

Antitumor effects and mechanisms of *Ganoderma* extracts and spores oil

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Abstract. *Ganoderma lucidum* is a popular herbal medicine used in China to promote health. Modern studies have disclosed that the active ingredients of *Ganoderma* can exhibit several effects, including antitumor effects and immunomodulation. The present study evaluated the antitumor effects of self-prepared *Ganoderma* extracts and spores oil, and investigated the possible underlying mechanisms by observing the effects of the extracts and oil on topoisomerases and the cell cycle. The results showed that *Ganoderma* extracts and spores oil presented dose-dependent inhibitory effects on tumor cells. The half maximal inhibitory concentration (IC₅₀) values of *Ganoderma* extracts on HL60, K562 and SGC-7901 cells for 24 h were 0.44, 0.39 and 0.90 mg/ml, respectively; for *Ganoderma* spores oil, the IC₅₀ values were 1.13, 2.27 and 6.29 mg/ml, respectively. In the *in vivo* study, the inhibitory rates of *Ganoderma* extracts (4 g/kg/d, intragastrically) on S180 and H22 cells were 39.1 and 44.6%, respectively, and for *Ganoderma* spores oil (1.2 g/kg/d, intragastrically) the inhibitory rates were 30.9 and 44.9%, respectively. *Ganoderma* extracts and spores oil inhibited the activities of topoisomerase I and II. *Ganoderma* spores oil was shown block the cell cycle at the transition between the G1 and S phases and induce a marked decrease in cyclin D1 levels in K562 cells, with no significant change in cyclin E level. These results suggest that the *Ganoderma* extracts and spores oil possessed antitumor effects in the *in vitro* and *in vivo* studies. The antitumor mechanisms of the extracts and spores oil were associated with inhibitory effects on topoisomerase I and II activities, and for *Ganoderma* spores oil, the antitumor effects may also be associated with decreased cyclin D1 levels, thus inducing G1 arrest in the cell cycle.

Introduction

Ganoderma lucidum, also called *Ganoderma* or Lingzhi, is one of most frequently used fungi in Chinese medicine. Modern pharmacological and clinical studies have confirmed that *Ganoderma* contains abundant biologically active substances in its fruiting body, mycelia and spores, and possesses variable functions, including immunomodulation, anti-aging, reducing blood lipids, anti-viral and anti-tumor activities (1-6). The present study examined the antitumor activity of a mixture of aqueous and ethanol extracts of the *Ganoderma* fruiting body and *Ganoderma* spores oil, which was extracted from broken spores by supercritical CO₂ extraction technology and explored the possible underlying mechanisms. DNA topoisomerases are a class of enzymes involved in the regulation of DNA supercoiling. Topoisomerase overexpression has been linked to a number of human malignancies and is the target for numerous chemotherapeutic agents (7). In the event that topoisomerases are blocked, the cell encounters problems during transcription of the DNA and during cell division. The widely-used anti-tumor drug, camptothecin, blocks the relaxing action of class I topoisomerases and induces significant G1 cell cycle arrest (8). A previous study indicated that the active components of *Ganoderma* exhibited inhibition of topoisomerases (9). The present study examined whether *Ganoderma* extracts and spore oil affected the cell cycle and topoisomerases I and II.

Materials and methods

Preparations of *Ganoderma* extracts and spores oil. *Ganoderma* extracts (GanoHerb™) and *Ganoderma* spores oil were provided by Fujian Xianzhilou Biological Science and Technology Co., Ltd. (Fuzhou, China). *Ganoderma* extract, a brown powder, was dissolved in double distilled water to prepare solutions of various concentrations, which were brown suspensions. *Ganoderma* spores oil was a soft capsule with 0.5 g/0.5 ml golden oil in each capsule. The stock solution of *Ganoderma* spores oil were prepared using double distilled water that contained 6 μl/ml (v/v) Tween 80.

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Cell lines and culture. The human acute myeloid leukemia HL-60, human chronic myeloid leukemia K562, human gastric carcinoma SGC7901, murine sarcoma S180 and murine

hepatoma H22 cell lines were purchased from the Shanghai Cell Institute of the Chinese Academy of Sciences (Shanghai, China). All tumor cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and antibiotics (100 kU/l penicillin and 100 mg/l streptomycin) at 37°C in a humidified atmosphere of 5% CO₂.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay. The cell cytotoxicity of *Ganoderma* spores oil *in vitro* was detected by MTT assay. The MTT assay was based on the ability of live cells to utilize thiazolyl blue and convert it into dark blue formazan. Exponentially growing cells (2x10⁴/well) were seeded into 96-well plates and treated with a series of concentrations of *Ganoderma* spores oil (0.31, 0.63, 1.25, 2.5, 5 and 10 mg/ml) for 24 h. Control cells were exposed to double distilled water containing the same concentration of Tween 80. Subsequently, 20 µl of 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 h. The dark blue formazan crystals were solubilized in lysis buffer containing 10% sodium dodecyl sulfate (SDS), 5% isobutyl alcohol and 12 mmol/l HCl, and the optical density (OD) was detected at 492 nm with a spectrophotometer. The rate of growth inhibition was calculated according to the formula: Inhibition rate (%) = (1 - mean OD value of treated group/mean OD value of control group) x 100. The half maximal inhibitory concentration (IC₅₀) value was calculated by linear regression method.

Trypan blue exclusion method. The cell cytotoxicity of *Ganoderma* extract *in vitro* was assessed using the trypan blue exclusion method. Briefly, exponentially growing cells were treated with a series of concentrations of *Ganoderma* extract (0.22, 0.44, 0.67, 0.89 and 1.10 mg/ml) for 24 h. Control cells were exposed to double distilled water. Then cells were dyed with 0.4% trypan blue. Viable (trypan blue dye-excluding) cells were counted using a light microscope. Inhibition rate was calculated according to the formula: Inhibition rate (%) = (1 - viable cells in treated group / viable cells in control group) x 100.

Antitumor effects in vivo. A total of 60 ICR mice (6-8 weeks old; male and female; body weight, 18-20 g) were obtained from the Animal Centre of Fujian Medical University (Fuzhou, China) and maintained in specific pathogen-free conditions. Animals were exposed to a 12 h light/dark cycle and fed a standard diet. All animal studies were approved by the ethics committee of Fujian Medical University. S180 or H22 tumor cells in suspension (1x10⁶ cells in 200 µl solution) were carefully inoculated intradermally into the left axilla of mice. The tumor-bearing mice were then randomly assigned to treatment experimental groups (n=10 per group). At 24 h following the tumor inoculation, mice were intragastrically administrated with i) extract of *Ganoderma*, 4 g/kg; ii) extract of *Ganoderma*, 2 g/kg; iii) extract of *Ganoderma*, 1 g/kg; iv) *Ganoderma* spores oil, 1.2 g/kg; v) fluorouracil (5-FU), 25 mg/kg (positive control); or vi) 0.9% NaCl solution 0.2 ml/10 g (negative control) once per day for 10 consecutive days. At the end of the experiments (day 12), mice were weighed and then sacrificed by cervical dislocation. The tumors, thymus glands and spleens

of the mice were excised carefully and weighed. The following formulae were used: i) Tumor suppression rate (%) = (1 - mean tumor weight in treated group / mean tumor weight in control group) x 100; ii) Thymus index = 100 x thymus weight / body weight; and iii) Spleen index = 100 x spleen weight / body weight.

Catalytic activity of topoisomerases. The catalytic activity of topoisomerases was measured as previously described (10). K562 cells (10⁷) were washed with phosphate-buffered saline (PBS) and treated with lysis buffer containing 20 mM Tris, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 25 mM KCl, 5 mM MgCl₂, 250 mM sucrose and 0.5% NP40 (pH 7.2) for 10 min at 4°C, then centrifuged at 4,000 x g for 2 min. The pellet was resuspended in 50 µl of extraction buffer containing 20 mM Tris, 1 mM EGTA, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT) and 400 mM NaCl (pH 7.2) and incubated for 30 min at 4°C. After centrifugation at 22,000 x g for 15 min, the supernatant containing extracted topoisomerases was obtained and its protein concentration was determined using the Bradford Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions, prior to storage at -20°C.

The catalytic activity of topoisomerase II in *Ganoderma* extracts was evaluated as follows: The total reaction volume was 10 µl, containing 2.5 µl 4X reaction buffer [50 mM Tris (pH 8.0), 120 mM KCl, 0.5 mM DTT, 0.5 mM adenosine triphosphate (ATP), 10 mM MgCl₂ and 30 µg/ml bovine serum albumin (BSA; Beyotime Institute of Biotechnology)], 0.1 mM ATP (Promega Corporation, Madison, WI, USA), 0.2 µg pBR322 DNA (Promega Corporation), various amounts of topoisomerase II-containing crude extract and double distilled water to make the total volume up to 10 µl. Following incubation at 37°C for 30 min, the reaction was stopped by the addition of 5 µl of a solution (50 mM EDTA, 50% glycerol and 0.25 mg/ml bromophenol blue) maintained at 4°C. Samples were separated through a 1% agarose gel. After staining with ethidium bromide, gels were photographed under ultraviolet (UV) illumination and analyzed using Syngene Genesnap Tools software (version 3.00.22; Syngene; Synoptics Ltd., Cambridge, UK).

The catalytic activity of topoisomerase I in *Ganoderma* extracts was evaluated as follows: The total reaction volume was 10 µl, containing 1 µl 10X reaction buffer [10 mM Tris-HCl (pH 7.9), 0.15 M NaCl, 1 mM EDTA, 0.1 mM spermidine, 0.1% BSA and 5% glycerol], 0.2 µg pBR322 DNA, various amounts of topoisomerase I-containing crude extract and double distilled water to make the total volume up to 10 µl. The rest of the procedures were the same as the aforementioned topoisomerase II method.

The inhibitory effects of *Ganoderma* extracts and *Ganoderma* spores oil on topoisomerase I and II were then detected. After selecting the amounts of crude extract that unwound 0.2 µg pBR322 DNA to establish topoisomerase I and topoisomerase II reaction systems, 1, 2 and 4 mg/ml extracts and 4.5, 9 and 18 mg/ml spores oil was added to each reaction system and double distilled water was used to make the total volume up to 10 µl. Following incubation at 37°C for 30 min, the reaction was stopped and separated through 1% agarose

Table I. Growth inhibition rate of *Ganoderma* extracts on tumor cell lines.

Cell lines	Inhibitory rate, %					IC ₅₀ , mg/ml
	1.10 mg/ml	0.89 mg/ml	0.67 mg/ml	0.44 mg/ml	0.22 mg/ml	
K562	97.34	90.55	85.71	40.48	9.52	0.44
HL60	100.00	95.35	95.20	56.16	12.33	0.39
SGC-7901	73.56	38.92	18.37	3.43	-1.64	0.90

IC₅₀, half maximal inhibitory concentration.

Table II. Growth inhibition rate of *Ganoderma* spores oil on tumor cell lines.

Cell lines	Inhibitory rate, %						IC ₅₀ , mg/ml
	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.63 mg/ml	0.31 mg/ml	
K562	83.14	74.47	70.21	52.63	32.31	11.49	1.13
HL60	89.62	87.76	55.50	15.50	8.88	3.54	2.27
SGC-7901	68.31	41.27	12.84	7.64	-1.30	2.67	6.29

IC₅₀, half maximal inhibitory concentration.

gel. The staining gel was photographed under ultraviolet (UV) illumination and analyzed using Syngene Genesnap Tools software (version 3.00.22; Syngene).

Cell cycle analysis. The cell cycle was analyzed by flow cytometry. K562 cells were seeded in 6-well plates at a density of 1×10^5 /ml, and treated with 1.25 and 2.5 mg/ml *Ganoderma* spores oil for 12 h. Then cells were washed twice with PBS and fixed in 70% ethanol at 4°C overnight. Prior to analysis, cells were washed and resuspended in PBS, then incubated with 10 mg/ml RNase A for 3-5 min and 50 µg/ml propidium iodide (Sigma-Aldrich) at 4°C for 30 min in a dark chamber. The cell cycle distribution (percentage of cells in various phases) was analyzed by flow cytometry (Epics XL; Beckman Coulter, Inc., Brea, CA, USA) using Multicycle software (version 3.11; Phoenix Flow Systems; San Diego, CA, USA).

Western blot analysis. K562 cells (1×10^7) were washed with PBS and then treated with lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM phenylmethane sulfonyl fluoride, 1 mM aprotinin and 1% NP40 for 30 min at 4°C. Following centrifugation at $11,000 \times g$ for 20 min, the protein concentration of the supernatant was determined; equal amounts (30 µg) of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and then electro-transferred onto nitrocellulose membranes in transfer buffer. The membrane was blocked with 5% BSA for 2 h and then incubated with monoclonal mouse anti-human cyclin D1 (cat. no. sc-20044; 1:500), monoclonal mouse anti-human cyclin E (cat. no. sc-247; 1:500) and polyclonal rabbit anti-human actin (cat. no. sc-7210; 1:2,000) primary antibodies (Santa Cruz Biotechnology Inc., Dallas, TX, USA) in blocking solution at 4°C for 2 h, followed by extensive washing with PBS twice. The membranes were then

incubated with alkaline-phosphatase conjugated goat anti-mouse (cat. no. sc-2047; 1:2,500) and goat anti-rabbit (cat. no. sc-2034; 1:2,000) IgG secondary antibodies (Santa Cruz Biotechnology Inc.) in blocking solution at room temperature, followed by washing with Tris-buffered saline 3 times. The target proteins became visible following the addition of Alkaline Phosphatase Substrate Solution BCIP/NBT (Promega Corporation).

Statistical analysis. Statistical analysis of the data was performed with the Student's *t*-test. Data were expressed as the mean ± standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Inhibitory effects on tumor cells in vitro. The anti-proliferative activities of *Ganoderma* extracts and spores oil on the K562, HL60 and SGC-7901 cell lines were determined. Using the trypan blue exclusion method, dose-dependent inhibitory effects were observed when the *Ganoderma* extract concentrations were in the range of 0.22-1.10 mg/ml. The IC₅₀ value of *Ganoderma* extracts for the K562, HL60 and SGC-7901 cells were 0.44, 0.39 and 0.90 mg/ml, respectively (Table I).

The MTT assays revealed that treatment with *Ganoderma* spores oil concentrations of 0.31-10.0 mg/ml caused dose-dependent cytotoxicity in K562, HL60 and SGC-7901 cells, with IC₅₀ values of 1.13, 2.27 and 6.29 mg/ml, respectively (Table II).

Suppression of tumor growth in vivo. The effects of intragastric administration of *Ganoderma* extracts and spores oil on S180 cells in mice were examined (Fig. 1; Table III). Table III shows that the 4 g/kg extracts and 1.2 g/kg oil groups resulted in significant inhibitions of tumor growth by comparing the average

Table III. Inhibitory effects of *Ganoderma* extracts and spores oil on S180 in mice.

Groups ^a	Body weight, g			Tumor weight, g	Inhibitory rate, %
	Start	End	Difference		
Control	21.1±1.3	26.3±2.7	5.2±1.7	1.4±0.5	-
Extracts, 4 g/kg	21.6±1.2	26.3±2.4	4.7±1.8	0.9±0.5	39.1 ^b
Extracts, 2 g/kg	21.6±0.7	26.6±2.0	5.0±1.8	1.2±0.5	19.0
Extracts, 1 g/kg	21.6±0.7	26.3±1.6	4.7±1.6	1.2±0.4	15.6
Oil, 1.2 g/kg	21.1±1.0	25.9±2.3	4.8±2.2	1.0±0.4	30.9 ^b
5-FU, 25 mg/kg	21.7±0.8	22.0±2.6	0.3±2.4 ^c	0.7±0.2	54.1 ^c

Data are expressed as mean ± standard deviation. ^aNo. of mice per group (n=10). ^bP<0.05, ^cP<0.01 vs. control; Student's *t*-test. 5-FU, fluorouracil.

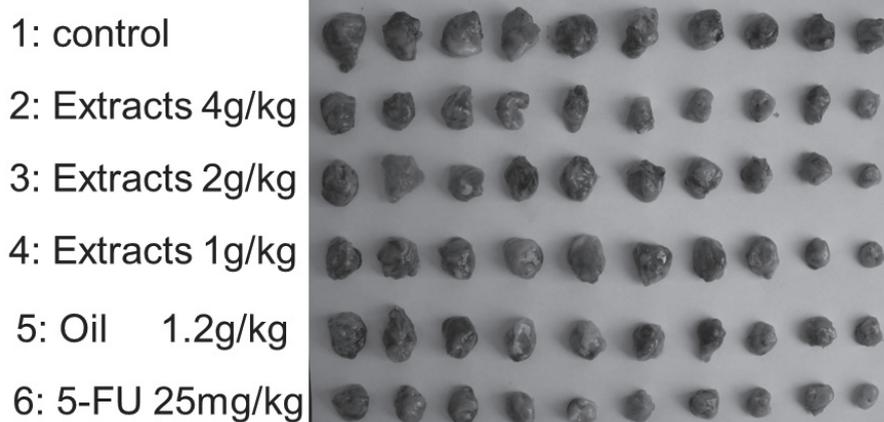


Figure 1. Tumor volume of S180 in mice influenced by *Ganoderma* extracts and spores oil. The tumor volumes in the 4 g/kg extracts group and 1.2 g/kg oil group were significantly smaller compared with the control group. 5-FU, fluorouracil.

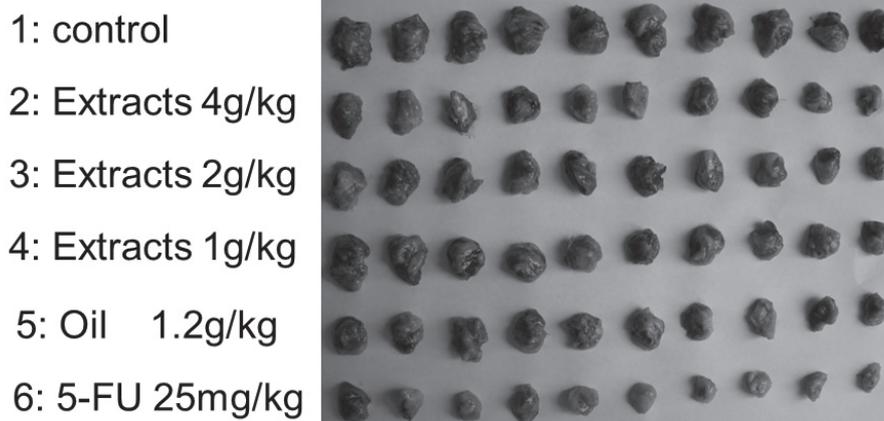


Figure 2. Tumor volume of H22 in mice affected by *Ganoderma* extracts and spores oil. All the *Ganoderma* extracts groups and the *Ganoderma* spores oil group exhibited potent inhibitory effects on H22 tumor volume. 5-FU, fluorouracil.

tumor weight with the control group at the end of the experiment ($P=0.013$ and $P=0.032$, respectively); however, the inhibitory rates of the 1 and 2 g/kg extract groups were 15.6 and 19.0%, respectively ($P=0.253$ and $P=0.170$, respectively). The average body weights were not significantly different among treatment groups, with the exception of the 5-FU group (positive control).

The suppressive effects of *Ganoderma* extracts and spores oil on H22 cells in mice are shown in Fig. 2 and Table IV. Data in Table IV showed that *Ganoderma* extracts and spores oil exhibited relatively strong inhibitory effects on H22 cells in mice. The inhibitory rates of the 1, 2 and 4 g/kg extract groups on H22 were 28.8, 38.5 and 44.6%, respectively ($P=0.008$,

Table IV. Inhibitory effects of *Ganoderma* extracts and spores oil on H22 in mice.

Groups ^a	Body weight, g			Tumor weight, g	Inhibitory rate, %
	Start	End	Difference		
Control	19.9±1.0	29.8±1.6	9.9±1.7	2.0±0.4	-
Extracts, 4 g/kg	19.6±1.2	26.9±2.2	7.3±1.7 ^b	1.1±0.2	44.6 ^c
Extracts, 2 g/kg	19.6±1.2	29.2±3.4	9.7±3.3	1.2±0.3	38.5 ^c
Extracts, 1 g/kg	19.3±0.7	27.4±2.5	8.1±2.5	1.4±0.5	28.8 ^c
Oil, 1.2 g/kg	19.5±1.2	27.2±3.6	7.8±2.8	1.1±0.2	44.9 ^c
5-FU, 25 mg/kg	19.1±1.1	25.0±2.2	5.9±2.1 ^c	0.7±0.2	64.8 ^c

Data are expressed as mean ± standard deviation. ^aNo. of mice per group (n=10). ^bP<0.05, ^cP<0.01 vs. control; Student's *t*-test. 5-FU, fluorouracil.

Table V. Effect of *Ganoderma* extracts and *Ganoderma* spores oil on immunity indexes of mice bearing S180.

Groups	Spleen index	Thymus index
Control	11.47±1.88	2.13±0.59
Extracts, 4 g/kg	13.31±2.89	2.79±0.67 ^a
Extracts, 2 g/kg	13.17±3.69	2.83±0.59 ^a
Extracts, 1 g/kg	13.75±1.95 ^a	2.92±0.75 ^a
Oil, 1.2 g/kg	12.29±2.55	2.51±0.85
5-FU, 25 mg/kg	10.57±3.74	1.21±0.50 ^b

Data are expressed as the mean ± standard deviation. ^aP<0.05, ^bP<0.01 vs. control; Student's *t*-test. 5-FU, fluorouracil.

Table VI. Effect of *Ganoderma* extracts and *Ganoderma* spores oil on immunity indexes of mice bearing H22.

Groups	Spleen index	Thymus index
Control	13.67±2.89	3.26±0.73
Extracts, 4 g/kg	16.48±4.25	2.89±0.78
Extracts, 2 g/kg	15.39±3.78	3.10±0.86
Extracts, 1 g/kg	14.25±3.84	2.86±0.61
Oil, 1.2 g/kg	14.65±4.03	2.60±0.78
5-FU, 25 mg/kg	9.32±2.26 ^a	1.32±0.77 ^b

Data are expressed as the mean ± standard deviation. ^aP<0.05, ^bP<0.01 vs. control; Student's *t*-test. 5-FU, fluorouracil.

Table VII. Effects of *Ganoderma* spores oil on K562 cell cycle.

Groups ^a	G1 phase, %	S phase, %
Control	32.7±0.5	55.7±0.6
Oil, 1.25 mg/ml	39.9±1.3	42.3±0.2 ^b
Oil, 2.5 mg/ml	43.7±2.2 ^b	38.7±3.2 ^b

Data are expressed as the mean ± standard deviation. ^aNo. of samples per group (n=3). ^bP<0.05 vs. control; Student's *t*-test.

P=0.008 and P=0.002, respectively, compared with control group), and of the 1.2 g/kg oil group was 44.9% (P=0.004). The average body weights were significantly decreased in the 4 g/kg extracts (P=0.038) and 5-FU (P=0.002) groups compared with the control group.

Effects on immunity indexes of mice bearing tumors. Table V shows that *Ganoderma* extracts increased the immunity indexes of mice bearing S180: The spleen index of the 1 g/kg extracts group was significantly increased compared with the control group (P=0.041); the thymus indexes in the 1, 2 and 4 g/kg extracts groups were all improved significantly (P=0.029, P=0.021 and P=0.038, respectively); while in the 5-FU group, the thymus index was decreased significantly (P=0.002). *Ganoderma* spores oil exerted no significant effects on spleen index and thymus index.

After treatment with *Ganoderma* extract and spores oil, there were no significant changes in the immunity indexes of mice bearing H22 cells (Table VI).

Inhibitory effects on topoisomerases activities. The activities of topoisomerase I and II are determined by the conversion of a fixed amount of supercoiled DNA into relaxed and broken DNA. Topoisomerase inhibitors could decrease the amount of relaxed and broken DNA and increased supercoiled DNA by inhibiting the catalytic activities of topoisomerases. The minimal concentration of crude topoisomerases from K562 cells required to fully relax 0.2 μg pBR322 DNA was used to test *Ganoderma* extracts and spores oil.

The results showed that the *Ganoderma* extracts and spores oil inhibited the activities of topoisomerase I and II. Treated with *Ganoderma* extracts, 2 mg/ml, topoisomerase I and II were inhibited, as reflected by the increased amount of supercoiled DNA and decreased amount of relaxed and broken DNA (Fig. 3). When *Ganoderma* spores oil, 4.5 mg/ml, was added into the reaction system of topoisomerase I, supercoiled DNA was observed. As the concentration of *Ganoderma* spores oil was increased, the inhibition of topoisomerase in dose-dependent manner was observed. Using *Ganoderma* spores oil, 18 mg/ml, the activity of topoisomerase II was then suppressed (Fig. 4).

Effect of Ganoderma spores oil on cell cycle. The cell cycle was analyzed by flow cytometry. As shown in Table VII,

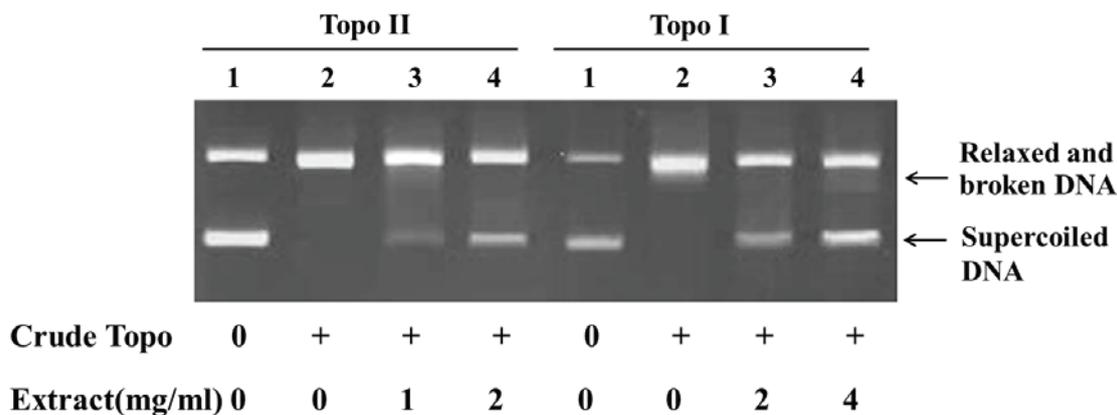


Figure 3. Effects of *Ganoderma* extracts on Topo I and II mediated pBR322 DNA relaxation. *Ganoderma* extracts showed inhibitory effects on Topo I and II at a dose of 2 mg/ml. As dosage increased, 4 mg/ml extracts exhibited an even stronger effect on Topo I. Topo, topoisomerase.

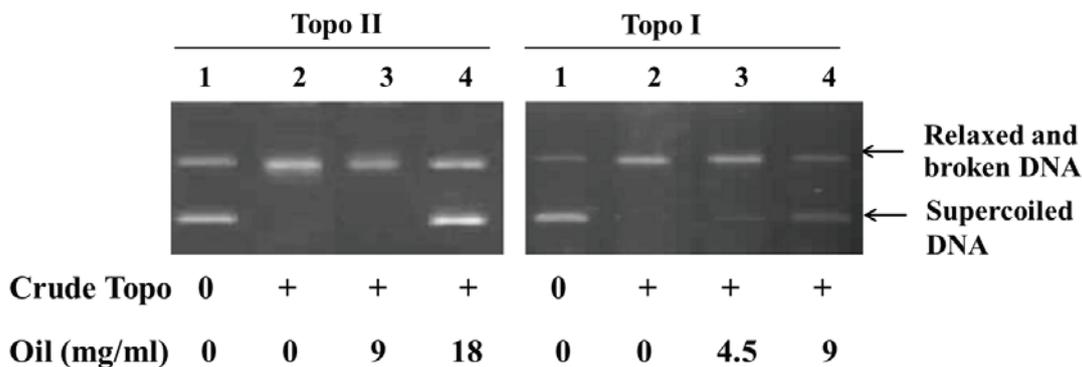


Figure 4. Effects of *Ganoderma* spores oil on Topo I and II mediated pBR322 DNA relaxation. *Ganoderma* spores oil showed a dose-dependent inhibitory effect on Topo I. At the dose of 18 mg/ml, Topo II was also suppressed. Topo, topoisomerase.

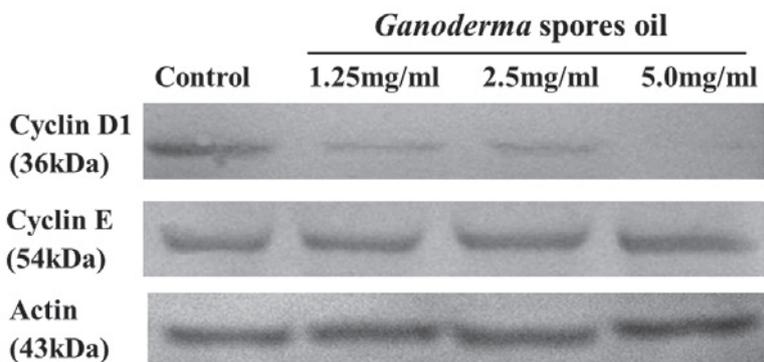


Figure 5. Level of cyclin D1 and E in K562 cells following treatment with various concentrations of *Ganoderma* spores oil for 12 h. The level of cyclin D1 was downregulated by *Ganoderma* spores oil, 1.25-5.0 mg/ml. Actin was used as a loading control.

Ganoderma spores oil induced a G1 arrest in K562 cells. With the treatment of *Ganoderma* spores oil, 1.25 mg/ml, for 12 h, the percentage of S cells in K562 was decreased from 55.7 ± 0.6 to 42.3 ± 0.2 ($P=0.038$). When the concentration of *Ganoderma* spores oil was raised to 2.5 mg/ml, the percentage of S cells was further decreased to 38.7 ± 3.2 ($P=0.017$), and the percentage of G1 cells rose from 32.7 ± 0.5 to 43.7 ± 2.2 ($P=0.022$).

Results of *Ganoderma* spores oil on cyclin D1 and cyclin E. Cyclin D1 and E are important regulators of G1-S phase cell cycle transition. The expression levels of cyclin D1 and E were determined by Western blotting. The results showed that after the treatment of *Ganoderma* spores oil 1.25-5.0 mg/ml for 12 h, cyclin D1 expression in K562 decreased, but cyclin E expression exhibited no obvious change (Fig. 5).

Discussion

Recently, the effect of *Ganoderma* on tumors has been increasingly studied. The present study revealed that *Ganoderma* extracts and spores oil inhibited the growth of human leukemia cells (K562 and HL60) and human gastric carcinoma cells (SGC-7901) in a dose-dependent manner. In addition, *Ganoderma* extracts and spores significantly suppressed the growth of the S180 and H22 transplant tumors in mice. Therefore, *Ganoderma* extracts and spores oil demonstrated definite antitumor effects in the *in vitro* and *in vivo* studies.

Since ancient times, *Ganoderma* has been widely used as a popular herbal medicine for the promotion of health (11). Numerous previous studies examined the immunomodulatory activities of *Ganoderma* (12,13). By detecting the immunity indexes of mice bearing S180 or H22 cells, *Ganoderma* extracts were concluded to have a certain effect on improving immune function, while *Ganoderma* spores oil had no significant effect on the spleen or thymus indexes of mice. One of the main components of *Ganoderma* extract is a polysaccharide that has been reported as immune function enhancer (12-15). As there were few polysaccharides (water-soluble substances) in the *Ganoderma* spores oil, the spores oil exhibited no evident effect on immunity. The present study also indicated that the antitumor effects of *Ganoderma* may be safer compared with 5-FU, which resulted in the decreased body weight and immunity indexes of mice (Tables I and II).

To investigate the possible mechanism of *Ganoderma* extracts and spores oil, the effects of extracts and spores oil on topoisomerases and the effect of spores oil on the cell cycle were examined.

DNA topoisomerases are a class of enzymes involved in the regulation of DNA supercoiling. Type I topoisomerases change the degree of supercoiling of DNA by causing single-strand breaks and religation, whereas type II topoisomerases cause double-strand breaks. These two activities are particularly crucial during DNA transcription and replication, when the DNA helix must be unwound to allow proper function of large enzymatic machinery. Cancer chemotherapy takes advantage of this finding, using drugs that block topoisomerases to kill rapidly-dividing cancer cells. For instance, the widely-used anthracycline drugs, such as doxorubicin and daunorubicin, attack class II topoisomerases and the plant toxin, camptothecin, blocks the relaxing action of class I topoisomerases (16).

Li *et al* (9) reported that Chinese *Ganoderma lucidum* essence, containing *Ganoderma lucidum* extracted powder and sporoderm-broken spores powder, could inhibit the activities of topoisomerase I and II. Consistent with these reports, the results of the present study showed that the catalytic activities of topoisomerase I and II were inhibited by *Ganoderma* extracts and spores oil. This finding indicates that topoisomerase I and II may be target for the actions of *Ganoderma* extracts and spores oil.

Extracting *Ganoderma* spores oil from broken spores by supercritical CO₂ extraction technology has been developed in recent years, and several studies have since examined the antitumor effect of *Ganoderma* spores oil (17,18). In the present study, a G1 arrest was detected in K562 cells treated with *Ganoderma* spores oil. *Ganoderma* extracts are in a brown suspension and cannot be detected by flow cytometry.

Ganoderma spores oil mainly contains triterpenes and fatty acids, and Tang *et al* (19) reported that ganoderic acid T, a triterpenoid chemical, markedly inhibited the proliferation of a highly metastatic lung cancer cell line by apoptosis induction and cell cycle arrest at the G1 phase. Therefore, the cell cycle blocking effect of *Ganoderma* spores oil was assumed to mainly be associated with triterpenes.

To the best of our knowledge, the present study described, for first time, that the level of cyclin D1 decreased in *Ganoderma* spores oil-treated K562 cells. Cyclin D1 is critical for the G1-S transition of the cell cycle and induces G1 arrest when its level is too low. Therefore, a decrease in cyclin D1 levels may be suggested as one of the reasons for the G1 arrest of the K562 cell cycle, which resulted in the inhibition of cell growth in the presence of *Ganoderma* spores oil. The active substances in *Ganoderma* are worth studying further.

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