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Antihepatoma and Liver Protective Potentials of *Ganoderma Lucidum* (靈芝 Ling Zhi) Fermented in a Medium Containing Black Soybean (黑豆 Hēi Dòu) and *Astragalus Membranaceus* (生黃耆 Shēng Huáng Qí)

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ABSTRACT

The antihepatoma activity and liver protective function of the fermentation products (5 L fermenator) of *Ganoderma lucidum* (GL; 靈芝 Ling Zhi) cultivated in a medium containing black soybean (BS; 黑豆 Hēi Dòu) and *Astragalus membranaceus* (AM; 生黃耆 Shēng Huáng Qí) at different fermentation temperatures were investigated in this study. Hep 3B cells pretreated with lovastatin were used to study the antihepatoma activity, and possible active components were analyzed by reverse-phase high-performance liquid chromatography. Carbon tetrachloride (CCl_4)-induced primary rat hepatocyte injury was further used to evaluate the liver protective activity of the fermentation products. While all the GL broth filtrates do not inhibit the growth of Hep 3B cells, the ethanolic extract from GL-2 mycelia (GL-2-mE), cultivated in the medium containing BS (50 g/L) and AM (20 g/L) at 24°C for 11 days showed the best antihepatoma activity (IC_{50} 26.6 μg/mL) than the other ethanolic extracts from GL mycelia, GL fruiting body, BS, and AM did. The antihepatoma activities were correlated with some unknown active components in these samples. Furthermore, GL-2-mE (100 μg/mL) without harmful effect on the growth of normal primary rat hepatocytes significantly maintained cell viability, reduced lactate dehydrogenase leakage, lowered lipid peroxidation, and increased glutathione peroxidase and glutathione *S*-transferase activities in the CCl_4-induced damaged primary rat hepatocytes.

Key words: Antihepatoma activity, *Astragalus membranaceus*, Black soybean, Fermentation, *Ganoderma lucidum*, Liver protective function

INTRODUCTION

Ganoderma lucidum (GL; 靈芝 Ling Zhi) has been used extensively in traditional Chinese medicine in East Asia for years. Triterpenoids, bitter metabolites in the GL fruiting bodies and spores, exhibit many biological activities and pharmacological functions such as antihepatoma activity, antitumor activity, antioxidation activity, antihuman immunodeficiency virus activity, antiallergic activity, antihypertensive activity, hepatoprotection, and inhibition of cholesterol synthesis and platelet aggregation.[1-3] The polysaccharides from GL fruiting bodies were also reported to possess antitumor, hypoglycemic, and immunomodulatory activities.[4,5] Black soybean (BS; 黑豆 Hēi Dòu), the seeds of *Glycine max* (L.) Merrill, containing isoflavones, anthocyanins, and soy protein was investigated for its carcinogenesis-inhibitory, cholesterol-lowering, antioxidation, and immunomodulatory effects.[6-9] The root of *Astragalus membranaceus* (AM; 生黃耆 Shēng Huáng Qí), a traditional Chinese medicine, has many biological activities such as

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immunomodulation, antiviral activity, antioxidation activity, cancer therapy, and prevention of cardiovascular diseases due to its active constituents, including polysaccharides, saponins, and flavonoids.[10-14] It was reported that isoflavone glucosides are converted into isoflavone aglycones, which are easily absorbed from the small intestine, after the soybean extracts are cultivated with the GL mycelia for 15 days, and the mixture of isoflavone aglycones and polysaccharides have antiangiogenic activities *in vivo*.[15]

The liver is the major organ in humans for metabolism, detoxification, and in the antioxidation system. Globally, hepatocellular carcinoma (HCC) is the most frequently occurring cancer and the leading cause of cancer death. Liver carcinogenesis is characterized by multistep carcinogenesis. In addition to viral infection and environmental agents, including aflatoxin B1, smoking, and alcohol, recent studies identified several potential factors that may contribute to the pathogenesis of HCC, such as oxidative stress, chronic inflammation, and genetic diseases.[16] Carbon tetrachloride (CCl_4) is a hepatotoxin because it is metabolized in the liver by the cytochrome P450 oxidase system resulting in the production of reactive radicals that induce liver injury.[17,18] In addition, lovastatin, a 3-hydroxy-3-methylgutaryl-CoA reductase inhibitor, is used to clinically treat hypercholesterolemia, and it effectively arrests tumor cells in the G1 phase through the mevalonate/cholesterol pathway and p21/p27 induction.[19] Mevalonate-demanding Hep 3B cells are more sensitive to growth arrest and apoptosis by a combination treatment with lovastatin and other anticancer agents, including taxol and *Ganoderma* triterpenoids.[1] Therefore, lovastatin-pretreated Hep 3B cells were used as the high-throughput antihepatoma screening system in this study.

In this study, we were interested in submerged fermentation of GL under various conditions, including in a medium containing BS and AM and at different temperatures, and interactions between GL and BS or AM were expected to produce fermentation products with antihepatoma activity and liver protective function. The potential of a combination treatment with lovastatin and the GL fermentation products was evaluated, and possible active components in each sample were analyzed by performing reverse-phase high-performance liquid chromatography (HPLC). Furthermore, we studied the effects of the fermentation products with the best antihepatoma activity on the antioxidation and detoxification systems in normal and CCl_4-induced injured primary rat hepatocytes.

MATERIALS AND METHODS

Materials and preparation of samples

The GL (BCRC 36123) fermentation products were cultured at difference fermentation conditions as listed in [Table 1]. BS was purchased from Tainan County Hseija Fruit and Vegetable Transporting and Marketing Association (Tainan, Taiwan). AM was purchased from Microbio Co., Ltd. (Taipei, Taiwan). The fermentation product of GL fermentation was separated into broth (b) and mycelia (m). The broth was sterilized at 121°C for 30 min and then filtered through a 0.22-μm filter. The broth filtrates were lyophilized. The GL (BCBC 36123) fruiting body (GL-36123-fb) was obtained from Prof. Shean-Shong Tzean, Department of Plant Pathology and Microbiology, National Taiwan University (Taipei, Taiwan). One gram of dry mycelia or fruiting bodies of GL, BS, or AM was mixed with 95% ethanol (20 mL) and incubated at 30°C for 24 h with shaking. The ethanolic extracts were evaporated to dryness under vacuum. The yields of GL-1-mE, GL-2-mE, GL-3-mE, GL-4-mE, GL-36123-fbE, BSE, and AME were 23.2, 22.8, 23.5, 8.3, 2.3, 21.3, and 10.6 g/100g dry matter, respectively.

Chemicals

Methanol (Tedia Co., OH, USA) and ethanol (95%) (Echo Co., Taipei, Taiwan) were used. Lovastatin was obtained from Wako Pure Chemical Industries Ltd. (Japan). Dulbecco's modified Eagle medium (DMEM), antibiotic-antimycotic solution, MEM nonessential amino acid solution, L-15 medium, insulin, transferrin, fetal bovine serum (FBS), and penicillin-streptomycin solution were purchased from Gibco Laboratories (Grand Island, NY, USA). Collagenase (type I) from Worthington Biochemical Co. (Lakewood, NJ, USA) and Percoll from Pharmacia LKB (Piscataway, NJ, USA) were used. N, N-dimethylfluoramide (DMF) was purchased from LabScan (Dublin, Ireland). We obtained bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), collagen, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glutathione reductase (GRd), iodoacetic acid, N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), oxidized glutathione (GSSG), phosphotungstic acid, reduced glutathione (GSH), β-nicotinamide adenine dinucleotide (β-NADH), β-nicotinamide adenine dinucleotide phosphate (β-NADPH), sodium azide (NaN3), silymarin, sodium dodecyl sulfate (SDS), 1,1,3,3-tetraethoxypropane, 2-thiobarbituric acid, and trypsin-ethylenediaminetetraacetic acid (EDTA) solution from Sigma Chemical (St Louis, MO, USA).

Culture of hepatoma cells

Human hepatoma Hep 3B cells were kindly given by Dr. Shiao (Department of Life Science, Chang Gung University, Taoyuan, Taiwan). Cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 100 μM nonessential amino acids (pH 7.0) at 37°C in 5% CO_2 and 90% relative humidity.

Antihepatoma activity assay

According to the methods of Shiao,[1] Hep 3B cells were cultured in 96-well plates at a density of 5×10^3 cells/100 μL medium/well. After 24 h of incubation, the medium in the 96-well plate was replaced by 100 μL serum-free DMEM containing 0 or 10 μM lovastatin. After another 48 h of incubation, the medium was replaced by 100 μL new serum-free DMEM containing various concentrations of the sample (the ethanolic extracts were previously dissolved in DMSO, and the final concentration of DMSO was 0.5%) for 48 h. Then the medium was discarded, and 25 μL MTT solution (5 mg/mL in phosphate-buffered saline [PBS]) and 100 μL serum-free DMEM were added to each well and reincubated for an additional 4 h. MTT lysis buffer (20 g SDS in 50 mL DMF and 50 mL water) (100 μL) was added to dissolve the formazan crystals formed. Then the plates were read at 570 nm in a microplate reader (Anthos 2001, Salzburg, Austria). Cells of the sample that were not exposed to lovastatin served as the control at 100% survival.

Determination of major components by reverse-phase HPLC

A model L-7100 solvent-delivery system (Hitachi, Tokyo, Japan) equipped with a model L-2200 autosampler (Hitachi), a model L-7420 UV-VIS detector (Hitachi), and a Peak-ABC chromatography data handling system (JiTeng, Singapore) were used in the HPLC analysis. Separation was performed using a prepacked reverse-phase column (Cosmoail $5C_{18}$, 25 × 0.46 cm I.D.; Nacalai Tesque, Kyoto, Japan). According to the method by Chyr and Shiao,[20] multi-step linear-gradient elution with a binary solvent system of methanol and water was employed, and a constant concentration of acetic acid (0.5%, v/v) was used. Ultraviolet detection at 243 nm was performed.

Isolation and culture of hepatocytes

Male Narl: SD rats (6-8 week old) were purchased from the National Laboratory Animal Center, Taipei, Taiwan. The rats were housed in plastic cages with an artificial 12-h light/dark cycle, and they were given food (Purina 5001 rodent chow diet, St. Louis, MO, USA) and water *ad libitum*. The method of hepatocyte isolation has been described.[21-23] Hepatocytes were plated at a density of 1×10^6 cells/2 mL in L-15 medium containing 18 mM HEPES, 5 μg/mL insulin, 5 μg/mL transferrin, 1 mg/mL galactose, 1 mM dexamethasone, 100 units/mL penicillin, 100 mg/mL streptomycin, 5 ng/mL Na_2SeO_3, and 0.2% BSA) in 35 mm collagen-precoated culture dishes (NUNC, Denmark) and incubated in a humidified incubator (NUAIRE, Plymouth, MN, USA) at 37°C in ambient atmosphere. The culture medium was replaced with fresh medium 4 h after plating. The medium was changed using the same culture medium but containing 2 g/L BSA instead of FBS at 24 h after plating.

Treatment of hepatocytes

At 24 h after plating, hepatocytes (1×10^6) were treated with various concentrations of samples that were previously dissolved in DMSO (the final concentration of DMSO was 0.5%) for 24 h. In terms of CCl_4-induced injury in hepatocytes, cells (1×10^6) were coincubated with 10 mM CCl_4, which was previously dissolved in 99.5% ethanol (the final concentration of ethanol was <1%) and various concentrations of samples that were previously dissolved in DMSO (the final concentration of DMSO was 0.5%) for 1 h. The culture medium was removed after treatment, and the hepatocytes were washed with cold PBS. Then the cells were removed with a cell scraper and collected for further analysis.[24]

Lactate dehydrogenase leakage of hepatocytes

The effect of samples on the viability of primary hepatocytes was evaluated by lactate dehydrogenase (LDH) leakage.[25] After collecting the cell-free medium, hepatocytes were lysed using 20 mM potassium phosphate buffer (1.090 g/L KH_2PO_4 and 2.090 g/L K_2HPO_4; pH 7.0) containing 0.5% Triton X-100. Cells were collected and centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was used to analyze the LDH activity. Samples were mixed in 1 mL of Krebs–Henseleit buffer (0.322 g/L $CaCl_2$, 6.9 g/L NaCl, 0.36 g/L KCl, 0.13 g/L KH_2PO_4, 0.295 g/L $MgSO_4 \cdot 7H_2O$, and 2 g/L $NaHCO_3$; pH 7.4) containing 20 g/L BSA, 0.2 mM NADH, and 1.36 mM pyruvate. The rate of change in absorbance at 340 nm due to NADH oxidation was recorded. Intracellular and extracellular LDH activities were assayed, and the percentage of leakage was determined.

Lipid peroxidation

According to the method by Fraga, *et al*.,[26] thiobarbituric acid-reactive substance (TBARS) level was measured to evaluate the condition of lipid peroxidation. The samples or standards (1,1,3,3-tetramethoxy propane) were mixed with 20 mM potassium phosphate buffer, 3% SDS solution, 0.1 N hydrochloric acid, 10% phosphotungstic acid, and 0.7% thiobarbituric acid (TBA), and then heated in boiling water for 30 min. The reactive products were extracted with n-butanol and assayed with a fluorescence spectrophotometer at 515 nm excitation and 555 nm emission. The results were compared with a standard curve expressed in nmole TBARS/mg protein.

Analysis of GSH and glutathione-related enzymes

The GSH content in the hepatocytes was assayed as described by Reed, *et al.*[27] Samples were prepared by adding 1 mL of 1 mol/L perchloric acid containing 2 mmol/L 1,10-phenanthroline to each dish. The acid-soluble GSH in the hepatocellular supernatant was measured by performing HPLC. Glutathione peroxidase (GPx) activity was assayed using hydrogen peroxide as the substrate.[28] According to the method by Bellomo, *et al*.,[29] GRd activity was assayed. Using CDNB as the substrate, glutathione *S*-transferase (GST) activity was assayed by the method by Habig, *et al.*[30] Protein concentration of the samples was assayed according to the method by Lowry *et al.*[31]

Cell viability of hepatocytes using MTT assay

The hepatocytes were cultured in 96-well collagen-precoated plates at a density of 5×10^4 cells/100 μL of culture medium/well and incubated in a humidified incubator at 37°C in ambient air. The culture medium was replaced with fresh medium 4 h after plating. At 24 h after plating, the hepatocytes were treated with 10 mM CCl_4 and various concentrations of the samples. After 1 h treatment, the culture medium was removed, and the hepatocytes were washed with cold PBS. Then the cell viability was determined using the MTT assay.

Statistical analyses

Statistical analyses were performed using one-way analysis of variance and Duncan's multiple-comparison test (SAS Institute, Cary, NC, USA) to determine the significant differences among the mean values ($P < 0.05$).

RESULTS

Effect of fermentation products on hepatoma cells

The effects of GL broth filtrates and ethanolic extracts from GL mycelia on hepatoma Hep 3B cells pretreated with lovastatin are shown in [Figure 1]. Compared with the control, the cell viability of Hep 3B cells (the lovastatin-treated control) exposed to lovastatin for 48 h and then serum-free medium for another 48 h was 80.6% of the control. In general, none of the GL broth filtrates at 100 μg/mL effectively inhibited the growth of Hep 3B cells. Silymarin as a positive control and the ethanolic extract from GL fruiting bodies (GL-36123-fbE) at 100 μg/mL exhib-

ited good abilities to decrease cell viability by 67.5% and 93.9% of the control, respectively. GL-1, -2, and -3 were fermented in medium containing 50 g/L BS and 20 g/L AM at different temperatures [Table 1], and their ethanolic extracts (GL-1, -2, and -3-mE) at 100 μg/mL exhibited stronger antihepatoma activities resulting in <40% cell viability of the control compared with silymarin and GL-36123-fbE. Neither the broth filtrate nor the mycelia ethanolic extract from GL-4, which was cultivated in a medium without BS or AM inhibited Hep 3B cell growth. The ethanolic extracts of BS (BSE) and AM (AME) at 100 μg/mL did not significantly decrease the cell viability. It suggested that BS and AM improved the antihepatoma activities of the fermentation products after cultivating with BS and AM. Furthermore, the effects of the ethanolic extracts of GL mycelia with better antihepatoma activity at various concentrations on Hep 3B cells pretreated with lovastatin were investigated, and the result of each sample appeared to be dose dependent. The half maximal inhibitory concentration (IC_{50}) values of silymarin and GL-36123-fbE were 51.8 and 41.6 μg/mL, respectively. GL-2-mE showed the lowest IC_{50} value (26.6 μg/mL) among the four fermentation products.

Determination of major components by reverse-phase HPLC

The profiles of major components in the samples analyzed by reverse-phase HPLC are shown in [Figure 2]. The results showed that there were some compounds in GL-1-mE, GL-2-mE, GL-3-mE, and GL-4-mE, but not in GL-1-b, GL-2-b, GL-3-b, and GL-4-b. Total area of peaks 1-7 in the HPLC profile of GL-2-mE was larger than that of GL-1-mE, GL-3-mE, or GL-4-mE [Supplementary Table 1]. It also suggested that these compounds may be the major contributors to their antihepatoma potentials on the basis of Pearson correlation coefficients [Supplementary Table 1]. As compared with the HPLC profiles of the ethanolic extracts of GL fermentation products, BS and AM might promote the formation of active components in the GL fermentation product since some major components cannot be detected in BSE and AME. In addition, the HPLC profiles are different between GL-36123-fbE and GL-2-mE. It suggested that different active compounds in the tested samples resulted in different antihepatoma effects, because the IC_{50} values of GL-2-mE against Hep 3B cells was less than that of GL-36123-fbE [Figure 1].

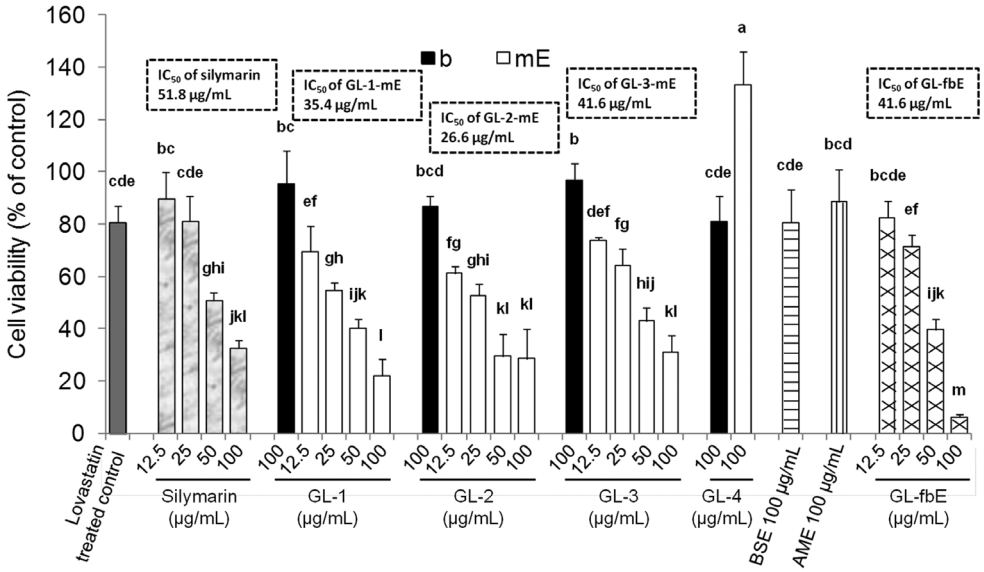

Figure 1. Effects of broth filtrates and ethanolic extracts of *Ganoderma lucidum* (GL) mycelia on the growth of Hep 3B cells pretreated with lovastatin. *Sample codes: BS, black soybean; AM, *Astragali membranaceus*; b, broth filtrate; m, mycelia; fb, fruiting body; E, ethanolic extract. Hep 3B cells pretreated with lovastatin for 48 h were treated with samples for another 48 h ($n = 3$). Cell viability was determined by MTT assay. Values bearing different letter are significantly different ($P < 0.05$)

Table 1. Fermentation conditions of Ganoderma lucidum fermentation products produced using black soybean and *Astragalus membranaceus* as part of the liquid fermentation medium

Sample[a]	Fermentation conditions							
	BS (g/L)	AM (g/L)	Temperature (°C)	Agitation (rpm)	Aeration (vvm)	Initial pH	Fermentation time (day)	Final mycelia yield (g/100 mL)
GL-1	50	20	18	50	0.75	6.0	11	0.565
GL-2	50	20	24	50	0.75	6.0	11	0.793
GL-3	50	20	30	50	0.75	6.0	11	2.212
GL-4	-	-	18	100	1.25	3.5	11	0.342

[a]All products were fermented by *Ganoderma lucidum* (BCRC 36123), The fermentation medium contains 2% glucose in a 5 L fermentor

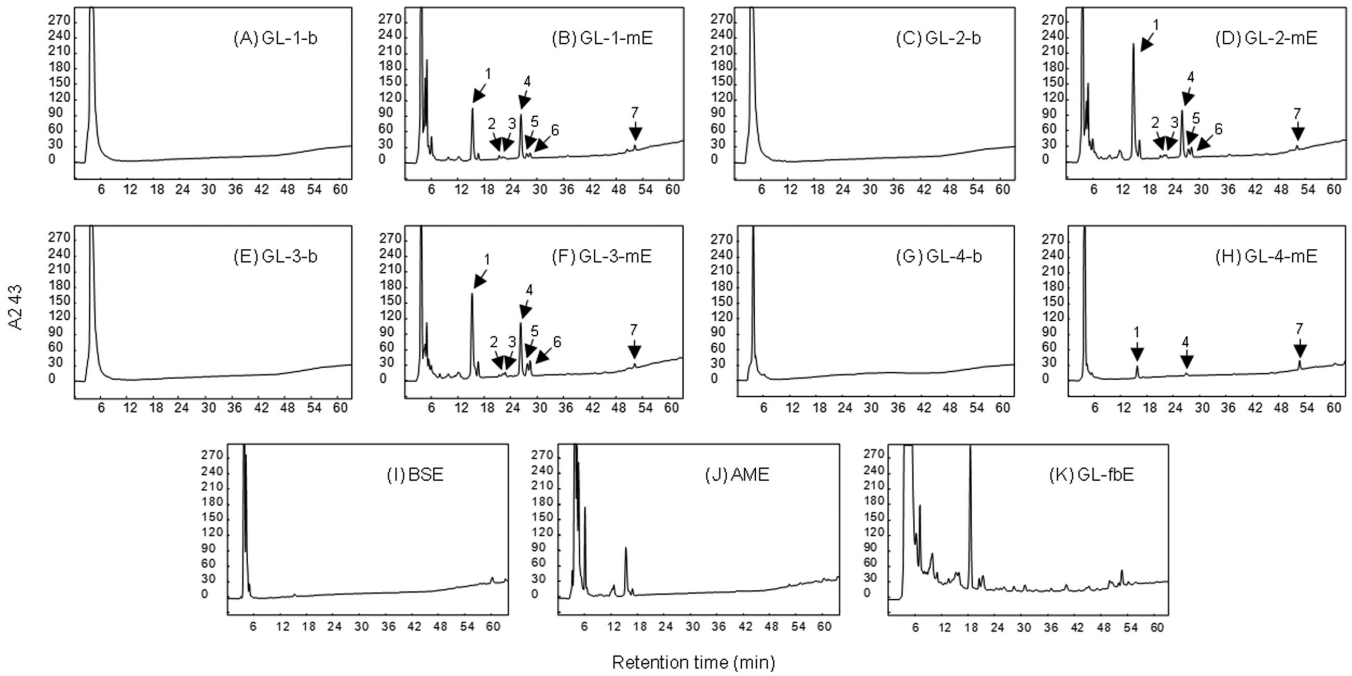

Figure 2. HPLC chromatograms of major components in GL-1-b (a), GL-1-mE (b), GL-2-b (c), GL-2-mE (d), GL-3-b (e), GL-3-mE (f), GL-4-b (g), GL-4-mE (h), BSE (i), AME (j), and GL-fbE (k). *Sample code: GL, *Ganoderma lucidum*; BS, black soybean; AM, *Astragali membranaceus*; b, broth filtrate; m, mycelia; fb, fruiting body; E, ethanolic extract. The concentration of each sample was 20,000 μg/mL. The retention time of peak 1 is 15.2 min, peak 2 is 22.0 min, peak 3 is 22.6 min, peak 4 is 26.1 min, peak 5 is 27.5 min, peak 6 is 28.3 min, and peak 7 is 52.1 min

Supplementary Table 1. Correlation of antihepatoma activity and major components existing in broth filtrates and mycelia ethanolic extracts of Ganoderma lucidum

Sample	Cell viability (% of control)[a,b]	Peak area							
	Peak no.	1	2	3	4	5	6	7	Total
GL-1-b	95.2±13.0	-	-	-	-	-	-	-	0
GL-1-mE	37.3±8.4	2608106	115952	40871	2327070	121584	84379	205822	5503784
GL-2-b	86.7±3.9	-	-	-	-	-	-	-	0
GL-2-mE	28.0±11.2	6655128	46566	50452	2693210	320514	271155	174290	10211315
GL-3-b	96.9±6.2	-	-	-	-	-	-	-	0
GL-3-mE	36.4±14.8	4306776	53073	117334	2970378	438309	419871	176825	8482566
GL-4-b	81.0±9.7	-	-	-	-	-	-	-	0
GL-4-mE	133.4±12.6	533467	-	-	106737	-	-	364219	1004423
Pearson correlation coefficients[c]		–0.8173	–0.7972	–0.8173	–0.8891	–0.7972	-0.7578	–0.0260	–0.7578
P value		0.0132	0.0178	0.0132	0.0031	0.0178	0.0294	0.9513	0.0294

[a]Effect of broth filtrates (b) and mycelia ethanolic extracts (mE) of G. lucidum (GL) (100 μg/mL) on the growth of Hep 3B cells pretreated with lovastatin. Cell viability was determined by MTT assay, [b]Each data represents mean±SD (n=3), [c]The peak areas were of higher negative correlation with cell viabilities indicated that the compounds might be of the major components for contributing antihepatoma activity

Effect of GL-2-mE on normal primary rat hepatocytes

After treatment with 50, 100, or 200 μg/mL of GL-2-mE for 24 h, the results of LDL leakage, lipid peroxidation (TBARS production), GSH content, and GSH-related enzyme activities in normal primary rat hepatocytes are shown in Table 2. It indicated that DMSO (0.5%) and GL-2-mE (<200 μg/mL) did not significantly affect the hepatocytes on the basis of the LDL leakage compared with the control, but GL-2-mE at 100 μg/mL tended to decrease more TBARS value and increase higher GSH level and GPx activity compared with the control (DMSO) than those of 200 μg/mL treatment. It also suggested that GL-2-mE possessed the capability of cytotoxic selectivity toward hepatoma cells rather than normal primary rat hepatocytes.

Effect of GL-2-mE on primary rat hepatocytes damaged by CCl_4

To study the liver protective activity of GL-2-mE, CCl_4 was further used to induce primary rat hepatocytes injury *ex vivo* [Figure 3 and Table 3]. DMSO (0.5%) and ethanol (1%) helped GL-2-mE and CCl_4 to dissolve in the medium, respectively, and they did not significantly affect normal primary rat hepatocytes, including morphology [Figure 3a and b], LDH leakage, cell viability, TBARS value, and GSH level [Table 3], or the activities of GPx, GRd, and GST [Table 3]. The results of morphological observations also indicated that CCl_4 could induce primary rat hepatocyte damage, including shrinkage, rupture, and death, compared with

Table 2. Effect of the ethanolic extract of GL mycelia (GL-2-mE) on lactate dehydrogenase (LDH) leakage, thiobarbituric acid-reactive species (TBARS) and glutathione (GSH) levels, and glutathione peroxidase (GPx), glutathione reductase (GRd) and glutathione S-transferase (GST) activities in normal primary rat hepatocytes[a,b]

Group	Dose (μg/mL)	LDH leakage (%)	TBARS (nmol/mg protein)	GSH (nmol/mg protein)	GPx	GRd	GST
					nmol/min/mg protein		
Control		0.8±0.8	0.53±0.07	85.4±15.7	160±17	55.3±8.2	580±75
Control (DMSO)		1.8±1.4	0.51±0.13	69.4±10.6	162±16	62.1±4.3	657±108
GL-2-mE	50	2.3±1.9	0.50±0.06	89.2±8.4	161±27	58.3±9.0	580±86
	100	2.0±1.4	0.44±0.05	88.0±2.3	181±33	60.7±13.0	620±41
	200	3.4±3.0	0.46±0.05	85.7±15.6	169±29	59.6±10.7	596±41

[a]Hepatocytes were isolated from 6 to 8 week-old male rats. Hepatocytes isolated from each animal were divided equally and cultured in medium containing various concentrations of GL-2-mE for 24 h. Values are expressed as mean±SD from 3 or 4 rats. [b]Results were not significantly different according to Duncan's multiple range test (P<0.05)

Table 3. Effect of the ethanolic extract of GL mycelia (GL-2-mE) on lactate dehydrogenase (LDH) leakage, cell viability, thiobarbituric acid-reactive species (TBARS) and glutathione (GSH), and glutathione peroxidase (GPx), glutathione reductase (GRd) and glutathione S-transferase (GST) activities levels in primary rat hepatocytes damaged by carbon tetrachloride[a, b]

Group	Dose (μg/mL)	LDH leakage (%)	Cell viability (%)	TBARS (nmol/mg protein)	GSH (nmol/mg protein)	GPx	GRd	GST
						nmol/min/mg protein		
Control		4.6±2.1*	100.0±4.9*	0.55±0.11*	91.0±16.7*	159±26*	44.5±6.6*	551±35*
Control (DMSO+ethanol)		4.0±1.7*	97.9±5.0*	0.52±0.15*	92.2±13.3*	145±11*	41.0±3.6	535±41*
CCl4		60.0±20.4	36.4±12.1	1.14±0.26	41.5±13.4	108±12	30.4±3.6	356±38
CCl4+silymarin	1	24.5±2.2*	58.5±21.4	1.08±0.30	56.0±15.2	102±31	31.9±6.0	342±41
	10	9.5±3.2*	73.6±15.2*	0.95±0.28	64.0±24.0	130±12	37.0±5.1	435±92
	100	3.3±1.9*	101.8±18.9*	0.59±0.10*	95.5±16.5*	154±15*	38.6±5.5	441±71
CCl4+GL-2-mE	1	51.3±16.2	57.7±24.4	1.02±0.11	66.0±19.2	100±29	29.3±7.0	322±45
	10	18.3±1.5*	68.0±20.8*	0.97±0.20	69.6±19.7	136±22	39.7±11.1	424±43
	100	11.2±3.8*	80.9±4.7*	0.61±0.13*	74.8±23.6	147±13*	41.3±10.7	458±43*

[a]Hepatocytes were isolated from 6 to 8 week-old male rats. Hepatocytes isolated from each animal were divided equally and cultured in medium containing 10 mM CCl_4 and various concentrations of GL-2-mE for 1 h. Values are expressed as the mean±SD from 3 or 4 rats. [b]*represents that the data is significant different (P<0.05) when compared with the CCl_4 treatment group.

the control (DMSO + ethanol) [Figure 3a-c]. Both silymarin and GL-2-mE at 100 μg/mL can prevent the hepatocytes from being damaged by CCl_4 on the basis of the morphology [Figure 3f and i].

Carbon tetrachloride increased LDH leakage and decreased the cell viability, which were complicated by increased TBARS values and reduced GSH levels in primary rat hepatocytes [Table 3]. Silymarin at 1 μg/mL reduced 36% of LDH leakage and increased cell viability by 22%, which were significantly changed by CCl_4 ($P < 0.05$), and even 100 μg/mL silymarin caused recovery of LDH leakage and cell viability to a normal condition and maintained the TBARS and GSH levels similar to those of the control. GL-2-mE at 10 and 100 μg/mL also decreased the LDH leakage increased by CCl_4 and maintained significantly more living hepatocytes ($P < 0.05$). GL-2-mE at 100 μg/mL significantly reduced TBARS values by 46% ($P < 0.05$) and slightly elevated the GSH level.

Furthermore, GPx, GRd, and GST activities in primary rat hepatocytes treated with CCl_4 and samples were detected [Table 3]. The results showed that CCl_4 treatment significantly decreased the activities of all the three enzymes ($P < 0.05$). Silymarin at 100 μg/mL significantly elevated GPx activity to 46 nmol/min/mg protein compared with CCl_4 treatment ($P < 0.05$), and GL-2-mE at 100 μg/mL significantly increased GPx and GST activities to 39 and 102 nmol/min/mg compared with CCl_4 treatment, respectively ($P < 0.05$).

DISCUSSION

Because cultivating GL fruiting bodies might take several months, and it is also difficult to control the quality, we were interested in submerged fermentation to produce fungal products with biological activity. Some investigations referred that different initial pH values or inoculation densities result in different productions of extracellular and intracellular polysaccharides or ganoderic acids in a fed batch fermentation of GL.[32,33] In the present study, we examined the antihepatoma activities of four GL fermentation products produced under different fermentation conditions, including medium with or without BS and AM and various fermentation temperatures.

Hep 3B cells pretreated with lovastain, a clinical hypercholesterolemic drug, was used to evaluate the antihepatoma potentials of GL fermentation products. It was reported that taxol or triterpenoids form *Ganoderma tsugae* more effectively inhibit the growth of lovastatin-pretreated Hep 3B cells.[1] It also suggested that Hep 3B cells pretreated with lovastatin are generally more sensitive to ethanolic extracts of mycelia than to broth filtrates, and GL-1, -2, and -3-mE have stronger inhibitive abilities than silymarin according to their IC_{50} values [Figure 1]. The GL fermentation product (GL-2) with the best antihepatoma activity was cultivated

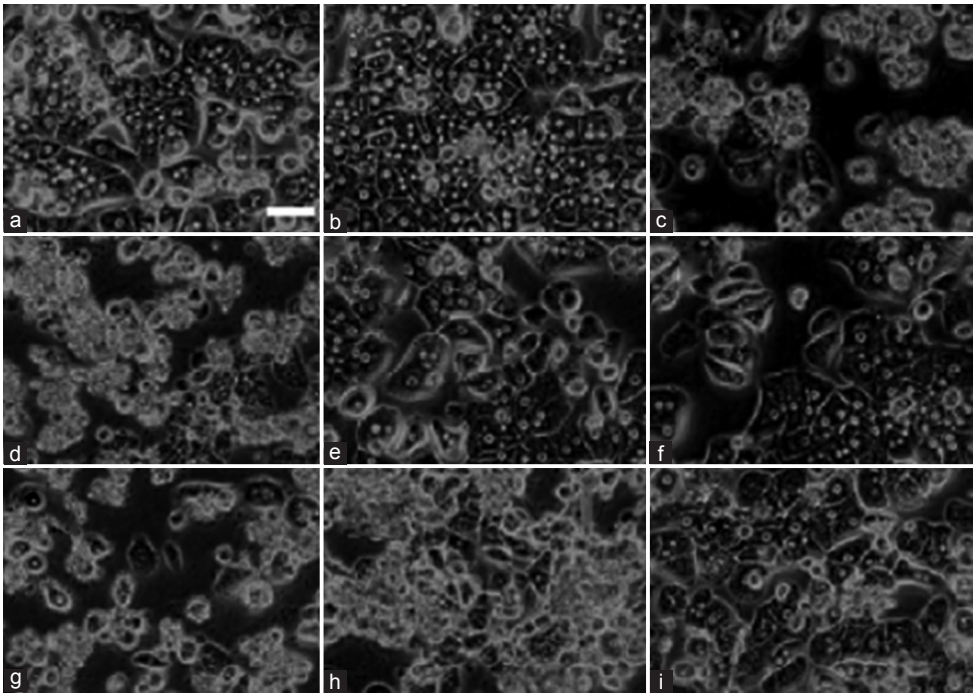

Figure 3. Effect of GL-2-mE on the morphology of primary rat hepatocytes damaged by CCl_4. *Hepatocytes were treated with 10 mM CCl_4 and various concentrations of samples for 1 h. a ~ i show the control, the control (DMSO + ethanol), CCl_4, CCl_4 + silymarin (1 μg/mL), CCl_4 + silymarin (10 μg/mL), CCl_4 + silymarin (100 μg/mL), CCl_4 + GL-2-mE (1 μg/mL), CCl_4 + GL-2-mE (10 μg/mL), and CCl_4 + GL-2-mE (100 μg/mL), respectively. Silymarin was the positive control. 100 × under an inverted-stage microscope equipped with phase contrast. "—": 50 μm

in a medium containing 50 g/L BS and 20 g/L AM at 24°C for 11 days, and it suggested that the main contributing constituents in the ethanolic extract of GL mycelia might be affected by fermentation temperature, fermentation medium composition, culture method, GL species, etc., [Figure 2]. In our study, the amounts of major components were not enough to identify their structures, but the HPLC results suggested that variable active compounds in the fermentation products and fruiting body resulted in lower IC_{50} values of GL-2-mE against Hep 3B cells compared with GL-36123-fbE [Figure 1]. It indeed has been reported that different triterpenoids isolated form GL fruiting body and fermentation products have different anticancer abilities.[34-36] Additionally, except for triterpenoids, other ingredients with antihepatoma activity might exist in the ethanolic extracts from GL fermentation products and need to be further confirmed; for example, ergosterol derivatives and long chain fatty acids.[37,38]

BSE, AME, and GL-4-mE, which were cultivated without BS or AM at 100 μg/mL could not inhibit the growth of Hep 3B cells, but BS and AM improved the antihepatoma activities of the fermentation products (GL-1, -2, and -3) after cultivation with BS and AM [Figure 1]. In our previous study, we also found that BS modified the antihepatoma activity of *Agaricus blazei* against Hep 3B and Hep G2 cells because it promoted the production of the active compounds.[39] It was reported that β-glucosidase from GL mycelia converted isoflavone glucosides into isoflavone aglycones when soybean extracts were cultivated with GL.[15] Therefore, we speculated that GL might convert the inactive components in BS or AM into active ones during the fermentation process in the present study. In addition, the formations of major components in GL fermentation products might be derived from the BS or AM. For example, soybean contains fatty acids, squalene, and phytosterol,[40] which could be the starting materials to biosynthesize active compounds such as cytotoxic steroids in *A. blazei* fermentation products.[41,42] In contrast, BS and AM might promote the production of active secondary metabolites in the GL fermentation products. Other components existing in BS or AM could also affect enzymatic activities for the biosynthesis of the major components, including trace elements, phenolic compounds, isoflavones, anthocyanins, saponins, etc.[43-48] Therefore, our study will be as an interesting reference for future investigators to study the change of the active compounds in the fungi fermentation products cultivated in a medium containing BS and AM.

The triterpene-enriched fraction, which was prepared from GL mycelia, inhibited the growth of human hepatoma Huh-7 cells, but not normal human Chang liver cells.[36] In order to evaluate the hepatoxicity of GL fermentation product with antihepatoma activity in this study, normal primary rat hepatocytes were treated with various concentrations of GL-2-mE, which exhibited the best inhibitive ability against Hep 3B cells. GL-2-mE (<200 μg/mL) caused no significant harmful influence on the hepatocytes, but at 100 μg/mL slightly increased GSH level and GPx activity resulted in lower lipid peroxidation compared with the control (DMSO) after 48 h treatment [Table 2]. Furthermore, CCl_4 is metabolized by the cytochrome P450 oxidase system resulting in reactive radicals that induce lipid peroxidation and reduce the content of reduced GSH and the activities of GSH-related enzymes in the liver.[17,18] Therefore, liver protective function of GL-2-mE was further evaluated using CCl_4-induced primary rat hepatocyte injury.

Both triterpenoids and polysaccharides from the hot-water extract of GL fruiting bodies can inhibit lipid peroxidation in erythrocyte cell membranes and liver mitochondria.[3] Ganoderic acids were also reported to retard CCl_4-induced liver damage in mice.[49] In our study, the cell viability of primary rat hepatocytes with CCl_4-induced injury were recovered after treatment with 100 μg/mL GL-2-mE, because it improved the GPx and GST activities, lowered lipid peroxidation, and reduced LDH leakage [Table 3]. It suggested that there might be some components with antioxidant activity or novel ability to regulate glutathione-related enzyme activities in GL-2-mE to protect primary hepatocytes from CCl_4-derived radicals.

In conclusion, this is the first study using BS and AM to cultivate GL in 5 L fermentation system with different fermentation temperatures to produce fermentation products with antihepatoma activity and liver protective effect. The ethanolic extract of GL fermentation mycelia (GL-2-mE) cultivated in medium containing BS (50 g/L) and AM (20 g/L) at 24°C for 11 days exhibited better antihepatoma activity than the ethanolic extract of GL-36123 fruiting body because of the difference in possible active components. GL-2-mE (<200 μg/mL) did not cause any damage in normal primary rat hepatocytes, whereas it at 100 μg/mL obviously prevented hepatocytes from CCl_4-induced injury. Further investigation of the constituents in the GL fermentation products with different fermentation conditions is needed to clarify the contributors to and mechanisms of the biological activities. These results provide important information for the development of health food for human beings.

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