Cytotoxic Activities of 9,11-Dehydroergosterol Peroxide and Ergosterol Peroxide from the Fermentation Mycelia of *Ganoderma lucidum* Cultivated in the Medium Containing Leguminous Plants on Hep 3B Cells

Yu-Kuo Chen,[†] Yueh-Hsiung Kuo,[‡,§,∥,⊥] Been-Huang Chiang,[†] Jir-Mehng Lo,[#] and Lee-Yan Sheen*,[†,§]

[†]Graduate Institute of Food Science and Technology and [‡]Department of Chemistry and [§]Center for Food and Biomolecules, National Taiwan University, Taipei, Taiwan 106, [∥]Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan 115, [⊥]Tsuzuki Institute for Traditional Medicine, College of Pharmacy, China Medical University, Taichung, Taiwan 404, and [#]Biomedical Engineering Research Laboratory, Industrial Technology Research Institute, Hsinchu, Taiwan 300

The objective of this study was to investigate the cytotoxicity of the ethanolic extract of mycelia from *Ganoderma lucidum* (EMG) cultivated in a medium containing leguminous plants *Glycine max* (L.) Merr. and *Astragalus membranaceus* on human hepatocellular carcinoma cells (Hep 3B) and to isolate the active components from EMG. The results indicated that EMG induced cytotoxicity in a dose- and time-dependent manner, and the cells treated with EMG for 24, 48, and 72 h had IC_{50} values of 156.8, 89.9, and 70.1 μg/mL, respectively. Furthermore, EMG was fractionated into seven fractions (F1−F7). We found that F5 and F6 had higher growth inhibitory effects on Hep 3B cells than the other fractions, and F6 possessed enough amounts (about 2.1 g) to carry out a more detailed study. F6 caused a sub-G1 peak rise and DNA fragmentation in Hep 3B cells and was further separated by high-performance liquid chromatography to obtain two active compounds, 9,11-dehydroergosterol peroxide [9(11)-DHEP] (compound **1**) and ergosterol peroxide (EP) (compound **2**). The IC_{50} values of 9(11)-DHEP and EP based on the cell viability of Hep 3B were 16.7 and 19.4 μg/mL, respectively.

KEYWORDS: *Ganoderma lucidum*; leguminous plants; fermentation product; ergosterol peroxide; antihepatoma activity; Hep 3B cells; apoptosis

INTRODUCTION

Liver cancer is one of the leading causes of cancer death in Asian countries (*1*). To maintain liver health is, therefore, an important issue in Asia. *Ganoderma lucidum*, a basidiomycetes mushroom, has been used as a functional food to promote health and longevity in China for thousands of years. Many researches have reported that *G. lucidum* might contribute to potential bioactivities, including antioxidant (*2*), antimicrobial (*3*), anti-inflammatory (*4*), antitumor (*5*), hepatoprotective (*6*), antiviral (*7*), antidiabetic (*8*), and immunomodulating (*9*) activities. Most reports are focused on the bioactivities of the fruiting body of *G. lucidum*; however, cultivation of the fruiting body might take numerous months. A submerged culture has been considered a useful method for producing a large amount of fermentation products with similar biological activities as the fruiting body.

G. lucidum possesses several bioactive components, including polysaccharides, triterpenoids, sterols, proteins, unsaturated fatty acids, nucleosides, alkaloids, vitamins, and essential minerals (*10*), which are claimed to have beneficial properties for the normalization and stabilization of the body and to prevent several diseases (*11*). However, existing research on the bioactive components of *G. lucidum* is mainly focused on polysaccharides and triterpenoids; other compounds are not well-investigated.

Glycine max (L.) Merr. (black soybean) is a soybean cultivar with black seed coat, but contains different contents of anthocyanins and phenolic compounds from soybean. Some studies reported that black soybeans have antioxidant activities against the LDL oxidation and radical scavenging (*12, 13*), and anticancer activities (*14*). *Astragalus membranaceus* is a popular herb used in the traditional Chinese medicine to replenish vital energy. Modern pharmacological studies revealed that *A. membranaceus* has immunostimulant and antifibrotic properties (*15, 16*). A few reports showed that some active components and biological activities were enhanced in fungal fermentation in the medium containing leguminous plants (*17, 18*). Our previous report also showed that black soybean can promote antihepatoma activity in the fermentation product of *Agaricus blazei* (*19*). However, the antihepatoma activity of fermentation products of

---

*To whom correspondence should be addressed. Tel: 886-2-33664129. Fax: 886-2-23620849. E-mail: lysheen@ntu.edu.tw.

G. lucidum cultivated in the medium containing leguminous plants [*G. max* (L.) Merr. and *A. membranaceus*] has not yet been studied.

The objectives of this study were to investigate the antihepatoma cell activities of the fermentation products of *G. lucidum* cultivated in a medium containing leguminous plants. Furthermore, we isolated the active components from the ethanolic extract of mycelia from *G. lucidum* (EMG) and studied their mechanism of the antihepatoma action.

MATERIALS AND METHODS

Materials and Chemicals. Antibiotic−antimycotic (AA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nonessential amino acids (NEAA), and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Gibco (Grand Island, NY). Ethanol (95%) was purchased from Echo Chemical (Taipei, Taiwan). Ethyl acetate, *n*-hexane, methanol, and thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). A QIAamp DNA Mini Kit was purchased from Qiagen (Hilden, Germany). *N,N*-Dimethylfluoramide (DMF) was purchased from Laboratory-Scan (Dublin, Ireland). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), trypsin-ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), propidium iodide (PI), and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Fermentation Mycelia of *G. lucidum*. *G. lucidum* hyphae were inoculated into a basic medium consisting of 5% *G. max* (L.) Merr., 2% *A. membranaceu*, and 2% glucose in a 200 L fermentor, and the cultivation was performed at an agitation rate of 50 rpm, an aeration rate of 0.75 vvm, and 24 °C for 12 days. The mycelia were then collected by centrifugation and subsequently lyophilized.

Preparation and Fractionation of Ethanolic Extracts. Dried mycelia were ground into powder and passed through a 40 mesh sieve. The extraction method used was based on that reported by Su et al. (*19*). One kilogram of dry powder was extracted with 20 L of 95% ethanol at 30 °C for 24 h. The EMG was concentrated to dryness under reduced pressure, and the yield was 6.8 g/100 g dry weight of mycelia. EMG (26 g) was mixed with silica gel (30 g) and subjected to separation on a silica gel column chromatography (Si gel 60, 40−63 m, Merck) (40 cm × 9.5 cm i.d.) with successive elution by *n*-hexane/ethyl acetate and ethyl acetate/methanol gradient. The flow rate of eluting solvent was maintained at 1 L/h. After silica gel column chromatography, the fractions were analyzed by TLC (silica gel 60 F_{254}, Merck). The TLC plates were developed with various ratios of *n*-hexane/ethyl acetate and then treated with H_2SO_4 (10%) for detection. Fractions having the same TLC pattern were combined into one fraction; thus, seven main fractions (F1−F7) were obtained. The fraction (F6) that showed greater cytotoxicity on Hep 3B cells and had higher yield was chromatographed on a silica gel column with a dichloromethane/ethyl acetate gradient system to yield subfractions. The subfractions were further separated by high-performance liquid chromatography (HPLC) (Shimadzu RID-10A) using a Phenomenex Luna silica column (5 *μ*m particles, 250 mm × 10 mm i.d., Torrance, CA) and eluted with 4% dichloromethane/acetone and 25% ethyl acetate/*n*-hexane to obtain compounds **1** and **2**, respectively.

Identification of Compounds 1 and 2. The structures of compounds **1** and **2** were identified by spectroscopic methods, including UV, IR, MS, and ^{1}H NMR. The UV spectra were recorded on a spectrophotometer. IR spectra were measured on a Perkin-Elmer 983 G infrared spectrometer (Wellesley, MA). ESI-MS spectra were obtained on a LCQ-Fleet mass spectrometer (ThermoFinigan, San Jose, CA). [1]H spectra (in CDCl$_3$) were performed on a Bruker AM-400 instrument (Bruker, Rheinstetten, Germany). The contents of compounds **1** and **2** in crude EMG were analyzed by a RP-HPLC system. A Supelcosil C18 reversed-phase column (150 mm × 4.6 mm i.d., Bellefonte, PA) was used for all applications and was housed in a temperature-regulated compartment maintained at 30 °C. For gradient elution, mobile phase A consisted of 20% water and 0.5% acetic acid in methanol, and mobile phase B consisted of 0.5% acetic acid in methanol.

Cell Line and Culture Conditions. Human hepatoma cell line (Hep 3B, BCRC 68001) was a gift from Dr. M. S. Shiao (Department of Medical Research and Education, Taipei Veterans Hospital, Taipei, Taiwan). Hep 3B cells were cultured in DMEM (pH 7.0), supplemented with 10% FBS, 100 units/mL AA, and 0.1 mM NEAA, and were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Upon reaching 60−70% confluence in full medium, cells were subcultured at a 1:4 or higher ratio using 1 mL of trypsin-EDTA solution and were fed fresh growth medium.

Cell Viability Assay. Inhibition of cell viability was measured using a modified MTT assay (*20*). Hep 3B cells were seeded at a density of 5×10^3 cells/100 *μ*L DMEM/well on a 96 well plate. After they were incubated for 24 h to allow the cells to adhere to the bottom of the culture plate, the medium was replaced by 100 *μ*L of serum-free DMEM containing different concentrations of samples for 24, 48, or 72 h of treatment, each in triplicate. The samples were dissolved in DMSO, and the final ratio of DMSO in the medium was 0.2%. At the end of the treatment, the medium was discarded, and 25 *μ*L of MTT solution and 100 *μ*L of serum-free DMEM were added to each well and then incubated at 37 °C for 4 h. Subsequently, 100 *μ*L of MTT lysis buffer (20% SDS and 50% DMF, in water) was added to each well to dissolve the formazan converted from tetrazolium salt, and plates were incubated for another 14−16 h at 37 °C. Then, the absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay plate reader (Anthos 2001, Salzburg). Inhibition of cell viability was represented as a percentage of growth of DMSO-treated cells (control), and the concentration of sample required to inhibit 50% of cell growth (IC_{50}) was determined by interpolation from the dose response.

Cell Cycle Distribution Assay. Cell cycle distribution was analyzed by FACScan flow cytometer (Becton Dickinson, San Jose, CA) with ModFit LT Ver. 2.0 (Verity Software House, Topsham, ME). Briefly, Hep 3B cells were plated on a six well plate and treated with various concentrations of samples for 48 h. The cells were then harvested by trypsinization, washed with ice-cold phosphate-buffered saline (PBS), and gently fixed in 70% ice-cold ethanol at −20 °C for at least 24 h. The cells were then collected by centrifugation and resuspended in PBS containing 40 *μ*g/mL PI, 0.1 mg/mL RNase A, and 0.1% Triton X-100 in a dark room at room temperature for 30 min. Subsequently, cells were filtered before they were analyzed with FACScan flow cytometer, and the results were analyzed by ModFit LT Ver. 3.0 software. The proportion of sub-G1 hypodiploid cells was quantitated and represented as a percentage of apoptotic cells.

DNA Fragmentation Assay. Likewise, Hep 3B cells were treated with samples at different concentrations. Two days after treatment, adherent cells were harvested by trypsinization and were washed with cold PBS and pelleted by centrifugation. DNA was extracted from cell pellets using QIAamp DNA mini kit, and then, the DNA fragmentation pattern was analyzed by electrophoresis in a 2% agarose gel at 50 V. The agarose gel was stained with ethidium bromide (1 *μ*g/mL) and photographed after electrophoresis.

Nuclear Morphology Observation. To examine the nuclear morphological changes, approximately 3.25×10^5 cells/well of Hep 3B cells in a six well plate with 20 *μ*g/mL of compounds **1** or **2** were incubated in an incubator for 48 h. After treatment, cells were washed three times with PBS following fixation in 3% formaldehyde for 15 min. Then, the cells were maintained in PBS solution containing 0.1% Triton X-100 for 15 min after they were washed twice with PBS. Cells were stained by 1 *μ*g/mL DAPI, a DNA-binding fluorescent dye, in a dark room at 37 °C for 30 min and then photographed by fluorescence microscope (*21, 22*).

Statistical Analysis. All experiments were performed in triplicate and presented as means ± standard deviations (SDs). Statistical analyses were performed using one-way analysis of variance and Duncan's multiple comparison tests (SAS Institute Inc., Cary, NC) to determine significant differences among means ($P < 0.05$).

RESULTS

Effect of Ethanolic Extract from Fermentation Mycelia of *G. lucidum* on Hep 3B Cells. Figure 1 shows the effect of EMG on the growth of human hepatocellular carcinoma cells (Hep 3B). Hep 3B cells were treated with various concentrations (25, 50, 100, 150, and 200 *μ*g/mL) of EMG for 24, 48, and 72 h, and the results indicated that EMG markedly reduced cell viability of Hep 3B in
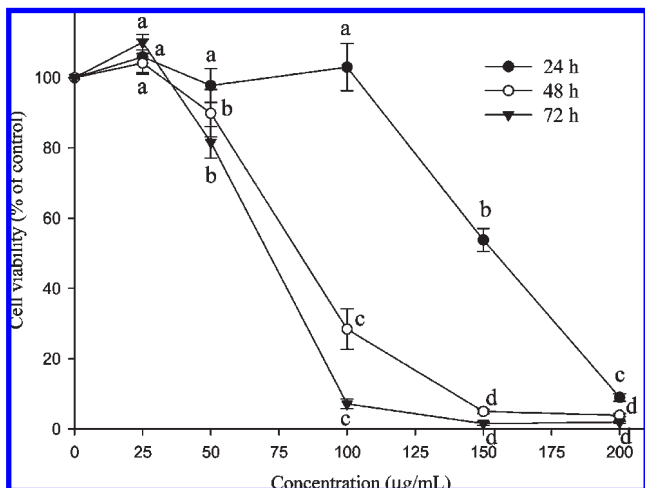
Figure 1. Effect of EMG on cell viability of Hep 3B cells. Cells were treated with various concentrations of EMG for 24, 48, and 72 h. Values are expressed as means ± SDs.
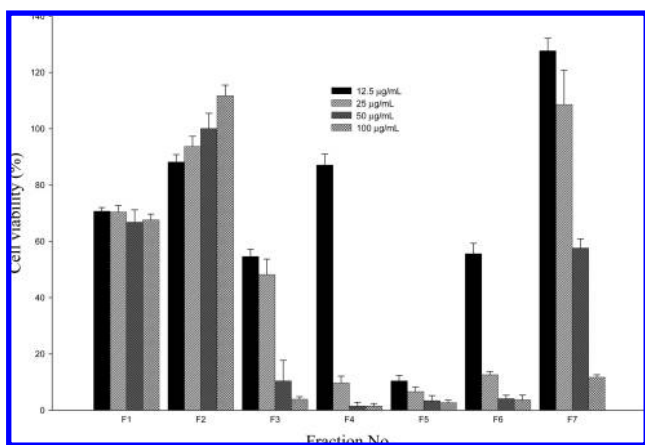

Figure 2. Cytotoxicity against Hep 3B cells of the various fractions obtained from EMG. Cells were treated with various concentrations of fractions for 48 h. Values are expressed as means ± SDs.

a dose-dependent manner. Besides, a time-dependent decrease in the cell viability of Hep 3B was also found, and the IC_{50} values of EMG against Hep 3B cells treated for 24, 48, and 72 h were 156.8, 89.9, and 70.1 μg/mL, respectively. Moreover, the observation of morphological changes (cell shrinkage and floating cells) of Hep 3B cells, the typical morphological characteristics of apoptosis, induced by treating with a higher dose of EMG showed the inhibition effect of EMG on Hep 3B cells (data not shown).

Antihepatoma Activities of Fractions Eluted from EMG. To isolate and identify the components with antihepatoma activity, EMG (26 g) was further subjected to column chromatography on silica gel with successive elution of *n*-hexane/ethyl acetate and ethyl acetate/methanol gradient. From the results of TLC, the collected fractions eluted from the column that showed the same pattern were combined into one fraction, and seven main fractions were collected. The cytotoxicity of seven fractions on Hep 3B cells was evaluated by MTT assay, and the results are shown in **Figure 2**. It was found that fraction 5 (F5) and fraction 6 (F6) had higher cytotoxic effects on Hep 3B cells. When treated with F5 and F6 at a concentration of 25 μg/mL for 48 h, the cell viabilities of cell growth were 6.6 and 12.6%, respectively. However, the amount of F6 (2.14 g) is much higher than F5 (0.29 g) and possesses enough amount to assay the antihepatoma mechanism and to identify the active components.

To investigate whether the decrease in cell viability by F6 was due to apoptosis, we analyzed the effect of F6 on cell cycle progression of Hep 3B cells. F6 was found to affect cell cycle distribution in Hep3B cells (**Figure 3**). An increase in G2/M phase population and an obvious sub-G1 peak (apoptosis %) were observed after incubation with 10 μg/mL F6 for 48 h, and the proportion of cells in the sub-G1 peak was increased in a dose-dependent manner. Furthermore, the induction of DNA fragmentation by F6 in Hep 3B cells was also assessed. DNA fragmentation is a marker of apoptosis that revealed a ladder pattern. **Figure 4** shows that the treatments with 20 and 40 μg/mL F6 for 48 h, the internucleosomal cleavage of the genomic DNA resulting in DNA ladder, was observed in Hep 3B cells.

Purification and Identification of Active Compounds 1 and 2. The active fraction, F6, was chromatographed on a silica gel column with a dichloromethane/ethyl acetate gradient system to yield subfractions. The subfractions were further applied to a silica column of preparative HPLC and eluted with 4% dichloromethane/acetone and 25% ethyl acetate/*n*-hexane to obtain compounds **1** and **2**, respectively. **Figure 5** shows the structures of compounds **1** and **2**, and they were identified as follows, on the basis of literature data (*23*).

Compound **1**: 9,11-dehydroergosterol peroxide [9(11)-DHEP], $C_{28}H_{42}O_3$. ESI-MS *m/z* (CID 25%): 427 [M + H]$^+$, 409, 367, 356, 339, 303, 189. 5α,8α-Epidioxy-22*E*-ergosta- 6,9(11),22-trien-3β-ol. Colorless needles. UV/vis λ_{max} (nm): 313, 325, 340. IR ν_{max} (KBr) cm^{-1}: 2950, 2871. ^{1}H NMR (CDCl$_3$, 400 MHz): δ 0.71 (3H, s, H-18), 0.79 (3H, d, *J* = 6.8 Hz, H-27), 0.81 (3H, d, *J* = 6.8 Hz, H-26), 0.89 (3H, d, *J* = 6.8 Hz, H-28), 0.97 (3H, d, *J* = 6.8 Hz, H-21), 1.06 (3H, s, H-19), 3.99 (1H, m, H-3), 5.13 (1H, dd, *J* = 8.4, 15.4 Hz, H-22), 5.21 (1H, dd, *J* = 7.2, 15.4 Hz, H-23), 5.40 (1H, d, *J* = 6.0 Hz, H-11), 6.26 (1H, d, *J* = 8.6 Hz, H-7), 6.57 (1H, d, *J* = 8.6 Hz, H-6).

Compound **2**: ergosterol peroxide (EP), $C_{28}H_{44}O_3$. ESI-MS *m/z* (CID 25%): 429 [M + H]$^+$, 411, 369, 358, 341, 305, 191. 5α, 8α-Epidioxy-22*E*-ergosta-6,22-dien-3β-ol. Colorless needles. UV/vis λ_{max} (nm): 283, 294. IR ν_{max} (KBr) cm^{-1}: 2960, 2878. ^{1}H NMR (CDCl$_3$, 400 MHz): δ 0.71 (3H, s, H-18), 0.79 (3H, d, *J* = 6.6 Hz, H-26), 0.81 (3H, d, *J* = 6.6 Hz, H-27), 0.86 (3H, s, H-19), 0.88 (3H, d, *J* = 6.8 Hz, H-28), 0.97 (3H, d, *J* = 6.8 Hz, H-21), 3.95 (1H, m, H-3), 5.11 (1H, dd, *J* = 8.0, 15.2 Hz, H-22), 5.18 (1H, dd, *J* = 7.6, 15.2 Hz, H-23), 6.21 (1H, d, *J* = 8.4 Hz, H-7), 6.48 (1H, d, *J* = 8.4 Hz, H-6).

9(11)-DHEP and EP were quantified by RP-HPLC. The contents of 9(11)-DHEP and EP in crude EMG were 42.9 and 8.9 mg/g dry weight, respectively.

Cytotoxicity of 9(11)-DHEP and EP. Purified 9(11)-DHEP or EP was added to the medium for Hep 3B cells culture. After 48 h of incubation, both 9(11)-DHEP and EP showed notable cytotoxic effects on Hep 3B cells. As shown in **Figure 6**, a dose-dependent decrease in the cell viability was observed, and the IC_{50} treated with 9(11)-DHEP and EP for 48 h was 16.7 (39.2 μM) and 19.4 μg/mL (45.3 μM), respectively. When the treatment of concentration was higher than IC_{50}, the growth inhibition effect of 9(11)-DHEP on Hep 3B cells was greater than that of EP. When treated at the concentration of 20 μg/mL, 9(11)-DHEP and EP had reduced cell viability of Hep 3B cells to 27 and 46% of control, respectively.

Determination of DNA Fragmentation Induced by 9(11)-DHEP and EP. Likewise, Hep 3B cells were treated with 20 μg/mL of 9(11)-DHEP or EP for 48 h, and then, cell pellets were harvested for isolation of DNA. Subsequently, the DNA fragmentation pattern was analyzed on agarose gels by electrophoresis. The results showed that DNA fragmentations were observed in
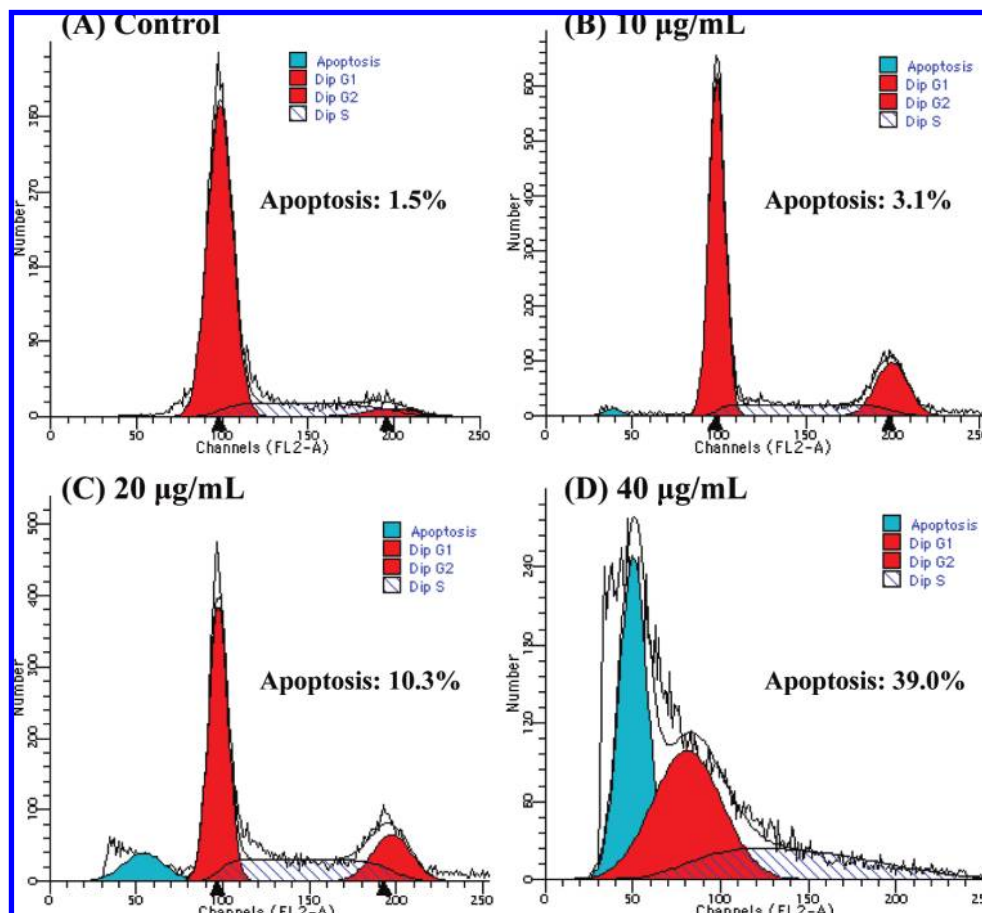
Figure 3. F6 from EMG caused a sub-G1 peak rise in Hep 3B cells. Cells were treated with various concentrations of F6 for 48 h: (**A**) 0.2% DMSO as control, (**B**) 10 μg/mL F6, (**C**) 20 μg/mL F6, or (**D**) 40 μg/mL F6.
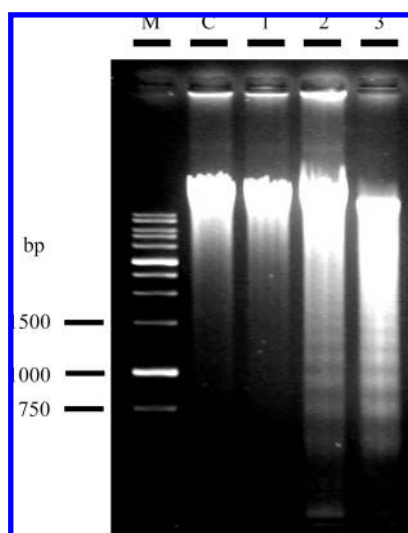

Figure 4. Induction of DNA fragmentation by F6 from EMG in Hep 3B cells. Cells were treated with various concentrations of F6 for 48 h, and DNA fragmentation was analyzed by electrophoresis in 2.0% agarose gel: DNA marker (lane M); cells were treated with 0.2% DMSO as a control (lane C), 10 μg/mL F6 (lane 1), 20 μg/mL F6 (lane 2), or 40 μg/mL F6 (lane 3).

Figure 5. Structures of 9,11-dehydroergosterol peroxide (**A**) and ergosterol peroxide (**B**).

the nuclei of control cells were round and intact, whereas condensed and fragmented chromatin were observed in cells after 20 μg/mL 9(11)-DHEP or EP treatment. These appearances suggest that apoptosis is induced by 9(11)-DHEP and EP.

DISCUSSION

Submerged culture is considered a useful method for the production of mycelia and various active metabolites including ganoderic acids, polysaccharides, and other low molecular weight compounds (*24−26*). In this study, we used leguminous plants, *G. max* (L.) Merr. and *A. membranaceus*, as ground substances of

9(11)-DHEP and EP treatments, whereas no DNA ladder was detected in 0.2% DMSO-treated cells (control) (**Figure 7**).

Effect of 9(11)-DHEP and EP on Nuclear Morphology of Hep 3B Cells. After 48 h of continuous incubation, effects of 9(11)-DHEP and EP on nuclear morphology of Hep 3B cells were detected by a DAPI staining method. As shown in **Figure 8**,
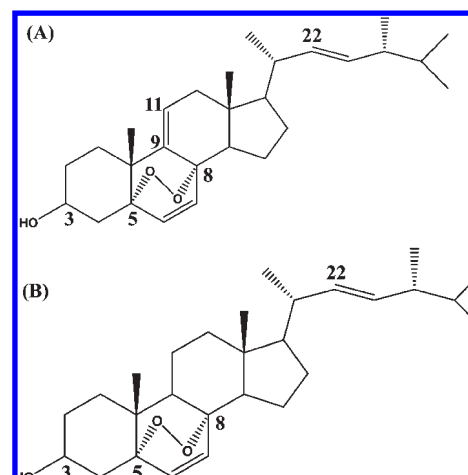
Article

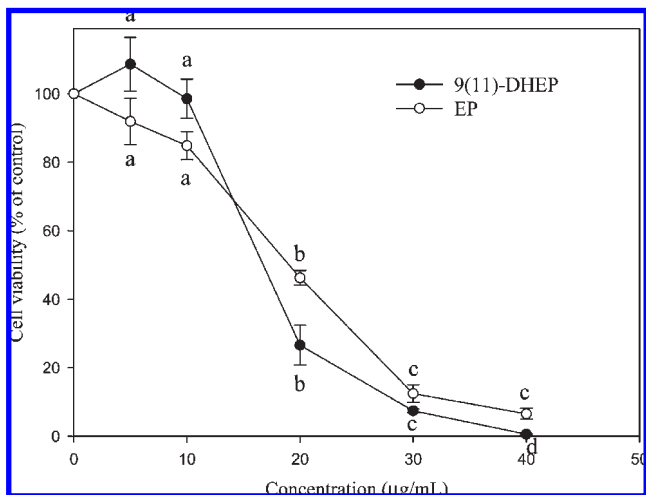*J. Agric. Food Chem.,* Vol. 57, No. 13, 2009 **5717**

Figure 6. Effects of active components isolated from EMG on the cell viability of Hep 3B cells. Cells were treated with various concentrations of 9(11)-DHEP (●) or EP (○) for 48 h. Values are expressed as means ± SDs.

Figure 8. Induction of nuclear condensation and fragmentation by 9(11)-DHEP and EP in Hep 3B cells. (**A**) Cells were treated with 0.2% DMSO as a control, (**B**) 20 μg/mL 9(11)-DHEP, or (**C**) 20 μg/mL EP for 48 h. The DAPI stain showed 9(11)-DHEP- or EP-induced condensed and fragmented nuclei (400× under fluorescence microscope).
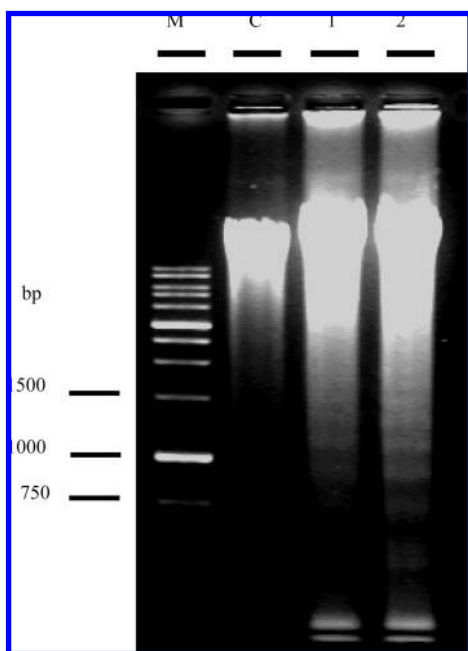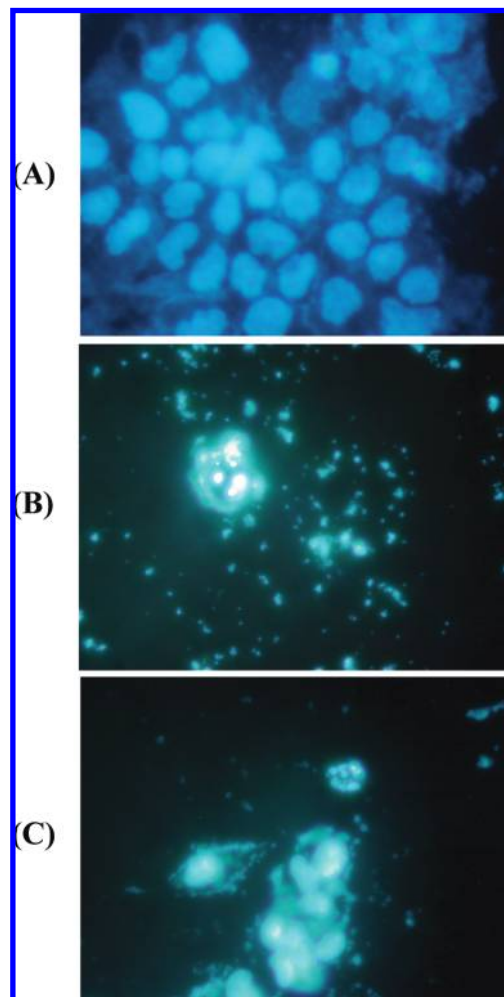

Figure 7. Induction of DNA fragmentation by 9(11)-DHEP and EP isolated from EMG in Hep 3B cells. Cells were treated with 9(11)-DHEP and EP for 48 h: DNA marker (lane M); cells were treated with 0.2% DMSO as a control (lane C), 20 μg/mL EP (lane 1), or 20 μg/mL 9(11)-DHEP (lane 2).

medium to ferment *G. lucidum* and to evaluate the antihepatoma activity of ethanolic extract of the fermentation product. Subsequently, the active components of *G. lucidum* were isolated and identified. We found that the fermentation products (mycelia or broth) of *G. lucidum* cultured without *G. max* (L) Merr. and *A. membranaceus* did not possess cytotoxicity against Hep 3B cells even with a concentration of ethanolic extract higher than 100 μg/mL (data not shown). Nevertheless, the addition of *G. max* (L.) Merr. and *A. membranaceus* during fermentation enhanced the anithepatoma activity of EMG (**Figure 1**), whereas no cytotoxicity was found in broth (data not shown). A few researches reported that some compounds and bioactivities were changed in fungal fermentation in the medium containing leguminous plants (*17*, *18*, *27*). Apparently, *G. max* (L) Merr.- and *A. membranaceus*-containing medium might enrich the active

components in the mycelia, resulting in improvement of anithepatoma activity of *G. lucidum*.

EMG was further separated into seven main fractions by chromatography, and F6 (fraction 6) was identified as the most active fraction of anithepatoma cells. As shown in **Figure 3**, F6 was found to increase the proportion of sub-G1 of Hep 3B cells, which were stained with DNA-specific fluorescent dye, PI, in a dose-dependent manner. Furthermore, DNA fragmentation assay was used to reveal whether the cytotoxic effect of F6 was due to apoptosis. Apoptosis has been characterized biochemically by the production of DNA fragments resulting from the activation of an endonuclease. This produces a characteristic pattern of DNA cleavage into oligonucleosomal fragments of multiples of 180−200 base pairs, which are integer fragments (a DNA ladder), when the DNA from the apoptotic cells is subjected to gel elcectrophoresis (*28*, *29*). Treated with F6 for 48 h, the internucleosomal cleavage of the genomic DNA resulting in DNA ladder was observed in Hep 3B cells (**Figure 4**). According to the above results, F6 from EMG might mediate its cytotoxicity by inducing cell apoptosis.

We further isolated two components from the active fraction F6; compounds **1** and **2** were recognized as 9(11)-DHEP and EP, respectively. **Figure 6** shows a dose-dependent decrease in the cell viability of Hep 3B cells treated by 9(11)-DHEP and EP for 48 h.

Chen et al.

Moreover, human embryonic intestinal epithelial cell line INT 407 was used to confirm whether the antiproliferative effects of 9(11)-DHEP and EP are specific to cancer cells. The cell viability of INT 407 cells was higher than 20% even treated with 40 μg/mL 9(11)-DHEP and EP [0.6 and 6.7% in Hep 3B cells treated with 9(11)-DHEP and EP, respectively] (data not shown). This reveals that the cytotoxicity of 9(11)-DHEP and EP was a little less in normal immortalized cells than that of cancer cells. EP has been reported to be transformed from ergosterol by photo-oxidation with singlet oxygen (*30*), while it can also be synthesized by biological oxidation of ergosterol, and there is a strong correlation between ergosterol content and fungal dry mass (*31*). It seems that the addition of *G. max* (L.) Merr. and *A. membranaceu* could increase the amount of ergosterol via generated more mycelial dry mass of *G. lucidum* (data not shown), but further investigations on this should be performed. EP is widely distributed in edible and medicinal mushrooms including *Cordyceps sinensis*, *Pleurotus eringii*, *Hypsizigus marmoreus*, *Sarcodon aspratus*, and *Ganoderma lipsiense* (*32−36*). Some studies have shown that ergosterol and its peroxides might possess several bioactivities, such as reducing inflammation, antioxidation, immunosuppression, and antitumor activities (*36−39*). Kobori et al. (*40*) reported that EP suppresses LPS-induced inflammatory responses through inhibition of NF-κB and C/EBPβ transcriptional activity and phosphorylation of MARPKs and inhibits the growth of some cancer cells to induce apoptosis of HL60 human leukemia cells (*34*). 9(11)-DHEP possesses one more double bond than EP, and it was reported to have a higher bioactivity than EP and inhibited HT29 cells growth by inducing CDKN1A expression, thus causing cell cycle arrest and apoptosis (*41*).

Furthermore, we investigated whether 9(11)-DHEP and EP can induce DNA fragmentation of Hep 3B cells. As the results show in **Figure 7**, the DNA ladder was observed in the treatment of 9(11)-DHEP and EP. In addition, condensed and fragmented chromatin was observed in cells after 20 μg/mL 9(11)-DHEP or EP treatment (**Figure 8**). These results suggest that 9(11)-DHEP and EP inhibit Hep 3B cells growth by inducing apoptosis. To the best of our knowledge, this study is the first report to show that 9(11)-DHEP and EP isolated from *G. lucidum* can inhibit cell growth of hepatoma cells through apoptosis.

In conclusion, EMG cultivated in the medium containing *G. max* (L) Merr. and *A. membranaceus* shows notable inhibition effects on Hep 3B cells, and its active fraction F6 could increase the proportion of sub-G1 cells and cause DNA fragmentation in Hep 3B cells. DNA fragmentation and condensed chromatin were also observed in Hep 3B cells when treated with the active compounds, 9(11)-DHEP and EP, isolated from EMG. Thus, it is suggested that 9(11)-DHEP and EP might be the active components in EMG that induced apoptosis in hepatoma cells. From this viewpoint, further work is required to clarify the molecular mechanism of apoptosis induced by 9(11)-DHEP and EP.

ABBREVIATIONS USED

EMG, ethanolic extract of mycelia from *Ganoderma lucidum*; IC$_{50}$, the concentration of sample required to inhibit 50% of cell growth; 9(11)-DHEP, 9,11-dehydroergosterol peroxide; EP, ergosterol peroxide.

ACKNOWLEDGMENT

LITERATURE CITED

(1) Tsukuma, H.; Tanaka, H.; Ajiki, W.; Oshima, A. Liver cancer and its prevention. *Asian Pac. J. Cancer Prev.* **2005**, *6*, 244–250.

(2) Thyagarajan, A.; Jiang, J.; Hopf, A.; Adamec, J.; Sliva, D. Inhibition of oxidative stress-induced invasiveness of cancer cells by *Ganoderma lucidum* is mediated through the suppression of interleukin-8 secretion. *Int. J. Mol. Med.* **2006**, *18*, 657–664.

(3) Yoon, S. Y.; Eo, S. K.; Kim, Y. S.; Lee, C. K.; Han, S. S. Antimicrobial activity of *Ganoderma lucidum* extract alone and in combination with some antibiotics. *Arch. Pharm. Res.* **1994**, *17*, 438–442.

(4) Ko, H. H.; Hung, C. F.; Wang, J. P.; Lin, C. N. Antiinflammatory triterpenoids and steroids from *Ganoderma lucidum* and *G. tsugae*. *Phytochemistry* **2008**, *69*, 234–239.

(5) Gao, Y.; Gao, H.; Chan, E.; Tang, W.; Xu, A.; Yang, H.; Huang, M.; Lan, J.; Li, X.; Duan, W.; Xu, C.; Zhou, S. Antitumor activity and underlying mechanisms of ganopoly, the refined polysaccharides extracted from *Ganoderma lucidum*, in mice. *Immunol. Invest.* **2005**, *34*, 171–198.

(6) Zhang, G. L.; Wang, Y. H.; Ni, W.; Teng, H. L.; Lin, Z. B. Hepatoprotective role of *Ganoderma lucidum* polysaccharide against BCG-induced immune liver injury in mice. *World J. Gastroenterol.* **2002**, *8*, 728–733.

(7) Li, Y.; Yang, Y.; Fang, L.; Zhang, Z.; Jin, J.; Zhang, K. Antihepatitis activities in the broth of *Ganoderma lucidum* supplemented with a Chinese herbal medicine. *Am. J. Chin. Med.* **2006**, *34*, 341–349.

(8) Ni, T.; Hu, Y.; Sun, L.; Chen, X.; Zhong, J.; Ma, H.; Lin, Z. Oral route of mini-proinsulin-expressing *Ganoderma lucidum* decreases blood glucose level in streptozocin-induced diabetic rats. *Int. J. Mol. Med.* **2007**, *20*, 45–51.

(9) Ji, Z.; Tang, Q.; Zhang, J.; Yang, Y.; Jia, W.; Pan, Y. Immunomodulation of RAW264.7 macrophages by GLIS, a proteopolysaccharide from *Ganoderma lucidum*. *J. Ethnopharmacol.* **2007**, *112*, 445–450.

(10) Paterson, R. R. Ganoderma—A therapeutic fungal biofactory. *Phytochemistry* **2006**, *67*, 1985–2001.

(11) Zhou, X.; Lin, J.; Yin, Y.; Zhao, J.; Sun, X.; Tang, K. Ganodermataceae: Natural products and their related pharmacological functions. *Am. J. Chin. Med.* **2007**, *35*, 559–574.

(12) Xu, B. J.; Chang, S. K. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *J. Food Sci.* **2007**, *72*, S159–166.

(13) Takahashi, R.; Ohmori, R.; Kiyose, C.; Momiyama, Y.; Ohsuzu, F.; Kondo, K. Antioxidant activities of black and yellow soybeans against low density lipoprotein oxidation. *J. Agric. Food Chem.* **2005**, *53*, 4578–4582.

(14) Liao, H. F.; Chou, C. J.; Wu, S. H.; Khoo, K. H.; Chen, C. F.; Wang, S. Y. Isolation and characterization of an active compound from black soybean [*Glycine max* (L.) Merr.] and its effect on proliferation and differentiation of human leukemic U937 cells. *Anticancer Drugs* **2001**, *12*, 841–846.

(15) Lee, Y. S.; Han, O. K.; Park, C. W.; Suh, S. I.; Shin, S. W.; Yang, C. H.; Jeon, T. W.; Lee, E. S.; Kim, K. J.; Kim, S. H.; Yoo, W. K.; Kim, H. J. Immunomodulatory effects of aqueous-extracted *Astragali radix* in methotrexate-treated mouse spleen cells. *J. Ethnopharmacol.* **2003**, *84*, 193–198.

(16) Sun, W. Y.; Wei, W.; Wu, L.; Gui, S. Y.; Wang, H. Effects and mechanisms of extract from *Paeonia lactiflora* and *Astragalus membranaceus* on liver fibrosis induced by carbon tetrachloride in rats. *J. Ethnopharmacol.* **2007**, *112*, 514–523.

(17) Miura, T.; Yuan, L.; Sun, B.; Fujii, H.; Yoshida, M.; Wakame, K.; Kosuna, K. Isoflavone aglycon produced by culture of soybean extracts with basidiomycetes and its anti-angiogenic activity. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 2626–2631.

(18) Lin, Y. W.; Chiang, B. H. Anti-tumor activity of the fermentation broth of *Cordyceps militaris* cultured in the medium of *Radix astragali*. *Process Biochem.* **2008**, *43*, 244–250.

(19) Su, Z. Y.; Hwang, L. S.; Kuo, Y. H.; Shu, C. H.; Sheen, L. Y. Black soybean promotes the formation of active components with anti-hepatoma activity in the fermentation product of *Agaricus blazei*. *J. Agric. Food Chem.* **2008**, *56*, 9447–9454.

Article

J. Agric. Food Chem., Vol. 57, No. 13, 2009 **5719**

(20) Zhao, W.; Kridel, S.; Thorburn, A.; Kooshki, M.; Little, J.; Hebbar, S.; Robbins, M. Fatty acid synthase: A novel target for antiglioma therapy. *Br. J. Cancer* **2006**, *95*, 869–878.

(21) Lin, S. S.; Huang, H. P.; Yang, J. S.; Wu, J. Y.; Hsai, T. C.; Lin, C. C.; Lin, C. W.; Kuo, C. L.; Gibson Wood, W.; Chung, J. G. DNA damage and endoplasmic reticulum stress mediated curcumin-induced cell cycle arrest and apoptosis in human lung carcinoma A-549 cells through the activation caspases cascade- and mitochondrial-dependent pathway. *Cancer Lett.* **2008**, *272*, 77–90.

(22) Lin, C. C.; Lin, S. Y.; Chung, J. G.; Lin, J. P.; Chen, G. W.; Kao, S. T. Down-regulation of cyclin B1 and up-regulation of Wee1 by berberine promotes entry of leukemia cells into the G2/M-phase of the cell cycle. *Anticancer Res.* **2006**, *26*, 1097–1104.

(23) Gunatilaka, A. A. L.; Gopichand, Y.; Schmitz, F. J.; Djerassi, C. Minor and trace sterols in marine invertebrates. 26. Isolation and structure elucidation of nine new 5α,8α-epidoxy sterols from four marine organisms. *J. Org. Chem.* **1981**, *46*, 3860–3866.

(24) Fang, Q. H.; Zhong, J. J. Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderma lucidum*. *Biotechnol. Prog.* **2002**, *18*, 51–54.

(25) Zhong, J. J.; Tang, Y. J. Submerged cultivation of medicinal mushrooms for production of valuable bioactive metabolites. *Adv. Biochem. Eng. Biotechnol.* **2004**, *87*, 25–59.

(26) Lin, J. T.; Liu, W. H. *o*-Orsellinaldehyde from the submerged culture of the edible mushroom *Grifola frondosa* exhibits selective cytotoxic effect against Hep 3B cells through apoptosis. *J. Agric. Food Chem.* **2006**, *54*, 7564–7569.

(27) Hung, Y. H.; Huang, H. Y.; Chou, C. C. Mutagenic and antimutagenic effects of methanol extracts of unfermented and fermented black soybeans. *Int. J. Food Microbiol.* **2007**, *118*, 62–68.

(28) Oberhammer, F.; Wilson, J. W.; Dive, C.; Morris, I. D.; Hickman, J. A.; Wakeling, A. E.; Walker, P. R.; Sikorska, M. Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *Eur. Mol. Biol. Org. J.* **1993**, *12*, 3679–3684.

(29) Park, S. Y.; Yang, H. C.; Moon, J. Y.; Lee, N. H.; Kim, S. J.; Kang, J. H.; Lee, Y. K.; Park, D. B.; Yoo, E. S.; Kang, H. K. Induction of the apoptosis of HL-60 promyelocytic leukemia cells by *Eurya emarginata*. *Cancer Lett.* **2004**, *205*, 31–38.

(30) Trigos, A.; Ortega-Regules, A. Selective destruction of microscopic fungi through photo-oxidation of ergosterol. *Mycologia* **2002**, *94*, 563–568.

(31) Pasanen, A. L.; Yli-Pietila, K.; Pasanen, P.; Kalliokoski, P.; Tarhanen, J. Ergosterol content in various fungal species and biocontaminated building materials. *Appl. Environ. Microbiol.* **1999**, *65*, 138–142.

(32) Yaoita, Y.; Yoshihara, Y.; Kakuda, R.; Machida, K.; Kikuchi, M. New sterols from two edible mushrooms, *Pleurotus eryngii* and *Panellus serotinus*. *Chem. Pharm. Bull.* **2002**, *50*, 551–553.

(33) Xu, M. L.; Choi, J. Y.; Jeong, B. S.; Li, G.; Lee, K. R.; Lee, C. S.; Woo, M. H.; Lee, E. S.; Jahng, Y.; Chang, H. W.; Lee, S. H.; Son, J. K. Cytotoxic constituents isolated from the fruit bodies of *Hypsizigus marmoreus*. *Arch. Pharmacal Res.* **2007**, *30*, 28–33.

(34) Takei, T.; Yoshida, M.; Ohnishi-Kameyama, M.; Kobori, M. Ergosterol peroxide, an apoptosis-inducing component isolated from *Sarcodon aspratus* (Berk.) S. Ito. *Biosci., Biotechnol., Biochem.* **2005**, *69*, 212–215.

(35) Rosecke, J.; Konig, W. A. Constituents of various wood-rotting basidiomycetes. *Phytochemistry* **2000**, *54*, 603–610.

(36) Bok, J. W.; Lermer, L.; Chilton, J.; Klingeman, H. G.; Towers, G. H. Antitumor sterols from the mycelia of *Cordyceps sinensis*. *Phytochemistry* **1999**, *51*, 891–898.

(37) Yazawa, Y.; Yokota, M.; Sugiyama, K. Antitumor promoting effect of an active component of Polyporus, ergosterol and related compounds on rat urinary bladder carcinogenesis in a short-term test with concanavalin A. *Biol. Pharm. Bull.* **2000**, *23* 1298–1302.

(38) Zhang, Y.; Mills, G. L.; Nair, M. G. Cyclooxygenase inhibitory and antioxidant compounds from the mycelia of the edible mushroom *Grifola frondosa*. *J. Agric. Food Chem.* **2002**, *50*, 7581–7585.

(39) Takaku, T.; Kimura, Y.; Okuda, H. Isolation of an antitumor compound from *Agaricus blazei* Murill and its mechanism of action. *J. Nutr.* **2001**, *131*, 1409–1413.

(40) Kobori, M.; Yoshida, M.; Ohnishi-Kameyama, M.; Shinmoto, H. Ergosterol peroxide from an edible mushroom suppresses inflammatory responses in RAW264.7 macrophages and growth of HT29 colon adenocarcinoma cells. *Br. J. Pharmacol.* **2007**, *150*, 209–219.

(41) Kobori, M.; Yoshida, M.; Ohnishi-Kameyama, M.; Takei, T.; Shinmoto, H. 5α,8α-Epidioxy-22E-ergosta-6,9(11),22-trien-3β-ol from an edible mushroom suppresses growth of HL60 leukemia and HT29 colon adenocarcinoma cells. *Biol. Pharm. Bull.* **2006**, *29*, 755–759.