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Evaluation of Antiproliferative Activities and Action Mechanisms of Extracts from Two Species of *Ganoderma* on Tumor Cell Lines

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The antiproliferative activities on tumoral cells, namely, human breast cancer (MCF-7 and MDA-MB-231), hepatoma (HepG2) and myeloid leukemia (HL-60), of ethanolic extracts from two species of *Ganoderma*, *G. lucidum* and *G. sinense*, were investigated. Though both extracts had certain antiproliferative activities, their chemical characteristics, including nucleosides, triterpenoids and sterols, were significantly different. Their effects on MDA-MB-231 cells were further studied using apoptotic detection and cell cycle analyses. As a result, both had apoptosis induction through the alternation of mitochondrial transmembrane depolarization, though no triterpenoids were detected in ethanolic extract of *G. sinense*. Furthermore, the two extracts from *G. lucidum* and *G. sinense* could arrest cell cycle at different phases. This study showed that ethanol extracts of both *G. lucidum* and *G. sinense* have antitumoral proliferation effect through both apoptosis pathway and cell cycle arrest effect, and some other compounds such as sterols and/or nucleosides may contribute to their activity besides triterpenoids.

KEYWORDS: ***Ganoderma lucidum*; *Ganoderma sinense*; antiproliferation; cell cycle; apoptosis**

INTRODUCTION

Ganoderma is a family of well-known medicinal mushrooms, which is called Lingzhi in China because it is considered as "Fairy Grass" which could relive the life. To date, more than 120 species of *Ganoderma* have been reported in the world, 98 species of which could be found in China. However, only *G. lucidum* and *G. sinense* (**Figure 1**) are listed as Lingzhi in Chinese Pharmacopoeia, which is used for replenishing Qi to calm the mind and relieve cough and dyspnea (*1*). Modern studies have revealed that Lingzhi contain a variety of chemical ingredients, and triterpenes are believed to be among the active compounds with antitumor activity (*2*−*4*). However, our previous study showed that the content of triterpenes was significantly different between two species of *Ganoderma*, *G. lucidum* and *G. sinense*, used as Lingzhi (*5*). Actually, little or no triterpene was detected in *G. sinense* (*5, 6*). Therefore, comparison of chemical components and pharmacological activities of two species of *Ganoderma* is helpful to elucidate the active components and mechanism of the therapeutic effects of Lingzhi. Indeed, the antiproliferative effects against tumoral cells and immunomodulatory effects of aqueous extracts of *G. lucidum* and *G. sinense* were examined, which suggested that the potency of both extracts was similar (*7*). However, water has poor extraction efficiency to triterpenes in Lingzhi, which cannot be used for evaluation of their effects rationally.

In this study, the antiproliferative effects of ethanolic extracts of *G. lucidum* and *G. sinense* on four tumoral cells, namely, human breast cancer (MCF-7 and MDA-MB-231), hepatoma (HepG2) and myeloid leukemia (HL-60) cell lines, were investigated. Their action mechanism on MDA-MB-231 cells and active compounds were also hypothesized based on their chemical characteristics.

MATERIALS AND METHODS

Chemicals and Cell Cultures. *G. lucidum* (Leyss. ex Fr.) Karst. and *G. sinense* Zhao, Xu et Zhang, were obtained from Shandong province of China. The voucher specimens of *Ganoderma* were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau, China.

Acetonitrile for LC was purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA). Ergosterol, uracil, cytidine, uridine, guanosine, and adenosine were purchased from Sigma (St. Louis, MO). Ganoderic acid A was purchased from Chromadex (California), ganoderic acid C2 and F were purchased from International Laboratory (Santa Anna, CA).

The tumoral cell lines were purchased from ATCC (Manassas, VA). Cell culture medium was purchased from Invitrogen (Carlsbad, CA). MCF-7, MDA-MB-231, HepG2 and HL-60 cell lines were cultured in medium RPMI 1640 containing 10% (20% for HL-60) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37 °C with 5% CO_2. Human

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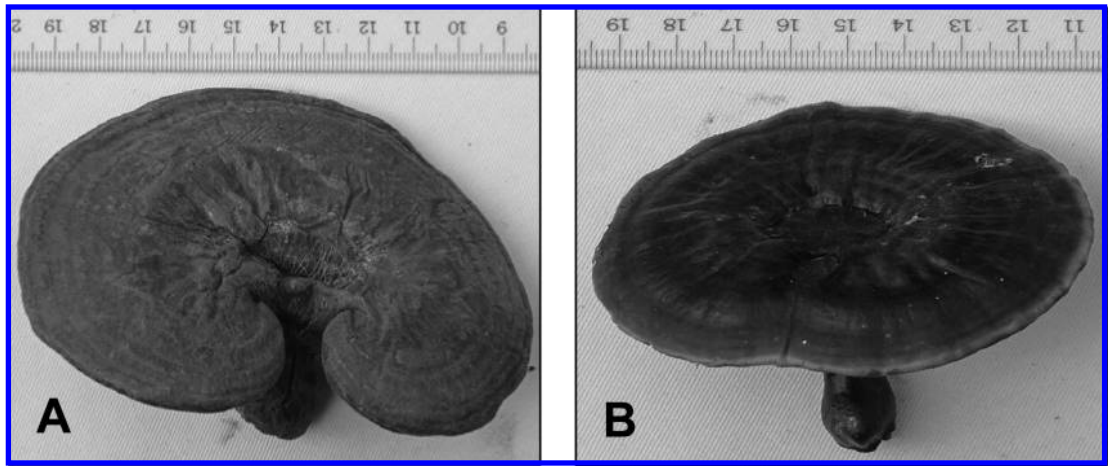

Figure 1. The raw materials of (**A**) *Ganoderma lucidum* and (**B**) *G. sinense*.

Table 1. Antiproliferative Effects of Ethanol Extracts from *G. lucidum* (GL-E) and *G. sinense* (GS-E) on Four Tumoral and Human Foreskin Fibroblasts (Hs68) Cells

samples	Hs68	HepG2	HL-60	MCF-7	MDA-MB-231
GL-E					
test range (μg/mL)	100−300	50−320	20−240	120−300	20−160
linear range (μg/mL)	120−300	50−320	20−200	160−300	20−160
regression equation	$y = 0.5665x - 75.222$	$y = 0.244x + 10.264$	$y = 0.345x + 3.4127$	$y = 0.529x - 66.192$	$y = 0.484x + 15.336$
R^2	0.920	0.970	0.947	0.974	0.963
IC_{50} (μg/mL)	209.6	162.6	136.3	219.5	71.6
95% confidence interval of IC_{50} (μg/mL)	181.2−240.1	137.8−187	106.3−165.4	192.5−248.1	56.18 −81.5
GS-E					
test range (μg/mL)	100−560	200−600	20−320	50−400	40−360
linear range (μg/mL)	200−560	400−600	40−320	120−400	160−320
regression equation	$y = 0.1927x - 28.964$	$y = 0.158x - 22.851$	$y = 0.197x + 1.6834$	$y = 0.238x - 30.958$	$y = 0.175x + 8.529$
R^2	0.952	0.995	0.939	0.960	0.990
IC_{50}(μg/ml)	378.3	462.0	245.7	339.7	237.5
95% confidence interval of IC_{50} (μg/mL)	320.1−445.2	402.4−510.4	299.7−302.1	296.2−396.5	181.2−289

foreskin fibroblasts cell (Hs68), which was used as normal cell control, was cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 °C with 5% CO_2.

Preparation of *Ganoderma* Extracts. The two species of *Ganoderma* used in this study were cut into small pieces, and dried in an oven (50 °C) for 24 h before extraction. Each *Ganoderma* species (60 g) was extracted with 1000 mL of absolute ethanol in reflux for 2 h. After filtration, the solvent of filtrates were removed under vacuum evaporation (Rotavapor R-200/205, Büchi, Switzerland) and then lyophilized (Modulyo D-230, Thermo Savant, USA). Finally, the extracts of *G. lucidum* (GL-E) and *G. sinense* (GS-E) were obtained with weights of 2.1 and 1.8 g, respectively. The ethanolic extracts were dissolved in DMSO to make stock solution at the concentration of 80 mg/mL. The final concentration of DMSO was controlled at 0.5% in the treated and control groups.

Cell Proliferation Assay. Cells were seeded in 96-well microplates (2×10^4 cells/well in 100 μL of medium). The extracts were added to the cells in serial concentrations and incubated for 48 h. Thirty microlilters of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (USB, Ohio) solution (5 mg/mL in PBS) was added to each well and further incubated for 4 h. Then the supernatant (the plate of HL-60 need centrifugation at 350*g* for 5 min first) was removed, and DMSO (100 μL) was added to dissolve the formed formazan crystals. The plate was then read in a microplate reader (1420 Multilabel counter victor^3, Perkin-Elmer, MA) at 570 nm. The cells treated with the same concentration of DMSO and MTT solution with DMSO (without cells and medium) were used as control and blank, respectively.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay. MDA-MB-231 cells were seeded in 25 cm^2 flasks. The medium was changed after incubation of 24 h, and the cells were treated with serial concentrations of GL-E and GS-E, respectively. Then the cells were incubated for an additional 48 h before harvest. The TUNEL assay was performed according to the manufacturer's instructions (Apo-BrdU TUNEL Assay Kit, Molecular Probes, Leiden, The Netherlands). Cells were fixed with 1% paraformaldehyde (PFA) in PBS on ice for 15 min. For a further fixation step, 70% ethanol was added and cells were kept on ice overnight. After 3 h of labeling at 37 °C with the DNA-labeling solution, cells were incubated with Alexa Fluor 488 conjugated anti-BrdU antibodies for 30 min at room temperature. Cells were analyzed using flow cytometry (BD FACSCanto, San Jose, CA).

Analysis of Mitochondrial Membrane Potential. MDA-MB-231 cells were seeded and incubated in six-well plates for 24 h. The medium of each well was discarded, and the cells were treated with serial concentrations of GL-E and GS-E, respectively. Then the cells were incubated in a humidified incubator (37 °C with 5% CO_2) for an additional 24 h. Finally, the cells were collected and centrifuged, the cell pellet resuspended in 1.0 mL of medium (1 μg/mL JC-1) for 20 min at 37 °C and 5% CO_2 in the dark, and analyzed using flow cytometry by excitation at 488 nm and emission at 530 and 590 nm to detect both green and red fluorescent signals in the FL1 and FL2 channels, respectively. Data were acquired and analyzed by BD FACSDiva 6.0 software.

Cell Cycle Analysis. MDA-MB-231 cells were seeded in a 25 cm^2 flask with a density of 2×10^5 cells/mL, the medium was changed after 24 h, and the cells were treated with serial concentrations of GL-E and GS-E for 24 and 48 h, respectively. The adherent cells were washed with PBS, then 300 μL of trypsin was incubated with cells for 3 min at 37 °C with 5% CO_2, and then 900 μL of medium was added to stop trypsinization and collect the cells. After centrifugation at 350*g* for 5 min at 4 °C, the cell pellet was obtained. The cell pellet was then resuspended and fixed with 1 mL of cold 70% ethanol at 4 °C overnight. The cell pellet was collected again by centrifugation at 350*g* for 5 min at 4 °C. Finally, 0.5 mL of

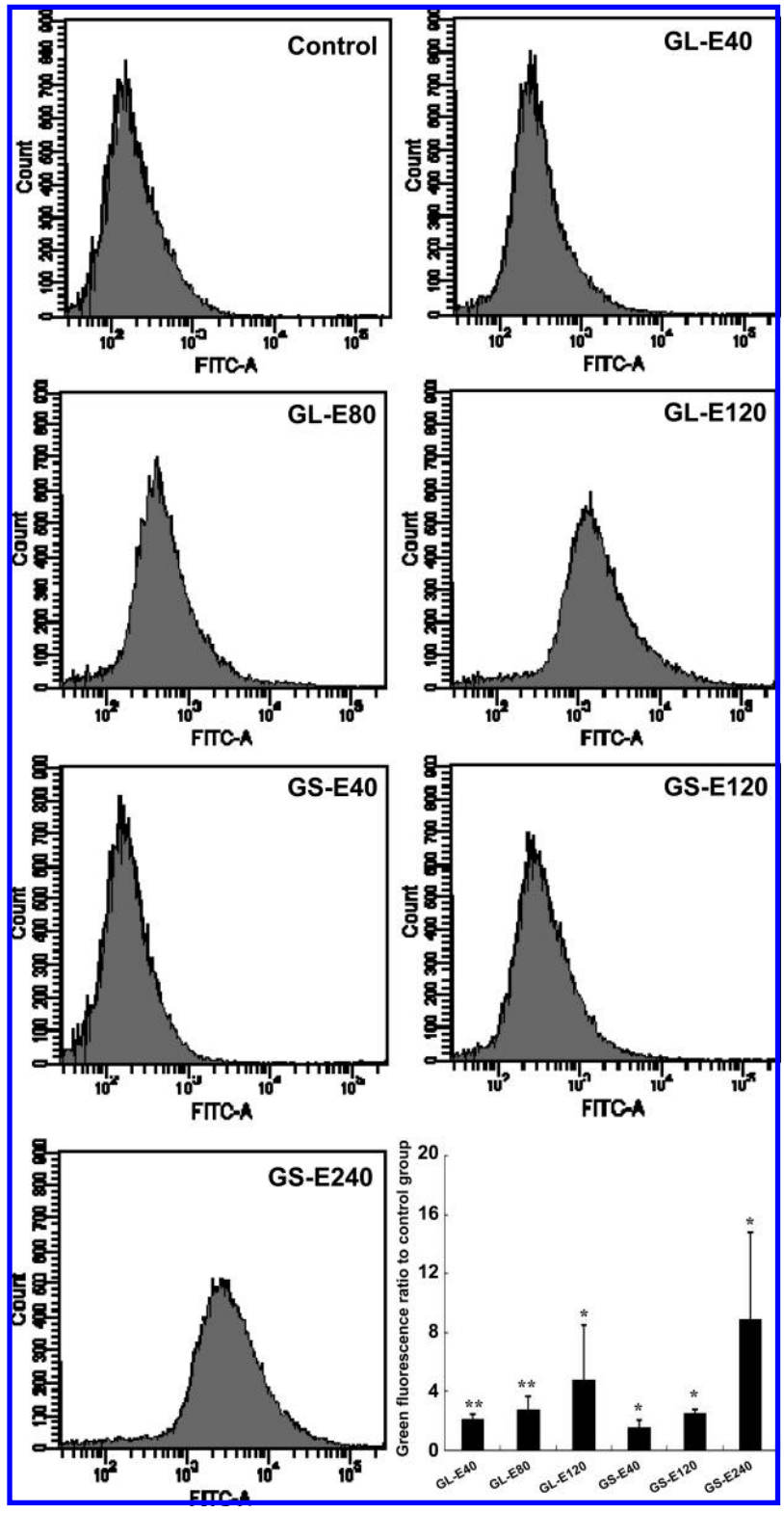

Figure 2. MDA-MB-231 cells, treated with different concentrations of ethanol extracts from *Ganoderma lucidum* (GL-E) and *G. sinense* (GS-E), were analyzed by flow cytometry after staining with TUNEL Assay Kit for 48 h. The *X*-axis indicates green fluorescence intensity on a logarithmic scale, and the *Y*-axis indicates the number of events. MDA-MB-231 cells treated without (Control) or with 40 μg/mL (GL-E40), 80 μg/mL (GL-E80) and 120 μg/mL (GL-E120) of ethanol extracts from *G. lucidum*, or 40 μg/mL (GS-E40), 120 μg/mL (GS-E120) and 240 μg/mL (GS-E240) of ethanol extracts from *G. sinense*, respectively. Data expressed as mean $\pm$ SD from three independent experiments. * $p < 0.05$ and ** $p < 0.01$ versus control.

propidium iodide (PI) stain solution was added to the samples, and the samples were then analyzed using flow cytometry. The data were analyzed with Modfit LT 3.0 software (Verity Software House, Topsham).

Chemical Analysis of *Ganoderma* Extracts. The compounds in *Ganoderma* extracts were analyzed using HPLC, which was performed on an Agilent 1200 series LC/MSD VL trap system (Agilent Technologies, Palo Alto, CA), equipped with a vacuum degasser, a binary pump, an autosampler, a diode array detector (DAD) and an ion trap mass spectrometer with electrospray ionization (ESI) interface connected to a Agilent LC/MSD Trap Software (version 4.2). An Agilent ZORBAX Aq C18 column (5

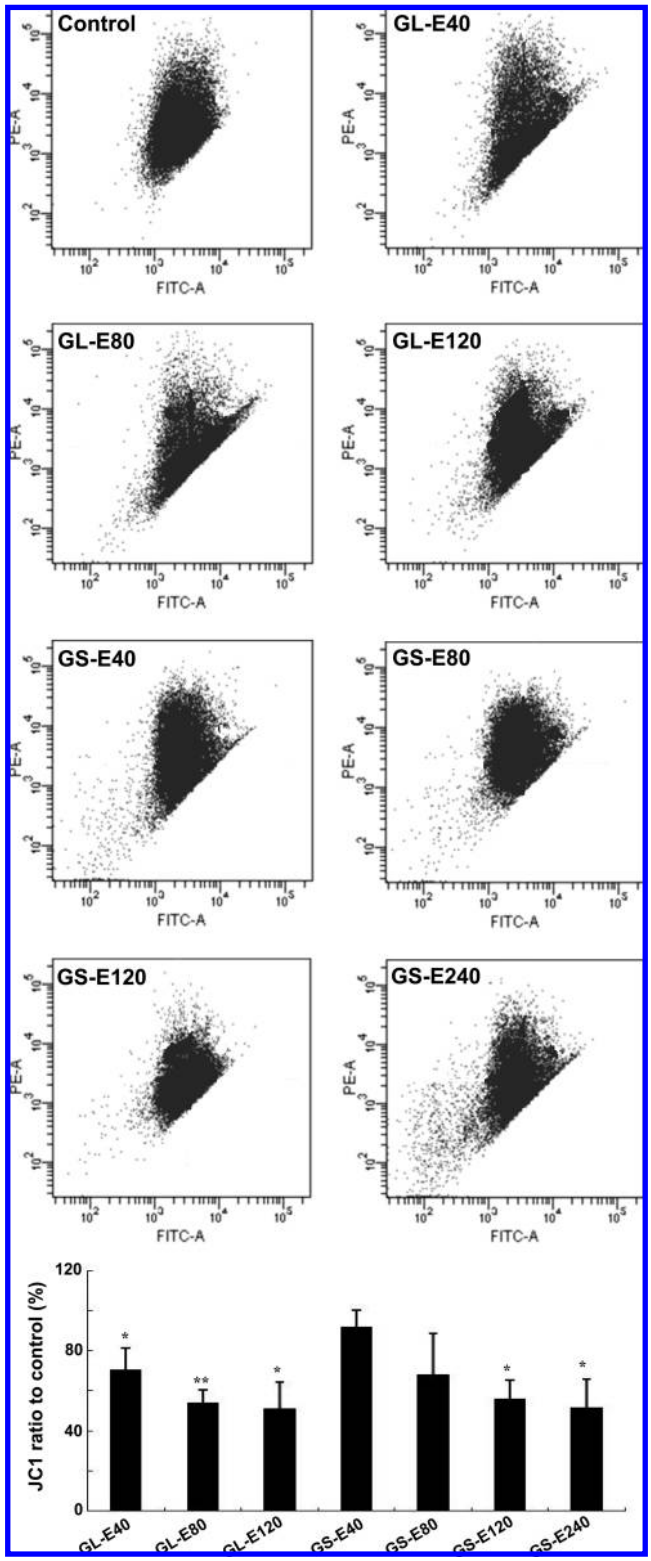

Figure 3. Analysis of change of mitochondrial transmembrane potential in MDA-MB-231 cells. MDA-MB-231 cells were stained with JC-1 probe, and analyzed using flow cytometry. Red fluorescence and green fluorescence were measured by FL2 and FL1 channel, respectively. Red fluorescence indicates intact mitochondrial potential while green fluorescence indicates breakdown of mitochondrial potential. The ratio of intensity of FL2 to FL1 ($\Delta\Psi$m) indicates the change of mitochondrial transmembrane potential, which present as JC-1 ratio ($\Delta\Psi m_{treatment}/\Delta\Psi m_{control} \times 100\%$, *I*). Data expressed as mean ± SD from three independent experiments. * $p < 0.05$ and ** $p < 0.01$ versus control. The group labels were the same as in **Figure 2**, besides the cells treated with 80 μg/mL (GS-E80) of ethanol extracts from *G. sinense*.

μm, 4.6 mm × 250 mm) with a ZORBAX Aq C18 column guard column (5.0 μm, 4.6 mm × 12.5 mm) was used. The separation was achieved using gradient elution with 5 mM ammonium acetate solution (A) and acetonitrile (B): 0−25 min, 0%−1% B; 25−45 min, 25%−26% B; 45−100 min, 26%−30% B; 100−110 min, 30%−40% B; 110−145 min, 40%−100% B; 145−150 min, 100% B isocratic. The flow rate was set at 1.0 mL/min. The column temperature was maintained at 30 °C, and DAD detection was at 260 nm. An aliquot of 15 μL of *Ganoderma* extract solution (5 mg/mL) obtained from above was injected for HPLC analysis. MS spectra were acquired in positive ion mode. The full scan mass spectra were obtained from *m*/*z* 100 to 1200. ESI-MS conditions were as follows: drying gas, N_2, 8.5 L/min, temperature, 320 °C, nebulizer pressure, 35 psi and compound stability 50%. ESI-MS/MS parameters: collision energy was set at 2 V, isolation width 4, fragment amplification 1.0.

Statistical Analysis. Data are expressed as mean ± SD from at least 3 independent experiments. IC_{50} was calculated by the software CraphPad Prism 5.0 (San Diego, CA). Differences between groups were determined by Student's *t* tests. $P < 0.05$ was considered significant.

RESULTS

Antiproliferative Effects of the Extracts. Assays for proliferative response in four tumor cells lines (HepG2, HL-60, MCF-7 and MDA-MB-231) and human foreskin fibroblasts (Hs68) were performed to evaluate the antiproliferative effects of the extracts from two *Ganoderma* species. Under the experimental conditions (48 h), both extracts of *Ganoderma* exhibited growth inhibitory effects on four tumor cells, but the antiproliferation of the ethanol extract of *G. lucidum* was significantly stronger than that of the extract of *G. sinense* (**Table 1**). Human normal fibroblastic cell, Hs68, was used as a control to determine whether their inhibition on the cancer cell proliferation was selective. As the result, the inhibition of GL-E (120 μg/mL) on HepG2, HL-60, MCF-7, MDA-MB-231 and Hs68 was 39.8%, 44.8%, 2.0%, 82.5% and 2.0%, respectively, while GS-E (200 μg/mL) inhibited HepG2, HL-60, MCF-7, MDA-MB-231 and Hs68 at the ratio of 15.5%, 41.1%, 11.26%, 45.5% and 4.11%, respectively. The results suggested that the ethanol extracts of *Ganoderma* had more selectivity on the MDA-MB 231 cell. Therefore, the MDA-MB-231 cell was further investigated.

Proapoptotic Effect of the Ethanolic Extracts. Apoptosis of MDA-MB-231 treated with the extracts was examined by TUNEL assay (Apo-BrdU TUNEL Assay Kit), which was used to detect the DNA fragmentation of advanced stage apoptosis cells by exploiting the fact that the DNA breaks and expose a large number of 3′-hydroxyl ends, at 48 h. A significant number of cells containing DNA strand breaks were found after treatment with the extracts of *Ganoderma* by flow cytometry analysis (**Figure 2**). At the same concentrations (40 μg/mL and 120 μg/mL), the fluorescence intensity of the GL-E group was 2 times and 4.8 times, and that of the GS-E group was 1.6 times and 2.5 times, respectively, that of control, which indicated that the proapoptotic effect of GL-E was stronger than that of GS-E. However, at around the concentration of their IC_{50} of antiproliferative activity, the fluorescence intensity of the GL-E group (80 μg/mL) is less than half of that of the GS-E group (240 μg/mL).

Analysis of Mitochondrial Membrane Potential for Apoptotic Effect. Alteration of the mitochondrial transmembrane potential, $\Delta\Psi$m, is one of the pathways inducing apoptosis. Mitochondrial injury was assessed by JC-1 dye, a mitochondrial potential sensor (Molecular Probes). The accumulation of JC-1 in the mitochondria causes the fluorescence emission shift from green to red. Thus, the intact mitochondria are

Table 2. Effect of Ethanol Extracts of *Ganoderma* on Cell Cycle of MDA-MB-231 Cells ($n = 3$)[a]

	G1		S		G2	
groups	24 h	48 h	24 h	48 h	24 h	48 h
control	40.49 ± 2.24	41.26 ± 2.80	57.44 ± 1.11	56.2 ± 2.27	2.06 ± 2.91	2.54 ± 2.34
GL-E, 40 μg/mL	46.38 ± 0.70*	45.94 ± 1.78	50.26 ± 5.45	49.61 ± 2.91*	3.36 ± 4.75	4.45 ± 4.63
GL-E, 80 μg/mL	49.58 ± 2.94*	50.14 ± 4.56*	46.84 ± 4.23*	45.94 ± 2.41**	3.56 ± 5.03	3.93 ± 3.21
GL-E,120 μg/mL	53.47 ± 5.64*	52.87 ± 5.36*	43.41 ± 5.00*	42.70 ± 3.14**	3.12 ± 4.41	4.44 ± 3.26
GS-E, 40 μg/mL	43.48 ± 2.67	47.56 ± 3.16	52.32 ± 1.04**	44.16 ± 2.83**	4.21 ± 3.68	8.28 ± 2.73*
GS-E, 80 μg/mL	44.53 ± 2.76	46.13 ± 1.73	50.18 ± 3.57*	45.41 ± 0.91**	5.29 ± 4.88	8.46 ± 2.61*
GS-E, 120 μg/mL	44.16 ± 2.23	45.82 ± 3.47	51.76 ± 2.72*	43.19 ± 0.75**	4.07 ± 3.87	11.00 ± 3.54*
GS-E, 240 μg/mL	43.15 ± 10.07	38.47 ± 6.87	50.21 ± 2.94*	49.88 ± 2.80*	6.64 ± 7.24	11.65 ± 4.58*

[a] GL-E and GS-E are the ethanolic extracts of *G. lucidum* and *G. sinense*, respectively. *$p < 0.05$, **$p < 0.01$.

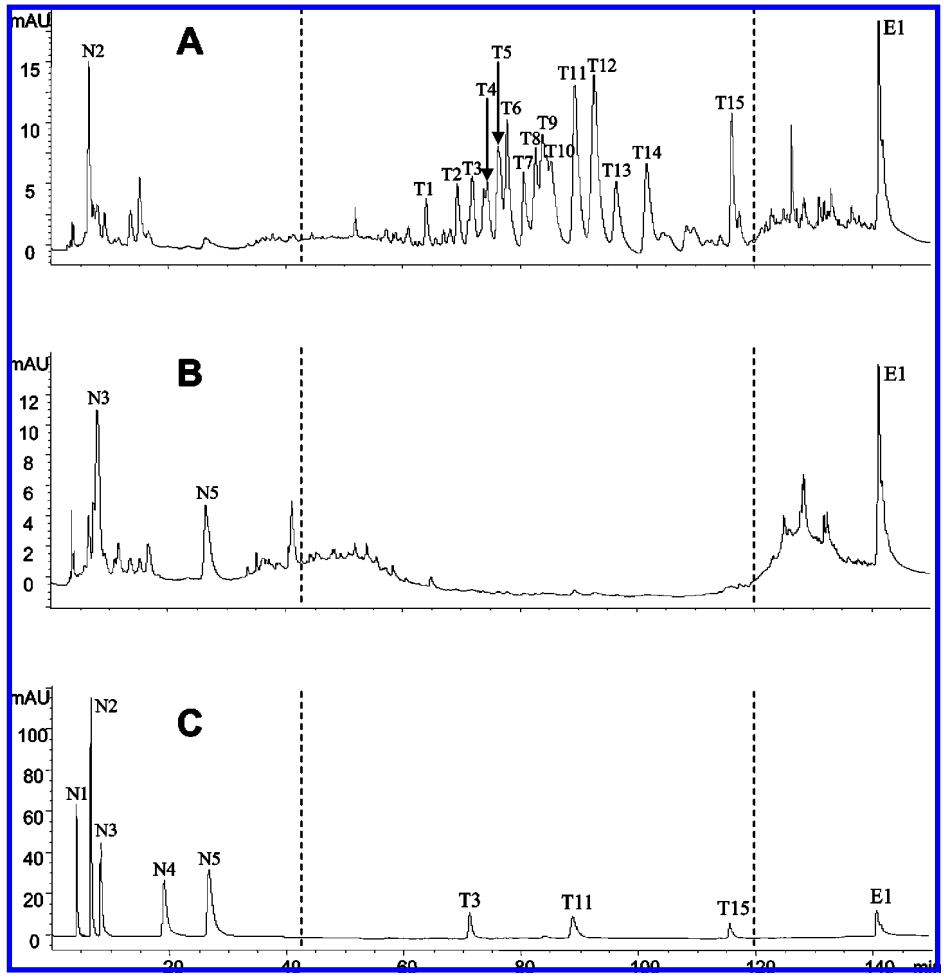

Figure 4. The chromatograms of ethanol extracts of (**A**) *G. lucidum*, (**B**) *G. sinense,* and (**C**) mixed standards.. N1, uracil; N2, cytidine; N3, uridine; N4, guanosine; N5, adenosine; T1, unknown; T2, unknown; T3, ganoderic acid C2; T4, ganoderic acid C6; T5, ganoderic acid G; T6, ganoderenic acid B; T7, ganoderic acid B; T8, ganoderenic acid K; T9, lucidenic acid E; T10, ganoderic acid K; T11, ganoderic acid A; T12, ganoderic acid H; T13, ganoderenic acid D; T14, ganoderic acid D; T15, ganoderic acid F; and E1, ergosterol.

indicated by a higher ratio of red to green fluorescence compared to that of the depolarized mitochondria. After treated with a series of concentration of GL-E or GS-E for 24 h, the red/green fluorescence in MDA-MB-231 cells significantly decreased (**Figure 3**).

Effects of the Extracts on Cell Cycle. The effects of different concentrations of GL-E and GS-E on cell-cycle progression of MDA-MB-231 cells was investigated after 24 h and 48 h. Treatment of cells with GL-E and GS-E led to obvious time-dependent changes in the cell cycle profiles (**Table 2**). GL-E treatment resulted in a significant increase percentage at the G1 phase, along with a dramatic decrease cell population of the S phase, while 48 h after GS-E treatment, a significant accumulation of cells in G2 phase with concomitant losses from S phase was observed. These results could be explained by the hypothesis that GL-E decreased G1-phase progression through the cell cycle or G1/S phase transition, and GS-E induced S/G2 transition or cell cycle arrest at the G2 phase.

Chemical Characteristics of the Extracts. For chemical characteristics, nucleosides, triterpenoids and sterols were investigated. The typical chromatograms of the mixed standards and different extracts of two species of *Ganoderma* are shown in **Figure 4**. The identification of the peaks was performed by comparing their MS data with the standards or references (*8*), which are listed in **Table 3**.

Table 3. The MS Data of Triterpenoids in *Ganoderma*

peaks	retention time (min)	MS (*m*/*z*)	identification
T1	63.9	531 [M − H]$^-$, 513, 401	unknown
T2	69.3	475 [M − H]$^-$, 457, 301	unknown
T3	71.7	517 [M − H]$^-$, 499, 431	ganoderic acid C2
T4	74.4	529 [M − H]$^-$, 511, 467	ganoderic acid C6
T5	76.2	531 [M − H]$^-$, 513, 469	ganoderic acid G
T6	77.8	513 [M − H]$^-$, 495, 451	ganoderenic acid B
T7	80.7	515 [M − H]$^-$, 497, 453	ganoderic acid B
T8	82.7	571 [M − H]$^-$, 533	ganoderenic acid K
T9	83.8	515 [M − H]$^-$, 473	lucidenic acid E
T10	84.9	573 [M − H]$^-$, 555, 469, 265	ganoderic acid K
T11	89.4	515 [M − H]$^-$, 497, 479, 435	ganoderic acid A
T12	92.6	571 [M − H]$^-$, 553, 511, 467	ganoderic acid H
T13	96.4	511 [M − H]$^-$, 493, 449	ganoderenic acid D
T14	101.6	513 [M − H]$^-$, 495, 451, 247	ganoderic acid D
T15	116.1	569 [M − H]$^-$, 551, 509, 465	ganoderic acid F

DISCUSSION

Ganoderma, a well-known traditional Chinese medicinal fungus, has been a favorite oriental medicine for centuries. In this study, the antiproliferative effects on tumoral cells and chemical characteristic of ethanolic extracts from two species of *Ganoderma*, *G. lucidum* and *G. sinense*, used as Lingzhi in China were investigated. The results showed that both *G. lucidum* and *G. sinense* had antitumor proliferation effect, but the former was more potent than latter. Actually, many studies on *G. lucidum* have demonstrated its antitumor activities but few reports are focused on *G. sinense* (*7*, *9*−*12*), even if both *G. lucidum* and *G. sinense* are the official species of Lingzhi recorded in Chinese Pharmacopoeia (2005) and they are considered to have the same therapeutic effects. Generally, triterpenoids and polysaccharides have been considered as the major active components in *Ganoderma*, which were also thoroughly investigated (*13*). Indeed, triterpenoids from *G. lucidum* have cytotoxicity (*3*, *4*, *14*), antiangiogenic (*15*), proapoptotic (*16*) and cell cycle arrest (*16*, *17*) activities. Our studies showed that triterpenoids were richer in ethanol extract of *G. lucidum* than in that of *G. sinense*. Therefore, the former have more potent antiproliferative activity on tumoral cells than the latter, which supported the previous study (*18*). Actually, the extracts with triterpenoids of *G. lucidum* possess antitumor activity through similar ways, such as antiangiogenic (*19*), proapoptotic (*20*, *21*), cell cycle arrest (*18*, *20*) and tumor invasion inhibition (*22*) actions. However, the extracts of *G. sinense* almost contained nothing of triterpenoids, which suggested that the active components and mechanism of their antiproliferative action on tumoral cells were, at least partially, different from those of *G. lucidum*. Ideally, the cell type of the control cell should be the same as that of the tumoral cell for investigation of the selectivity of a drug. However, it is difficult to perform. Actually, a different cell type such as Hs68 used in this study also can give information for the selectivity of the cytotoxic activity of drugs (*7*, *23*, *24*).

It is very interesting that ethanol extracts of both *G. lucidum and G. sinense* contain similar sterols. Ergosterol, one of the chemical components from mycelium cells, is the predominant sterol found in most fungi. It is also the common component in the two species of *Ganoderma*. Actually, ergosterol analogues also have multiple pharmacological activities, such as cytotoxic action (*25*, *26*). The anticancer property of phytosterols has also been reported (*27*). Phytosterols can induce apoptosis and stimulate ceramide cascades in transformed cell lines in vitro. Significant induction of cellular apoptosis following β-sitosterol supplementation has been observed in MDA-MB-231 cells (*28*). Other phytosterols including solamargine are potent inducers of apoptosis in human erythroleukemia HEL and human hepatocytic Hep3B cells (*29*). Phytosterols also affect cell cycle kinetics. In tissue culture studies of MDA-MB-231 cells, β-sitosterol induced cell cycle arrest at the G2/M transition (*30*). After continuous β-sitosterol supplementation, 43% of the breast carcinoma cells were in G2 compared to 12 and 24% of cells maintained in cholesterol or vehicle supplemented media, respectively. Generally, phytosterols are not potent inhibitors of cell cycle progression, but rather they act as weak inhibitors that over prolonged periods of exposure subtly regulate the activity of proteins and the expression of genes involved in cell growth and apoptosis. The antiproliferative property of sterols in *Ganoderma* needs further investigation.

The sensitivity of two human breast cancer cells, MDA-MB-231 and MCF-7 cells, to the inhibition of *Ganoderma* extracts is significantly different. This differential action may be resulted from the hormonal status in the cells. A recent work has shown that the inhibitory effects of *G. lucidum* on the proliferation of estrogen-dependent (MCF-7) and estrogen independent (MDA-MB-231) breast cancer cells are attributed to the modulation of the estrogen receptor (ER) and NF-κB signaling (*31*). Another report suggested that the aqueous extracts of *Ganoderma*, with stronger immunopotentiating activities in mouse splenic lymphocytes, could significantly inhibit cell proliferation in human breast cancer cell lines MCF-7 and MDA-MB-231 (*7*). These interesting results imply that the effects of ethanolic extracts of *Ganoderma* may depend on the estrogen-related pathways. Furthermore, nucleosides are rich in both *G. sinense* and *G. lucidum* (*32*, *33*). These compounds in *Ganoderma* and their activities should be further investigated.

In summary, our study shows that both ethanol extracts of *G. lucidum* and *G. sinense* have antitumor proliferation effects through both apoptosis pathway and cell cycle arrest effect, and some other compounds may contribute to their activity besides triterpenoids. Further study on *G. sinense* is need.

ABBREVIATIONS USED

$\Delta\Psi$m, mitochondrial transmembrane potential; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HPGPC, high performance gel permeation chromatography; HPLC, high performance liquid chromatography; IC_{50}, 50% of inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

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