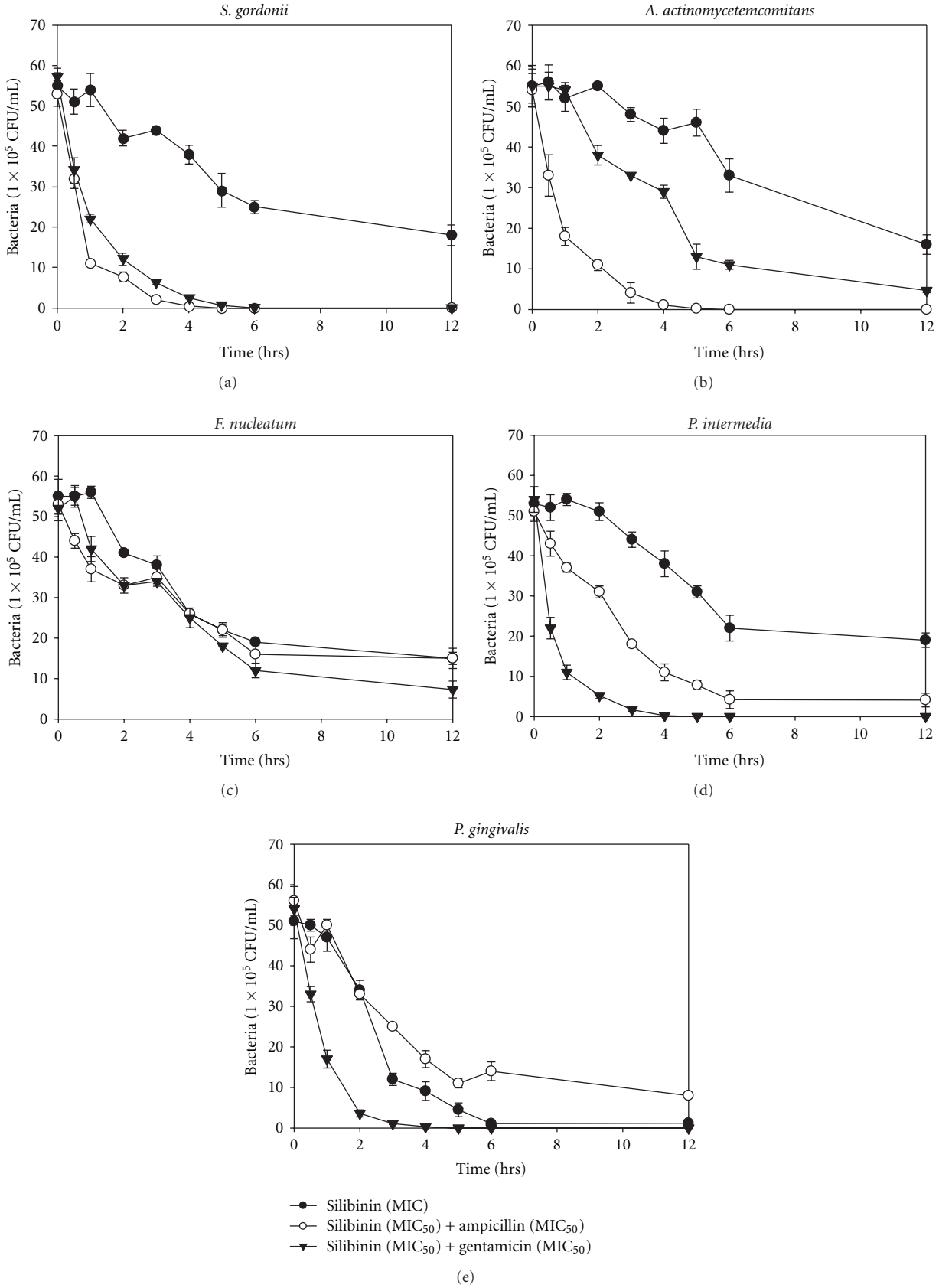

Figure 2: Time-kill curves of MIC of silibinin alone and its combination with MIC_{50} of Amp or Gen against *S. gordonii*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia,* and *P. gingivalis*. Bacteria were incubated with silibinin (•), silibinin + Amp (◦), and silibinin + Gen (▼) over time. Data points are the mean values ± S.E.M. of six experiments. CFU: colony-forming units.

Table 3: Synergistic effects of the silibinin with gentamicin against oral bacteria.

Strains	Agent	MIC/MBC (μg/mL)		FIC	FICI[2]	Outcome
		Alone	Combination[1]			
S. mutans ATCC 25175[3]	Silibinin	0.2/0.4	0.05/0.1	0.25/0.25	0.5/0.5	Synergistic/synergistic
	Gentamicin	8/16	2/4	0.25/0.25		
S. sanguinis ATCC 10556	Silibinin	0.4/0.4	0.1/0.2	0.25/0.5	0.375/0.75	Synergistic/additive
	Gentamicin	64/64	8/16	0.125/0.25		
S. sobrinus ATCC 27607	Silibinin	0.4/0.8	0.05/0.2	0.125/0.25	0.375/0.5	Synergistic/synergistic
	Gentamicin	4/8	1/2	0.25/0.25		
S. ratti KCTC 3294[4]	Silibinin	0.8/0.8	0.2/0.4	0.25/0.5	0.5/0.625	Synergistic/additive
	Gentamicin	16/32	4/4	0.25/0.125		
S. criceti KCTC 3292	Silibinin	0.8/1.6	0.2/0.4	0.25/0.25	0.375/0.375	Synergistic/synergistic
	Gentamicin	8/16	1/2	0.125/0.125		
S. anginosus ATCC 31412	Silibinin	0.8/1.6	0.2/0.2	0.25/0.125	0.375/0.375	Synergistic/synergistic
	Gentamicin	32/32	4/8	0.125/0.25		
S. gordonii ATCC 10558	Silibinin	0.1/0.2	0.025/0.05	0.25/0.25	0.375/0.5	Synergistic/synergistic
	Gentamicin	32/32	4/8	0.125/0.25		
A. actinomycetemcomitans ATCC 43717	Silibinin	1.6/3.2	0.4/0.8	0.25/0.25	0.5/0.5	Synergistic/synergistic
	Gentamicin	4/8	1/2	0.25/0.25		
F. nucleatum ATCC 51190	Silibinin	3.2/6.4	0.8/1.6	0.25/0.25	0.5/0.5	Synergistic/synergistic
	Gentamicin	2/4	0.5/1	0.25/0.25		
P. intermedia ATCC 25611	Silibinin	1.6/3.2	0.4/0.8	0.25/0.25	0.5/0.375	Synergistic/synergistic
	Gentamicin	16/32	4/4	0.25/0.125		
P. gingivalis ATCC 33277	Silibinin	0.8/0.8	0.1/0.2	0.125/0.25	0.375/0.375	Synergistic/synergistic
	Gentamicin	256/512	64/64	0.25/0.125		

[1]The MIC and MBC of the silibinin with gentamicin.
[2]The fractional inhibitory concentration index (FIC index).
[3]American Type Culture Collection (ATCC).
[4]Korean collection for type cultures (KCTC).

by the inhibition of RNA and protein synthesis rather than by attacking the bacterial membrane [25, 26]. The bacterial effect of silibinin with ampicillin or gentamicin against oral bacteria was confirmed by time-kill curve experiments. The silibinin (MIC or MIC_{50}) alone resulted in a rate of killing increasing or not changing in CFU/mL at time-dependent manner, with a more rapid rate of killing by silibinin (MIC_{50}) with ampicillin (MIC_{50}) or gentamicin (MIC_{50}) (Figures 1 and 2). A strong bactericidal effect was exerted in drug combinations.

4. Conclusion

These findings suggest that silibinin fulfills the conditions required of a novel cariogenic bacteria and periodontal pathogens, particularly bacteroides species, drug and may be useful in the future in the treatment of oral bacteria.

Acknowledgments

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Research Article

Biocompatibility Evaluation of a New Hydrogel Dressing Based on Polyvinylpyrrolidone/Polyethylene Glycol

Esmaeil Biazar,[1] Ziba Roveimiab,[2] Gholamreza Shahhosseini,[3] Mohammadreza Khataminezhad,[4] Mandana Zafari,[5] and Ali Majdi[5]

[1] *Department of Chemistry, Tonekabon branch, Islamic Azad University, Tonekabon, Iran*
[2] *Young Researchers Club, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[3] *Nuclear Agriculture Research Department, Agricultural, Medical and Industrial Research School, Nuclear Science and Technology Institute, Iran*
[4] *Department of Microbiology, Tonekabon branch, Islamic Azad University, Tonekabon, Iran*
[5] *Young Researchers Club, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran*

Correspondence should be addressed to Esmaeil Biazar, e.biazar@tonekaboniau.ac.ir

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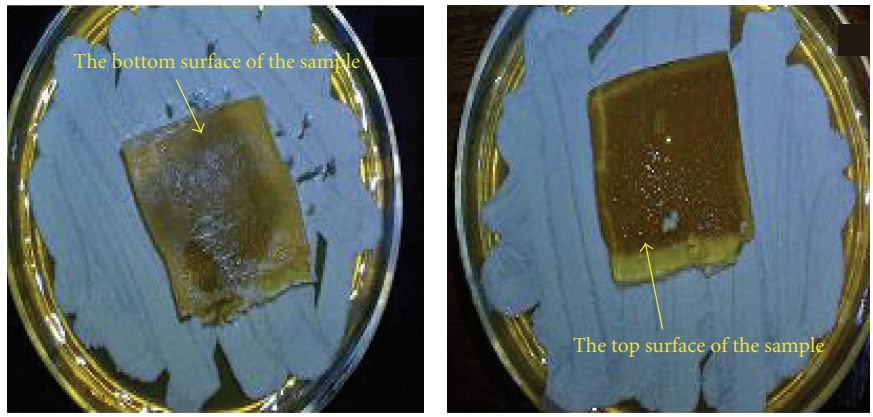
The composition of the dressings is based on polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), and agar. The electron beam irradiation technique has been used to prepare hydrogel wound dressings. The *in vitro* biocompatibility of the hydrogel was investigated by check samples (hydrocolloid Comfeel), antibacterial test (*Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia Coli* k12), anti fungal test (*Candida Albicans*) and cytotoxicity test (Fibroblast L929). Results have shown cell attachment characteristics and nontoxicity of all samples. Antibacterial testing also showed that the antibacterial effect of the hydrogel sample to the check sample increased to 30%. Also, investigation of antifungal analysis did not show any trace of fungi growth on the surface of the hydrogel, whereas antifungal effect did not observe on the surface of the check sample. Finally, this hydrogel sample showed a good *in vitro* biocompatibility.

1. Introduction

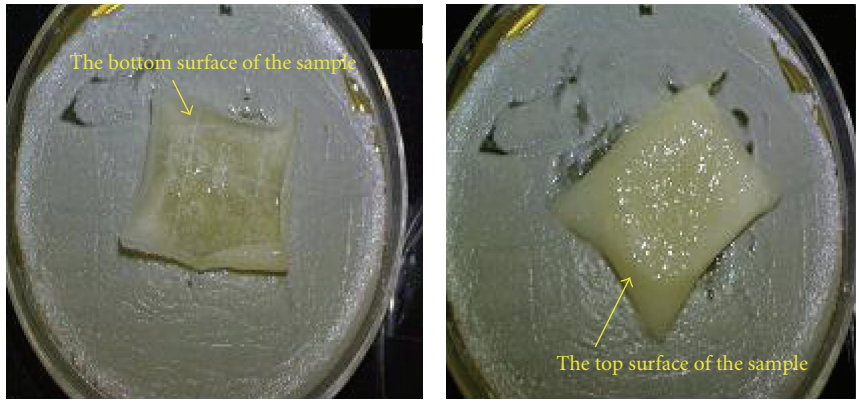
Bed sore is considered a wound resulted due to exerting high and protracted capillary pressures on the surface of the skin long time and can lead to the necrosis of the soft tissues [1, 2]. Among of these necrotic areas the pressure ulcers, burnt ulcers, as well as diabetic wounds can be mentioned [3, 4]. One way for healing these wounds is using of dressings. Conventional dressings like gauze and cotton have been increasingly applied. Modern dressing has been used in the past two decades provided the humid healing of the wounds [5]. They have possessed most of the characteristics of an ideal dressing. Transparent dressing [6], hydrogel [7], alginate [8], and foam and hydrocolloids [9] are some examples of dressing for wound healing. Hydrogels are provided in two shape planes and shapeless gels. Hydrogels contain large amounts of water and the jelly substance constructed the polymer network [10, 11]. Other examples of hydrogels are polyethylene oxide or polyvinyl pyrrolidone, carboxyl methyl cellulose, alginate, collagen, and other materials [11]. Also, PEG and PVP are hydrogel that can be used as wound dressing [12].

In vitro biocompatibility tests of dressings include cytotoxicity and antimicrobial tests (antibacterial and antifungal). Also, in vivo tests include irritation, sensitization, implantation, acute and chronic toxicity, and systemic toxicity. Fungi and bacteria are the resistant factors for fast wound healing. In our previous work [13], the application of the electron accelerator (Rhodotron TT200) for preparation of hydrogel dressings with polyvinyl pyrrolidone, polyethylene glycol, agar, and water composition was investigated. The effect of some parameters such as gel fraction and maximum swelling on the properties of the dressing demonstrated that hydrogel has the proper physical and mechanical properties. In this research, in vitro biocompatibility of hydrogel samples has been compared with the hydrocolloid (Coloplast Ltd.



(a) (b)

Figure 1: Hydrogel sample: (a) The bottom surface of the sample no growth was detected; (b) The top surface which showed the lack of full growth of fungi.



(a) (b)

Figure 2: Transparent Comfeel sample: (a) The bottom surface which showed the growth of fungi; (b) The top surface where fungi has completely grown on it.

Comfeel plus Hydrocolloid dressing, England) as check and control sample through standard cytotoxicity (ISO 10993), antibacterial, and antifungal tests.

2. Experimental

2.1. Materials. The hydrogel samples (PEG, PVP, agar, and water) have been prepared by the Radiation Processing Center in Yazd as follows [13]. PVP (BASF, MW 1/4, 90000), PEG (BASF, MW 1/4, 200), agar (Difco) and water have been used to prepare the hydrogel. First, an aqueous solution of these materials has been prepared, and then, a homogeneous solution has been formed by solving and mixing the materials in a constant temperature. The solutions have been formed as wound dressings in the molds. After cooling down the solution, a gel structure with high viscosity has been manufactured. Gel samples are irradiated under a proper dose (600 kGy/min) and radiation energy of 10 MeV in Electron Accelerator for crosslinking. Finally, the samples have been sterilized under an appropriate dose of radiation. To assess the cytotoxicity of hydrogel samples, check sample (Comfeel: hydrocolloids contain CMC and calcium alginate; NHS: ELM351 Coloplast, Britain ltd.), polystyrene control (TCPS) and fibroblast cells (L929) were used. The *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* bacteria (Iran Pastor Institute), and *Candida albicans* fungi (Iran Pastor Institute) were used to assess antimicrobial effect.

2.2. Antifungal Analysis. A cell suspension was prepared by *C. albicans* and 8–10 cc physiologic serum in hemolysis tube. 64230 cells per each microliter of the suspension were put on the neobar lam. Then, a part of this suspension was poured into the petridish and cultured on the Sabroe Dextrose agar (SDA) with 0.05% chloramphenicol. The wound dressings of Comfeel and the hydrogel samples (20 samples with the best physical properties) were cut out (1.5×1.5 cm) and placed on the culture media. *C. albicans* was placed on the (SDA) media along with dressing in the incubator at 35°C for 3–5 days.

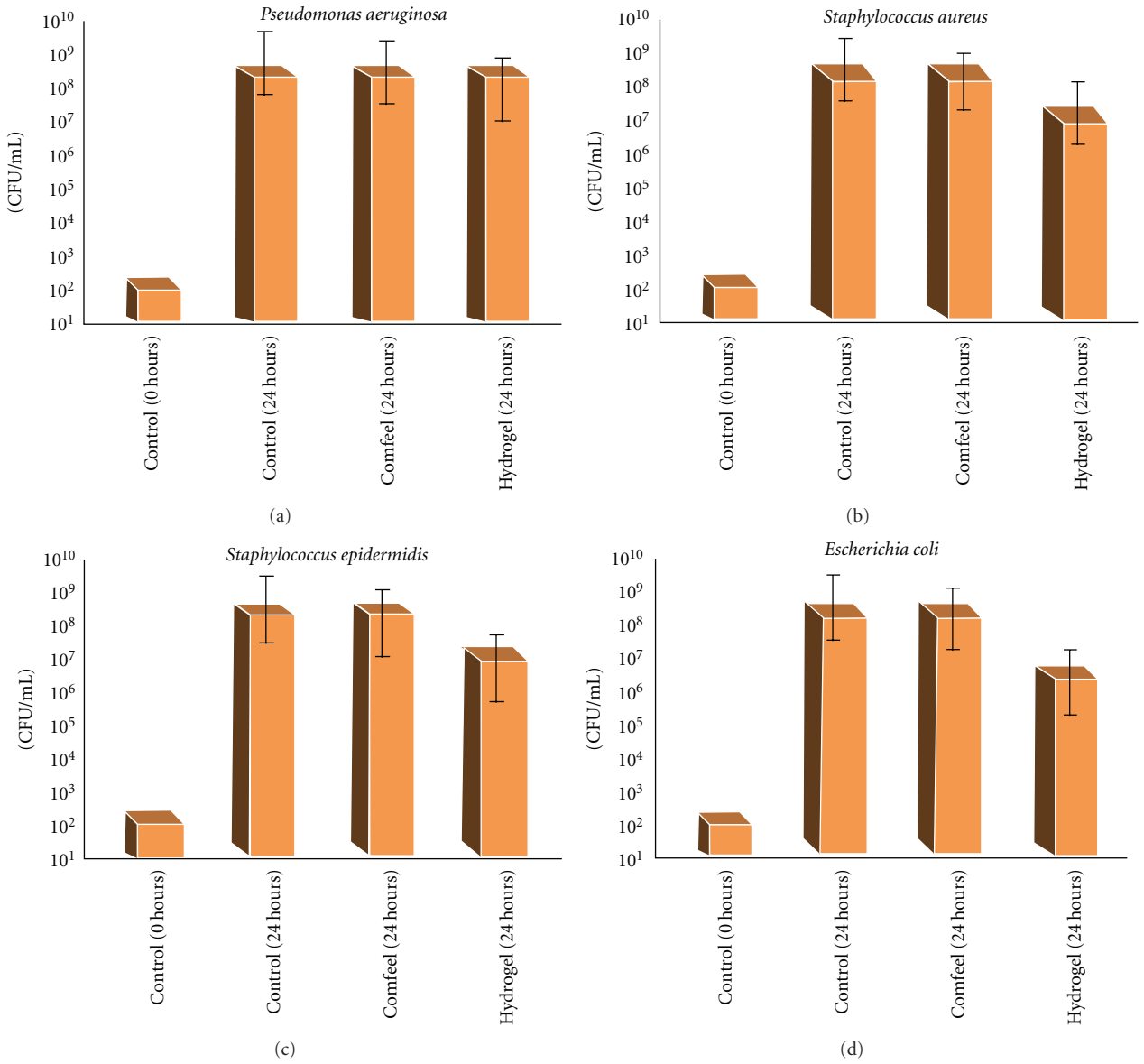
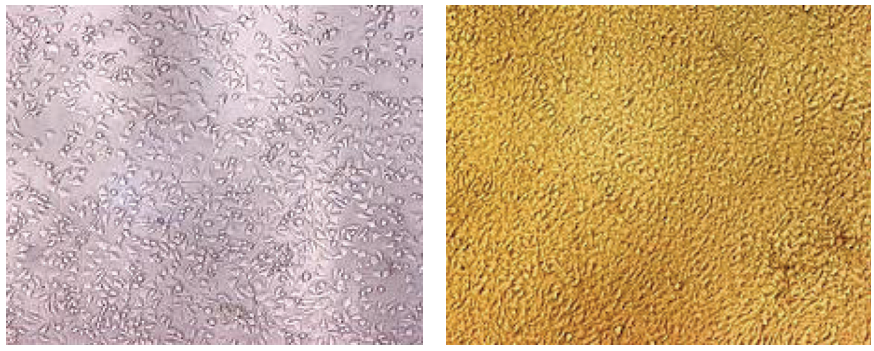
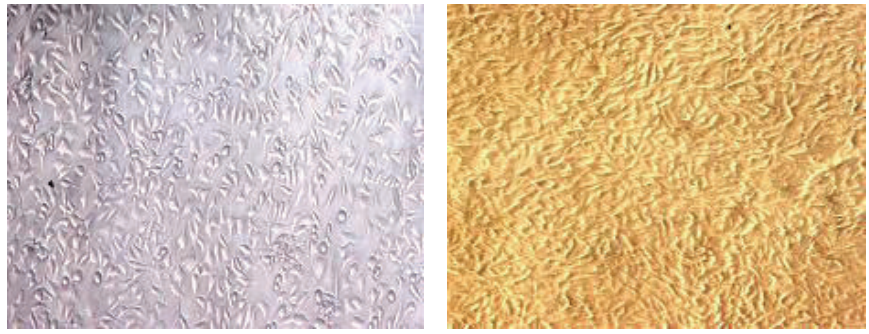


Figure 3: The number of microorganisms, control (a) after 0 hours, control (b) after 24 hours, comfeel (c) after 24 hours, and hydrogel (d) after 24 hours.



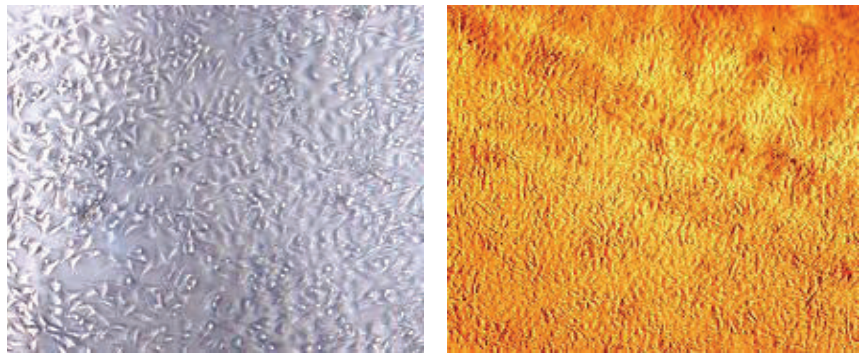
(a) (b)

Figure 4: Fibroblast cells growth on the control sample (TCPS) (100×): (a) After 24 hours; (b) After 48 hours.



(a) (b)

Figure 5: Fibroblast cells growth on the (Comfeel) control sample (100×): (a) After 24 hours; (b) After 48 hours.



(a) (b)

Figure 6: Fibroblast cells growth on the hydrogel sample (100×): (a) After 24 hours; (b) After 48 hours.

2.3. Antibacterial Analysis. A suspension of each four bacteria (*S.epidermidis*, *S.aureus*, *P. aeruginosa*, and *E. coli* K12) was prepared in broth from fresh colonies after overnight incubation, and the turbidity was adjusted to the 0.5 McFarland standard (1.5×10^8 c.f.u./mL). A part of this bacterial suspension (10 mL) was added to each vial containing the dressing. Control broths with bacterial inoculation were also included. The vials were then incubated with agitation at 35°C in a water bath. 10 mL of the bacterial broth were sampled from each vial at specific time intervals (0, 24 h), and serial 10-fold dilutions for each aliquot were prepared in broth. Duplicate aliquots (25 mL) of each of the serially diluted samples were spread on plates. The plates were then incubated overnight at 35°C and colonies counted (c.f.u./mL). The dilutions that allowed quantification (10–150 colonies) were counted, and the mean counts calculated. vials, containing the antimicrobial dressings as well as the control dressing (Comfeel sample) together with the culture and the broth controls, were included in each experiment for each organism. Plate counts were measured in triplicate, and each experiment was repeated three times to obtain a mean value of c.f.u. counts. Results were evaluated by K square statistical analysis ($P < 0.05$).

2.4. Cytotoxicity Analysis. The fibroblast cell suspension (L929) was prepared from the mouse tail. The surface of the Comfeel sample (check) was well cleaned by using cotton and alcohol as well as the hydrogel samples. Pieces (0.5 × 0.5 cm) of Comfeel and the main sample were cut and placed in one of the Petri dish wall (each one separately) by using a sterilized pincer. 3 cc of the cell suspension was removed by pipette and poured on the Comfeel and the main samples. Then, all of the samples were placed in the Memmert incubator at 37°C for 24 and 48 hours, separately. The samples in the polystyrene Petri dish were removed from the incubator after 24 and 48 hours and were studied by Nikon Eclipse Ts-100 photonic microscope (100×).

3. Results and Discussion

3.1. Antifungal Analysis. The results of the growth effect of Candida albicans fungi onto two hydrogel samples containing polyvinyl pyrrolidone, polyethylene glycol, agar, water, and the Comfeel as check sample are provided as follow.

The results in Table 1 have shown that fungi clearly grow onto the Comfeel surface (control sample), due to the lack of antifungal effect, but fungal growth is negative for hydrogel sample. Also, the results of the fungi growth on the check and hydrogel sample are shown in Figures 1 and 2, and they confirmed the results of Table 1. Figures 1(a) and 1(b) showed the bottom and the top surface of hydrogel sample

Table 1: Antifungal analysis results.

Sample	*C. albicans*									
	1	2	3	4	5	6	7	8	9	10
Hydrogel sample	−	−	−	−	−	−	−	−	−	−
Comfeel sample	+	+	+	+	+	+	+	+	+	+

K Square analysis: $P < 0.05$.

effect on fungi growth, respectively. Also, Figures 2(a) and 2(b) showed the bottom and the top surface of the control sample effect on fungi growth, respectively, as well.

Figure 1 revealed the hydrogel sample with the lack of full growth of fungi on the surface of the hydrogel (Figure 1(a) showed only some fungi on the surface). Figures 2(a) and 2(b) demonstrated the full growth of fungi onto the surface of Comfeel (check sample).

3.2. Antibacterial Analysis. The bactericidal activities of the antimicrobial dressings against the *S.epidermidis*, *S.aureus*, *P. aeruginosa*, and *E. coli* K12 was indicated by a reduction in bacterial counts presented as $\log_{10}$ c.f.u. (colony forming units) mL^{-1} over time. The Results have shown that the hydrogel sample relative to the check sample was able to remove up to 30% of *S. epidermidis*, *S. aureus,* and *E. coli* K12 after 24 hours. But on *P. aeruginosa,* the situation was different; *P. aeruginosa* possess an increasing ability to resist antibacterial agent and Its intrinsically resistant to antimicrobial agents might be because of its low permeability of its cell wall and Its genetic capacity to express a wide repertoire of resistance mechanisms so no effect was detected on *P. aeruginosa*. Antibacterial test of two samples revealed a better biocompatibility of the hydrogel sample in comparison with Comfeel check sample that show in the Figure 3. Antibacterial test of two samples revealed a better biocompatibility of the hydrogel sample in comparison with Comfeel check sample.

3.3. Cytotoxicity Analysis. The cytotoxicity of hydrogel, Comfeel check sample and polystyrene control Petri dish were studied by fibroblast cells (1929) of mouse tail. Figure 4 showed the fibroblast cells growth on the polystyrene control Petri dish (TCPS). The growth of fibroblast cells on the Comfeel and hydrogel samples are shown in Figures 5 and 6.

Figures 4(a) and 4(b) clearly demonstrated the fibroblast cells growth on the polystyrene control Petri dish (TCPS). As shown in Figures 5 and 6, the fibroblast cells are well clung and grown on the check (Comfeel) and hydrogel samples did not significantly differ to control sample. These results have shown a proper biocompatibility and viability of the hydrogel sample.

4. Conclusion

In this study, the hydrogel biocompatibility was compared with Comfeel sample. *In vitro* biocompatibility assessments included cytotoxicity, antifungal, and antibacterial tests, which were the most important *in vitro* tests for bed sore wounds dressings. According to the observations of the cytotoxicity analysis, both of the hydrogel and the check samples showed good biocompatibility. It means that attachment or viability cells of both hydrogel and check sample are appropriate. The results of antibacterial test showed a rather better biocompatibility of the hydrogel sample than the Comfeel check sample. Also, observations of antifungal test demonstrated the lack of attachment and fungal growth on the surface of the hydrogel sample unlike the Comfeel check sample.

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Research Article

Protection of SH-SY5Y Neuronal Cells from Glutamate-Induced Apoptosis by 3,6′-Disinapoyl Sucrose, a Bioactive Compound Isolated from Radix Polygala

Yuan Hu,[1] Jie Li,[2] Ping Liu,[1] Xu Chen,[3] Dai-Hong Guo,[1] Qing-Shan Li,[3] and Khalid Rahman[4]

[1] *Department of Clinical Pharmacology and Pharmacy, Center of Pharmacy, Chinese PLA General Hospital, Beijing 100853, China*
[2] *Department of Obstetrics and Gynecology, Chinese PLA General Hospital, Beijing 100853, China*
[3] *School of Pharmaceutical Science, Shanxi Medical University, Taiyuan 030001, China*
[4] *Faculty of Science, School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK*

Correspondence should be addressed to Ping Liu, liupingpla@126.com

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The neuroprotective effects of 3,6′-disinapoyl sucrose (DISS) from Radix Polygala against glutamate-induced SH-SY5Y neuronal cells injury were evaluated in the present study. SH-SY5Y neuronal cells were pretreated with glutamate (8 mM) for 30 min followed by cotreatment with DISS for 12 h. Cell viability was determined by (3,4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assay, and apoptosis was confirmed by cell morphology and flow cytometry assay, evaluated with propidium iodide dye. Treatment with DISS (0.6, 6, and 60 μmol/L) increased cell viability dose dependently, inhibited LDH release, and attenuated apoptosis. The mechanisms by which DISS protected neuron cells from glutamate-induced excitotoxicity included the downregulation of proapoptotic gene Bax and the upregulation of antiapoptotic gene Bcl-2. The present findings indicated that DISS exerts neuroprotective effects against glutamate toxicity, which might be of importance and contribute to its clinical efficacy for the treatment of neurodegenerative diseases.

1. Introduction

Although neurological insults are diverse in nature, there are common mechanisms of cell injury, and glutamate toxicity plays an integral role in a variety of neurobiology of disorders such as Parkinson's disease, Alzheimer's disease, epilepsy ischemic stroke, anxiety, and depression [1, 2]. Glutamate is the major fast excitatory neurotransmitter in the mammalian central nerve system. Thus far, a distinct glutamate-induced cell death pathway has been identified. The excitotoxic pathway relies on hyperactivation of glutamate receptors [3]. Besides, it has been proposed that the combination of antidepressant drugs that elevate the noradrenergic neurotransmission with drugs that modify the glutamatergic system could be an option in treating depression [4].

3,6′-disinapoyl sucrose (DISS, Figure 1(a)) is the active oligosaccharide ester component found in the root of *Polygala tenuifolia* Willd (Radix Polygala). Recorded as "YuanZhi" in the Pharmacopoeia of the People's Republic of China, the root has been used in traditional medicine as, among other things, an expectorant, tonic, tranquillizer, and antipsychotic agent. Results from previous studies indicated that DISS had notable antidepressant effects in pharmacological depression models, an action closely related to the potentiation of central 5-hydroxytryptamine (5-HT) and norepinephrine (NE) systems [5]. Our recent study also found that DISS increased expression in the hippocampus of three noradrenergic-regulated plasticity genes (laminin, CAM-L1, and CREB) and one neurotrophic factor (BDNF) [6]. Based on our results, we hypothesized that DISS might have neuroprotective property. Thus, the authors used the human neuroblastoma SH-SY5Y cell line, with a low degree of differentiation and metabotropic glutamate receptors [7], utilized glutamate as an insult to induce SH-SY5Y neuronal

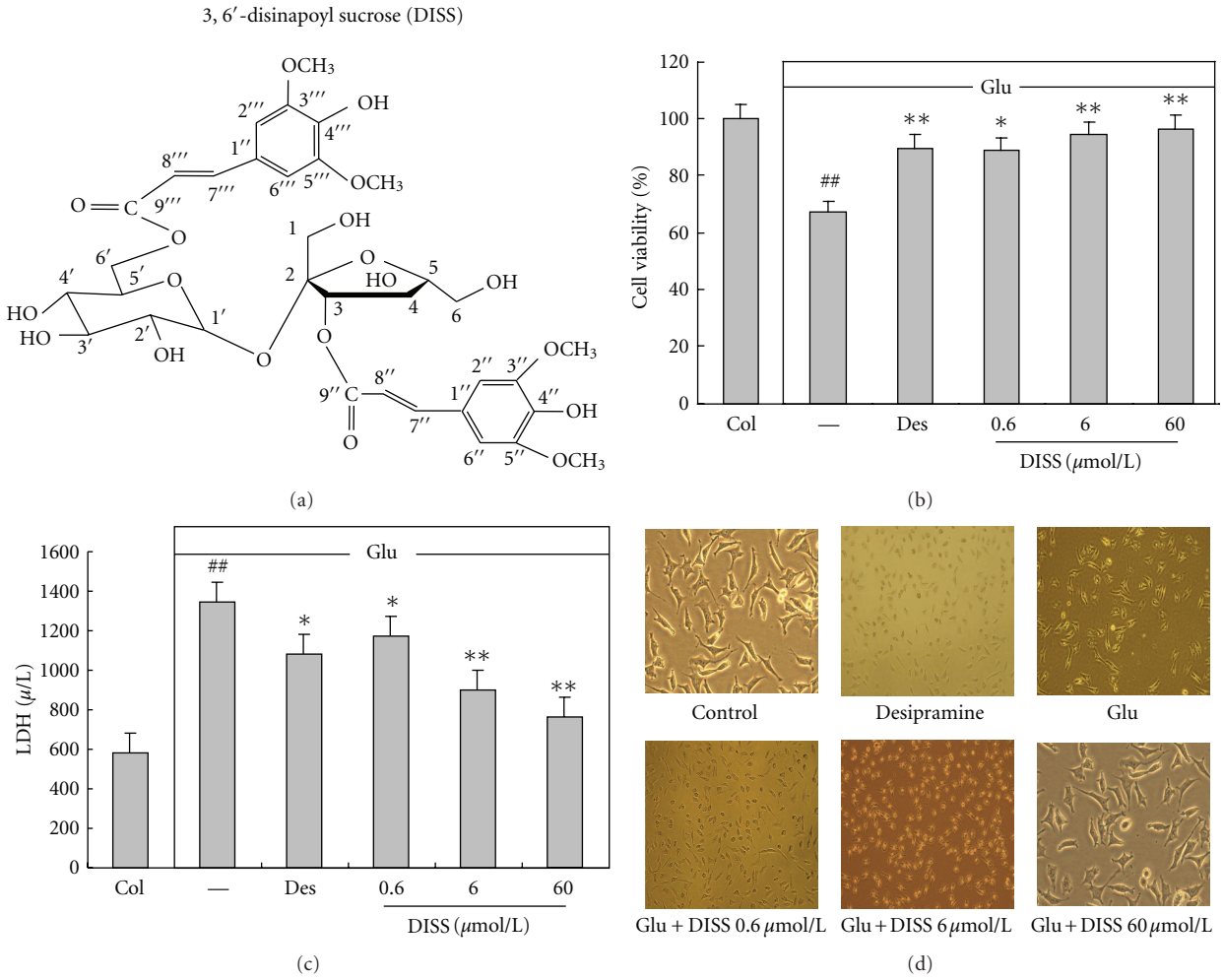

Figure 1: Protective effects of DISS on glutamate- (Glu-) induced cytotoxicity in SH-SY5Y cells. (a) Chemical structure of 3,6′-disinapoyl sucrose (DISS). (b) Effects of DISS treatment on SH-SY5Y cells viability decrease induced by glutamate (8 mM) exposure. (c) Effects of DISS treatment on lactate dehydrogenase (LDH) release of SH-SY5Y cell damaged by glutamate. (d) Effect of DISS on glutamate- (Glu-)induced morphological alterations in SH-SY5Y cells. All data were expressed as mean ± S.D. of three experiments. $^{\#\#}P < 0.01$, compared with normal cultures; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Glu alone group. Co: Control; Des: desipramine, which is positive drug.

cell injury, and aimed to investigate the neuroprotective effects of DISS on glutamate-induced apoptosis as well as its mechanisms.

2. Resource and Preparation of DISS

DISS, used in present study, with a purity of over 90%, was extracted from the roots of *P. tenuifolia,* which were purchased from Traditional Chinese Medicinal (TCM) pharmacy, Chinese People's Liberation Army (PLA) General Hospital (Beijing, China); a voucher specimen (NU-80617) was deposited in the Herbarium there. Three-month air-dried roots (965.27 g) were extracted with 60% EtOH (8 : 1) at room temperature for 2 weeks. The dry extract obtained was then subjected to open column chromatography (CC) packed with macroporous resin (1300 Version). The 50% aqueous-ethanol fraction was concentrated under reduced pressure using a rotary evaporator and lyophilized into powders, further chromatographed on the silica gel column, and eluted by $CHCl_3$–MeOH–H_2O to get DISS [8]. The structures were identified by a combination of spectral methods (UV, IR, MS, and NMR), with purity of over 90%.

2.1. Cell Culture and Treatment. The human neuroblastoma SH-SY5Y was provided by Department of Obstetrics and Gynecology in Chinese PLA General Hospital. The human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin in a humid atmosphere of 5% CO_2 and 95% air at 37°C. SH-SY5Y cells were plated in plates. Confluent SH-SY5Y cells were washed twice with D-Hanks solution before the addition of 0.25% trypsin-EDTA. The flask was left for 2-3 min at room temperature(close to 20°C), after which the cells were detached, resuspended in full medium, counted, and seeded into 96-well plates at a density of

1×10^4 cells/well in normal growth medium. After 24 h, the cells were completely attached to the well bottom. Then cells were exposed to L-Glutamate (Glu) for 0.5 h, followed by treatment with various concentrations of DISSs (0.6, 6, and 60 μmol/L) and desipramine as a positive drug. DISS and glutamate were dissolved in dimethyl sulfoxide (DMSO) before added in cell. Final drug concentrations were obtained by dilution of stock solutions in experimental media. Final concentrations of DMSO were always less than 0.01%, which was proved to have no effects on cell viability. The absorbance was read at 570 nm with DMSO as the blank. The DISS dose range was chosen from previous results on preliminary experiments. Cell viability and LDH activity were measured after 12 h incubation at 37°C.

2.2. Cell Viability. Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay [9]. Briefly, after 0.5 h exposure to Glu, 20 μL of MTT (2 mg/mL in PBS) were added to each well and the cells were incubated at 37°C for 4 h. The supernatants were aspirated carefully, 150 μL of DMSO were added to each well to dissolve the precipitate, and absorbance was measured at 570 nm using a microplate reader (Spectra MR, Dynex, USA). Cell death was determined by measuring the lactate dehydrogenase (LDH) activity using commercially available kits from the Nanjing Jiancheng Bio-company (Nanjing, China).

2.3. Flow Cytometric Detection of Apoptotic Cells. After treatment, 5×10^6 cells were trypsinized, washed in PBS, and centrifuged at 1000 g for 5 min. Then cells were fixed in 70% ethanol overnight. The pellet was rinsed twice and resuspended in 0.5 mL PBS (containing 50 μg/mL RNaseA), incubated for 30 min at 37°C. PI (50 μg/mL) was added, mixed gently, and incubated for 30 min at 4°C in dark. The samples were then read in a Becton Dickinson flow cytometer (USA) at 488 nm excitation. A 600 nm bandpass filter for PI detection was used. Ten thousand cells in each sample were analyzed, and the percentage of apoptotic cells accumulating in the sub-G1 peak was calculated by CellQuest software.

2.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from cells cultured in the 25 cm^2 plastic flasks with 5×10^6 cells using Trizol reagent (Gibco BRL) as described by the manufacturer. And its reverse transcribtion to cDNA was performed using iScript cDNA Synthesis Kit (Bio-Rad, Calif, USA), according to the manufacturer's protocol. Human β-actin, bcl-2, and Bax primers were synthesized by BM (Biomed, China) according to the following sequences [10]. β-actin: forward 5′-GGACATCCGCAAAGACCTGTA-3′, reverse 5′-ACATCTGCTGGAAGGTGGACA-3′; bcl-2: forward 5′-TTTGAGTTCGGTGGGGTCATC-3′, reverse 5′-CCAGGAGAAATCAAACAGAGG-3′; bax: forward 5′-TTTGCTTCAGGGTTTCATCC- 3′, reverse 5′-GCCACTCGGAAAAAGACCTC-3′. SsoFast EvaGreen Supermix (Bio-Rad, Calif, USA) was used for real-time PCR to detect abundance of PCR products among samples. Standard curves were generated for each gene, and transcript values were calculated relative to dilution series of cDNA as described in Bio-Rad iQ5 System (Calif, USA). Target quantities were normalized to 18S ribosomal RNA, calibrated using control values, and defined as a value of "1.0". All quantities were expressed as n-fold relative to the calibrator (control).

2.5. Statistical Analysis. Data are presented as the mean ± S.D. Statistical comparisons were made by one-way ANOVA followed by Tukey's post hoc test. Values of $P < 0.05$ and $P < 0.01$ were considered significant.

3. Result

3.1. Effect of DISS on Cell Viability and LDH Release. As shown in Figures 1(b) and 1(c), 8 mM of Glu was used to induce SH-SY5Y cell injury, whose group was only 67.28 ± 1.2% viable cells and increased the LDH level as compared to control cells, while treating the cells with DISS at different concentrations (0.6, 6, and 60 μmol/L) increased the viability of cells and inhibited LDH release, both with dose-dependent. Moreover the effects of DISS could also be confirmed by the morphological observation (Figure 1(d)). There was a significant injury in SH-SY5Y cells after treatment with Glu, including the disappearance of cellular processes, decrease of the refraction, and falling to pieces. The damage in groups of DISS-treated cells was greatly decreased.

3.2. Flow Cytometry Assay. The nuclear staining assay was used to evaluate the morphological changes of apoptosis in SH-SY5Y cells. As shown in Figure 2, control cells without the treatment with Glu exhibited uniformly dispersed chromatin and intact cell membrane. The cells, treated with 8 mM Glu, increased the percentage of apoptotic cells from 0.96% to 4.93%, compared to control cells. However, in 0.6, 6, and 60 μmol/L DISS-treated cells, cell apoptosis induced by Glu was markedly decreased to 2.58%, 2.36%, and 1.95%, respectively.

3.3. Effect of DISS on the Expression of Bcl-2 and Bax and in Glu-Induced Cells. The effects of DISS on the expression of the apoptotic genes Bax and Bcl-2 were also examined in Glu-injured SH-SY5Y cells. As shown in Table 1, Glu enhanced 2-fold expression of Bax increased, on the contrary, it decreased the levels of Bcl-2 comparison with the normal control group. However, DISS (60 μmol/L) treatment inhibited the increase of Bax and the decrease of Bcl-2 dramatically at 12 h of glutamate exposure.

4. Discussion

Glutamate, a major excitatory amino acid neurotransmitter in central nervous system, mediates several physiological processes. An increasing body of evidence indicates the important role of the glutamatergic system in the pathophysiology of depression. Firstly, depressed patients exhibit elevated levels of glutamate both in plasma and the limbic

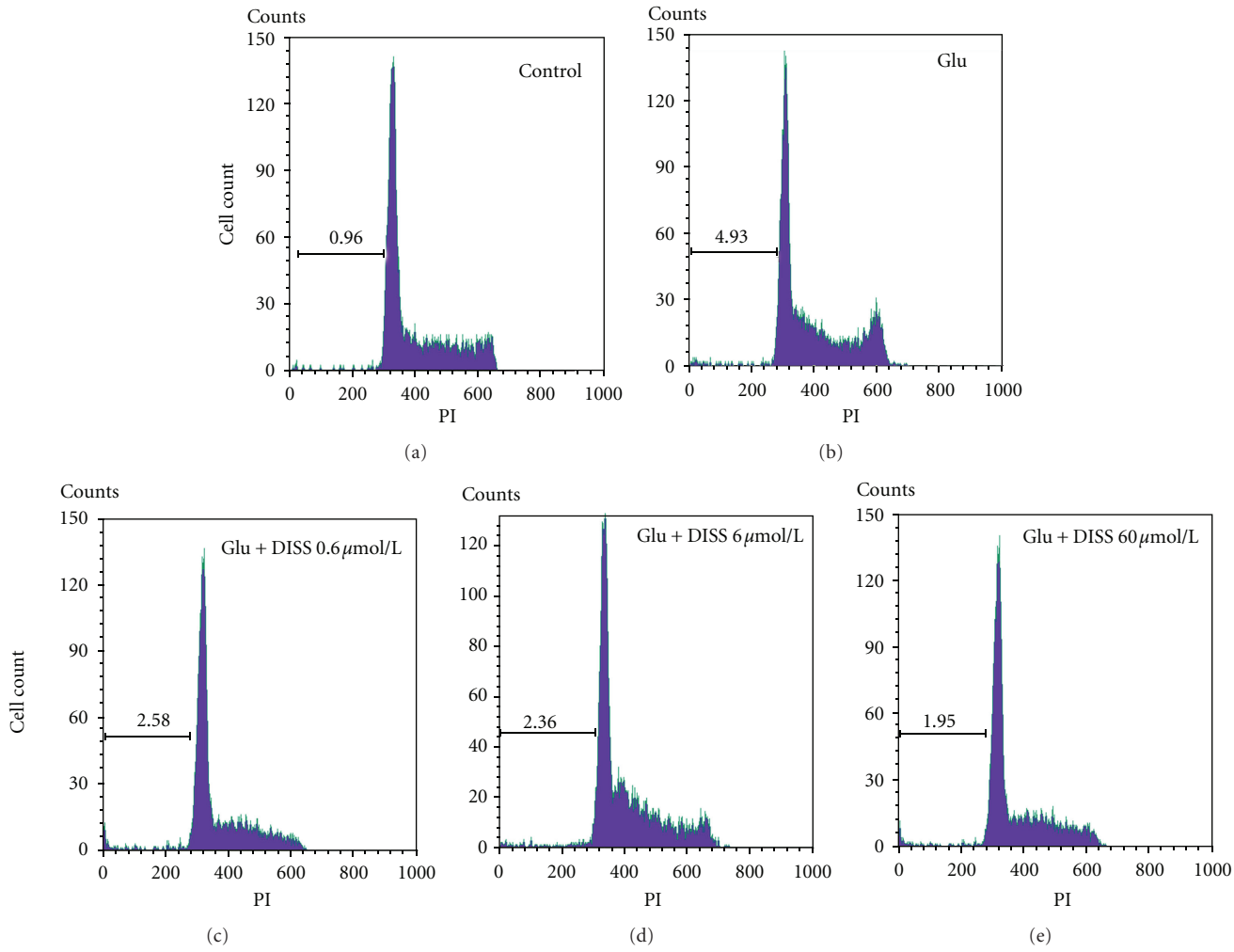

Figure 2: Effect of DISS against glutamate- (Glu-) induced neurotoxicity in cultured SH-SY5Y cells by flow cytometric DNA analysis. The sub-G1 peaks were determined by flow cytometry. (a) Control, (b) Glu alone, (c) Glu + 0.6 μmol/L DISS, (d) Glu + 6 μmol/L DISS, (e) Glu + 60 μmol/L M DISS. Bar(|—|) represents a sub-G1 or hypodiploid DNA fraction.

Table 1: Effect of DISS on Bax and Bcl-2 expression in SH-SY5Y cells after Glu exposure. β-actin was used as an internal control. Results are from three independent experiments.

	Control	Glu	Glu + DISS		
		(8 mM)	(8 mM + 0.6 μmol/L)	(8 mM + 6 μmol/L)	(8 mM + 60 μmol/L)
Bax	1 ± 0.249	2.317 ± 0.280$^{\#\#}$	2.081 ± 0.316	1.921 ± 0.458	1.597 ± 0.351**
Bcl-2	1 ± 0.109	0.467 ± 0.025$^{\#\#}$	0.631 ± 0.018	0.743 ± 0.028	0.825 ± 0.016**

$^{\#\#}P < 0.01$, compared with normal cultures; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Glu alone group.

brain areas, which are believed to be involved in mood disorders [11]. Additionally, it has been shown that chronic treatment with antidepressants of different mechanisms reduces the glutamate release in rats [12, 13].

DISS is the active oligosaccharide ester component and has been proven to have antidepressant [5, 6], cerebral protective, and cognition-improving effects [14]. In the present study, we demonstrated that cotreatment with DISS protected SH-SY5Y neuronal cells against glutamate insult, with maximal neuroprotection being observed at 60 μmol/L.

Similarly, flow cytometry detects apoptotic cells with fragmented nuclei, which are also called sub-G1 cells. DISS treatment significantly reduced the apoptotic cells induced by Glu. We also investigated whether DISS has any effect on the transcriptional level of Bax and Bcl-2 in Glu-treated cells. Proapoptotic gene Bax, antiapoptotic gene Bcl-2 mRNA expression levels were measured by Real-Time PCR. It was reported that Bax and Bcl-2, the two main members of Bcl-2 family, play a key role in the mitochondrial pathway of apoptosis. Bax has been implicated in promoting cell

apoptosis, whereas Bcl-2 in inhibiting apoptosis [15, 16]. Our results indicate that DISS provides neuroprotection partly by the dramatic inhibition of bax overexpression induced by glutamate and increasing antiapoptotic bcl-2 gene expression.

In summary, DISS could provide neuroprotection in glutamate-induced cell injury model. The protective effects of DISS were related to modulating apoptosis-related gene expression, by downregulating the synthesis of proapoptotic Bax and upregulating antiapoptotic bcl-2 expression. Further studies on the neuroprotection effects of DISS in primary neuronal cell injury model, mediated via MAPK-CREB-BDNF/TrkB signaling pathway, are currently under way to evaluate the detail neuroprotective mechanism of therapeutic efforts of DISS.

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