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Inhibitory Effects of *Ganoderma lucidum* on Tumorigenesis and Metastasis of Human Hepatoma Cells in Cells and Animal Models

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Metastasis is considered to be the major cause of death in patients with cancers, and hepatocellular carcinoma (HCC) is a highly metastatic cancer. *Ganoderma lucidum*, a well-known mushroom with various biological effects, is a functional food known to contain lucidenic acid. The objectives of this study were to investigate the anti-invasion effect of a lucidenic acid-rich *G. lucidum* extract (GLE) on human hepatoma HepG2 cells as well as the antiproliferative and antimetastatic effects of GLE in human hepatoma cells implanted into ICR-*nu*/*nu* mice. Phorbol-12-myristate-13-acetate (PMA)-induced invasion and matrix metalloproteinase (MMP)-9 expression levels of HepG2 cells were reduced by GLE treatment in a dose-dependent manner. The inhibitory effects of GLE on MMP-9 expression proceeded by inhibiting the phosphorylation of extracellular signal-regulated kinase (ERK1/2) and protein kinase B in the cytosol as well as reducing activator protein-1 and nuclear factor-κ B levels in the nucleus of HepG2 cells. In a human tumor xenograft model, a dose−response inhibition was observed in the average size, volume, and weight of tumors upon oral administration of GLE. The number of metastatic tumor-bearing mice, the number of affected organs, and the number of tumor foci as well as the MMP-2 and -9 activities in serum of mice were also significantly suppressed by oral administration of GLE. These results suggest that the lucidenic acid-rich GLE could serve as a chemopreventive agent for the tumorigenesis and metastasis of highly invasive hepatoma cells.

KEYWORDS: ***Ganoderma lucidum*; invasion; lucidenic acid; metastasis; MMP-9 activity**

INTRODUCTION

Ganoderma lucidum, also called lingzhi, is a well-known mushroom with various biological effects and has been used in China for thousands of years as a functional food for health and longevity purposes. The pharmacological and clinical applications of the aqueous/ethanol extracts of *G. lucidum* include the prevention or treatment of various types of human diseases, such as allergy, bronchitis, hyperglycemia, inflammation, nephritis, hepatopathy, arthritis, hypertension, neurasthenia, and chronic hepatitis (*1*, *2*). It is also used to reduce the likelihood of tumor invasion and metastasis and to prevent the occurrence or recurrence of various types of cancers (*1*, *3*). The potential nutritional and medicinal values of *G. lucidum* have attracted a great deal of attention in the search for its bioactive components and underlying mechanism.

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Cancer metastasis refers to the spread of cancer cells from the primary neoplasm and the growth of secondary tumors at sites distant from a primary tumor. Metastasis is responsible for the majority of failures in cancer treatment and is also the major cause of death in patients with a variety of cancers (*4*). Therefore, in addition to minimizing the growth of existing tumors, treatments that limit their spread to new sites and block invasion into new tissues have been sought to enhance the survival of cancer patients (*5*). The degradation of environmental barriers such as the extracellular matrix (ECM) and basement membrane by various proteolytic enzymes is involved in the invasion and metastasis of various cancer cells. Type IV collagen is a major constituent of the basement membrane, and the matrix metalloproteinases (MMP)-2 and MMP-9 are enzymes with the ability to degrade collagen. These enzymes are highly expressed in various malignant tumors, and the levels of expression are closely related to tumor progression and metastasis of cancer cells. Secretion of MMP-9 has been reported in lung, colon, and breast cancers (*6*). Overexpression of MMP-9 has been reported to be associated

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with the capsular infiltration of hepatocellular carcinoma (HCC) and the growth of small HCCs (*7*, *8*). MMP-9 expression associated with the invasion of human hepatic carcinoma cells was also observed in a previous study (*9*). In addition, MMP-2 expression has been found in A549 cells, a highly metastatic human lung cancer cell line (*10*). Therefore, these two MMPs are presumed to be associated with the progression and invasion of different types of cancer cells.

Hepatocellular carcinoma is one of the most common malignancies in the world as a whole, as well as in Taiwan specifically. HCC has been proved to possess a strong tendency to rapidly invade portal veins and to invade the major hepatic vein and the inferior vena cava occasionally (*11*). HCC is a hypervascular tumor, in which venous invasion is a common and important risk factor for tumor metastasis. It is known that the expression of MMP-9 can promote the growth of small HCCs (*7*, *8*). In recent years, the relationship between MMP-9 expression and HCC invasion has been demonstrated by clinical studies (*12*). Therefore, several MMP inhibitors (e.g., BAY12-9566, KB-R7785, *N*-biphenyl sulfonyl-phenylalanine hydroxamic acid) were used in clinical trials to prevent tumor growth and metastasis (*13*, *14*). In a previous study (*9*), four triterpenoids including lucidenic acids A, B, C, and N had been isolated and identified from the fruiting body of a *G. lucidum* strain (YK-02). The crude triterpenoids and lucidenic acids extracted from *G. lucidum* (YK-02) are 3 and 10 times, respectively, higher than those of common *G. lucidum* reported by Nishitoba et al. (*15*) and Wu et al. (*16*). The results revealed that YK-02 is a strain of *Ganoderma* with high contents of triterpenoids and lucidenic acids. These four lucidenic acids showed an inhibitory effect on the phorbol 12-myristate 13-acetate (PMA)-induced invasion of HCC by suppressing MMP-9 expression (*9*, *17*). However, little is known regarding the anti-invasive and antimetastatic activities of the *G. lucidum* (YK-02) extract (GLE) as well as their underlying mechanisms on HCC in vitro and in vivo. In the present study, we prepared an extract (GLE) from the fruiting body of lucidenic acid-rich *G. lucidum* to investigate its inhibitory effect on the proliferation and PMA-induced invasion of HepG2 cells in a cell model. A human hepatoma cell subcutaneous transplantation and an experimental metastasis model using immunodeficient nude mice were also used to verify the antitumor and antimetastatic activity of GLE in vivo.

MATERIALS AND METHODS

Materials and Chemicals. The *G. lucidum* strain (YK-02), which was preserved in the Biotechnology Research and Development Institute (Double Crane, Tainan Hsien, Taiwan), was grown to the fruiting body stage according to the protocol of Chen et al. (*18*). It was identified by Dr. Shen-Hwa Wu (National Museum of Natural Science, Taiwan) using morphological characterization and DNA profiles. PMA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Tedia Co. (Fairfield, OH). Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone Inc. (Logan, UT). Fetal bovine serum (FBS) was purchased from Gibco BRL Co. (Grand Island, NY). The antibodies for total and phosphorylated MAPK/ERK1/2, p38 MAPK, SAPK/JNK, Akt (PKB), c-Jun, c-Fos, and NF-κB (p65) were purchased from Cell Signaling Technology Inc. (Boston, MA). β-Actin and lamin B monoclonal antibodies were purchased from BioVision Inc. (Mountain View, CA). Goat anti-rabbit IgG (H&L) horseradish peroxidase conjugated antibody and goat anti-mouse IgG (H&L) horseradish peroxidase conjugated antibody were purchased from Chemicon International Inc. (Billerica, MA).

Preparation of Extract from *G. lucidum* (GLE). The dried fruiting body of *G. lucidum* (YK-02) was ground into powder and extracted with absolute ethanol (1:10, w/v) in a shaking incubator at room temperature for 8 h. After cooling, the extract was filtered, and the filtrate was evaporated to dryness under vacuum using a rotary evaporator and then stored at −20 °C until use. GLE was reconstituted in DMSO for subsequent experiments. The final concentration of DMSO was controlled under 0.5% in the control and each test group.

Determination of Lucidenic Acids in GLE. The content of lucidenic acids in GLE was determined as the method described by Chen and Chen (*19*). Briefly, after extraction and purification according to the previous paper (*9*), 42 g of crude triterpenoids was obtained from 1 kg of *G. lucidum* (YK-02) (extraction yield = 4.2 g/100 g). Five grams of crude triterpenoids was dissolved with 5 mL of 50% ethanol and then subjected to semipreparative reverse-phase HPLC (Hitachi, Tokyo, Japan). Semipreparative HPLC was performed on a Hitachi 6050 pump equipped with a Hitachi L-4200 UV−vis detector and a Hitachi D2500 integrator. Detection wavelength was set at 252 nm. A column of Lichrosorb RP-18 (Merck Hibar, 7 μm, 250 × 25 mm) was used. The mobile phase was acetonitrile/2% acetic acid = 1:3 for the first 80 min and then changed to 1:2. Flow rate was set at 7.8 mL/min. The eluted peaks were collected and concentrated in a rotary evaporator and were stood for 4−8 days for crystallization and identification.

Cell Culture. Human hepatoma cells (HepG2 cells) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). According to BCRC protocol, cells were grown in DMEM supplemented with 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.37% (w/v) $NaHCO_3$, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. In the invasive and metastatic experiments, the cells were cultured in a serum-free medium.

Cell Viability Assay. Cell viability was determined with an MTT assay. HepG2 cells were seeded onto 96-well plates at a concentration of 1×10^5 cells/well in DMEM without FBS. After 24 h of incubation, the cells were treated with various concentrations of GLE and further incubated for 24−72 h. The controls were treated with H_2O alone. The dye solution [10 μL; 5 mg/mL dye in phosphate-buffered saline (PBS)] was added to each well for an additional 60 min of incubation at 37 °C. After the addition of DMSO (100 μL/well), the reaction solution was incubated for 30 min in the dark. The absorbances at 570 and 630 nm (reference) were recorded with a Fluostar Galaxy plate reader. The percent viability of the treated cells was calculated as follows:

$$(A_{570\text{nm}} - A_{630\text{nm}})_{\text{sample}} / (A_{570\text{nm}} - A_{630\text{nm}})_{\text{control}} \times 100$$

Cell Invasion Assay. HepG2 cells were detached from the tissue culture plates, washed with PBS, resuspended in serum-free DMEM medium (5×10^4 cells/200 μL) in the presence or absence of compound (PMA and GLE), and then seeded onto the upper chamber of Matrigel-coated filter inserts (8 μm pore size) purchased from BD Biosciences (San Jose, CA). Serum-free DMEM medium (500 μL) was added to the lower chamber. After 24 h of incubation, filter inserts were removed from the wells and the cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed with methanol for 10 min and stained with Giemsa dye for 1 h, and then the cells that had invaded the lower surface of the filter were counted under a microscope.

Gelatin Zymography. HepG2 cells were incubated in serum-free DMEM with or without GLE in the presence of indicated PMA concentrations for a given time, and the conditioned media were collected as samples. The unboiled samples were separated by electrophoresis on 8% sodium dodecyl sulfate (SDS)−polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed twice in washing buffer (2.5% Triton X-100 in dH_2O) for 30 min at room temperature and were then incubated in reaction buffer (10 mM $CaCl_2$, 0.01% NaN_3, and 40 mM Tris-HCl, pH 8.0) at 37 °C for 12 h. Bands corresponding to activity were visualized by negative staining using Coomassie Brilliant blue R-250 (Bio-Rad Laboratories, Richmond, CA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was prepared from HepG2 cells using the 3-Zol (Trizol) reagent (MDBio, Inc., Piscataway, NJ) and performed by following the manufacturer's instructions. For RT-PCR, 4 μg of total cellular RNA was used as template in a 20 μL reaction solution that contained 4 μL of dNTPs (2.5 mM), 2.5 μL of oligo dT (10 pmol/μL), and RTase (200 units/μL).

This reaction was performed at 42 °C for 1 h. The resulting cDNA (5 μL) was amplified by PCR with the following primers: MMP-9 (269 bp), 5′-CACTGTCCACCCCTCAGAGC-3′ (sense) and 5′-GCCACTTGTC-GGCGATAAGG-3′ (antisense); MMP-2 (474 bp) 5′-GGCCCTGTCAC-TCCTGAGAT-3′ (sense) and 5′-GGCATCCAGGTTATCGGGGAT-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 309 bp), 5′-TCCCTCAAGATTGTCAGCAA-3′ (sense) and 5′- AGATCCACAA-CGGATACATT-3′ (antisense). PCR amplification was performed under the following conditions: 35 cycles of 94 °C for 1 min, 63 °C for 1 min (for MMP-9 and GAPDH) or 62 °C (for MMP-2), 72 °C for 2 min, followed by a final incubation at 72 °C for 10 min.

Preparation of Cell Lysates and Nuclear Fractions. Cell lysates and nuclear fractions were prepared using a Nuclear Extraction Kit (Panomics, Redwood City, CA). Briefly, harvested cells (1×10^6 cells/6 cm plate) were washed twice with 5 mL of cold 1 × PBS. A 0.5 mL aliquot of buffer A solution was added to each plate. The plate was transferred to an ice bucket on a rocking platform at 150 rpm for 10 min. The treated cell suspension was transferred to a sterilized Eppendorf tube and centrifuged at 14000*g* for 3 min at 4 °C. The supernatant (cytosolic fraction) was removed, and the pellet was kept on ice. A 75 μL aliquot of buffer B solution was added to each pellet. Pellets were vortexed at maximum speed for 10 s, and the Eppendorf tubes were shaken on a rocking platform at 150 rpm for 2 h. After centrifugation at 14000*g* for 5 min at 4 °C, the supernatant was nuclear extracted and stored at −80 °C until use.

Western Blotting. Ten microgram samples of total cell lysates or nuclear fractions were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto a polyvinylidene fluoride (PVDF) membrane using Bio-Rad Mini Protean electrotransfer system (Mini-Protean, Bio-Rad Laboratories, Richmond, CA). The blot was subsequently blocked with 5% skim milk in PBST for 1 h and probed with antibodies to total and phosphorylated MAPK/ERK1/2, p38MAPK, SAPK/JNK, Akt (PKB). β-Actin, NF-κB (p65), c-Jun, c-Fos, and lamin B were detected with their respective specific antibodies overnight at 4 °C. Detection was performed with an appropriate peroxidase-conjugated secondary antibody at room temperature for 1 h. Intensive PBS washing was performed after each incubation. After the final PBS wash, the signal was visualized by an enhanced chemiluminescence (ECL) detection system and Kodak X-OMAT Blue Autoradiography Film. The protein content was determined according to the Bradford assay (*20*) with bovine serum albumin as a standard.

Experimental Animals and Grouping. Male ICR-*nu*/*nu* strain nude mice (5 weeks old) were purchased from BioLASCO Co. (Taipei, Taiwan). HepG2 cells were injected in 6-week-old male nude mice (15−20 g), which were kept in filter-top cages in a room maintained at 25 ± 1 °C with 60% humidity. Sterilized food and water were accessible ad libitum. The 40 experimental mice were equally randomized into five groups: positive control (C+), negative control (C−), and high (800 mg/kg), medium (200 mg/kg), and low (50 mg/kg) dose groups. Three groups of experimental mice (n = 8 for each group) were given the indicated dose of GLE orally once daily for 68 days starting on the first day after hepatoma cell implantation. The C+ group (n = 8) received the equivalent volume of normal saline (or distilled water) in the same way. The C− group was normal mice without implanted hepatoma cells and with normal feed. All experimental procedures involving animal studies were conducted in accordance with the National Institutes of Health (NIH). This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (NCHU), Taichung, Taiwan.

Subcutaneous and Tail Vein Injection of Tumor Cells. Subconfluent cultures of HepG2 cells were detached by trypsinization. The cells were washed three times with serum-free DMEM. Cells were suspended at a concentration of 1×10^8 cells/mL in serum-free DMEM and then stored on ice for injection. Each nude mouse was implanted with 0.1 mL (1×10^7) cells by subcutaneous injection (with a 24-gauge needle) into the left flank or by tail vein injection (with a 26-gauge needle) with a 1 mL syringe.

Measurement of Antitumor and Anti-invasive Effects of GLE in Immunodeficient Nude Mice. Tumor volume was measured once every other day using a caliper when the first tumors formed. Tumor volume was estimated according to the following formula: tumor volume (mm^3) = length × width^2/2. All of the mice were sacrificed at day 68 after tumor implantation. Tumor inhibitory efficiency (%) = (1 − mean tumor volume of the GLE administration group/mean tumor volume of the C+ group) × 100%. Blood samples were chilled in test tubes containing heparin. Tumor tissue, liver, lung, heart, spleen, and kidney specimens were quickly removed, weighed, and taken for routine pathology and electron microscopy observation, including changes in morphology and tumor status.

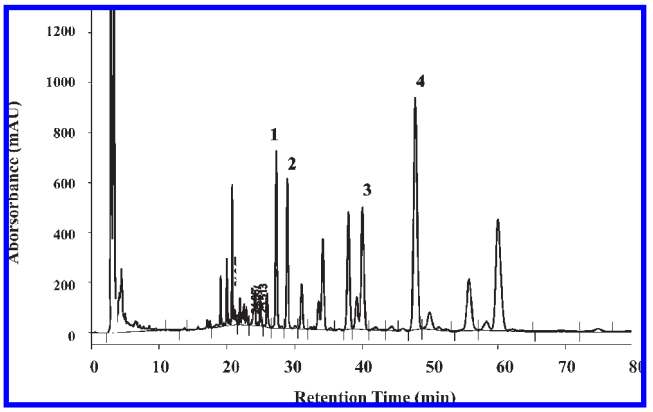

Figure 1. Reverse-phase HPLC chromatogram of ethanolic extract of *G. lucidum* (YK-02). Peaks 1, 2, 3, and 4 are lucidenic acids C, N, B, and A, respectively.

Statistical Analysis. Data are presented as the mean ± SD of three independent measurements. Differences between variants were analyzed using Student's t test for unpaired data. Values of $p < 0.05$ (∗) or $p < 0.01$ (∗∗) were regarded as statistically significant.

RESULTS

Content of Lucidenic Acids in GLE. The compounds of peaks 1, 2, 3, and 4 in the reverse-phase HPLC chromatogram of *G. lucidum* (YK-02) ethanolic extract (**Figure 1**) were identified as lucidenic acids C, N, B, and A, respectively. The structures and identification of these lucidenic acids were published in a previous study (*9*). In this study, the extraction yields of lucidenic acids A, B, C, and N separated from 1 kg *G. lucidum* (YK-02) were 2320, 1010, 850, and 680 mg, respectively. It is revealed that the GLE used in the present study was a lucidenic acid-rich sample as in the previous study (*9*).

Effect of GLE on the Viability of HepG2 Cells. The effect of GLE on the viability of HepG2 cells was determined by an MTT assay, which is used for measuring the metabolic activity of a cell. The absorbance decrease in this assay could be a consequence of either cell death or the reduction of cell proliferation. HepG2 cells were treated with GLE at various concentrations (0, 0.01, 0.1, and 1 mg/mL) for 24 h, and the viability of cells was not significantly reduced. The viability of HepG2 cells was decreased by 23 and 21% after incubation with GLE (1 mg/mL) for 48 and 72 h, respectively, as compared with controls (**Figure 2**). The result indicated that GLE at high concentration or extended incubation time could inhibit proliferation of the cells or be cytotoxic to the cells.

GLE Inhibits PMA-Induced Invasion of HepG2 Cells and Suppresses MMP-9 Expression at a Transcriptional Level. Quantitative analyses by a cell invasion assay with Matrigel-coated filter inserts showed that the invasion of HepG2 cells was increased approximately 2.2-fold upon PMA (200 nM) treatment. This PMA-induced invasion was inhibited by GLE treatment in a dose-dependent manner (**Figure 3A**). Because ECM degradation is crucial for cell invasion, the effect of GLE on MMP-2 and MMP-9 of HepG2 cells was analyzed by gelatin zymography to determine if MMP-2, MMP-9, or both were active in the GLE-inhibited invasion of HepG2 cells. As shown in **Figure 3B**,

GLE could reduce PMA-induced MMP-9 activity in a dose-dependent manner, but MMP-2 activity was not affected by PMA or GLE treatment. By comparison of the results in panels **A** and **B** of **Figure 3**, we found that the inhibition of GLE on PMA-induced invasion was correlated with MMP-9 activity in HepG2 cells. These results suggested that GLE inhibited PMA-induced invasion of HepG2 cells through suppressing MMP-9 activity. To further evaluate whether the inhibitory effect of GLE on MMP-9 activity in HepG2 cells takes place at the mRNA level, a semiquantitative RT-PCR analysis was performed. After treatment with various noncytotoxic concentrations (0, 0.1, 0.5, and 1 mg/mL) of GLE, mRNA levels of MMP-9 were significantly reduced in a dose-dependent manner while that of the internal control (GAPDH) remained unchanged (**Figure 3C**). The results revealed that GLE might regulate the expression of MMP-9, at least partially, on the transcriptional level.

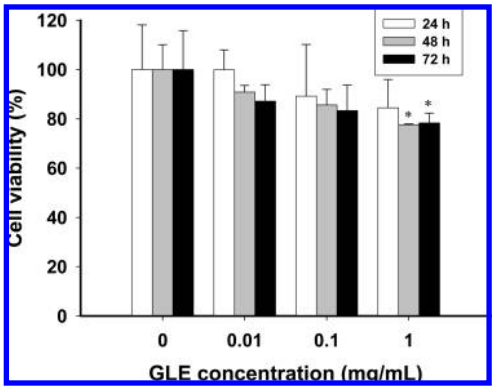

Figure 2. Effect of GLE on the viability of HepG2 cells by MTT assay. Results are expressed as percentage of cell viability of the untreated group.

GLE Inhibits PMA-Induced MMP-9 Expression in HepG2 Cells by Suppressing Phosphorylation of MAPK/ERK1/2 and Akt as well as Suppression of PMA-Stimulated NF-κB and AP-1 Activities. Because GLE was found to possess inhibitory effects on PMA-induced MMP-9 expression in HepG2 cells, the effects of PMA and GLE on the MAPK and PI3K/Akt pathways were investigated by Western blotting to clarify the underlying

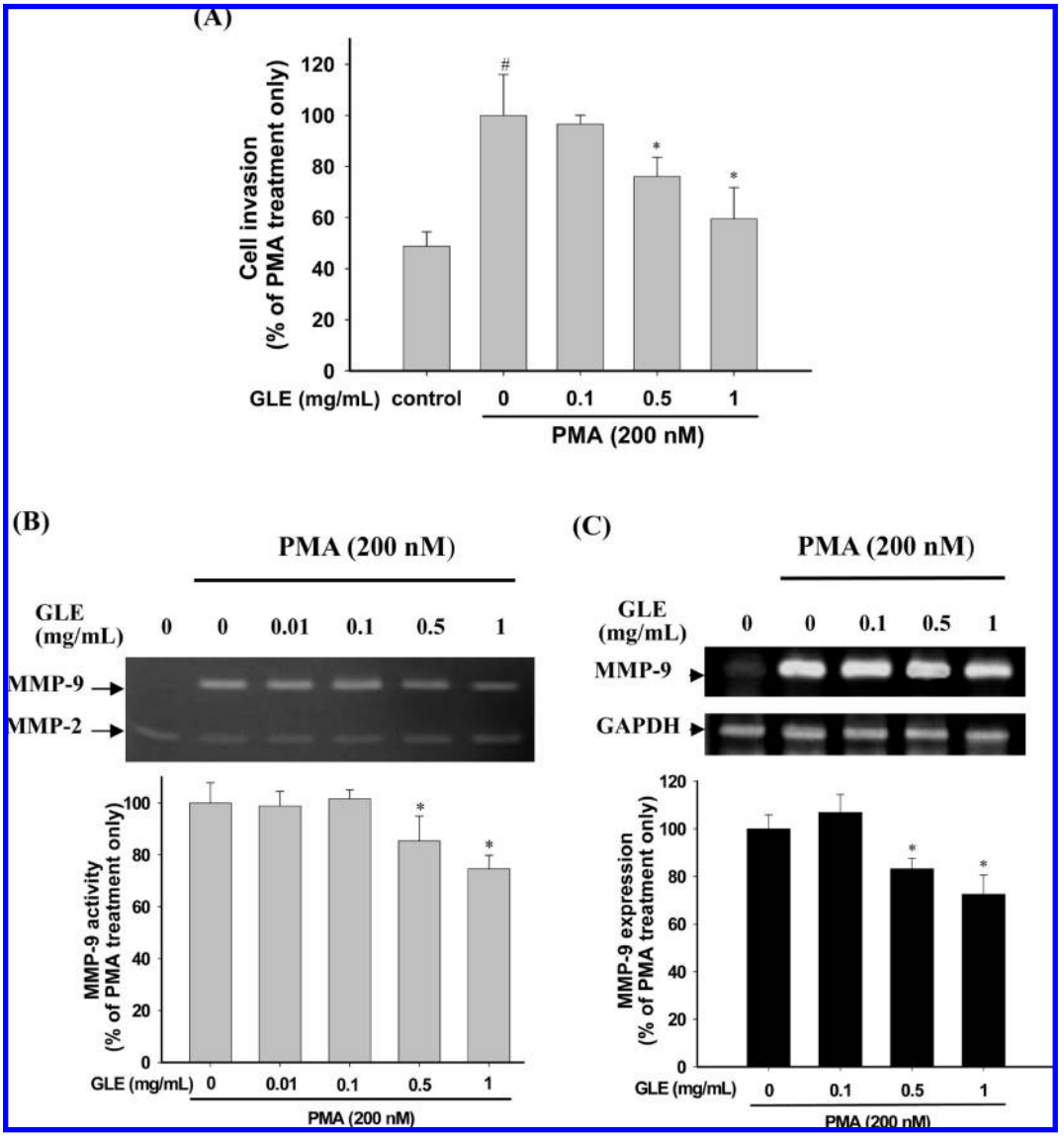

Figure 3. Dose-dependent inhibitory effects of GLE on PMA-induced invasion, MMP-9 activity, and MMP-9 mRNA expression of HepG2 cells. HepG2 cells were incubated in a serum-free medium containing PMA and GLE for 24 h. (**A**) The invasive ability of HepG2 cells was determined by a Matrigel invasion assay. (**B**) MMP-9 activity of HepG2 cells was determined by gelatin zymography. (**C**) The RNA extracted from HepG2 cells was subjected to a semiquantitative RT-PCR. GAPDH was used as an internal control. The final PCR products were quantified by densitometric analysis with the density of PMA treated being 100%. #, $p < 0.01$, indicates significant differences from the untreated group; ∗, $p < 0.05$, indicates significant differences from the PMA treatment alone.

mechanisms. The results of RT-PCR and Western blots showed that GLE inhibited PMA-induced MMP-9 expression (**Figure 3C**) through suppression of the phosphorylation of ERK1/2 and Akt (**Figure 4A**) in HepG2 cells. Treatment with GLE (0.5 mg/mL) resulted in 37 and 32% decreases in phosphorylation of ERK and Akt, respectively, as seen by comparing the densitometric analyses of blots from cells treated with PMA and GLE to those of cells treated with PMA alone. Neither PMA nor GLE affected SAPK/JNK and p38 MAPK significantly (**Figure 4A**). It is known that the AP-1 and NF-κB proteins are the downstream targets of MAPK signaling and that the expression of the *MMP-9* gene is regulated by interaction of AP-1 and NF-κB with their binding sequences on the *MMP-9* gene promoter in the nucleus. Here, the effects of PMA and GLE on the protein levels of NF-κB and AP-1 in nucleus of HepG2 cells were examined. HepG2 cells were treated with various noncytotoxic concentrations of GLE (0, 0.1, 0.5, and 1 mg/mL) in the presence of PMA (200 nM) for 24 h, and the nuclear extracts were analyzed by Western blotting to measure the levels of NF-κB, c-Jun, and c-Fos. As shown in **Figure 4B**, the levels of PMA-induced NF-κB, c-Jun, and c-Fos in the nucleus were decreased by GLE treatment in a dose-dependent manner.

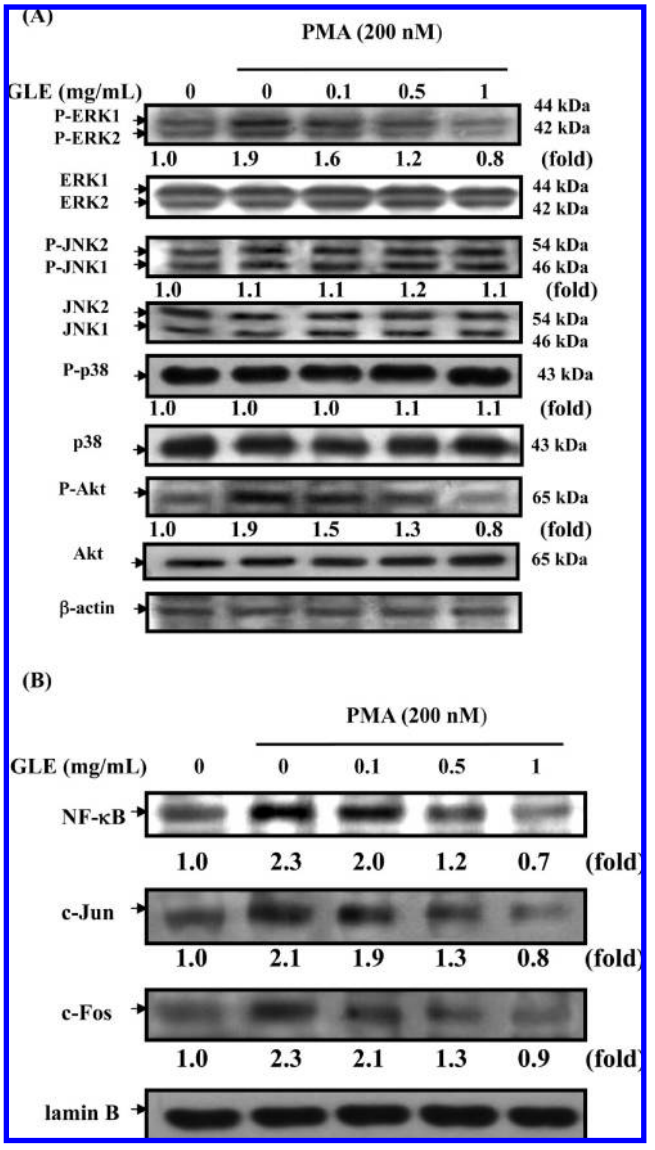

Figure 4. Inhibitory effect of GLE on the phosphorylation of ERK1/2 and Akt at the protein level (**A**) and on expression levels of NF-κB, c-Jun, and c-Fos (**B**). HepG2 cells were cultured in serum-free media containing PMA and GLE for 24 h, and then the cell lysates and the nuclear extracts were subjected to Western blot analysis. Levels of these proteins were subsequently quantified by densitometric analyses, and the relative density was compared with the untreated group.

Inhibitory Effect of GLE on Tumorigenesis in Nude Mice. In **Figure 2**, GLE exerted its antiproliferative activity on HepG2 cells after incubation at a concentration of 1 mg/mL for 48 and 72 h. To further investigate the effect of GLE on tumor growth in vivo, HepG2 cells were injected subcutaneously into the left flank of nude mice (6 weeks old). Experimental animals were treated with GLE as described under Materials and Methods. As shown in **Table 1**, tumor growth was observed in each group of mice. However, the numbers of mice with tumor growth in GLE treatment groups were lower than those in the positive control group. Tumor volume was measured once every other day using a caliper when the first tumors formed. The results shown in **Figure 5A** indicate that the growth of tumors in nude mice was significantly inhibited by treatment with GLE at doses of 200 and 800 mg/kg. The tumor was excised surgically at the time of sacrifice. The visible tumor sizes are shown in **Figure 5B**,**C**. The actual average tumor volume and average tumor weight at the end of the experiment showed a significant reduction in the 200 (60 and 89% inhibition, respectively) and 800 (98 and 99% inhibition, respectively) mg/kg GLE treatment groups as compared with the positive control group (**Table 1**).

Inhibitory Effect of GLE on Tumor Metastasis in Nude Mice. After the HepG2 cells were transferred into nude mice by tail vein injection, visible tumor foci in liver (**Figure 6AI**) and lung (**Figure 6BI**) tissue of positive control and GLE treatment groups were found by macroscopic observation on day 68. The growth of hepatoma cells was confirmed by histological examination (hematoxylin/eosin staining) of liver (**Figure 6AII**) and lung (**Figure 6BII**) sections with tumor foci obtained from the nude mice. The characteristics of hepatoma cells including large nucleus, high nucleus/cytoplasma ratio, irregular nuclear shape, dense nuclear dyeing, and variable nuclear size (*21*, *22*) were observed in these organ sections. As compared with the positive

Table 1. Inhibitory Effect of GLE on Growth of HCC Tumor Xenografts in Nude Mice

group[a]	HCC growth no. of mice (no.) (%)	tumor av vol [e] (mm^3)	inhibition (%)	tumor av wt [e] (mg)	inhibition (%)
control (−)[b]	0 (0)				
control (+)[c]	5 (63)	3498	0	2320	0
GLE-treated[d] (mg/kg of bw)					
50	1 (13)	3609	−3	2400	−5
200	1 (13)	1397	60	260	89
800	1 (13)	87	98	30	99

[a] The number of mice in each group ($n = 8$). [b] Control (−) indicates normal nude mice with normal feed. [c] Control (+) indicates HCC injected nude mice without GLE treatment. [d] GLE, *G. lucidum* extract. The indicated doses of GLE were administered orally for 68 consecutive days, starting the day after implantation of tumor cells. [e] Measurement at the day sacrifice.

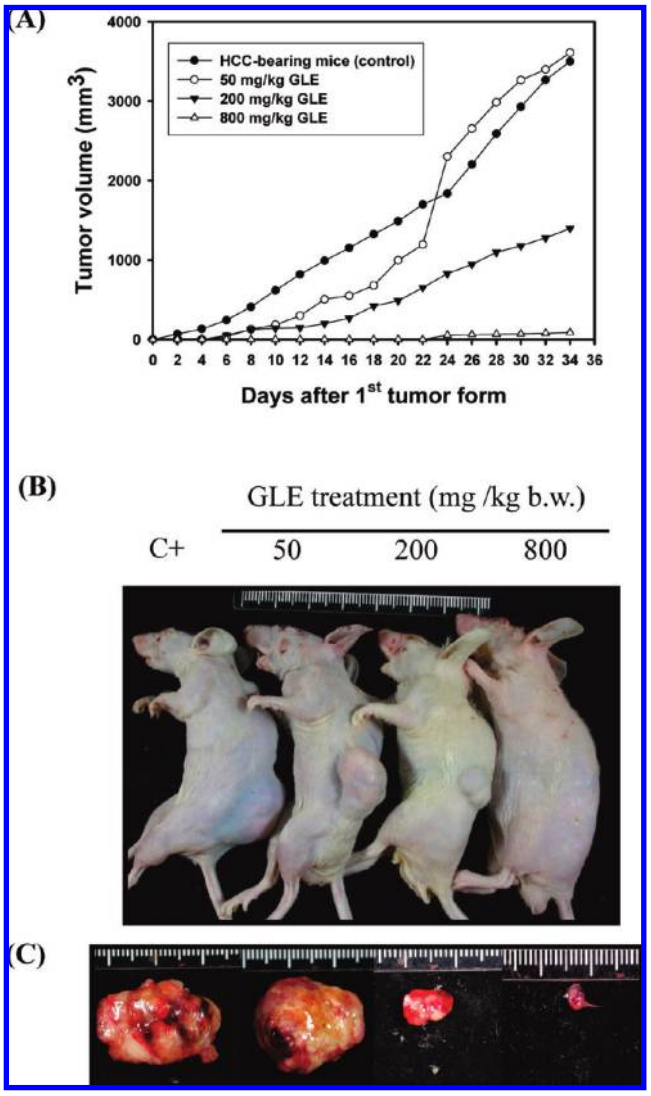

Figure 5. Suppression of tumorigenesis by GLE treatment in HCC-bearing nude mice. The indicated doses of GLE were administered orally for 68 consecutive days, starting the day after the implantation of tumor cells. Control (C+) mice were administered distilled water orally alone on the same schedule. (**A**) Inhibitory effect of GLE treatment for 68 days on tumor volume in HCC-bearing nude mice. Tumor volumes were directly measured with calipers each 2 days after the first tumor formed and calculated by the formula shown under Materials and Methods. (**B**) Visible tumor sizes on day 68 after injection of indicated HepG2 cells. (**C**) Photographs of representative tumors removed from mice on day 68.

control group, a significant suppressive effect on tumor metastasis in nude mice by the oral administration of GLE is shown in **Table 2**. Metastasis of hepatoma cells was observed in 4 mice (4/8, 50%) from the positive control group, whereas only 2 (2/8, 25%), 2 (2/8, 25%), and 1 (1/8, 12.5%) mice showed tumor metastasis in the low (50 mg/mL), medium (200 mg/mL), and high (800 mg/mL) dose groups, respectively. GLE treatment decreased the number of organs with metastatic tumors, and the number of foci in these organs was also smaller than the positive control group (**Table 2**). Interestingly, MMP-9 activity in the serum of nude mice was increased after HepG2 cell injection and was significantly decreased by oral administration of GLE in a dose-dependent manner. Under the same conditions, changes in the levels of MMP-2 activity in the serum were not as obvious as the observed changes in MMP-9 activity (**Figure 7**).

DISCUSSION

G. lucidum has been used to inhibit tumorigenesis and reduce the invasion and metastasis of various types of cancers, including hepatoma for many years; however, the bioactive component and underlying mechanism remain unclear. In this study, the cytotoxicity of the GLE on HepG2 cells was shown after co-incubation over 48 h. Liu et al. (*23*) presented a similar result on the antiproliferative effects of *G. lucidum* ethanolic extract. According to the result, the time of GLE treatment on HepG2 cells in anti-invasive assay was controlled at <48 h. The relationships between PMA stimulation, MMP expression, and cell invasion in various cancers have been well-documented. It has been reported that PMA stimulates the migration and invasion of HCC 7721 cells (*24*) and that PMA also induces MMP-9 expression in an HCC Malavu cell line (*25*). Taken together, MMP-9 expression up-regulation by stimulators appears to induce invasion in some cell lines. Here, we have shown that PMA stimulates cell invasion through induction of MMP-9 expression in HepG2 cells (**Figure 3**). It has been demonstrated that lucidenic acids A, B, C, and N inhibited the PMA-induced invasion of hepatoma cells by suppressing MMP-9 expression in our previous studies (*9*, *17*). In the present study, we found that the treatment of PMA-induced HepG2 cells with GLE also exerted an inhibitory effect on cell invasion (**Figure 3A**) and MMP-9 expression (**Figure 3C**) in a dose-dependent manner. Through Western blotting assays, it was found that treatment of PMA-induced HepG2 cells with GLE could inhibit the phosphorylation of ERK1/2 and Akt (**Figure 4A**). Taken together, these results suggest that the inhibitory effect of GLE on PMA-induced MMP-9 expression is through the inactivation of ERK1/2 and Akt phosphorylation, which results in the inhibition of cell invasion in HepG2 cells. The transcription of the *MMP-9* gene is regulated by upstream promoter sequences, including AP-1, NF-κB, stimulatory protein-1 (Sp1), and polyoma virus enhancer activator-3 (PEA3) binding sites (*26*, *27*). Among these binding sites, the AP-1 and NF-κB elements are centrally involved in the induction of the *MMP-9* gene associated with the invasion of tumor cells activated by PMA and cytokines. In the present study, the expression levels of AP-1 (c-Jun and c-Fos) and NF-κB in HepG2 cells were reduced by GLE treatment (**Figure 4B**). Interestingly, lucidenic acid was also shown to have an inhibitory effect on the PMA-induced invasion of human hepatoma cells through inactivation of the MAPK/ERK signal transduction pathway and reduction of the promoter binding activities of NF-κB and AP-1 in a previous study (*17*). These results suggested that lucidenic acid might be the bioactive component in *G. lucidum* producing anti-invasive activity in HepG2 cells.

Tumor invasion and metastasis are complex processes that include cell proliferation, cell migration, proteolytic degradation of ECM, invasion of the circulatory system, cell adhesion, and growth of tumors at metastatic sites (*28*, *29*). In agreement with the results of our in vitro model, the inhibitory effects of GLE on metastasis and tumor growth were further verified in vivo. Tumorigenesis (**Table 1**) and metastasis (**Table 2**) were both significantly inhibited by oral administration of GLE at a daily dose of up to 200 mg/kg in experimental animals. The effective dose (200 mg/kg) of GLE used in a mouse with a body weight of 20 g converted to the daily intake of an adult human with a body weight of 60 kg of approximately 1.32 g. The dosage is available via human dietary supplementation. On the basis of the lucidenic acid content in *G. lucidum* that we described under Results, the bioavailabilities of lucidenic acids A, B, C, and N from the putative daily dose of GLE were 73, 32, 27, and 21 mg, respectively. MMPs have been reported to play

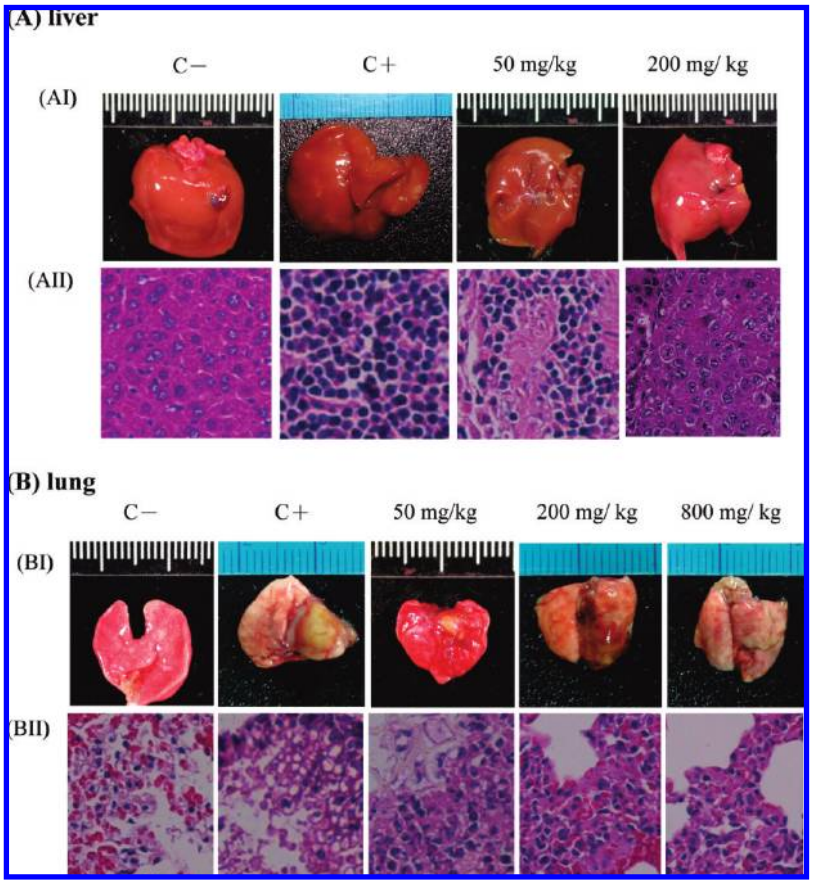

Figure 6. Inhibitory effect of GLE treatment for 68 days on tumor metastasis in nude mice: visible tumor foci of liver (**AI**) and lung (**BI**) on day 68 after transfer of HepG2 cells via tail vein injection; histological examination of the liver (**AII**) and lung (**BII**) sections with tumor foci obtained from nude mice sacrificed at day 68 after inoculation of HepG2 cells (hematoxylin/eosin staining, 200×). "C −" indicates normal mice with normal feed. "C +" indicates administration of distilled water orally on the same schedule. The indicated doses of GLE were administered orally for 68 consecutive days, starting the day after the injection of tumor cells.

Table 2. Inhibitory Effect of GLE on HCC Metastasis in Nude Mice

	metastasis		
group[a]	no. of mice[e] (%)	no. of organs[e]	no. of foci[e]
control (−)[b]	0 (0)	0	0
control (+)[c]	4 (50)	5	16
GLE-treated [d] (mg /kg of bw)			
50	2 (25)	2	5
200	2 (25)	3	4
800	1 (13)	1	2

[a] Number of mice in each group ($n = 8$). [b] Control (−) indicates normal nude mice with normal feed. [c] Control (+) indicates HCC injected nude mice without GLE treatment. [d] GLE, *G. lucidum* extract. The indicated doses of GLE were administered orally for 68 consecutive days, starting the day after injection of tumor cells. [e] Measurement at the day of sacrifice.

a major role in promoting tumor metastasis and angiogenesis (*30*). Tumor-derived and host stroma-derived MMPs have also been demonstrated to be associated with tumor growth (*31*). Enhanced expression of MMP-9 has been shown to be associated with the progression and invasion of tumors (*6*, *32*). It has also been reported that the overexpression of MMP-9 results in capsular infiltration of HCC and growth of small HCC (*7*, *8*). Some artificial MMP inhibitors have been developed and are undergoing clinical trials for antitumor or antimetastasis uses (*13*, *14*, *33*). Here, we demonstrate that MMP-9 activity in the serum of nude mice was increased by implantation of HepG2 cells and decreased upon GLE treatment. MMP-2 activity in serum may play a minor role for hepatoma formation or metastasis. It is known that the anticancer effects of *G. lucidum* might be derived from triterpenoids, polysaccharides, or immunomodulatory protein components. Hence, the lucidenic acids in GLE might be the mediator of antitumor effects in nude mice.

In the present study, the inhibitory effects of *G. lucidum* on tumorigenesis and metastasis of human hepatoma cells in vitro and in animal models were demonstrated. Our findings revealed that the potential antiproliferative and anti-invasive effects of *G. lucidum* on hepatoma cells were, at least partly, derived from lucidenic acid through the repression of MMP-9 transcriptional activity as well as the down-regulation of AP-1 and NF-κB expressions. These results also suggest that the use of *G. lucidum* as a functional food could be considered as an additional method for prevention of the tumorigenesis and metastasis of highly invasive hepatoma cells. *G. lucidum* might be an effective edible mushroom for chemoprevention of liver cancer.

ABBREVIATIONS USED

Akt, protein kinase B; AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ECM, extracellular matrix; ERK, extracellular signal regulated kinase; GLE, *G. lucidum* extract; HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

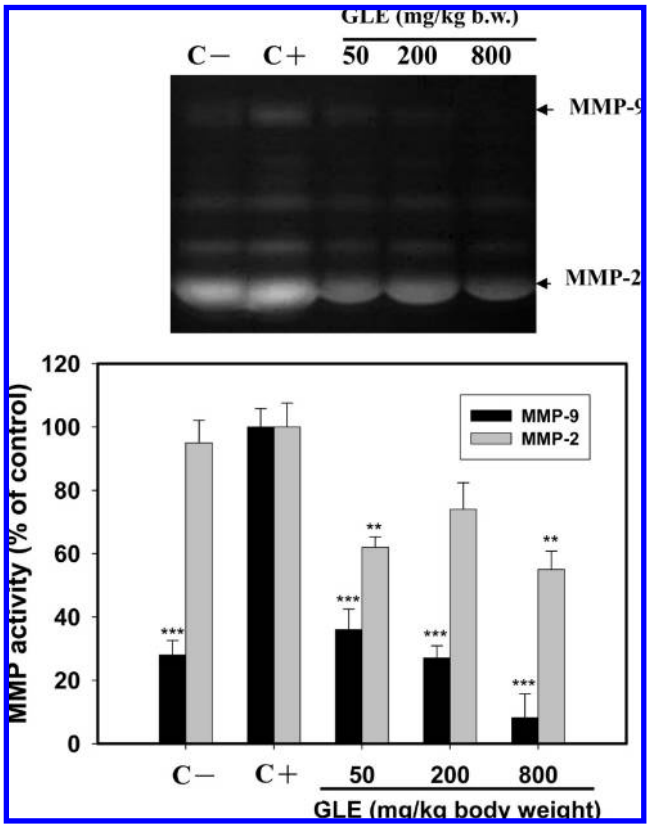

Figure 7. Effect oral administration of GLE on MMPs activity in serum of nude mice. "C −" indicates normal nude mice with normal feed. "C +" indicates HepG2 cells injected nude mice without GLE treatment. Results are expressed as percentage of MMPs activities of the control. $**$, $p < 0.01$, and $***$, $p < 0.001$, compared with the control.

NF-κB, factor-kappa B; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; PVDF, polyvinylidene fluoride; RT-PCR, reverse transcription Polymerase Chain Reaction; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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