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Effects of Lu-Do-Huang Extract (LDHE) on Apoptosis Induction in Human Hep3B Cells

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Abstract

Lu-Do-Huang (*Pracparatum mungo*) is a fermented mung beans (*Vigna radiata*) and has long been used as a traditional and functional food in Traditional Chinese Medicine, especially for treating a variety of liver disorders. The present study aimed to evaluate the apoptotic effects of Lu-Do-Huang ethanol extract (LDHE) on Hep3B cells, a human hepatoma cell line. A variety of cellular assays, flow cytometry and immunoblotting were used. Our results showed that LDHE significantly inhibited Hep3B cells growth. Additionally, the cell cycle assay showed that LDHE prevented Hep3B cell entry into S phase and led to an arrest of Hep3B cells in the G_0/G_1 phase. LDHE induced Hep3B cells to undergo apoptosis as determined through Hep3B cell morphology changes, increase of apoptotic bodies, apoptotic cells, DNA fragmentations and caspase activity. We further examined the protein expression of TRADD, FADD, and Bax to verify the possible apoptotic pathways. The results indicated that LDHE-induced apoptosis in Hep3B cells might mediate by an extrinsic signaling pathway leading to an induction of apoptosis in Hep3B cells. In conclusion, LDHE induced apoptosis and cell cycle arrest in Hep3B cells. Our data provide the evidences regarding the anti-hepatoma potential of LDHE in Hep3B cells.

Key Words: anti-hepatoma, apoptosis, caspase, FADD, Hep3B cell, Lu-Do-Huang, *P. mungo*, TRADD

Introduction

Hepatocellular carcinoma (HCC) is one of the most common primary malignant tumors of the liver, with the highest prevalence occurring in South Asia (23). Because of the multifocal nature of HCC, only approximately 20% of patients with HCC are considered respectable upon presentation of the case (1). Chemotherapy, therefore, has become the major form of treatment for patients with unrespectable HCC (16). Unfortunately, cancer cells that are resistant to chemotherapy will eventually dominate the cell population and cause mortality (10). Thus, there is an urgent effort being made to improve the therapeutic activity of anticancer agents and to develop new anticancer drugs, drug combinations, and chemotherapy strategies through the methodical and scientific exploration of a massive pool of low-toxicity synthetic, biological, and natural substances (7, 14, 28).

Since ancient times, mung beans have been known for their capacity to promote heat clearance, detoxification, summer heat relief, and diuresis. *Pracparatum mungo* is based on fermented mung beans (*Vigna radiata*), and is usually named Lu-Do-Huang

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in Taiwan. Lu-Do-Huang used to use a traditional and alternative medicine in Chinese. Recent studies have also found that mung beans and mung bean sprouts have anti-tumor and anti-cancer properties (6, 17, 21), immune system-regulation (5), antibacterial (30), antifungal activities (33), and liver-protection (31). Kuo *et al*. noted that *P. mungo* extract (PME) has a protective effect against acute hepatotoxicity induced by the administration of CCl_4 in rats, and that the hepatoprotective effects of PME may be due to both the inhibition of lipid peroxidation and the increase of antioxidant activity. PME also possesses antioxidant and melanogenesis inhibitory activities that support skin protection. The study also shows PME can enhance the enzymatic performance of superoxide dismutase, catalase and glutathione peroxidase in the liver (15).

The composition of the bacterial communities during manufacture Lu-Do-Huang has been studied by using high-throughput pyrosequencing analyses. The study revealed that there are 9 phyla, 264 genera, and 586 species of bacteria found through samples obtained at 7 points during fermentation. Family *Lactobacillaceae* and genus *Lactobacillus* emerged in highest abundance, while mung beans were inside bamboo sections (stages 1 and 2 of the fermentation process); *Lactobacillus plantarum* was broadly distributed among these samples. During stage 3, the bacterial distribution shifted to family *Porphyromonadaceae*, and *Butyricimonas virosa* became the predominant microbial component. Thereafter, bacterial counts decreased dramatically, and organisms were too few to be detected during stage 4.

An anticancer chemotherapy regimen triggers tumor cell death through the induction of apoptosis, and alterations in the apoptotic pathways may determine tumor resistance to the current therapeutic strategies (32). Hence, the identification and targeting of a given disabled pathway might provide the most successful approach for chemotherapy in the future. There are many compounds have been proved could induce apoptosis in different cancer cells (4, 18, 20, 29). Therefore, considering the continuing need for effective anticancer agents, medicinal plants might be a potential source of anticancer drugs in terms of both variety and mechanisms of action (24). Lu-Do-Huang has been known as a "sacred detoxification medicine" for thousands of years and is widely used for treating a variety of liver disorders in Chinese. While there exists few researches discussing the liver protection of Lu-Do-Huang, but there is no scientific evidence and possible mechanism to support anti-hepatoma ability of Lu-Do-Huang. In the current study, we first aimed to determine the phytochemical content by using high performance liquid chromatograph (HPLC). In order to investigate the effect of Lu-Do-Huang ethanol extract (LDHE) in Hep3B cells for cell death/apoptosis induction, the assay of cell cycle analysis, cell morphology, Annexin V/PI, and TUNEL were estimated in this research. Furthermore, we detected the expression of FADD, TRADD, caspase-8, caspase-3, and Bax, and cytochrome *c* release to elucidate the possible signaling pathway of apoptosis induction in Hep3B cells after LDHE treatment.

Materials and Methods

Sample Preparation

Lu-Do-Huang was purchased from a local company (Eight Princes Biotechnology Co. Ltd.) in Cha-yi, Taiwan, and then powdered. The manufacturing process to produce *P. mungo* (Lu-Do-Huang) can roughly be divided into four stages, as described in our recent study (3). The powder (100 g) was extracted with ethanol (90% ethanol, 1000 ml, two extractions) for 24 h at 4°C and then centrifuged at 500 × *g* for 20 min. The extract was filtered and then evaporated to dryness under reduced pressure in a rotary evaporator; the eventual yield was more than 10 g of crude ethanol extract. This crude ethanol extract was then lyophilized (EYMA Freeze Dryer, FDU-540, Tokyo, Japan). The final 0.96 g of LDHE was dissolved in phosphate buffered saline (PBS) for long-term storage at -20°C.

High Performance Liquid Chromatograph (HPLC) and Phytochemical Content Analysis

Chinese herbal medicine mixtures including *Ganoderma lucidum*, *Taiwanofungus camphoratus*, *Coptis chinensis* Franch., *Houttuynia cordata* Thunb., Chinese Angelica Root and *Glycyrrhiza uralensis* Fisch were added to freshly selected mung beans for 480 days in order to produce Lu-Do-Huang. Our previously study has been revealed that major compound of those Chinese medicine are berberine, ferulic and glycyrrhizin (11). Therefore we conduct the quantification of berberine, ferulic and glycyrrhizin levels in the extract of Lu-Do-Huang by using HPLC as described in previous studies (25-27). We also examined the nutrition components of Lu-Do-Huang and the mung bean according to the Chinese National Standards (CNS) criteria.

Cell Lines and Culture Conditions

Hepatocellular carcinoma cell line (Hep3B, BCRC 60434) was purchased from the Food Industry Research & Development Institute in Hsin Chu, Taiwan. The cells were maintained in DMEM medium (Hyclone, Logan, UT, USA) supplemented with 10%

FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were maintained in the logarithmic growth phase by routine passage every 2-3 days using 0.025% trypsin-EDTA treatment.

Measurement Cell Viability

The cells were cultured at a density of 5×10^5 cells/well and then spread evenly across a 6-well culture plate. At 24 and 48 h after cell culturing, the LDHE was added at various concentrations (50, 100, 500, 1000, 2000, and 4000 μg/ml), with 5 wells used for each concentration. The cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The relative amount of viable cells was determined by measuring the reduction of the MTT dye in live cells to blue formazan crystals at an optical density of 570 nm and was expressed as a percentage of the control samples without LDHE.

Cell Cycle Analysis

Hep3B cells (5×10^5 cells/ml) were seeded in 6-well culture plates and incubated overnight. The cells were then treated with LDHE at 1000 μg/ml for 24 and 48 h. The cells were harvested after incubation, washed, and fixed in 95% vol/vol ethanol at 4°C overnight. Before analysis, the cells were resuspended in phosphate-buffered saline (PBS) containing propidium iodide (PI, 50 μg/ml) and RNase A (100 μg/ml) at 4°C in the dark for 30 min. The cell cycle distribution was analyzed by flow cytometry (Cyflow D-48161; PARTEC), and data from 10,000 cells per sample were collected.

Annexin-V FITC/PI Staining Assay

Cells at a density of 5×10^5 were grown in 6-well plates, and the cells were incubated with 1000 μg/ml of LDHE extract for 2, 8, 24, or 48 h. The cells were then fixed and assayed per the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Trypsin was added for the Annexin V-FITC/PI staining, and centrifugation at 500 × *g* for 5 min was used to separate the cells. Annexin V Incubation Reagent was then applied per the manufacturer's instructions, and 400 μl binding buffer was added to the cells. The cells were then subjected to flow cytometry.

Terminal Deoxynucleotidyl Transferase-Mediated Dutp Nick-end Labeling (TUNEL) Assay

For the TUNEL assay, 1×10^5 cells were washed with PBS twice after the 2-, 8-, 24-, and 48-h treatment and centrifuged (500 × *g*) for 5 min. The cells were then fixed with 3.7% formaldehyde. Cytonin was incubated with the cells for 30 min, and then terminal deoxynucleotidyl transferase (TdT) labeling buffer was added. The cells were then incubated with a labeling reaction mix for 1 h at 37°C, with stop buffer applied at the end to terminate the reaction. Streptavidin-FITC working solution was incubated with the sample for 10 min, and the sample was analyzed using a flow cytometer (Cyflow D-48161, PARTEC, Münster, Germany).

Caspase Activity Assay

This assay was used to test whether caspase-3 or caspase-8 was activated in the Hep3B cells. Cells at a density of 1×10^6 cells/ml were thoroughly disrupted by sonication for 1 min at 4°C, followed by centrifugation (10,000 × *g*) for 30 min. Caspase-3 and caspase-8 activity was evaluated using a caspase-3,8 Colorimetric Assay Kit from R&D System according to the protocol provided by the manufacturer. A total of 1×10^6 cells were plated in 6-well plates and treated with 1000 μg/ml of LDHE. At 24 and 48 h after treatment, the cells were harvested and washed with PBS twice. Cell lysis buffer (25 μl/1×10^6 cells) was added to the samples, and the samples were cooled on ice for 10 min. The samples were centrifuged (8000 × *g*) for 1 min. A 25-μl aliquot of the supernatant was pipetted into a 96-well plate, then the following substances were added into each well: 50 μl reaction buffer, 5 μl DEVD-pNA, and 5 μl LETD-pNA. The plate was then incubated at 37°C for 3 h, and the caspase activity was determined using an ELISA reader set at 405 nm.

Cytochrome c *Release Analysis*

Cytochrome *c* release was observed using a Quantikine human cytochrome *c* kit. The cells were cultured in a 6-well plate with 1000 μg/ml of LDHE for 2, 4, 8, 12, and 24 h. PBS was used to wash the cells, and cell lysis buffer was incubated with the cells for one hour. The sample was centrifuged for 15 min (1000 × *g*), and the following solutions were incubated with the sample: 100 μl conjugate solution (horseradish peroxidase) for 2 h and a substrate solution (a combination of hydrogen peroxide and chromogen tetramethyl benzidine) for 20 min. The absorption rate at 450 nm was assessed using a microplate reader.

Western Blot Analysis

Cells were treated with 1000 μg/ml of LDHE for 24, 48, and 72 h. Whole-cell extracts were prepared by washing the cells with PBS and suspend-

Table 1. The phytochemical content of Lu-Do-Huang

	Content (μg/g)
Berberine	175.43 ± 2.65
Glycyrrhizin	34.58 ± 0.00
Ferulic acid	ND

Data are presented as the mean ± SD and representative of three independent experiments. The phytochemical content was quantified by HPLC. ND: non-detectable.

ing them in 250 μl of RIPA cell lysis buffer. After 15 min of cooling on ice, the mixtures were centrifuged (8000 × *g*) for 15 min, and the supernatants were collected as the whole-cell extracts. The protein concentration was determined with a protein assay dye reagent and a microplate reader set at a wavelength of 570 nm; BSA was used as the standard. The proteins were separated by SDS-PAGE using 1.5 mm discontinuous acrylamide and a protein loading dye heated to 100°C and then cooled in ice water. The voltage was set at 100 volts and then turned up to 120 volts when the sample entered the resolving gel. Protein expression was analyzed using the immunoblotting. The proteins were transferred to an NC membrane in transfer buffer (50 mM Tris base, 40 mM glycine (pH 8.3), and 10% methanol). TBST buffer (20 mM Tris-HCI, 137 mM NaCl, and 0.05% Tween 20) was used to wash the membrane. Primary antibodies against FADD, TRADD, and Bax were incubated for 1 day at 4°C or incubated for 1 h at 37°C. TBST buffer was used to wash the membrane, and the secondary antibody was added. The membrane was washed with TBST buffer, and the results were photographed with SYSTEM.

Statistical Analysis

The results were expressed as the mean standard deviation (SD). For multiple group comparisons, the significance of the differences among the treatment groups and their respective control groups was tested using a one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. Statistical significance was considered at $P < 0.05$.

Results

Quantification of Berberine, Glycyrrhizin and Ferulic Acid Levels of Lu-Do-Huang by HPLC

As shown in the Table 1, the amounts of berberine and glycyrrhizin in the Lu-Do-Huang extract were 175.43 ± 2.65 and 34.58 ± 0.00 μg/g, respectively. The ferulic acid was not detected in the test samples.

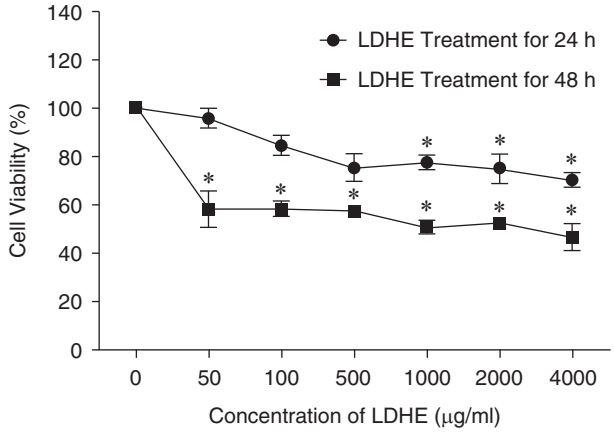

Fig. 1. The effects of different concentrations of LDHE on the cell viability of Hep3B cells. Data are presented as the mean ± SD and representative of three independent experiments. The cell viability was significantly lower than control when the Hep3B cells were treated more than 1000 μg/ml of LDHE at 24 h post treatment and more than 50 μg/ml of LDHE at 48 h post treatment. *$P < 0.05$ indicates a significant difference between the control and LDHE-treated cells.

LDHE Inhibits Hep3B Cells Proliferation

We first examined the effect of LDHE on the viability of the Hep3B cells in the presence of different concentrations of LDHE for 24 h (Fig. 1). Compared with the controls, the survival rates of the Hep3B cells treated with 50, 100, 500, 1000, 2000, and 4000 μg/ml LDHE were 95.7 ± 4.1%, 84.5 ± 4.0%, 75.3 ± 5.8%, 77.5 ± 2.7%, 74.8 ± 6.1%, and 70.2 ± 3.0%, respectively. With the addition LDHE for 48 h, the survival rates of the Hep3B cells treated with 50, 100, 500, 1000, 2000, and 4000 μg/ml of LDHE were 58.1 ± 7.5%, 58.2 ± 3.2%, 57.5 ± 1.7%, 50.5 ± 2.7%, 52.5 ± 0.3%, and 46.6 ± 5.4%, respectively. The results indicated that longer treatment times and increasing LDHE concentrations reduced the survival rates of Hep3B cells, particularly in the presence of 4000 μg/ml LDHE for 48 h. According to the cell viability test, there were mild changes in the cell survival rate after 24 h in culture with a concentration of 1000 μg/ml LDHE, whereas there was a greater change in the cell survival rate after 48 h in culture at various concentrations. Even though both 1000 μg/ml and 2000 μg/ml treatment are reach about 50% survival rate after 48 h incubation, but there is no significant change between 1000 μg/ml and 2000 μg/ml treatment. In order to reduce preparation time and cost, we chose lower concentration of 1000 μg/ml for following experiments.

LDHE Induced G_0/G_1 Arrest in Hep3B Cells

Previous studies have clearly illustrated that cell

Table 2. Changes in the cell cycle of Hep3B cells treated with 1000 μg/ml LDHE for 24 and 48 h

Time (h)	Cell cycle (%)			
	$subG_1$	G_0/G_1	S	G_2/M
Control	2.92 ± 1.31	67.15 ± 0.83	13.49 ± 1.33	12.28 ± 1.29
24	1.21 ± 0.72	75.46 ± 1.44	8.11 ± 2.87	11.14 ± 0.83
48	2.89 ± 0.88	74.11 ± 0.29	12.14 ± 1.43	7.99 ± 0.76

The percentage of $subG_1$ (apoptosis), G_0/G_1, S and G_2/M cells was calculated.

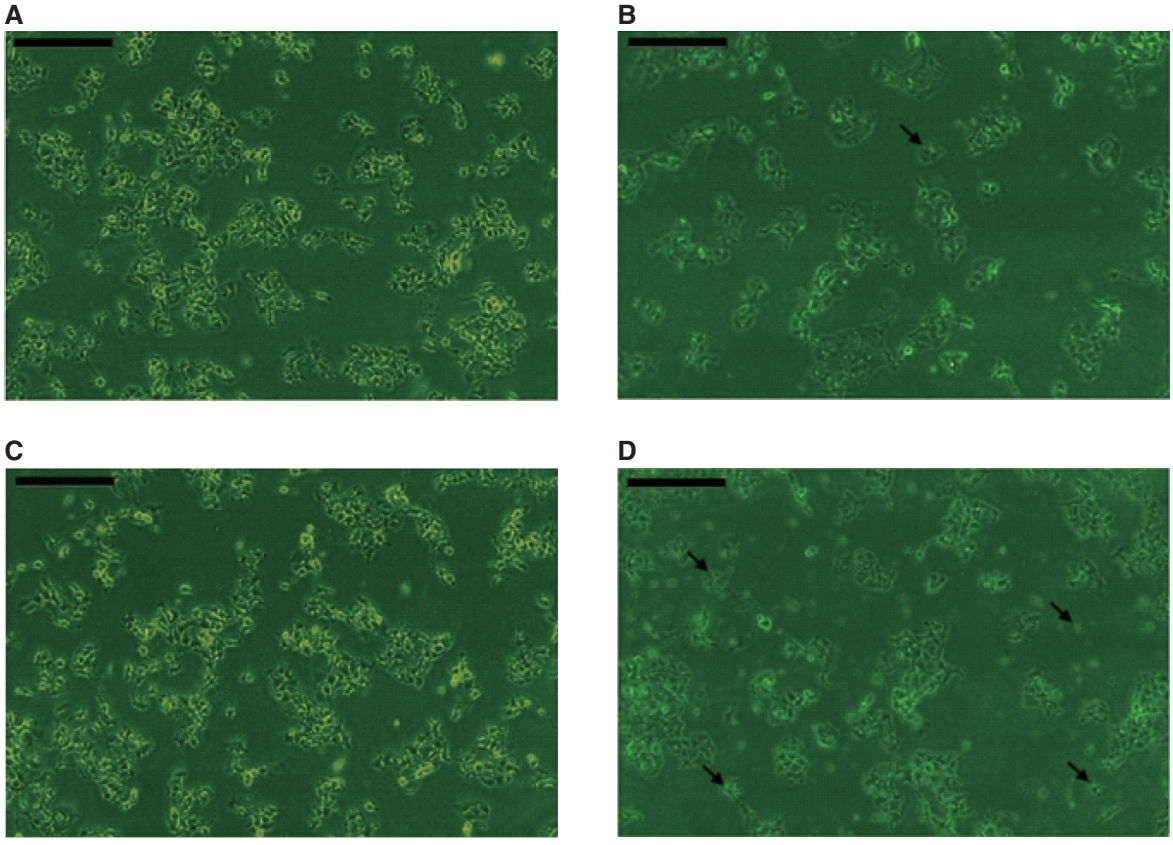

Fig. 2. Changes in the cell number and morphology of Hep3B cells treated with 1000 μg/ml LDHE. The photomicrographs were obtained using phase-contrast microscopy (×100). Cells were treated without or with LDHE for 24 and 48 h. (A) Control, 24 h (B) LDHE, 24 h (C) Control, 48 h (D) LDHE, 48 h treatment. Arrows indicate the apoptotic bodies. The scale bar is 200 μm.

proliferation and cell division are regulated by the cell cycle, which is composed of G_1, S, G_2, and M phases. We further investigated the effect of LDHE on cell cycle changes in the Hep3B cells using flow cytometry. LDHE at 1000 μg/ml induced apoptosis in the Hep3B cells after 24 and 48 h. The results in the Table 2 showed that the Hep3B cells did not inappropriately enter the S phase or arrest in the G_0/G_1 phase following the 1000 μg/ml LDHE treatment for 24 and 48 h (67.15 ± 0.83%, 75.46 ± 1.44%, and 74.11 ± 0.29% G_0/G_1 peaks for the control and 24 h and 48 h LDHE treatments, respectively). This result revealed that LDHE addition in the Hep3B cell culture could induce death in Hep3B cells.

LDHE Induced Apoptosis in Hep3B Cells

The Hep3B cells were treated with 1000 μg/ml LDHE for 24 and 48 h, and the cell morphology was observed under an inverted phase-contrast microscope and photographed. The number of LDHE-treated cells was reduced compared to the control cells as a function of the treatment time (Fig. 1). When visualized using inverted phase-contrast microscopy, the LDHE-treated Hep3B cells showed morphological changes including apoptotic bodies (indicated by the black

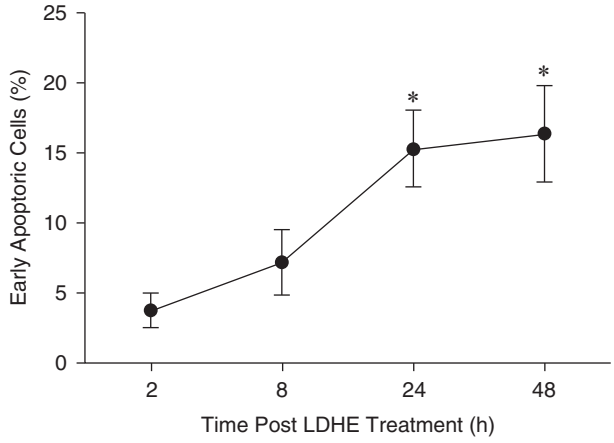

Fig. 3. Flow cytometric analysis of Hep3B cells using the Annexin-V/Propidium iodide assay to detect early apoptosis. Hep3B cells were cultured in the absence or presence of 1000 μg/ml LDHE. The percentage of Annexin-V positive/PI negative cells was significantly higher than control at 24 and 48 h post LDHE treatment. *$P < 0.05$ indicates a significant difference between the control and LDHE-treated cells.

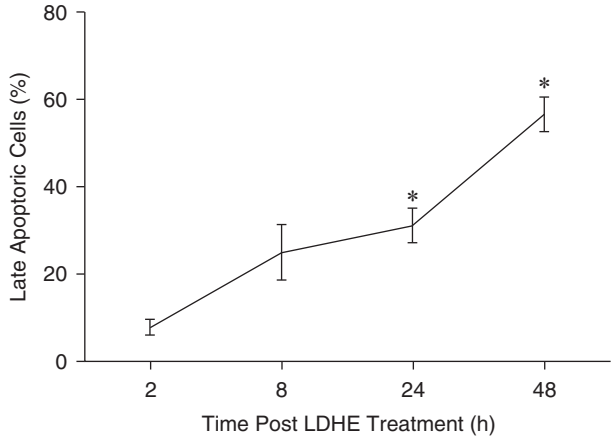

Fig. 4. Fluorescence intensity results of the flow cytometric analysis of Hep3B cells cultured in presence of 1000 μg/ml LDHE for 2, 8, 24, and 48 h using the TUNEL assay. The percentage of TUNEL positive cells was significantly higher than control at 24 and 48 h post LDHE treatment. *$P < 0.05$ indicates a significant difference between the control and LDHE-treated cells.

arrows in Fig. 2), cell shrinkage, and broken cells.

An Annexin V-FITC/propidium iodide (PI) double staining assay coupled with flow cytometry was used to confirm the early phase of apoptosis in Hep3B cells after treatment with LDHE. The result could help to identify the number of necrotic cells and apoptotic cells in the LDHE (dissolved in PBS)-treated group. The results showed that the number of apoptotic cells stained with Annexin V significantly increased in the presence of 1000 μg/ml LDHE (Fig. 3). After an 8 h incubation period, relatively few cells were apoptosis in the presence of LDHE (7.15 ± 2.33%), and more apoptotic cells were observed in the LDHE-treated group after 24 and 48 h of LDHE incubation (15.25 ± 2.75% and 16.35 ± 3.46%, respectively). These results suggest that 1000 μg/ml LDHE is able to induce early apoptosis in Hep3B cells.

Apoptotic bodies are produced from cells undergoing apoptosis and are associated with DNA fragmentation. TUNEL assay was used to further confirm the late phase apoptosis in LDHE treated cells. As shown in Fig. 4, more TUNEL-positive fluorescent cells were observed in the LDHE-treated group over time (2, 8, 24, and 48 h) compared to the untreated cells (the peaks gradually moved to the right over time). In total, 7.74 ± 1.33%, 24.98 ± 5.12%, 31.15 ± 3.52%, and 56.63 ± 3.77% of TUNEL-positive fluorescent cells were detected within the population of cells treated with 1000 μg/ml LDHE for 2, 8, 24, and 48 h, respectively (M2).

LDHE Induced Caspase Dependent Apoptosis

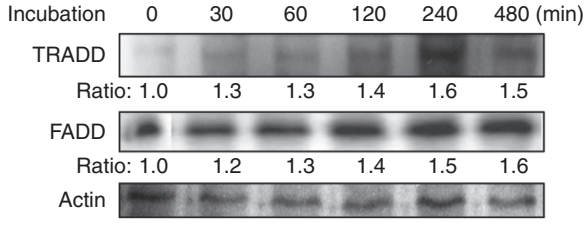

Fig. 5. Effect of 1000 μg/ml LDHE on TRADD and FADD protein expression in Hep3B cells after 0, 30, 60, 120, 240 and 480 min.

On the basis of our results on cell morphology change, Annexin V/PI assay, and TUNEL assay, LDHE could induce apoptosis in Hep3B cells. Therefore, we tried to investigate the possible signaling pathway of apoptosis whether related to the caspase dependent pathway. The expression of TRADD and FADD were monitored by Western blot analysis. Significantly increased levels of TRADD and FADD were observed in the Hep3B cells treated with 1000 μg/ml LDHE for 30, 60, 120, 240, and 480 min compared to the control cells (Fig. 5).

We also measured the activities of caspase-3 and caspase-8 in Hep3B cells treated with 1000 μg/ml LDHE for 24 h and 48 h by using a caspase activity kit. LDHE significantly induced both the caspase-3 and caspase-8 activities at 24 h and 48 h post treatment ($P < 0.05$). The fold changes to the LDHE-untreated control were 1.53 at 24 h and 1.8 at 48 h for caspase-3 and 1.21 at 24 h and 1.4 at 48 h for caspase 8 (Fig. 6). The results suggest that LDHE induced the

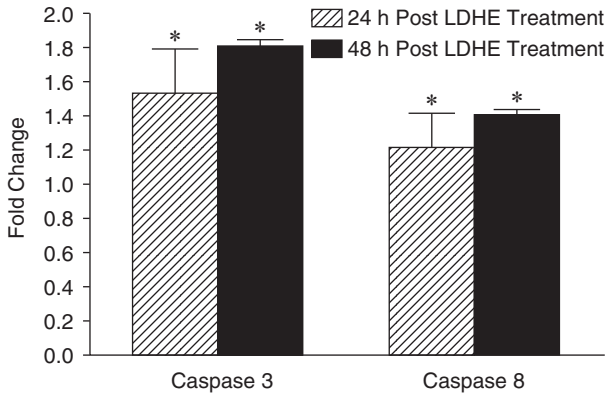

Fig. 6. Mean relative expression of caspase-3 and caspase-8 of Hep3B cells with 1000 μg/ml LDHE to non LDHE control after 24 and 48 h. $*P < 0.05$ indicates a significant difference between the control and LDHE-treated cells.

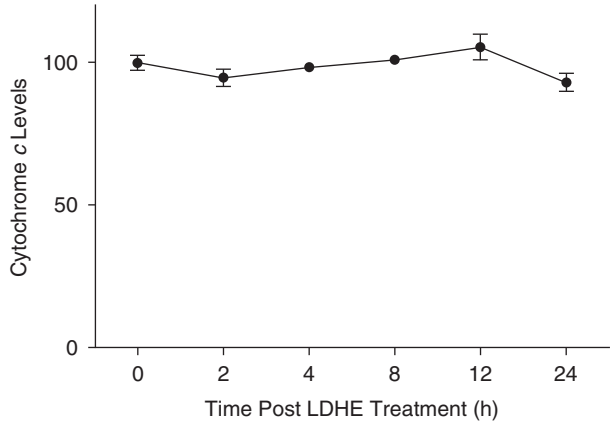

Fig. 7. Effect of 1000 μg/ml LDHE on cytochrome *c* release in Hep3B cells after 2, 4 and 24 h. The cytochrome *c* levels were relative to the LDHE-untreated control. Data are presented as the mean ± SD and representative of three independent experiments. No significant difference between the control and LDHE-treated cells at any time point.

activity of caspase-8, an upstream initiator caspase, which subsequently affects the activity of capase-3, a downstream effector caspase that could induces apoptosis in Hep3B cells.

On the other hand, Bax did not detected in 48 h post treatment (data not show). Consistently, the result of cytochrome *c* release assay indicated that the level of cytochrome *c* in LDHE-treated Hep3B cells was not significantly different from the LDHE-untreated control at 2 h, 4 h, 8 h, 12 h, and 24 h post LDHE treatment. LDHE at 1000 μg/ml did not induce cytochrome *c* release in Hep3B cells after 2 h or 24 h of treatment (Fig. 7).

These results suggested that LDHE could induced apoptosis *via* death-receptor to activate TRADD and FADD, and then to cleave caspase-8 followed by directly caspase-3 activation.

Discussion

The induction of apoptosis is the key to the success of plant products used as anticancer agents (8, 19). The apoptosis induction of LDHE were investigated in this study for the first time. In the present study, the characteristics of apoptotic cells were observed after treating Hep3B cells with LDHE, including the increase of apoptotic bodies (Fig. 2), apoptotic cells (Fig. 3), and evident DNA fragmentation (Fig. 4). LDHE may lead Hep3B cells to enter caspase dependent apoptosis through death receptor-mediated pathway. The mechanism of action of this extract might be apoptosis induction through the activation of TRADD/FADD expression and the activation of caspase-8. Caspase-8 regulated caspase-3 activation which caused DNA fragmentation and cell death.

In our study, we found LDHE containing berberine and glycyrrhizin (Table 1). Berberine is a major form of isoquinoline alkaloid derived from medicinal herbs such as *Hydrastis canadensis* (goldenseal), *Cortex phellodendri* (huangbai), and *Rhizomacoptidis* (huanglian), showing antibacterial and antipyretic activities (22). A recent study has demonstrated that the beneficial effects of berberine on suppressed proinflammatory responses through AMP-activated protein kinase (AMPK) activation in macrophages (9). Glycyrrhizin, a triterpene glycoside and a conjugative compound of enoxolone and glucuronic acid as a major active constituent of licorice (Glycyrrhizaglabra) root, has various pharmacological effects including anti-inflammatory, anti-viral, antioxidative, anti-liver cancer, immunomodulatory and hepatoprotec. The results of cell proliferation and cell cycle analysis show that human liver cancer cell line, Hep3B, drive toward cell death following LDHE treatment and suggest the anti-hepatoma ability of LDHE.

Apoptosis leads to characteristic cell changes, including cell membrane shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation (into a "ladder" pattern after agarose gel electrophoresis), and the exposure of N-acetyl glucosamine on the surface of apoptotic cells. The engulfment and clearance of apoptotic cells occurs as they are taken up by neighboring cells or macrophages (12). The changes in membrane composition lead to the extracellular exposure of phosphatidylserine (PS), an anionic phospholipid, residues, which occur in the early stage of apoptosis (7).

Apoptosis pathways can be classified to caspase dependent and caspase independent pathways. In the present study, we only focused on caspase depent apop-

tosis. Caspase family was known as an important role in several apoptosis pathways. Activated caspase-8 stimulates apoptosis *via* two different pathways. One pathway results from the fact that activated caspase-8 can directly cleave procaspase-3 to activate it. The other complex pathway involves caspase-8 cleaving Bid into tBid (truncated Bid), which sequentially induces Bax and translocates to the mitochondria where it triggers cytochrome *c* release. The released cytochrome *c* induces a series of biochemical reactions, also resulting in caspase-3 activation (2). As an executioner of apoptosis, caspase-3 can, in turn, activate such endonucleases as caspase-activated DNase (CAD), resulting in DNA breaks and cell death. Thus, the protein expression of caspase-3 is often used to determine directly whether cells have experienced apoptosis (7). In our study, the up-regulation of caspase-8 and caspase-3 suggests that LDHE induced caspase-dependent apoptosis.

Extrinsic or intrinsic inducers are required for the induction of caspase dependent apoptosis. The main apoptotic pathways are the extrinsic, or death receptor-mediated, pathway and the intrinsic, or mitochondrial stress-mediated, pathway. Previous studies have reported that Hep3B cells are Fas-deficient and that death receptor-mediated apoptosis in Hep3B cells is induced by activating membrane death receptors, such as TNFR or TRAIL-R (13). When the death receptors are activated, the downstream adaptor molecules, such as TRADD and FADD, are recruited, resulting in the recruitment of procaspase-8 to the receptor complex and leading to the formation of DISC (death-inducing signaling complex). After the exposure to LDHE, we found that the expression levels of the death receptors TRADD and FADD were significantly increased and that the activities of caspase-8 and caspase-3 were also significantly upregulated in the Hep3B cells. However, no significant increase in the amount of cytochrome *c* released from the mitochondria was detected. On the other hand, western blot analysis showed that the expression of Bax proteins were not significantly induced with the presence or absence of 1000 μg/ml LDHE treatment for 24 and 48 h (data not shown), and no significant increase in the amount of cytochrome *c* released from the mitochondria (Fig. 7). The results were indicating that LDHE did not affect mitochondria in a way that would lead to apoptosis, which proves that the LDHE-induced apoptosis in Hep3B cells is mediated by the death receptor-mediated pathway. When the membrane death receptors TNFR and TRAIL-R are activated, TRADD and FADD are recruited to the death receptors, resulting in the recruitment of procaspase-8 and the formation of DISC (death-inducing signaling complex). Activated caspase-8 subsequently activates caspase-3, resulting in the activation of caspase-activated DNase (CAD), which induces DNA breaks and apoptosis in Hep3B cells. In the present study, we discovered that the LDHE-induced caspase dependent apoptosis in Hep3B cells occurred *via* the death receptor-mediated pathway, not the mitochondria-mediated pathway.

These results demonstrated LDHE could induce apoptosis in caspase dependent pathway, but the caspase independent apoptosis might also contribute to apoptosis in Hep3B cells, too. Therefore, further studies will be needed to understand weather caspase independent pathway involve to induced apoptosis in Hep3B cells after LDHE treatment. However, these results revealed that Lu-Do-Huang may be a potential and promising natural resource to serve as a novel therapeutic agent in the treatment and/or prevention of HCC. This possible use of this extract warrants further investigation.

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