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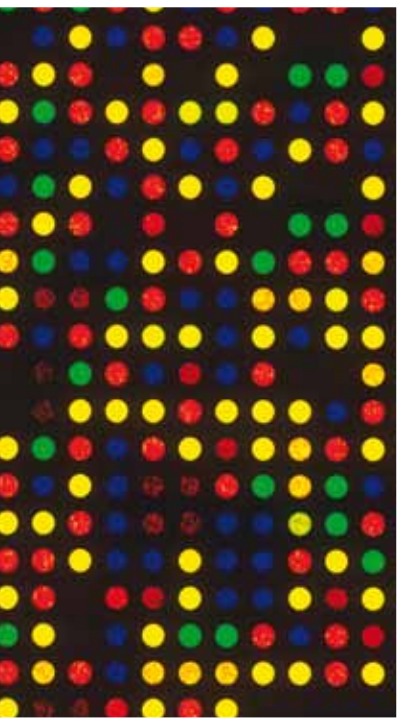
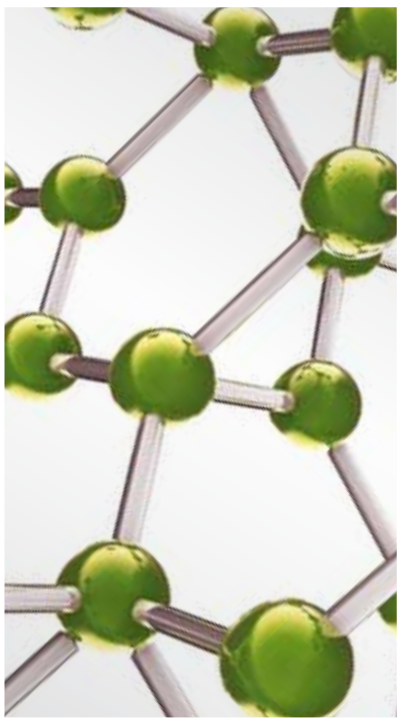
Complementary and Alternative Therapies for Liver Diseases

Guest Editors: Yong-Song Guan, Mohammad Ahmad Al-Shatouri, and Qing He





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Editorial

Complementary and Alternative Therapies for Liver Diseases

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Alternative and complementary therapies for liver disease are undergoing a rapid development in the fields of theoretical research, laboratory study, and clinical practice. However, their safety, efficacy, and mechanisms of action remain insufficiently understood and even controversial. And incidences of many types of liver disease are currently rising. So there is an urgent need for the clarification of their safety, efficacy, quality assurance, and reproducibility.

That a plant heals better than a tablet sounds marvelous, especially for such an organ as the liver which is complicated in both structure and function. Definite proof is exceptionally valuable for the establishment of methods to cure a group of disease with worldwide prevalence. The accumulated evidence on researches in liver disease will set a foundation for these cheaper, more holistic, more person-centered, or customizing therapies.

This special issue contains the research papers focusing on current general interest in these therapies, including everyday life utensils and nourishment. A number of materials used in these therapies are subject to our study, such as pollen, bee honey, plant extracts, concoctions, officinal TCM pellets, and herbal injections. Convincible evidence is presented from *ex vivo* and *in vivo* studies, as well as clinical trials for the support of their hepatoprotective, antiviral, and anticancer effects in the basis of pharmacological sciences, molecular biology, and molecular medicine.

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Research Article

Fermented Wheat Germ Extract Induced Cell Death and Enhanced Cytotoxicity of Cisplatin and 5-Fluorouracil on Human Hepatocellular Carcinoma Cells

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Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death worldwide. Due to the difficulties of early diagnosis, curative treatments are not available for most patients. Palliative treatments such as chemotherapy are often associated with low response rate, strong adverse effects and limited clinical benefits for patients. The alternative approaches such as fermented wheat germ extract (FWGE) with anti-tumor efficacy may provide improvements in the clinical outcome of current therapy for HCC. This study aimed to clarify antitumor efficacy of FWGE and the combination drug effect of FWGE with chemotherapeutic agents, cisplatin and 5-fluorouracil (5-Fu) in human HCC cells, HepG2, Hep3B, and HepJ5. The present study indicated that FWGE exhibited potential to suppress HepG2, Hep3B, and HepJ5 cells, with the half maximal inhibitory concentrations (IC_{50}) of FWGE were 0.494, 0.371 and 1.524 mg/mL, respectively. FWGE also induced Poly (Adenosine diphosphate ribose) polymerase (PARP) associated cell death in Hep3B cells. Moreover, the FWGE treatment further enhanced the cytotoxicity of cisplatin in all tested HCC cells, and cytotoxicity of 5-Fu in a synergistic manner in HepJ5 cells. Collectively, the results identified the anti-tumor efficacy of FWGE in HCC cells and suggested that FWGE can be used as a supplement to effectively improve the tumor suppression efficiency of cisplatin and 5-Fu in HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and stands in the second place of cancer death [1], particularly in eastern Asia and sub-Saharan Africa [2]. The curative treatments for early-stage HCC are liver transplantation, resection, or local ablation, but these approaches are not viable for patients with advanced [2, 3] and the recurrence rate is as high as 50% at 2 years after operation [4]. Due to the difficulties in early diagnosis of

HCC, about 70% of patients diagnosed with HCC are in advanced stage and unable to receive curative treatments [3]. In the development of alternative therapeutic approaches, palliative treatments such as chemoembolisation are suggested to show survival benefits in patients with advanced HCC [5, 6]. For example, although standard chemotherapeutic agents such as cisplatin and 5-Fluorouracil (5-Fu) administrated as systemic chemotherapy demonstrated no clinical benefit or improvement in survival [7, 8], hepatic arterial infusion and chemoembolisation with cisplatin and 5-Fu are considered potential therapeutic approaches for treating HCC [9]. In recent years, novel agents such as sorafenib also recommended for treating advance liver cancer [10]. Despite the development of therapeutic approaches for treating HCC, the mortality rate of patients with HCC still exceeds 90% worldwide [1]. Alternative treatments (e.g., components such as curcumin, resveratrol, silibinin isolated from natural products) that provide improvements in current clinical outcomes of HCC therapy are therefore in an urgent need [11].

The fermented wheat germ extract (FWGE), developed by Dr. Mate Hidvegi, is a nutrient supplement with medical value as demonstrated in a wide range of potential disease targets [12–14], including anti-tumor efficacy against many tumor types *in vitro* [15] and *in vivo* [16, 17]. Furthermore, some clinical studies also reported that the use of FWGE improved the overall survival in patients with colorectal cancer and skin melanoma. These data suggest that FWGE has the potential to provide benefits in cancer therapy [18, 19]. 2-methoxy benzoquinone and 2,6-dimethoxybenzene, the two major components of FWGE, are suggested to exert main biological properties of FWGE [14, 20]. Recent studies suggest that FWGE disrupts the anaerobic glycolysis and pentose cycle by targeting transketolase glucose-6-phosphate dehydrogenase, lactate dehydrogenase, and hexokinase [14, 21, 22], by which FWGE suppresses the allocation of precursors for DNA synthesis on tumor cells [13]. In the tumor cells of T-cell leukemia, FWGE treatment induced programmed cell death by interfering glycolysis and pentose cycle, resulting in cell cycle arrest and activation of the caspase-dependent Poly (Adenosine diphosphate ribose) polymerase (PARP) pathway [23]. The effects of FWGE combined with chemotherapeutic agents have been demonstrated on HCC, colorectal, ovarian, and breast cancer cells [16, 24, 25]. Results of these pioneer studies suggested that FWGE may enhance the cytotoxicity of cisplatin in ovarian cancer cells [24], and increase the efficacy of 5-Fu in colorectal cancer cells [15]. However, although the anti-proliferative effects of treatment with FWGE alone were demonstrated in human HCC and HepG2 cells [15], FWGE failed to enhance the cytotoxicity when combined with 5-Fu, Dacarbazine, or Adriblastina in the same cell lines [16]. Further clarification is required on the use of FWGE in combination with chemotherapeutic agents for HCC therapy.

Therefore, the aims of this study were to evaluate the antitumor effect of FWGE in human HCC cells, and to further clarify the effects of FWGE in combination with standard chemotherapeutic agents, cisplatin and 5-Fu. These data may provide a rational basis for the combined use of FWGE supplement and the development of therapeutic options in HCC therapy.

2. Materials and Methods

2.1. Cell Culture. Human hepatocellular carcinoma cell lines, HepG2, Hep3B, and HepJ5 were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) with 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a 5% CO_2 humidified incubator.

2.2. Cell Viability Assay and Microscopic Observation. HepG2, Hep3B, and HepJ5 cells were seeded into 96-well microplates at a density of 5×10^3 cells per well overnight and then treated with various concentrations of fermented wheat germ extract (FWGE, brand name Avemar, American BioSciences Inc, Blauvelt, NY, USA) for 48 or 72 hr. Cell viability of tumor cells in this study was mainly determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and further confirmed by cell size measurements using a Scepter cell counter (Merck Millipore Billerica, MA, USA). Tumor cells were seeded into 24-well plates at a density of 3×10^4 cells per well. After cultured overnight, cells were then exposed to serial dilutions of FWGE: 0, 0.25, 0.5 mg/mL for HepG2 cells, 0, 0.2, 0.4 mg/mL for Hep3B cells, and 0, 0.5, 1 mg/mL for HepJ2 cells. Cells were harvested by trypsinization 72 h after adding FWGE and cell number was counted by a Scepter cell counter.

To investigate the influence of FWGE combined with cisplatin and 5-fluorouracil (5-Fu, both agents were purchased from Sigma-Aldrich, St Louis, MO, USA), HepG2, Hep3B and HepJ5 cells were treated with various concentrations of cisplatin and 5-Fu with 0.5, 0.25 and 1 mg/mL FWGE. After 72 hr, cell viability was determined by MTT assay and morphology was observed with a Nikon Eclipse TS100 optical microscope (Nikon Instruments, Melville, NY, USA) and photographed at 100x magnification.

2.3. Western Blotting Analysis. HepG2, Hep3B, and HepJ5 cells (5×10^5 cells per dish) were seeded in 6 cm dishes overnight. After incubation with various concentrations of FWGE (as indicated), cells were harvested by RIPA buffer (150 mM NaCl, 50 mM pH 7.5 Tris-HCL, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/mL leupeptin, and 100 μg/mL aprotinin). The total protein concentrations from whole cell extracts were determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Each cell extract was then equalized to 30 μg and separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred onto a polyvinylidene fluoride membrane (Pall Corp., Port Washington, NY, USA) and probed with the primary antibodies, PARP (1 : 1,000, Cell Signaling Technology, Danvers, MA, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1 : 10,000, Abfroniter, Seoul, Korea), followed by donkey antirabbit horseradish peroxidase conjugated secondary antibody (1 : 10,000, Santa Cruz Biotechnology, Santa

Cruz, CA, USA). Immunoreactivity was then detected with a electrochemiluminescence western blotting detection kit (WesternBright, Advabsta, Menlo Park, CA, USA).

2.4. Statistical Analysis. Data from cell viability and semi-quantitative western blotting analysis were presented as mean ± stand derivation (SD). Statistical significance was analyzed one-way ANOVA when examining the dose dependent effect. Statistical analysis was performed by SPSS (SPSS Inc, Chicago, IL, USA).

CalcuSyn software (Biosoft, Cambridge, UK) was used for the statistical analysis of the half maximal inhibitory concentration (IC_{50}) determined by MTT assay and for the combined effects of FWGE with chemotherapeutic drugs. Statistical analysis of the combined drug effects with the CalcuSyn software is based on the median-effect method and evaluated by the combination index (CI) value [26], which is a useful tool for identifying synergistic, additive and antagonistic effects between components on cancer cells [27, 28].

3. Results

3.1. FWGE Treatment Induced Cell Death in Human Hepatocellular Carcinoma Cells. In present study, antiproliferative activity of a 48 or 72 hr continuous exposure to various concentrations of FWGE was evaluated in three human HCC cells, HepG2, hep3B and HepJ5 cells. As shown in Figures 1(a) to 1(c), anti-proliferative effects of FWGE in three tested tumor cells demonstrated a dose-dependent manner. In HepG2 and Hep3B cells, FWGE treatment for 72 hr resulted in a greater inhibitory effect on cell growth than 48 hr treatment. In contrast, FWGE treatment exerted a similar growth inhibitory effect in HepJ5 cells for 48 and 72 hrs. IC_{50} of FWGE were 0.494, 0.371 and 1.524 mg/mL for HepG2, Hep3B, and HepJ5 cells, respectively, suggesting that HepG2 and Hep 3B cells were more sensitive to FWGE treatment than HepJ5 cells (Table 1). Morphological changes observed in FWGE treated cells suggested that FWGE induced cell death rather than cell growth inhibition (Figures 1(d) to 1(e)). FWGE treatment also led to cell shrinkage in HepG2 and Hep3B cells and the presence of apoptosis body like vesicles around shrinking cells (Figures 1(d) and 1(e)), whereas FWGE increased the formation of lipid droplet like vesicles in HepJ5 cells (Figure 1(f)). Cell viability determined by MTT assay is based on the measurement of metabolic activity of mitochondrial oxidoreductases in survival cells, but may be biased when the metabolic activity of survival cells was disrupted by specific stress on mitochondria [29]. To further confirm the anti-proliferative effects of FWGE on tested tumor cells, cell size distribution was determined. Cell number counted by a Scepter cell counter was based on Coulter principle of impedance-based particle detection [30]. Cells with a diameter ranged between 10 to 22 μm were counted as survival cells. In Figure 2(a), survival cell numbers of HepG2, 3B, and J5 cells were significantly decreased in a dose-dependent manner after FWGE treatment for 72 hr. Moreover, FWGE treatment resulted in more cells with a much smaller size (6 to 8 μm) suggesting the accumulation of cell debris from dead cells (Figures 2(b) and 2(c)). The findings of Western blotting analysis also indicated activation of PARP in Hep3B cells treated with 0.25 mg/mL FWGE for 72 hr. These results together with morphological changes observed in FWGE treated cells indicated that FWGE was likely to trigger programmed cell death rather than inhibit proliferation of tumor cells.

Interestingly, cell viability determined by MTT assay was higher than counted by a Scepter cell counter. For example, the viability of HepG2 cells following 0.5 mg/mL FWGE treatment was 85% as determined by MTT assay, whereas the result counted by a Scepter cell counter was 50%. Similar results were observed in Hep3B and J5 cells. Hep3B and HepJ5 cells exposed to 0.2 and 0.5 mg/mL FWGE revealed 75% and 77% cell viability by MTT assay but were 35% and 65% as determined a Scepter cell counting. Together these results suggested that MTT assay may underestimate the anti-tumor efficiency of FWGE on HCC cells.

3.2. FWGE Enhanced Cytotoxicity of Chemotherapeutic Drugs on Human HCC Cells. For evaluating the effects of combination of FWGE and chemotherapeutic drugs on HCC cells, HepG2, 3B, and J5 cells were treated with various doses of cisplatin or 5-Fu with 0.5, 0.25 and 1 mg/mL FWGE, respectively. The FWGE doses used for each of cell lines were approximately 60% inhibition achieved in previous results (Figure 1). The use of IC_{60} of FWGE was to avoid over suppression of cell viability and resulted in the difficulty of evaluation on combination effect of FWGE and chemotherapeutic drugs. The viability of tumor cells exposed to cisplatin or 5-Fu alone or combinations with FWGE was shown in Figure 3. Results suggested that FWGE further decreased cell viability of HepG2 and Hep3B cells in the presence of cisplatin, and HepJ5 in the presence of 5-Fu. Subsequent to treatment, FWGE decreased the IC_{50} of cisplatin from 6.843 to 1.049, 4.436 to 1.111, and 15.785 to 6.021 μM in hepG2, Hep3B, and HepJ5 cells, respectively. In 5-Fu treated cells, co-treatment with FWGE only slightly decreased the IC_{50} of 5-Fu from 5.237 to 4.591 in HepG2 but not Hep3B cells (Table 1). Although HepJ5 was relatively resistant to 5-Fu treatment compared with HepG2 and Hep3B cells, FWGE combined with 5-Fu still led to a greater inhibition for HepJ5 cells (Figure 3 and Table 1). These results together suggested that cytotoxicity of cisplatin and 5-Fu may be further enhanced by co-treatment of FWGE. Combination index (CI) analyzed by Calcusyn software may help to identify the combination effects of FWGE with cisplain and 5-Fu on HCC cells. By which, synergistic, additive, and antagonistic effects were indicated by CI values of <1, =1, and >1. As shown in Table 2, FWGE treatment was found to result in additive effect in cells treated with 1 to 2 μM of cisplatin (CI closed to 1), and synergic effect in 5 to 20 μM of cisplatin (CI < 1) for HepG2 cells. Whereas synergic effects in 1 to 15 and 5 to 30 μM cisplatin were observed in Hep3B and HepJ5 cells, respectively. FWGE treatment also showed the synergic effect with 1 to 50 and 50 to 250 μM 5-Fu in HepG2 and HepJ5 cells. Antagonistic effect (CI > 1) was observed in Hep3B cells,

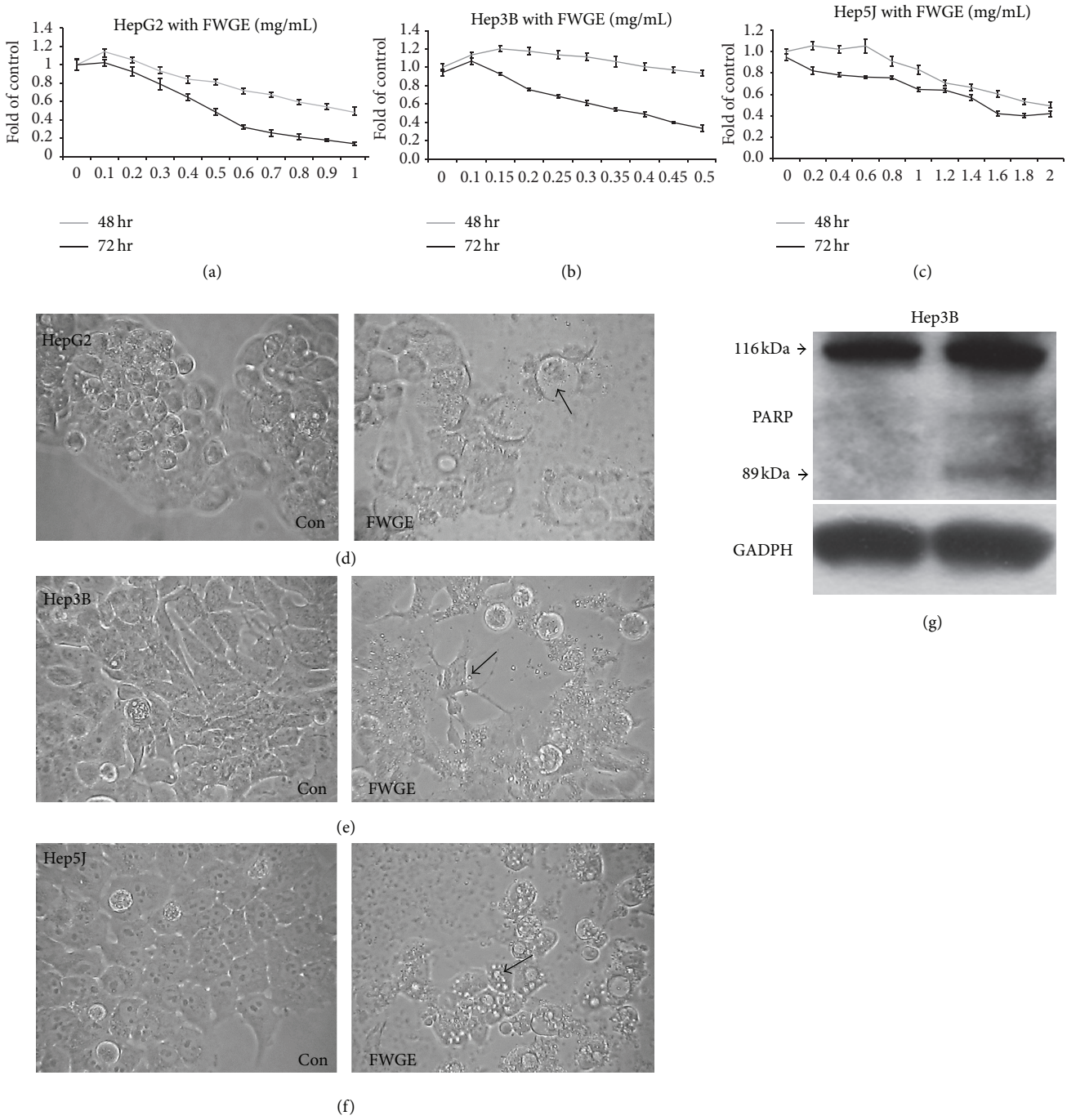

Figure 1: FWGE treatment inhibited cell growth of human HCC cells, HepG2, 3B, and J5. (a) to (c) cells were treated with a serial dilutions of FWGE in HepG2, 3B, and J5 cells for 48 or 72 hr. Cell viability was analyzed by MTT assay. Experiments were performed in triplicate at least and data were shown as mean ± SD. All cell lines showed dose-dependent effects at 72 hr after FWGE treatment in the MTT assay (one-way ANOVA, $P < 0.01$). (d) to (f) where FWGE-induced morphological changes in HepG2, Hep3B, and HepJ5 cells treated with 0.4, 0.25 and 1 mg/mL for 72 hr. Arrows indicated morphological changes in FWGE treated cells. (g) Western blotting analysis of PARP for Hep3B cells treated with 0.25 mg/mL FWGE for 72 hr. PARP was 116 KDa and cleaved PARP was 89 kDa.

but cell viability was similar in various tumor cells treated with 5-Fu alone and combined with FWGE (Figure 3(b)). According to CI analysis, cotreatment with FWGE may enhance cytotoxicity of cisplatin and 5-Fu, depending on cell types and administrated doses of chemotherapeutic agents in human HCC cells.

4. Discussion

The present study demonstrated that FWGE exhibited an effective anti-tumor activity against human HCC cells, particularly in HepG2 and Hep3B cells with IC_{50} values of 0.494 and 0.371 mg/mL. In contrast, HepJ5 was more resistant to

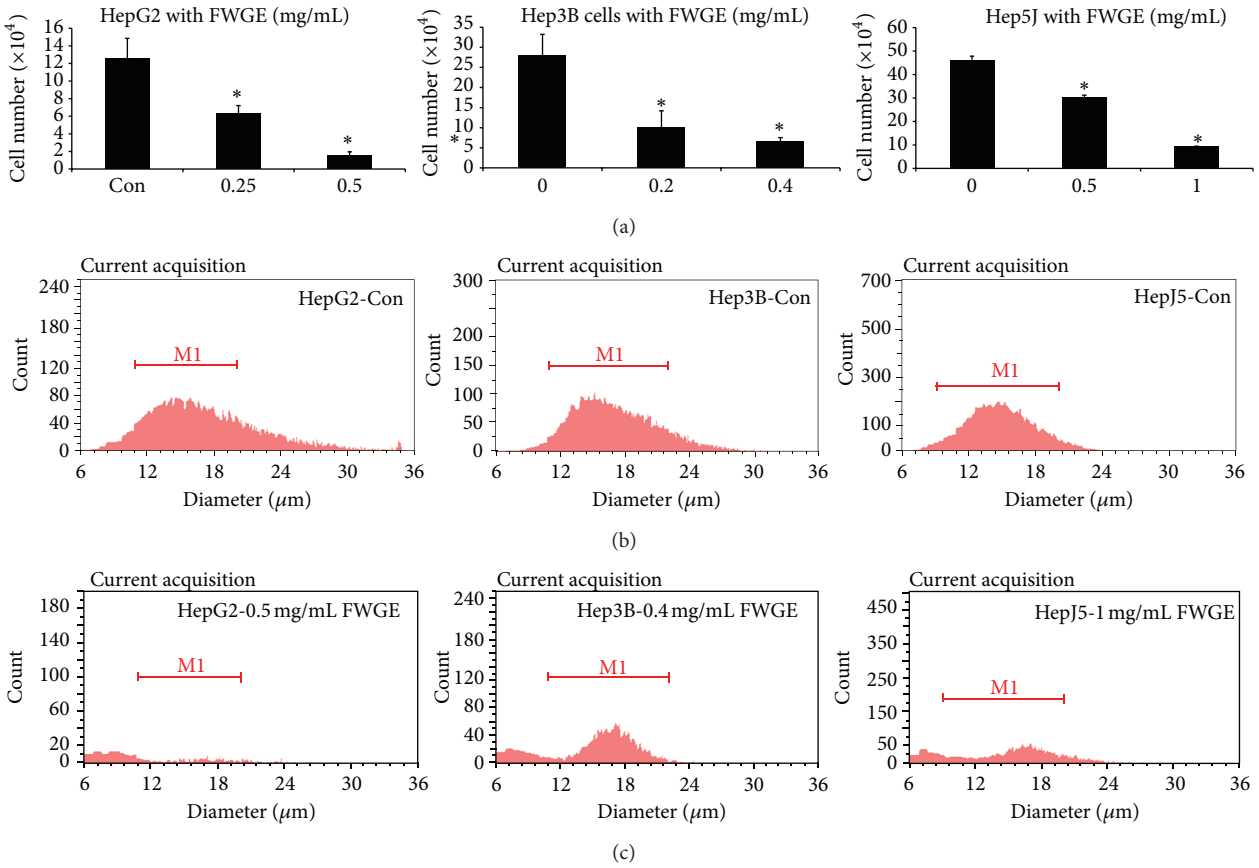

FIGURE 2: FWGE-induced cell death in HCC cells. (a) HepG2, Hep3B, and HepJ5 cells were treated by FWGE for 72 hr. Survival cells were determined by size measurements for the cells sized between 10 to 22 μm. (a) Survival cell number of FWGE treated HepG2, Hep3B, and HepJ5 cells after 72 hr. All cell lines showed dose-dependent effects following FWGE treatment (one-way ANOVA, $P < 0.01$ on HepG2 and Hep3B cells and $P < 0.05$ on HepJ5 cells). Experiments were performed in triplicate at least and data were shown as mean ± SD. (b) Cell size distribution of cells in control medium or (c) FWGE (0.5, 0.4 and 1.0 mg/mL in HepG2, Hep3B, and HepJ5 cells for 72 hr) treated cells.

FWGE treatment with an IC_{50} of 1.524 mg/mL. Our findings were comparable to previous studies indicating an IC_{50} of FWGE treatment for 96 hr was 0.33 mg/mL in HepG2 cells. Interestingly, cell viability determined by MTT assay significantly overestimated the survival of FWGE treated HCC cells compared with the measurements of cell size distribution, particularly for HepG2 (85% versus 56% of cell viability at 0.25 mg/mL FWGE) and Hep3B (75% versus 35% cell viability at 0.2 mg/mL). Since the cell viability measured by MTT assay depending on the colorimetrics of purple formazan converted by mitochondrial dehydrogenases [31], the FWGE pentose phosphate pathway disrupted by FWGE might lead to altered metabolic activity of mitochondria in HCC cells and resulted in an overestimated measurement of optic density. In HepJ5 cells, the difference between MTT assay and cell size distribution was smaller in HepG2 and Hep3B cells, suggesting that the FWGE treatment may cause less mitochondrial metabolic disruption in HepJ5 cells. Tung and colleagues suggested that hypoxia inducible factor 1-alpha (HIF 1-α) is overexpressed in HepJ5 cells and plays a protective role in mitochondrial mediated apoptosis [32]. This biological feature may help HepJ5 cells to evade destruction and death induced by FWGE.

TABLE 1: The half maximal inhibitory concentration (IC_{50}) of FWGE calculated in human HCC cells. IC_{50} were determined following treatment with FWGE alone or combined with chemotherapeutic drugs by MTT assay after 72 hr.

	HepG2		Hep3B		HepJ5	
FWGE (mg/mL)	0.494		0.371		1.524	
	Combination of FWGE (mg/mL)					
	0	0.5	0	0.25	0	1
Cisplatin (μM)	6.843	1.049	4.436	1.111	15.785	6.021
5-Fu (μM)	5.237	4.591	14.427	15.306	>500	70.571

FWGE treatment also resulted in obvious morphological changes in three HCC cell lines. In both HepG2 and Hep3B cells, cell shrinkage and formation of apoptotic body-like vesicles occurred after FWGE treatment, whereas lipid-droplet like vesicles was shown in cytoplasm of HepJ5. Cell size distribution of HCC cells ranged from 10 to 22 μm and shifted to 6~8 μm in cells treated with FWGE, suggesting that the HCC cells were broken down into fragments. These results together suggested FWGE induced cell death of HCC cells. Morphological changes observed in Hep3B

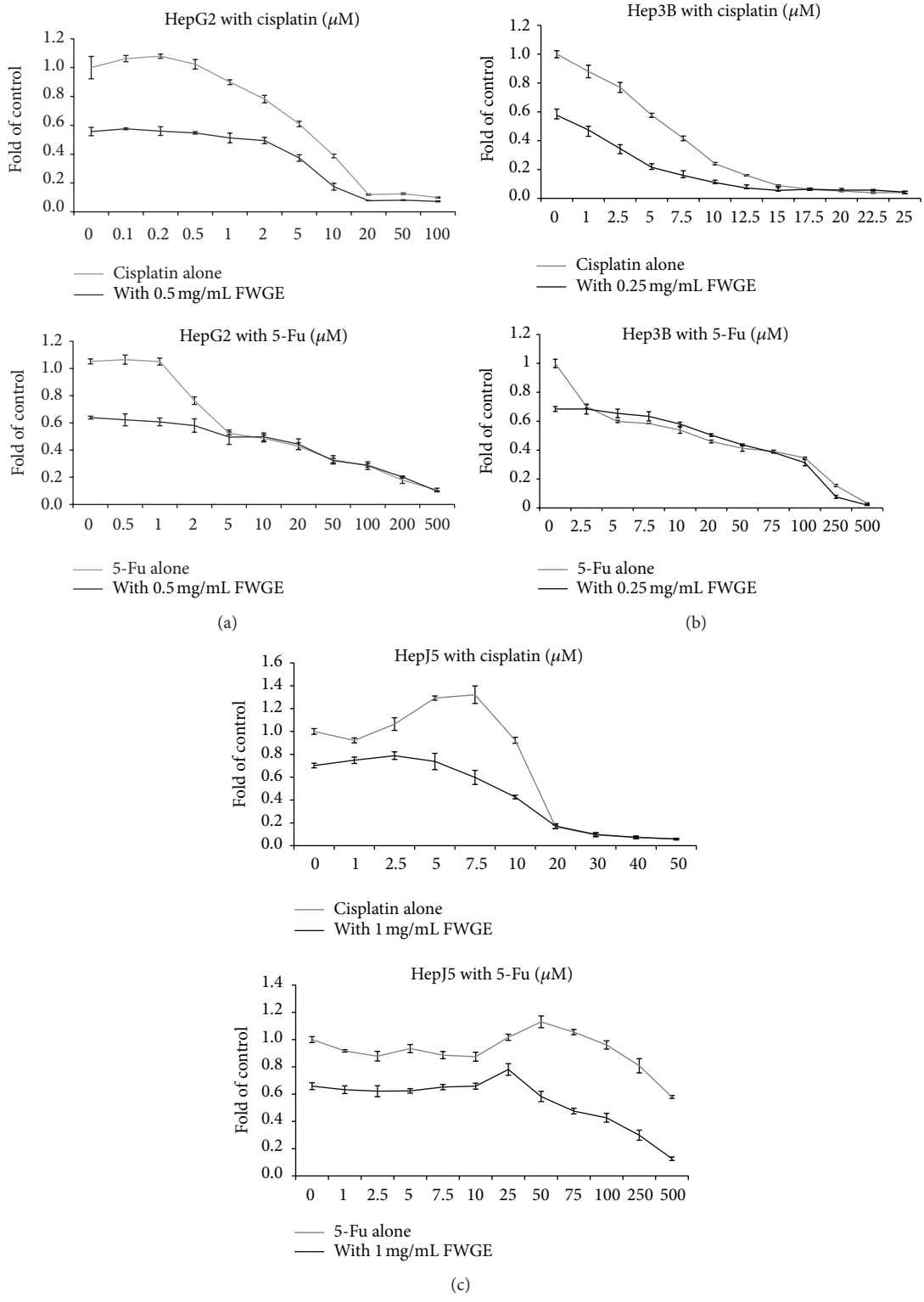

Figure 3: Combination of FWGE with cisplatin or 5-Fu in HepG2, Hep3B, and HepJ5 cells. (a) HepG2; (b) Hep3B; (c) HepJ5 cells. Cells were treated serial dilutions of cisplatin or 5-Fu alone or in combination of FWGE (0.5, 0.25 and 1 mg/mL FWGE on HepG2, Hep3B, and HepJ5 cells, resp.) for 72 hr. Cell viability was analyzed by MTT assay. Experiments were performed in triplicate at least and data were shown as mean ± SD.

Table 2: Analysis of combination effects of FWGE with cisplatin or 5-Fu in human HCC cells. Cell viability of FWGE treated cells with cisplain or 5-Fu for 72 hr were determined by MTT assay. Combination index (CI) was obtained by using Calcusyn software. CI < 1 indicated a synergistic effect, CI = 1 indicated an additive effect, and CI > 1 indicated an antagonistic effect.

	HepG2		Hep3B		HepJ5	
	Dose	CI	Dose	CI	Dose	CI
Combination of FWGE (mg/mL)	0.5		0.25		1	
Cisplatin (μM)	1	1.03	1	0.41	5	0.75
	2	1.05	2.5	0.51	7.5	0.64
	5	0.71	5	0.61	10	0.54
	10	0.41	7.5	0.71	20	0.52
	20	0.41	10	0.71	30	0.58
	50	1.03	12.5	0.68		
			15	0.69		
5-Fu (μM)	1	0.41	5	0.96	50	0.32
	2	0.34	7.5	1.25	75	0.35
	5	0.25	10	1.16	100	0.02
	10	0.41	20	1.44	250	0.01
	20	0.57	50	2.35		
	50	0.85	75	2.49		
	100	1.47	100	2.05		

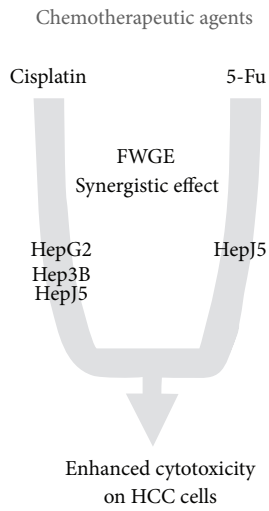

Figure 4: Conclusion on the combination effect of FWGE with chemotherapeutic agents, cisplain, and 5-Fu in HCC cells.

and G2 cells indicated that apoptosis might be involved in FWGE-induced cell death. Cleavage of PARP in Hep3B cells further confirmed this observation and suggested that FWGE induced HCC cell death was associated with PARP involved programmed cell death. Since FWGE induced cell death on T-cell leukemia cells was involved in caspase-PARP pathway [23], PARP activation is likely to play a critical role in FWGE induced cell death in tumor cells. On the other hand, the specific morphological change and formation of lipid-droplet like vesicles following FWGE treatment indicated a cell-specific response to FWGE treatment in HepJ5 cells. This lipid-droplet like vesicles was similar to autophagosome and therefore raised a doubt that a protective autophagic process was activated in HepJ5 cells to evade destruction and subsequent cell death induced by FWGE. Since the autophagy is a double-edge sword as a programmed cell death process or a preventing cell death from lethal stress, particularly in cancer cells [33], whether FWGE induces autophagy and the role of autophagy in HepJ5 cells may warrant further investigation.

FWGE is considered as a safe nutrient supplement in recommended dosage for medical purposes including cancer patients without observed adverse effects [14], though the clinical significance of FWGE as a drug component remains to be verified by more well-designed clinical trials [34]. FWGE was used as an adjuvant treatment with chemotherapy and radiotherapy for colorectal cancer and skin melanoma in some clinical studies [18, 19]. Therefore, the effects of FWGE combined with chemotherapeutic agents, cisplatin and 5-Fu, were tested in the present study to clarify the clinical potential of FWGE for HCC treatment. In HepG2 and Hep3B cells, cisplatin induced cytotoxicity was greatly enhanced by FWGE and resulted in decrease of the IC_{50} dosage (Table 1). Although IC_{50} was not available in HepJ5 cells, FWGE treatment still enhanced cisplatin-induced cytotoxicity in Hepj5, HepG2, and Hep3B cells. The CI analysis suggested that the effects of FWGE enhanced cisplatin-induced cytotoxicity was synergistic at 5–20, 1–15 and 5–30 μM for HepG2, Hep3B and HepJ5 cells, respectively. These data were coincident with the observation in human ovarian cancer cells treated with FWGE and cisplatin [24]. On the other hand, FWGE co-treatment with 5-Fu enhanced the cytotoxicity of 5-Fu at IC_{50} dosage in HepJ5 cells, but not HebG2 and Hep3B cells. CI analysis also indicated a synergistic effect of FWGE on 5-Fu in HepJ5 cells. Due to the high similarity of cell viability curves after treatment with FWGE and 5-Fu in HepG2 and

Hep3B cells, CI analysis was unable to clearly identify the combination effect of FWGE and 5-Fu in these cell lines. Nevertheless, FWGE with cisplain or 5-Fu demonstrated no antagonistic effect in tested HCC cells. Since FWGE activated programmed cell death in HCC cells, whether the pathway of FWGE related cell death also interacts with cisplatin or 5-Fu induced apoptosis requires further investigation.

5. Conclusion

FWGE exhibited an anti-tumor efficacy and induced cell death in human HCC cells, including HepG2, Hep3B, and HepJ5 cells. In combination with chemotherapeutic agents, cisplatin and 5-Fu, FWGE showed no antagonistic effect, and eventually enhanced cisplatin-induced cytotoxicity in HepG2, Hep3B, and HepJ5 cells, as well as 5-Fu-induced cytotoxicity in HepJ5 cells. The combination effect of cisplatin and 5-Fu were summarized in Figure 4. In conclusion, the results of this study suggest that FWGE is a potential adjuvant treatment to improve tumor suppression efficiency, particularly in treatment regimens using cisplatin and 5-Fu for patients with HCC.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Cheng-Jeng Tai and Wen-Ching Wang contributed equally to this work.

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Research Article

Jiao Tai Wan Attenuates Hepatic Lipid Accumulation in Type 2 Diabetes Mellitus

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Jiao Tai Wan (JTW), a Chinese herbal formula containing Rhizoma Coptidis and Cortex Cinnamomi, has been used for diabetic treatment for many years. The aim of this study was to determine the main components in JTW and to investigate the effects of JTW on hepatic lipid accumulation in diabetic rats and humans. JTW extract was prepared and the main components were assayed by HPLC. An animal model of diabetes mellitus was established and JTW was administered intragastrically. In the clinical study, diabetic patients with poor glycemic control were treated with JTW. Blood glucose and lipid parameters, liver histology, hepatic triglyceride content and lipogenic gene expression were examined. Our data demonstrated that JTW significantly improved hyperglycemia, hyperlipidemia and hepatic lipid accumulation in diabetic rats. This was accompanied by the down-regulation of acetyl coenzyme A carboxylase (ACC) and fatty acid synthase (FAS) protein expressions, and the up-regulation of AMP-activated protein kinase (AMPK) and phosphorylated-ACC (pACC) protein expressions in the liver tissues. Diabetic patients also exhibited decreases in their hepatic triglyceride content. The results suggest that JTW attenuates hepatic lipid accumulation in diabetic rats and humans. These beneficial effects are possibly associated with the inhibition of lipogenic gene expression in the liver.

1. Introduction

Obesity, the abnormal or excessive accumulation of fat within the body, has become a global epidemic secondary to high caloric food intake and sedentary life style [1]. Under physiological conditions, excessive free fatty acid (FFA) is converted to triglycerides or cholesterol esters and is then stored in lipid droplets inside adipocytes [2]. The initial deposition of triglycerides occurs in subcutaneous adipose tissue, and as the deposition increases in size, insulin resistance rises and limits further subcutaneous lipid accumulation. Triglycerides are then diverted to the visceral fat depot as well as to nonadipose tissue [3]. The latter is also known as ectopic lipid accumulation and may be linked to insulin resistance and β cell dysfunction [4, 5]. In the skeletal muscle and liver, fat deposition has been associated with the development of insulin resistance [6, 7]. In the pancreatic islets, FFA and triglycerides accumulation induce β cell functional impairment and lipoapoptosis [8]. Therefore, ectopic lipid accumulation is often associated with the development of type 2 diabetes mellitus (T2DM).

Excessive lipid accumulation in the liver of patients who drink little or no alcohol is called nonalcoholic fatty liver disease (NAFLD). NAFLD refers to a wide spectrum of liver diseases ranging from simple hepatic steatosis to nonalcoholic steatohepatitis to cirrhosis. NAFLD is highly prevalent in subjects with T2DM; approximately 70% of type 2 diabetic patients are estimated to have NAFLD [9]. This high prevalence rate makes the treatment of diabetes complex due to the liver damage and hepatotoxicity of oral

hypoglycemic drugs. To date, there has been no specific or effective treatment of NAFLD, especially in the patients with T2DM. A few clinical trials of drugs designed to reduce insulin resistance, including thiazolidinediones and metformin, appear to have some limited benefits [10, 11]. However, the beneficial effects of insulin sensitizers should be further confirmed by more multicenter randomized clinical trials.

Jiao Tai Wan (JTW) is a classical traditional Chinese prescription first mentioned in the book Han-Shi-Yi-Tong in the Ming Dynasty that consists of Rhizoma Coptidis (*Coptis chinensis* Franch, Ranunculaceae) and Cortex Cinnamomi(*Cinnamomum cassia* Presl, Lauraceae). Rhizoma Coptidis (huang lian in Chinese) mainly consists of various alkaloids including berberine, coptisine, palmatine, and epiberberine, whereas Cortex Cinnamomi (rou gui in Chinese) largely contains cinnamaldehyde and cinnamic acid [12, 13]. JTW is a common remedy to treat insomnia caused by the imbalance between the heart and kidney. However, recent research has determined that Rhizoma Coptidis and Cortex Cinnamomi both exert beneficial effects in diabetes [14–18]. We have also demonstrated that JTW is more effective than its single components in the treatment of rat hyperglycemia [19]. Given that fatty liver is highly prevalent in diabetes, we hypothesize that JTW may have the potential to alleviate hepatic fat accumulation in diabetes. Therefore, we investigated the effect of JTW on hepatic fat content in diabetic rats and humans.

2. Materials and Methods

2.1. Animals and Experimental Design. Male Wistar rats (8 weeks, 180 ± 20 g) were obtained from the Hubei Province Center for Disease Control and Prevention (Wuhan, China). The rats received a standard diet with free access to tap water and were maintained on a 12:12 h light-dark cycles in a humidity- and temperature-controlled environment. All procedures were performed in accordance with the Chinese guidelines for animal experiments (MSTPRC Directive of 1988, no. 88-2) and were approved by the local authorities.

Seven rats were selected randomly as the control group and were injected with saline into the tail vein. The remaining rats were injected with streptozotocin (STZ; Sigma Chemical Co. MO, USA; 30 mg/kg) into the tail vein. An oral glucose tolerance test (OGTT) was performed two weeks later. Impaired glucose tolerance (IGT) was diagnosed when the plasma glucose levels of rats at two time points were higher than the upper limit (4.62 mmol/L before glucose-loading, 7.56 mmol/L at 1 h after glucose-loading, and 6.29 mmol/L at 2 h after glucose-loading, resp., which is the 95% range of confidence calculated according to plasma glucose levels in the rats of control group) or when the plasma glucose levels at one time point exceeded the upper limit by more than 20% (5.54 mmol/L, 9.07 mmol/L, and 7.55 mmol/L, resp.). Thereafter, twenty-one rats with IGT were randomly divided into 3 groups. Rats receiving saline served as diabetic controls. Rats receiving JTW (3 g/kg/day) were taken as the JTW group, and rats receiving metformin hydrochloride (183 mg/kg/day) were taken as the metformin (Met) group. Oral gavage was performed once a day between 8:00 and 10:00 a.m. The doses were adjusted to the body weight, which was recorded once a week. Meanwhile, the high-fat chow (containing 72.7% standard laboratory rat chow, 20% lard, 5% egg yolk powder, 2% cholesterol, and 0.3% bile salts) was provided to the animals with IGT while the rats in the control group continued their standard diet. At the end of the 8-week period, overnight-fasted rats were weighed and then anaesthetized by pentobarbital sodium. Blood samples were obtained from the abdominal aorta. The liver was quickly excised and divided into two parts. One sample was immediately stored in liquid nitrogen, while the other was fixed with 4% paraformaldehyde.

2.2. Preparation of JTW. Rhizoma Coptidis and Cortex Cinnamomi used for animal experiments were obtained from the Traditional Chinese Medicine Company in Hubei Province (Wuhan, China). The voucher specimen was deposited at the herbarium of the Pharmacy Faculty at the Hubei University of Chinese Medicine and authenticated by Department of Pharmacognosy, Hubei University of Chinese Medicine (Wuhan, China). The rat doses of Rhizoma Coptidis and Cortex Cinnamomi were obtained by the conversion of the human doses (Chinese Pharmacopoeia, 2010) to rat equivalent doses based on body surface areas. The JTW preparation process was as follows: ninety grams Rhizoma Coptidis and forty-five grams Cortex Cinnamomi were mixed together. The mixture was decocted twice by refluxing it with water (1 : 10 w/v), 2 hours for the first time and 1 hour for the second time. The volatile oil was collected by the volatile oil extractor during the decocting procedure. The volatile oil and the solution obtained were concentrated to 450 grams. This extract represented the solution for rat administration and was stored at 4°C until use.

In the clinical study, we used another form of JTW for the convenience of oral administration. JTW, containing Rhizoma Coptidisand Cortex Cinnamomi concentrated granules, was purchased from China Resources Sanjiu Medical and Pharmaceutical Co., Ltd. Rhizoma Coptidis concentrated granules largely contain berberine (>104.0 mg/g, HPLC), coptisine (>26.0 mg/g, HPLC), palmatine (>26.0 mg/g, HPLC), and epiberberine (>17.0 mg/g, HPLC). Cortex Cinnamomi is mainly composed of cinnamaldehyde (>1.0 mg/g, HPLC) and cinnamic acid (>0.5 mg/g, HPLC). Each pack of JTW contains two grams of Rhizoma Coptidis concentrated granules (equal to six grams of Rhizoma Coptidis) and one gram of Cortex Cinnamomi concentrated granules (equal to three grams ofCortex Cinnamomi). Patients mixed the granules together by boiling water and drank two packs each day.

2.3. Determinations of the Contents of Berberine, Cinnamaldehyde, and Cinnamic Acid Contents in JTW. Berberine, cinnamaldehyde, and cinnamic acid, the main components in JTW extracts and concentrated granules, were all measured using the high-performance liquid chromatography (HPLC) method. HPLC (Waters, USA) equipped with Waters 600 Pump, and Waters 2487 Dual λ Absorbance Detector, and a XTerra RP18 chromatographic column (250 mm

× 4.6 mm, 5 μm particle size) was used. The extract of JTW was dissolved in methanol. To measure berberine, all analyses were performed at a column temperature of 35°C. The mobile phase was 30 : 70 (v/v) acetonitrile-water containing potassium dihydrogen phosphate (0.1 mol/L) and sodium hydroxide (0.05 mol/L), adjusted to pH 9.5 with triethanolamine. The flow rate was 0.9 mL/min and the wavelength of detection was set at 345 nm. To measure cinnamaldehyde, the analyses were performed at a column temperature of 22°C. The mobile phase was 55 : 15 : 25 : 5 (v/v/v/v) water, acetonitrile, methanol, and tetrahydrofuran. The flow rate was 0.6 mL/min and the wavelength of detection was set at 290 nm. To measure cinnamic acid, the analyses were performed at a column temperature of 25°C. The mobile phase was 65 : 15 : 15 : 5 (v/v/v/v) water, acetonitrile, methanol, and tetrahydrofuran, adjusted to pH 5.5 with glacial acetic acid. The flow rate was 0.9 mL/min and the wavelength of detection was set at 270 nm.

2.4. Oral Glucose Tolerance Test (OGTT). The rats were fasted overnight and given a single dose of 50% glucose solution one week before being sacrificed. The glucose solution was administered by gavage at a dose of 2.0 g/kg body weight. Blood samples for glucose measurement were obtained from the tail vein at 0 hour (before glucose-loading), 1 hour, and 2 hours (after glucose-loading) by glucose-oxidase method using a glucose monitor (LifeScan Inc., J&J Company, Milpitas, CA, USA).

2.5. Biochemical Analysis. The plasma levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), nonesterified fatty acid (NEFA), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined using commercial reagents (Jiancheng Bioengineering Institute, Nanjing, China). The fasting insulin concentration (FINS) was measured by radioimmunoassay kit (Northern Institute of Biotechnology, Beijing, China). The homeostasis model assessment index (HOMA-IR) was calculated using the formula of fasting glucose (mmol/L) × fasting insulin (μIU/mL)/22.5.

2.6. Liver Histology and Hepatic Triglyceride Content Measurements. Serial 4 μm thick paraffin sections of the liver were stained with hematoxylin-eosin (HE) for liver pathology. The morphology of the liver cells and inflammatory cells was observed under high-power magnification (×400). Neutral lipids were extracted from each aliquot using the Folch procedure with slight modifications [20]. Hepatic TG content (HTC) was measured with the same enzymatic kit used in the plasma analysis.

2.7. Western Blot Analysis. Protein lysates extracted from liver homogenates were subjected to 10% SDS-PAGE gel (100 v, 2 h–2.5 h) and then transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat milk in tris-buffered saline with Tween 20 (TBST) for 2 h at room temperature. The blocking solution was then removed, and the membranes were washed by TBST, followed by overnight incubation at 4°C with the primary antibodies (rabbit anti-rat AMPK, pAMPK, ACC, pACC, and FAS) (Abcam, Hong Kong, China) and β-actin (Gene Tex Inc., CA, USA). The membranes were then washed by TBST, followed by lucifugal incubation with the DyLight 800-labeled antibody to rabbit IgG (1 : 1000) (KPL Company, Hong Kong, China) at room temperature for 1 h. The membranes were washed in a lucifugal way 3 times with TBST. Protein bands were detected by the LI-COR Odyssey Infrared Fluorescent Scanner (Odyssey, Lincoln, USA). Band densities were determined by Bio-Rad Quantity One software v 4.62 and quantified as the ratio between the OD value of target band and the OD value of β-actin.

2.8. Clinical Intervention Study. Type 2 diabetic patients with poor glycemic control were enrolled in this study based on the following inclusion criteria: (1) aged 25–70 years; (2) use of stabilizing oral hypoglycemic drugs or lifestyle modification to control their blood glucose for at least 8 weeks; (3) fasting plasma glucose (FPG) 7.0–13.0 mmol or 2-hour postprandial blood glucose (2hPPG) 11.1–16.6 mmol/L and hemoglobin A1c (HbA1c) >6.5%; (4) no known acute or chronic disease based on history, physical examination, and standard laboratory tests; (5) alcohol consumption <20 g/day; and (6) no evidence of hepatitis A, B, or C as well as no evidence of autoimmune hepatitis, clinical signs or symptoms of inborn errors of metabolism, and no history of the use of toxins or drugs known to induce hepatitis. Exclusion criteria included thyroid disease, the use of antihypertensive agents that could possibly influence glucose metabolism (β-blockers and thiazides), the use of insulin, and women who were pregnant or lactating. The nature and potential risks of the study were explained to all subjects before obtaining their written informed consent. The experimental protocol was approved by the ethics committee of the Tongji Hospital affiliated with Tongji Medical College following the ethical principles outlined in the Declaration of Helsinki.

A total of forty patients with T2DM were enrolled. None of patients withdrew or dropped out of the study during the 12 weeks. The patients were randomly divided into two groups: JTW group (treated with one pack of JTW two times a day for 12 weeks) and the control group. Their previous diabetes management was maintained during the 12-week experimental period except for the addition of JTW in patients of the JTW-treated group. The following indicators before and after treatment were measured: body mass index (BMI), waist circumference, waist-to-hip ratio (WHR), blood pressure (BP), FPG, 2hPPG, HbA1c, plasma TC, TG, LDL-C, HDL-C, ALT, and AST.

All of the patients received proton magnetic resonance spectroscopy (^{1}H-MRS) scans of their livers before and after treatment, as described previously [21], which was acquired on a 1.5-T MRI scanner (GE Healthcare, USA) using a standard body coil and respiratory gating mode in our hospital. Spectroscopic data were processed using the SAGE software (GE Healthcare, USA). Chemical shifts were measured relative to water at 4.77 ppm. The methylene signal, which represents intracellular triglyceride, was measured at

1.40 ppm. The peak areas of the water (Sw) and fat resonance (Sf) were measured. HTC was calculated by dividing 100 times Sf by the sum of Sf and Sw. All spectra were analyzed by physicists who were unaware of any of the clinical data.

2.9. Statistical Analysis. Normally distributed data are presented as the mean ± SD, whereas nonnormally distributed data are presented as the median with the 25th and 75th percentiles in parenthesis. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's T3 test for data with equal variances not assumed. For data with equal variances assumed, ANOVA followed by LSD test was used. Nonparametric tests were used to compare median values between the groups. Calculations were performed using SPSS 14.0 software. Statistical significance was defined as P less than 0.05.

3. Results

3.1. The Content of Berberine, Cinnamaldehyde, and Cinnamic Acid in JTW. The contents of berberine, cinnamaldehyde, and cinnamic acid in the JTW extract used in the animal experiments were 23.03 mg/g, 3.12 mg/g, and 0.27 mg/g, respectively. In clinical trials, the contents of berberine, cinnamaldehyde, and cinnamic acid in the JTW concentrated granules were 79.51 mg/g, 13.39 mg/g, and 1.06 mg/g, respectively. The chromatograms of berberine, cinnamaldehyde, and cinnamic acid in JTW were presented in Figure 1.

3.2. The Effect of JTW on Body Weight, OGTT, Insulin Levels, and HOMA-IR in Diabetic Rats. As shown in Table 1, in diabetic rats, body weight, fasting and postprandial plasma glucose (FPG, 2hPPG), FINS, and HOMA-IR were all increased compared with the respective levels in the control group ($P < 0.05$, $P < 0.01$). In contrast to diabetic rats, the rats in the JTW- or metformin-treated group exhibited little gain in body weight as well as a significant reduction in plasma glucose, insulin levels, and HOMA-IR ($P < 0.05$, $P < 0.01$).

3.3. The Effect of JTW on Plasma Lipid Profiles in Diabetic Rats. As shown in Table 2, diabetic rats exhibited severe dyslipidemia characterized by elevated TC, TG, LDL-C, and NEFA levels as well as decreased HDL-C levels compared with control group ($P < 0.01$). However, the treatment with JTW or metformin significantly reversed these plasma lipid changes in diabetic rats ($P < 0.05$, $P < 0.01$).

3.4. The Effect of JTW on Plasma Liver Enzymatic Activity, HTC, and Liver Histology in Diabetic Rats. As shown in Table 3, diabetic rats exhibited a larger increase in plasma ALT, AST, and HTC ($P < 0.01$). These changes were accompanied by a larger increase in the severity of hepatic steatosis compared with controls as well as increased intrahepatic inflammatory cell infiltration (Figures 2(a) and 2(b)). However, JTW or metformin treatment significantly reduced ALT, AST, and HTC ($P < 0.05$, $P < 0.01$). The degree of fatty degeneration was decreased without obvious intrahepatic inflammatory cell infiltration (Figures 2(c) and 2(d)).

3.5. The Effect of JTW on the Liver Expression of Genes Related to Lipogenesis in Diabetic Rats. As shown in Figure 3, the hepatic expression of AMPK, pAMPK, and pACC in diabetic rats was decreased compared with controls. This decrease was accompanied by increased hepatic expression of ACC and FAS ($P < 0.05$, $P < 0.01$). However, treatment with JTW or metformin resulted in a marked increase in the hepatic expression of AMPK, pAMPK, and pACC as well as significant decreases in ACC and FAS proteins ($P < 0.05$, $P < 0.01$). JTW treatment did not change the ratio of pAMPK to AMPK expression but increased the ratio of pACC to ACC expression significantly ($P < 0.01$).

3.6. The Effect of JTW on Clinical Markers and HTC in Diabetic Patients with Poor Glycemic Control. No significant differences were noted between the baseline markers of diabetic patients in the JTW group and the control group. Patients with poor glycemic control were treated with JTW for 12 weeks. These patients exhibited decreasing trend in their BMI, waist circumference, WHR, FPG, 2hPPG, FINS, HbA1c, TC, TG, and LDL-C at the end of the treatment period ($P < 0.05$). In addition, HTC decreased significantly after the addition of JTW to the diabetic treatment but increased in the control group ($P < 0.05$). However, the levels of ALT, AST, and HDL-C did not change significantly (Table 4).

3.7. Adverse Effects. No serious adverse events were observed when JTW was used. Plasma creatinine concentrations and electrocardiography (ECG) were also monitored in the clinical study. No significant changes in plasma creatinine were observed during the 12 weeks of JTW treatment. No severe hypoglycemia was observed.

4. Discussion

Chinese medicine is an excellent alternative and complementary medicine in treating NAFLD. In traditional Chinese medicine, NAFLD falls into the category of symptoms caused by phlegm/dampness retention, qi/blood stasis, and spleen/kidney deficiency. Increasing evidence indicates that herbs that remove accumulated pathogenic phlegm and dampness are useful in treating NAFLD [22]. In Chinese medicine, Rhizoma Coptidis has the potential to remove dampness and Cortex Cinnamomican reinforce kidney deficiency, both of which are involved in the development of NAFLD. Additionally, the hypoglycemic and hypolipidemic effects of Rhizoma Coptidis and Cortex Cinnamomi have been confirmed by clinical practice and experimental studies [15, 23, 24]. Among the main constituents in JTW extract, berberine, cinnamaldehyde, and cinnamic acid have also been demonstrated to exhibit beneficial effects in attenuating disturbances of glucose and lipid metabolism [17, 25, 26]. However, the effect of JTW, which is the combination of these two herbs, on treating NAFLD has not been sufficiently reported. Thus, the present study aimed to explore the effect

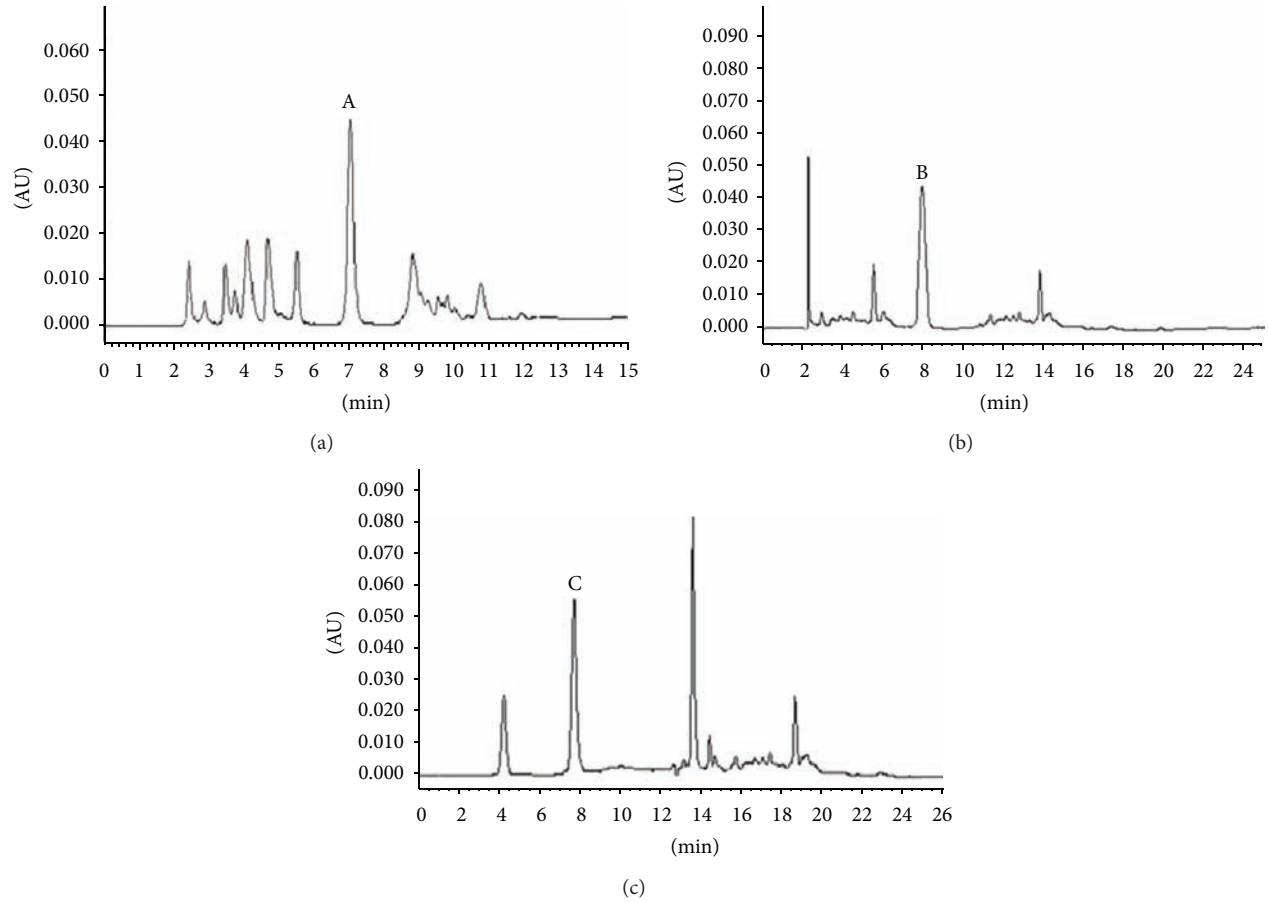

Figure 1: The chromatograms of berberine, cinnamaldehyde, and cinnamic acid in JTW. (a) The chromatogram of berberine in JTW (peak A is berberine), (b) the chromatogram of cinnamaldehyde in JTW (peak B is cinnamaldehyde), and (c) the chromatogram of cinnamic acid in JTW (peak C is cinnamic acid).

Table 1: The effect of JTW on body weight, OGTT, insulin levels, and HOMA-IR in diabetic rats.

Group	Body weight (g)	FPG (mmol/L)	1hPPG (mmol/L)	2hPPG (mmol/L)	FINS (μIU/mL)	HOMA-IR
Control	451 ± 28	6.20 ± 0.69	7.39 ± 0.50	7.10 ± 0.44	29.71 ± 10.21	9.10 ± 1.37
Diabetic	509 ± 52$^{\#}$	8.34 ± 0.81$^{\#\#}$	13.39 ± 4.80$^{\#\#}$	11.63 ± 1.93$^{\#\#}$	55.32 ± 19.18$^{\#\#}$	17.54 ± 4.37$^{\#\#}$
JTW	459 ± 40*	6.13 ± 0.54**	8.71 ± 0.78**	8.03 ± 0.82**	40.38 ± 11.86**	11.14 ± 1.93**
Met	435 ± 49*	6.30 ± 0.69**	8.17 ± 1.45**	7.76 ± 1.15**	41.15 ± 11.86**	11.47 ± 2.83**

Values are expressed as mean ± SD. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus diabetic group.

Table 2: The effect of JTW on plasma lipid profiles in diabetic rats.

Group	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	NEFA (mmol/L)
Control	3.16 ± 0.18	0.34 ± 0.03	1.20 ± 0.18	1.12 ± 0.09	0.81 ± 0.28
Diabetic	5.66 ± 0.77$^{\#\#}$	0.95 ± 0.28$^{\#\#}$	3.76 ± 1.12$^{\#\#}$	0.83 ± 0.13$^{\#\#}$	1.57 ± 0.35$^{\#\#}$
JTW	4.23 ± 0.34**	0.45 ± 0.03**	1.92 ± 0.45*	1.09 ± 0.21**	1.15 ± 0.30*
Met	3.88 ± 0.62**	0.45 ± 0.04**	1.58 ± 0.70**	1.07 ± 0.18**	1.02 ± 0.41**

Values are expressed as mean ± SD. $^{\#\#}P < 0.01$ versus control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus diabetic group.

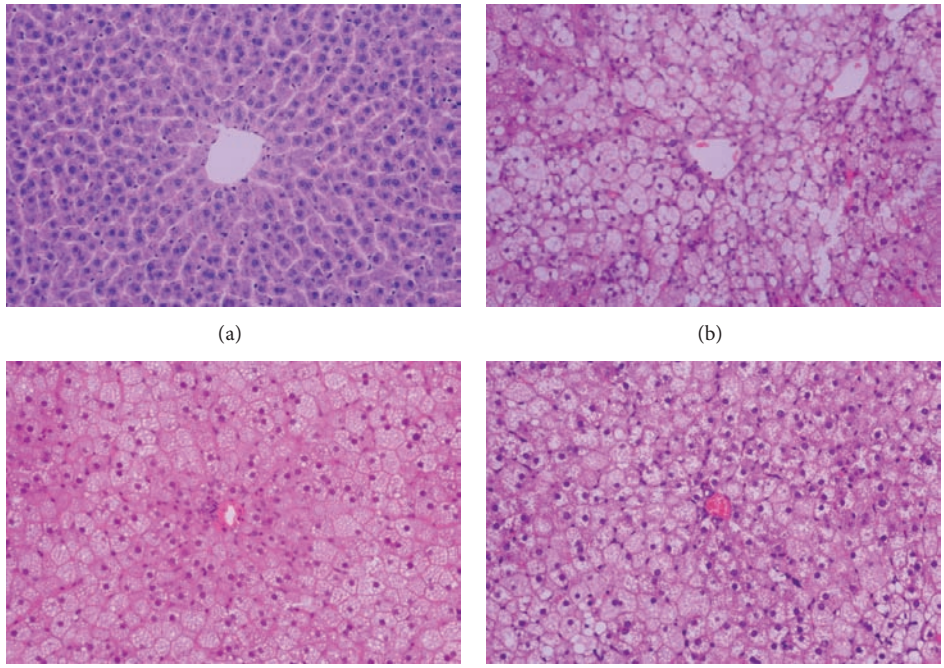

Figure 2: The effect of JTW on liver pathology in diabetic rats. Representative H&E stained images in liver sections from the control group (a), diabetic group (b), JTW group (c), and metformin group (d). Original magnification ×400.

Table 3: The effect of JTW on plasma liver enzyme activity and HTC in diabetic rats.

Group	ALT (U/L)	AST (U/L)	HTC (μmol/g)
Control	36.13 ± 0.39	18.13 ± 4.36	4.16 ± 2.54
Diabetic	58.62 ± 10.45$^{\#\#}$	29.62 ± 3.67$^{\#\#}$	12.30 ± 3.30$^{\#\#}$
JTW	41.43 ± 2.08**	21.98 ± 3.62**	8.35 ± 3.20*
Met	42.55 ± 3.17*	22.07 ± 3.19*	8.70 ± 3.22*

Values are expressed as mean ± SD. $^{\#\#}P < 0.01$ versus control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus diabetic group.

of JTW on hepatic lipid accumulation in rats and humans with T2DM.

In the animal study, the diabetic rats exhibited the characteristics of hyperglycemia, hyperinsulinemia, and hyperlipidemia. Plasma liver enzymatic activity and HTC also significantly increased. Liver histology demonstrated severe fatty degeneration and inflammation infiltration. The oral administration of JTW not only attenuated the biochemical disturbances in peripheral blood but also alleviated TG elevation and the morphological changes in liver tissues. We suggest that JTW could be an effective herbal prescription to treat nonalcoholic steatohepatitis of diabetic rats. These results were further confirmed by the clinical study, with the exception of the effects of JTW on liver histology and enzymatic activity. Invasive liver biopsy was not used in the clinical study to obtain histology data for ethical considerations. As an alternative, we applied noninvasive ^{1}H-MRS scans of liver as an assessment tool. Previous studies have demonstrated that, although this method gives no information of liver histology, HTC obtained by spectroscopy closely coincides with biopsy or autopsy-derived triglyceride concentrations [27]. Additionally, ALT levels do not correlate well with HTC of NAFLD severity in many reports. Patients with NAFLD and normal ALT values may also exhibit steatohepatitis and advanced fibrosis [28]. However, the preliminary data from our clinical study suggests that, at minimum, JTW has beneficial effects on blood glucose and lipid control and also exhibits efficacy in reducing hepatic lipid accumulation in diabetic patients.

With respect to the constituents of Rhizoma Coptidis and Cortex Cinnamomi, many substances have been identified. Among the constituents effective in treating diabetes, berberine (which is contained in Rhizoma Coptidis), cinnamaldehyde, and cinnamic acid (which are contained in Cortex Cinnamomi) have attracted significant attention [13, 29, 30]. Unlike berberine and cinnamic acid, cinnamaldehyde is difficult to detect in peripheral blood after taking cinnamon. This is likely due to the rapid oxidation of cinnamaldehyde to cinnamic acid by oxidase after absorption [31]. To date, there has been no direct clinical evidence supporting the efficacy of Rhizoma Coptidis and Cortex Cinnamomi or the above-mentioned constituents in reducing HTC. However, the potential efficacy of Cortex Cinnamomi extract and berberine has been identified in several experimental studies [32–34]. Berberine moderates glucose and lipid metabolism through a multipathway mechanism [35]. One of the possible mechanisms may be related to the activation of hepatic adenosine monophosphate-activated protein kinase (AMPK). AMPK is a serine/threonine protein kinase

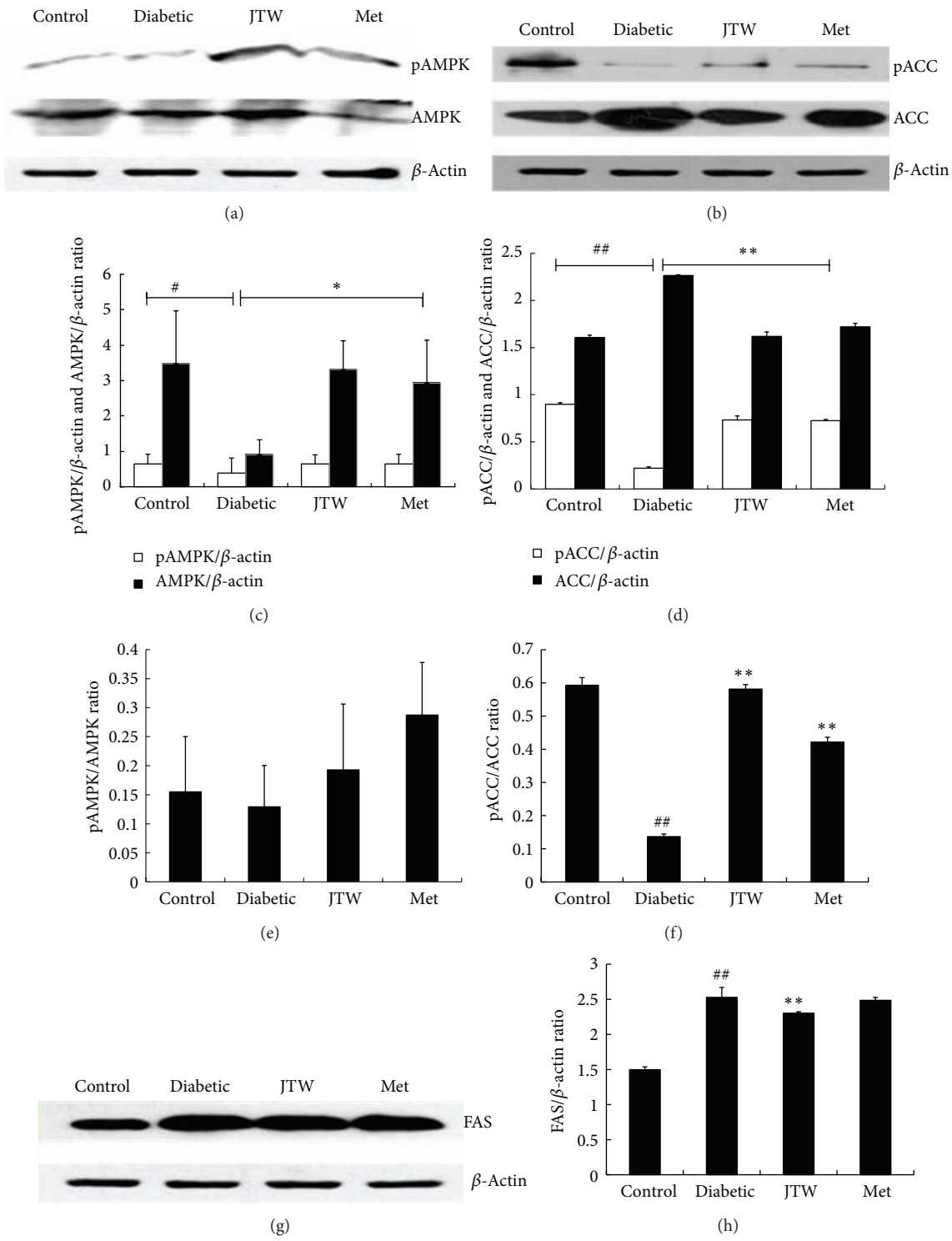

FIGURE 3: The effect of JTW on hepatic lipogenic gene expression in diabetic rats. pAMPK and AMPK protein expressions ((a), (c), and (e)), pACC and ACC protein expressions ((b), (d), and (f)), and FAS protein expression ((g), (h)). Values are expressed as mean ± SD. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus diabetic group.

that plays a central role in regulating cellular metabolism and energy balance [36]. Berberine, through AMPK activation, inhibits triglyceride synthesis and increases fatty acid oxidation by modulating downstream-signaling component expression and activity, leading to the alleviation of hepatosteatosis [37, 38]. In the present study, hepatic AMPK, pAMPK, and pACC protein expression were all upregulated in rats treated with JTW. This was accompanied by the down-regulation of ACC and FAS expressions. However, there was no change in the ratio of pAMPK to AMPK

Table 4: General characteristics and laboratory data of diabetic patients at baseline and 12 weeks after the therapy.

Group	Control		JTW	
	Before	After	Before	After
Subjects (M/F)	9/11		9/11	
Age (years)	46.1 ± 7.3		46.2 ± 6.1	
BMI (kg/m^2)	24.9 ± 3.5	24.7 ± 3.3	25.1 ± 3.6	22.9 ± 2.9$^{\#*}$
WC (cm) (F)	83.4 ± 5.7	83.3 ± 6.5	82.9 ± 5.8	80.6 ± 4.2$^{\#*}$
WC (cm) (M)	90.6 ± 7.0	90.5 ± 8.1	91.7 ± 6.7	89.1 ± 5.6$^{\#*}$
WHR (F)	0.87 ± 0.06	0.87 ± 0.05	0.88 ± 0.05	0.86 ± 0.03$^{\#*}$
WHR (M)	0.94 ± 0.07	0.93 ± 0.07	0.94 ± 0.04	0.91 ± 0.05$^{\#*}$
SBP (mmHg)	132 ± 22	130 ± 24	130 ± 20	130 ± 20
DBP (mmHg)	80 ± 12	79 ± 10	80 ± 10	79 ± 12
FPG (mmol/L)	7.64 (6.20, 9.37)	7.67 (6.41, 9.25)	7.89 (6.10, 9.22)	6.78 (4.01, 8.69)$^{\#*}$
2hPPG (mmol/L)	13.16 ± 4.15	13.21 ± 4.55	13.25 ± 4.09	10.55 ± 3.51$^{\#*}$
FINS (μIU/L)	7.59 (4.33, 10.42)	7.61 (4.57, 9.99)	7.52 (4.11, 12.92)	6.04 (4.20, 9.61)$^{\#*}$
HbA1c (%)	7.81 ± 1.61	7.80 ± 1.59	7.84 ± 1.33	6.91 ± 1.48$^{\#*}$
TC (mmol/L)	4.76 ± 0.73	4.82 ± 0.88	4.94 ± 0.83	3.89 ± 0.63$^{\#*}$
TG (mmol/L)	1.32 (1.05, 2.38)	1.31 (1.01, 2.23)	1.29 (1.02, 2.75)	1.18 (0.94, 2.13)$^{\#*}$
HDL-C (mmol/L)	1.24 (0.98, 1.58)	1.25 (1.03, 1.60)	1.27 (1.03, 1.90)	1.26 (0.96, 1.87)
LDL-C (mmol/L)	2.89 ± 0.82	2.90 ± 0.74	2.95 ± 0.76	1.87 ± 0.77$^{\#*}$
ALT (U/L)	26 (18, 35)	25 (17, 35)	21 (18, 40)	24 (17, 35)
AST (U/L)	22 (15, 31)	21 (16, 32)	19 (11, 35)	21 (10, 30)
HTC (%)	9.63 (5.87, 11.18)	10.79 (6.75, 19.99)$^{\#}$	9.81 (5.76, 15.09)	6.54 (4.82, 13.08)$^{\#*}$

Normally distributed data are presented as the mean ± SD; nonnormally distributed data are presented as the median with the 25th and 75th percentiles in parenthesis. $^{\#}P < 0.05$ versus pretreatment. $^{*}P < 0.05$ versus the control group.

expression although the ratio of pACC to ACC expression increased significantly. These data suggest that JTW could inhibit lipogenesis in liver tissues. Thus hepatic TG content decreased after JTW intervention.

However, one limitation of our study must be considered. The effect of JTW on the fatty liver was evaluated using different formulations in the animal and clinical studies. In the animal experiments, the traditional Chinese herbal decoction was used, whereas concentrated herbal granules were chosen in the clinical trial. These different preparations account for the differences in the content of berberine, cinnamaldehyde, and cinnamic acid in JTW detected by HPLC. However, the daily dosage of JTW intake in diabetic rats was nearly identical to that in diabetic patients. Herbal granules, extracted from herbs with modern pharmaceutical technology, are convenient for those who cannot make an herbal decoction every day. We believe that the application of JTW granules assures quality control in the clinical trials. Nonetheless, we cannot confirm that there are any other differences in the constituents of JTW without the process of boiling. However, we have provided preliminary data that suggest that JTW is promising in the treatment of NAFLD.

In summary, our study demonstrates that oral treatment with JTW attenuates hepatic lipid accumulation in diabetic rats and humans. These beneficial effects are possibly associated with the inhibition of lipogenic gene expression in the liver.

Abbreviations

ACC: Acetyl coenzyme A carboxylase
ALT: Alanine aminotransferase
AMPK: AMP-activated protein kinase
BMI: Body mass index
DBP: Diastolic blood pressure
FAS: Fatty acid synthase
FFA: Free fatty acid
FINS: Fasting insulin level
FPG: Fasting plasma glucose
HbA1c: Hemoglobin A1c
HDL-C: High-density lipoprotein cholesterol
^{1}H-MRS: Proton magnetic resonance spectroscopy
HOMA-IR: Homeostasis model assessment index
HPLC: High-performance liquid chromatography
2hPPG: 2-hour postprandial blood glucose
HTC: Hepatic triglyceride content
IGT: Impaired glucose tolerance
JTW: Jiao Tai Wan
LDL-C: Low-density lipoprotein cholesterol
Met: Metformin
NAFLD: Nonalcoholic fatty liver disease
NEFA: Nonesterified fatty acid
OGTT: Oral glucose tolerance test
pACC: Phosphorylated-ACC
pAMPK: Phosphorylated-AMPK

SBP: Systolic blood pressure
STZ: Streptozotocin
TC: Total cholesterol
T2DM: Type 2 diabetes mellitus
TG: Triglycerides
WC: Waist circumference
WHR: Waist-to-hip ratio.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Zhaoyi Huang and Xiaohu Xu contributed equally to this work.

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Research Article

Pectinesterase Inhibitor from Jelly Fig (*Ficus awkeotsang* Makino) Achene Inhibits Surface Antigen Expression by Human Hepatitis B Virus

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Pectinesterase inhibitor (PEI) isolated from jelly fig (*Ficus awkeotsang* Makino) is an edible component of a popular drink consumed in Asia. Hepatitis B virus (HBV) infection is prevalent in Asia, and current treatments for HBV infection need improvement. This study aimed to evaluate the effect of PEI on the surface antigen expression by HBV (HBsAg). Human hepatoma cell lines Hep3B and Huh7 served as in vitro models for assessing the cytotoxicity and HBsAg expression. A culture of primary hepatocytes cultured from mice served as the normal counterpart. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. HBsAg expression was evaluated by measuring HBsAg secretion into the culture medium using an enzyme-linked immunosorbent assay. The results showed that PEI did not affect the viability of the human hepatoma cell lines or primary mouse hepatocytes. PEI inhibited the expression of HBsAg in hepatoma cell lines harboring endogenous (Hep3B) and integrated (Huh7) HBV genomes in a concentration- and time-dependent manner, thus implicating a universal activity against HBV gene expression. In conclusion, it suggests that PEI from jelly fig inhibits the expression of human HBsAg in host cells without toxic effects on normal primary hepatocytes.

1. Introduction

Hepatitis B virus (HBV) infection is an important health issue because of its increasing prevalence. It is highly correlated with the development of cirrhosis and development of hepatocellular carcinoma, a malignancy with an extremely poor prognosis. The current antiviral agents used to treat HBV infection include lamivudine, adefovir, entecavir, and interferon- (IFN-) α [1]. However, the effectiveness of antiviral agents, particularly nucleoside analogs, is limited by drug resistance due to gene mutations in HBV caused by long-term administration [2, 3]. For example, lamivudine administration causes high rates of resistance due to emergence of HBV strains with the YMDD mutation [4]. Therapy with IFN-α for a finite duration is often interrupted by its toxic effects such as pyrexia, fatigue, headache, myalgia, depression, and myelosuppression [5]. These limitations compromise the therapeutic efficacy of current treatments for HBV infections. Clearly, it is important to develop a novel category of therapeutics for HBV infection based on an innovative strategy.

Jelly fig (*Ficus awkeotsang* Makino) is a native woody vine that grows extensively in Taiwan. A jelly curd produced from the water extract of the jelly fig achenes in the presence of calcium is a popular drink. Pectinesterase (PE) catalyzes the deesterification of pectin and converts the protopectin into soluble pectin and pectate. Previously, Jiang et al. reported that PE activity in a solution derived from intact achenes gradually increased and then decreased to almost zero during the 90 h extraction of PE [6]. Intriguingly, the PE activity of jelly fig achenes was eliminated when a homogenized solution of crushed achenes was used with the same PE extraction procedure. Therefore, some substances released from crushed achenes during PE extraction, that is, PE inhibitors (PEIs), inhibit the PE activity and decrease the PE-catalyzed reaction rate [6]. PEIs prepared from intact jelly fig achenes were previously separated and characterized as polypeptides with molecular weights of 3.5–4.5 kDa, comprising more than 50% histidine. These PEIs displayed marked competitive inhibition of the activity of PEs derived from fruits and vegetables [6]. The known biological activity of PEI includes growth inhibition in human leukemic U937 cells by inducing cell cycle arrest and apoptosis through intrinsic pathway involvement and caspase 3 activation [7]. However, the bioactivity of PEIs against viruses remains unknown.

This study aimed to examine the anti-HBV effect of a natural dietary constituent purified from jelly fig. Surface antigen expression by HBV in host cells served as the major endpoint. Primary hepatocytes isolated from mice were used as the normal counterpart to assess safety.

2. Materials and Methods

2.1. Preparation of PEI from Jelly Fig Achenes. The PEI solution was prepared from pectin-depleted jelly fig achenes by the Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan, as reported previously [6]. The yield of PEI extraction from jelly fig achenes was around 0.1% (w/w). In brief, achenes were rinsed in 20 volumes (w/v) of 5% NaCl solution repeatedly to wash out the major pectin and PE; subsequently, they were homogenized using a blender (6000 rpm) for 2 min. After being extracted using 15 parts (1/15, w/v) of distilled water for 5 h and centrifuged (10000 ×g, 10 min, 4°C), the supernatant was heated in a boiling water bath for 30 min to denature the residual PE. The crude PEI solution was concentrated using a rotary evaporator under reduced pressure (<100 mm Hg). The concentrates were extracted using 250 mL of ethyl acetate, and this was repeated four times. These concentrates (1000 mL) were evaporated under reduced pressure (<100 mm Hg) at room temperature to obtain the PEI concentrate. Further separations were performed by loading approximately 300 mg of the PEI concentrate with ethyl acetate into a 25 × 400 mm glass column packed with ToyoPearl HW-40S resin (Tosoh Bioscience LLC, Tokyo, Japan), by monitoring at 280 nm. Methanol elution [methanol : acetone : water (45 : 25 : 30, v/v/v)] at a flow rate of 1.5 mL/min was used to separate the PEI concentrate. The fourth pooled fractions with PE inhibitory activity (termed as PEI) were lyophilized and stored at −20°C until use. Cells were incubated in 35 mm Petri dishes at an initial concentration of 10^5 cells/mL in the presence of PEI (50–400 μg/mL) at 37°C in a humidified 5% CO_2 incubator.

2.2. Hepatoma Cells and Culture. Human hepatocellular carcinoma cell lines Hep3B and Huh7 cells harbor endogenous and integrated HBV genomes, respectively, with stable production of HBsAg and served as in vitro models of HBV replication [8, 9]. These hepatoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 10^5 I.U./L penicillin, 100 mg/L streptomycin, and 1,000 micromole/L L-glutamine in a humidified 5% CO_2 incubator at 37°C.

2.3. Preparation and Culture of Primary Mouse Hepatocytes. Primary hepatocytes were isolated from 6- to 8-week-old male BALB/c mice using the procedures described by Klaunig et al. with some modifications [10]. After anaesthetization and laparotomy, the portal vein was cannulated and perfused with calcium-free Hanks' balanced salt solution (HBSS) containing ethylene glycol-bis-(b-aminoethyl) N, N9-tetraacetic acid, and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid. This was followed by perfusion with HBSS containing collagenase for isolation of hepatocytes. After removing the liver, the hepatocytes were mechanically dissociated and filtered through a mesh and were isolated by gradient centrifugation with Percoll (GE Healthcare, Little Chalfont, UK) and washed. Cells were cultured further and plated with complete medium and adequate hepatocyte growth factor. The medium was changed regularly or as required.

2.4. Growth Inhibition and Cell Viability Assessment Using the MTT Assay. The viability of hepatoma cells was assessed using a tetrazolium dye colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] assay [11] and expressed as the MTT value of the experimental group divided by the MTT value of the untreated control group.

2.5. Assay to Determine Relative HBsAg Expression. Hepatoma cells were cultured in DMEM with 10% fetal bovine serum for 24 h and transferred to serum-free DMEM with or without PEI and incubated thereafter. The HBsAg secreted in the culture medium was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (General Biological, Taipei, Taiwan). The optical density values determined using ELISA were normalized with the cell viability. The detection of HBsAg in the sera of patients indicates an existing HBV infection and the risk of developing cirrhosis and hepatocellular carcinoma. Thus, HBsAg is a useful index for evaluating viral activity [12]. The relative HBsAg expression was determined using the following formula: (HBsAg/MTT) from PEI-treated cells/(HBsAg/MTT) from the untreated cells. (HBsAg/MTT) from the untreated control group was treated as a 100% expression. Measurement of viral antigen secretion by HBV host cells, such as HBsAg secretion by Hep3B cells, has been used in various studies to evaluate the effects of cytokine drugs on HBV.

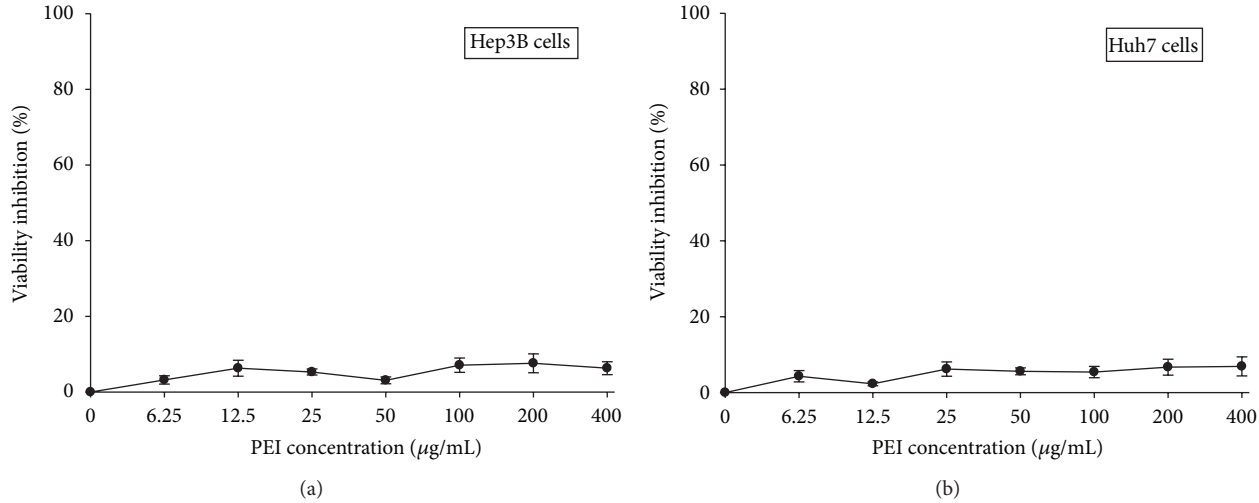

Figure 1: The viability of (a) Hep3B and (b) Huh7 human hepatoma cells with PEI treatment. Cells were treated with various concentrations of PEI for 48 h and then subjected to MTT assay. Triplicated data from separate experiments are expressed as mean ± SEM.

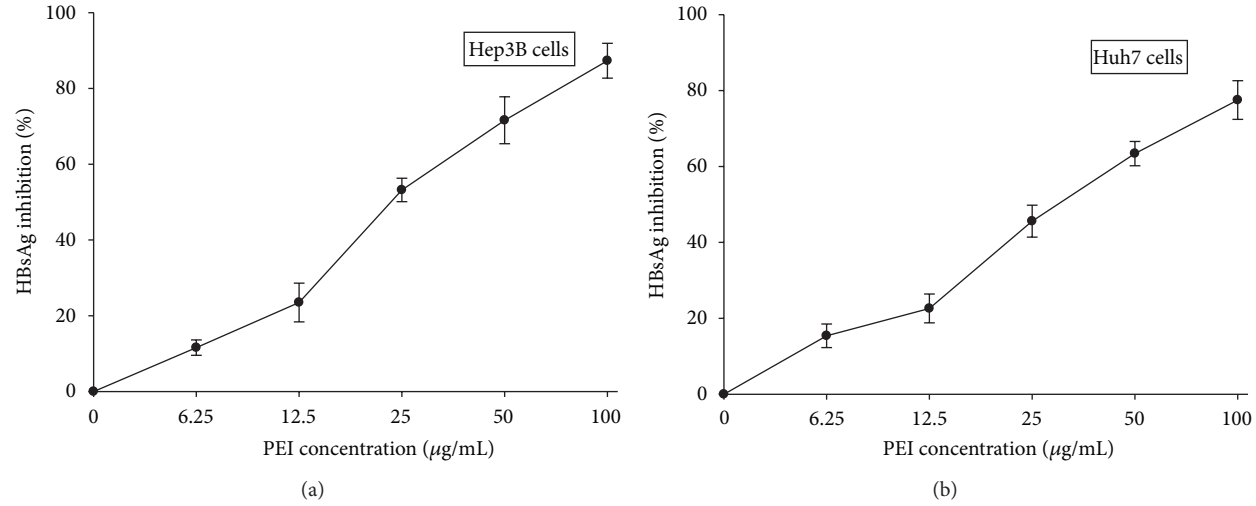

Figure 2: The relative HBsAg expression of (a) Hep3B and (b) Huh7 human hepatoma cells treated with various concentrations of PEI for 48 h. Triplicated data from separate experiments are expressed as mean ± SEM.

2.6. Statistical Analysis. Results are presented as mean ± SEM. Differences among the treatment groups were assessed by Student's t-test or ANOVA test. Statistical analyses were performed using the Statistical Package for the Social Sciences software package, v18.0 (SPSS Inc., Chicago, IL), and a P value of <0.05 was considered significant.

3. Results

3.1. Effect of PEI on the Viability of Hepatoma Cells. To determine the cytotoxicity of PEI on the human hepatoma cells, Hep3B and Huh7 cells were treated with various concentrations of PEI (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μg/mL) for 48 h and then subjected to MTT assay. As shown in Figures 1(a) and 1(b), PEI at concentrations of 0–400 μg/mL did not significantly affect the cell viability of Hep3B and Huh7 cells, respectively. Therefore, the biological activity of PEI in these cells was probably not due to cellular toxicity.

3.2. Reduction of the Relative HBsAg Expression in Human Hepatoma Cells by PEI. To examine the relative expression levels of HBsAg in human hepatoma cells exposed to PEI. Hep3B and Huh7 cells were treated with different concentrations of PEI (0, 6.25, 12.5, 25, 50, and 100 μg/mL) for 48 h in serum-free medium after serum starvation. The culture medium was then harvested and the amount of HBsAg was measured by ELISA. As shown in Figures 2(a) and 2(b), PEI suppressed the endogenously expressed HBsAg in Hep3B cells and suppressed the HBsAg produced from the stable clone of HBV DNA-integrated in human hepatoma Huh7 cells without endogenous HBV genomes. This inhibitory effect was concentration-dependent with 87.3% ± 4.6% and

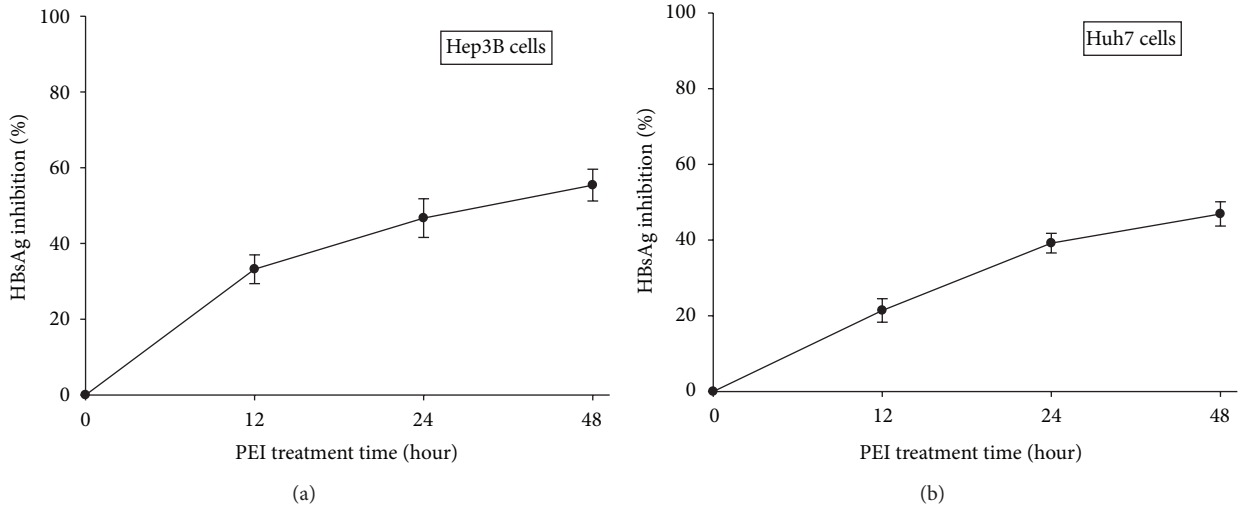

Figure 3: The relative HBsAg expression of (a) Hep3B and (b) Huh7 human hepatoma cells at 25 μg/mL of PEI for 12, 24, and 48 h. Triplicated data from separate experiments are expressed as mean ± SEM.

77.5% ± 5.1% reductions at 100 μg/mL of PEI in Hep3B and Huh7 cells, respectively (Figures 2(a) and 2(b)). In addition, at the 50% HBsAg-inhibitory concentration, that is, 25 μg/mL of PEI, a time-dependent effect (12, 24, and 48 h) was also demonstrated in Hep3B and Huh7 cells, as shown in Figures 3(a) and 3(b).

3.3. Effect of PEI on the Viability of Normal Hepatocytes. Primary cultured hepatocytes isolated from BALB/c mice served as a normal counterpart for assessment of PEI toxicity in normal tissue. Normal hepatocytes were treated with various concentrations of PEI (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μg/mL) for 48 h and then subjected to MTT assay. As shown in Figure 4, PEI at concentrations greater than those that effectively inhibited HBsAg expression in hepatoma cells did not significantly decrease the viability of normal hepatocytes.

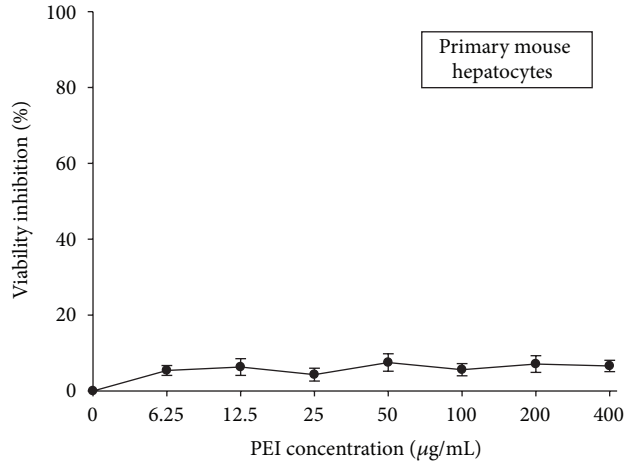

Figure 4: The viability of primary cultured hepatocytes with PEI treatment. Cells were treated with various concentrations of PEI for 48 h and then subjected to MTT assay. Triplicated data from separate experiments are expressed as mean ± SEM.

4. Discussion

We found that PEI isolated from jelly fig, a raw material found in a popular drink in Asia, inhibited the expression of human HBsAg in host cells without toxic effects on normal primary hepatocytes.

As mentioned previously, the current treatments for HBV are either toxic or have a tendency of resulting in resistance through gene mutations. We found that the naturally occurring PEI from jelly fig probably inhibits HBsAg expression without any toxicity to hepatocytes, which may facilitate the development of novel therapeutic agents. PEI from jelly fig comprises thermally stable polypeptides (3.5–4.5 kDa) with 57% basic amino acids, which inhibited the intrinsic carambola PE activity required for methanol reduction in wines [13]. It is unknown whether the bioactivity of PEI from jelly fig will be retained in the gastrointestinal tract after digestion. Thus, further in vivo tests are required.

The experimental models tested in this study comprised Hep3B cells that harbored endogenous HBsAg and a stable clone of HBV DNA-integrated Huh7 cells. PEI inhibited HBsAg expression in both cell lines, indicating its universal activity against HBsAg expression. However, this experimental model only provides a screening platform for anti-HBV agents [14]. Further molecular studies, including viral replication assays, are required to confirm whether PEI from jelly fig can inhibit HBV.

Normal liver toxicity assessment based on the in vitro hepatocyte viability showed no evidence of PEI hepatotoxicity. Currently used anti-HBV agents have various levels of toxicity [5], which further supports the potential development of PEI as a novel anti-HBV agent.

In clinical practice, chemotherapy-related HBV reactivation and subsequent fulminant failure is not rare in HBV-infected patients [15, 16]. The prophylactic administration of antiviral agents such as lamivudine has been shown to be effective [17]. Given that PEI is found in a safe and commonly used beverage, it is expected that PEI may be another option for prophylaxis against HBV reactivation in patients with cancer who already receive many chemotherapy agents.

5. Conclusions

In conclusion, PEI isolated from jelly fig may inhibit human HBsAg expression in host cells without any toxic effects on normal hepatocytes.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

High-Frequency Ultrasound Imaging to Evaluate Liver Fibrosis Progression in Rats and Yi Guan Jian Herbal Therapeutic Effects

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The animals used in liver fibrosis studies must usually be sacrificed. Ultrasound has been demonstrated to have the ability to diagnose hepatic fibrosis and cirrhosis in experimental small-animal models. However, few studies have used high-frequency ultrasound (HFU, 40 MHz) to monitor changes in the rat liver and other hollow organs longitudinally. In this study, liver fibrosis was induced by administering dimethylnitrosamine (DMN) in SD rats, aged 8 weeks, for three consecutive days per week for up to 4 weeks. A Chinese herbal medicine Yi Guan Jian (YGJ) was orally administered (1.8 g/kg daily) to DMN-induced liver fibrosis rats for 2 weeks. Compared with the normal control rats, rats treated with DMN for either 2 weeks or 4 weeks had significantly lower body weights, liver indexes and elevation of hydroxyproline, GOT, and GPT contents. YGJ herbal treatment remarkably prevented rats from DMN-induced liver fibrosis. The HFU scoring results among the normal controls, 2-week DMN-treated rats, 4-week DMN-treated rats, and combined 2-week YGJ therapy with 4-week DMN-treated rats also reached statistical significance. Thus, HFU is an accurate tool for the longitudinal analysis of liver fibrosis progression in small-animal models, and the YGJ may be useful in reversing the development of hepatic fibrosis.

1. Introduction

Hepatic fibrosis has been reported to be a response to necrosis and inflammation caused by various factors such as infection, intoxication, and detrimental factors. Hepatic fibrosis leads to the activation of Kupffer cells, mononuclear cells, and hepatic stellate cells, resulting in the degeneration of the hepatic cords. The persistence of the insult disrupts the normal hepatic architecture, leading to the development of regenerative nodules and vascular dysfunction, which are used to determine the cirrhosis stage [1, 2]. Liver biopsy is the major tool for diagnosing hepatic fibrosis or cirrhosis and assessing the severity of an injured liver. However, this procedure has several drawbacks, including its invasiveness, the likelihood of sampling errors, and the potential for medical complications.

Ultrasound has been demonstrated to have the ability to diagnose hepatic fibrosis and cirrhosis in experimental small-animal models [3–6]. However, the ultrasonic frequency used in these studies was always below 15 MHz, and the quality of the images was limited by the spatial resolution. High-frequency ultrasound (HFU) imaging, a recent developed

technology, allows the imaging of small laboratory animals at a very high resolution [7, 8]. The frequency of such systems is above 20 MHz, and the spatial resolution is less than 100 μm [8–10]. One of the most important advantages of this technology is the ability to image organs and tissue noninvasively in living animals. This ability allows disease progression in animal models to be monitored in longitudinal studies, which can shorten the observation time and reduce the number of sacrificed animals [11]. In our previous study, we identified the surgical anatomy of the rodent abdomen using high-frequency ultrasound (40 MHz) [12].

Traditional Chinese medicines (TCMs) are multi-ingredient extracts with low side effects in the treatment of chronic liver diseases [13, 14]. Yi Guan Jian (YGJ) decoction is a complex prescription of TCM consisting of 9 medical herbs [15, 16]. Although there is still no evidence-based clinical study, YGJ has been used to treat human liver fibrosis induced by hepatitis and has shown apparent efficacy in the reversal of liver fibrosis. The major active components of YGJ extract, ferulic acid, and catalpol, significantly inhibit the progression of hepatic fibrosis in carbon tetrachloride-(CCL4-) induced animal model study [17]. In addition, a more clinical representation of liver fibrosis model showed that the livers in dimethylnitrosamine- (DMN-) treated animals were shrunken with dark discoloration because of congestion [18]. However, the therapeutic effects of YGJ extract in the DMN-induced liver fibrogenesis have not been fully clarified.

In the present study, we used a commercially available high-frequency (40 MHz) ultrasound imaging system to detect and monitor the progression of hepatic fibrosis *in vivo* in the normal controls, 2-week DMN-treated rats, 4-week DMN-treated rats, and combined 2-week YGJ therapy with 4-week DMN-treated rats. The therapeutic effects of YGJ extract for liver fibrosis were extensively evaluated on histological examination, biochemical values, and hepatic fibrosis-related gene regulations.

2. Methods

2.1. Animal Model. Twenty male Sprague-Dawley (SD) rats (aged 8 weeks and weighing 250–300 g) were used. The animals were housed in an air-conditioned room at 23 ± 3°C with 55–60% relative humidity and a 12 h light/dark cycle. This study conformed to "The Guide for the Care and Use of Laboratory Animals" and was approved by the Ethics Committee of National Chung Hsing University (Approval no. 102-10). Two distinct models of experimental hepatic fibrosis were established in rats, one using DMN and one using normal saline (control group). In the DMN-induced fibrosis group, the rats were injected intraperitoneally with 1% DMN (10 mg/kg body weight) for three consecutive days per week for 2 weeks or 4 weeks as described previously [15, 19]. After treatment with DMN for 2 weeks, rats were orally administered 1.8 g/kg YGJ herbal extract (Koda Pharmaceutics Ltd., Taoyuan, Taiwan). The mock control group was given the same volume of phosphate-buffered saline (PBS) intraperitoneally for 4 weeks. The rats were sacrificed after 2 or 4 weeks of treatment with DMN or normal phosphate-buffered saline (PBS) or combined with YGJ herbal therapy.

2.2. High-Frequency Ultrasound (HFU) Examination. Animals were lightly anesthetized with 2.0–2.5% isoflurane vapor and placed in the supine position while breathing spontaneously. After the rats were anesthetized, the abdomen was shaved and further cleaned with a chemical hair remover to minimize ultrasound attenuation [19]. A commercially available HFU system (Visual Sonics Vevo 770, Toronto, Ontario, Canada) was used in this experiment. A transducer with a central frequency of 40 MHz, providing an axial resolution of 40 μm with a 14.6 mm field of view, was used to image the rat's abdominal organs. When beginning to perform the HFU analysis, ultrasound gel was put on the skin as a coupling fluid.

2.3. Longitudinal Evaluation of the Abdominal Organs by HFU. HFU was used to assess each rat on the first day of the 10th and 12th weeks of age. Each rat's abdomen was scanned carefully by three experienced researchers. The liver was examined to assess echogenicity, homogeneity, the surface of the margin, and intrahepatic vascular abnormalities. In addition, the size of the spleen and the presence or absence of ascites were also examined and recorded.

2.4. Ultrasound Scoring System. To evaluate the validity of HFU for the diagnosis of cirrhosis, we scored the ultrasonographic findings for the rats using a modified scoring system [20]. Items including the liver parenchyma echotexture, spleen size, ascites, vasculature, and the surface of the margin were used to score the severity of cirrhosis. The detailed scoring method is presented in Table 1.

2.5. Histological Examinations. To validate the *in vivo* imaging findings, rats were sacrificed, and their livers were excised immediately. To correlate the size and location of the previously observed lesions, visual inspection and *ex vivo* US of the liver were performed. The liver index (liver wet weight/body weight) and spleen index (spleen wet weight/body weight) were calculated. Liver tissue slices were fixed for 5 hours in Bouin's solution, followed by overnight exposure to 10% buffered formalin solution, pH 7.2, and then embedded in paraffin. Liver samples were sectioned (5 μm) and stained with hematoxylin and eosin (H&E) and picrosirius according to standard protocols [21–23]. Masson's trichrome and Sirius Red staining were used to assess collagen deposition [15].

2.6. Tissue Hydroxyproline and Blood Biochemical Assays. Serum glutamate oxaloacetate transaminase (GOT) and glutamic pyruvic transaminase (GPT) concentrations were determined using a JSCC kit (Roche, Indianapolis, IN, USA). The hepatic hydroxyproline content was measured as described previously [15].

2.7. Detection of Fibrogenesis-Related mRNA by Quantitative RT-PCR. The mRNA expression of the fibrogenesis genes, alpha-smooth muscle actin (α-*SMA*) and collagen alpha-1(1) (*collagen α1-I*) was determined by semiquantitative RT-PCR.

Table 1: Scoring system for high-frequency ultrasound (HFU) signs for the assessment of liver fibrosis.

Characteristic	US sign	Score
Parenchymal echotexture[a]	Homogeneous echotexture	0
	Heterogeneous echotexture	1
	Coarse echotexture	2
Spleen size[b]	Normal	0
	Enlarged	1
Ascites[c]	Absence	0
	Presence	1
Vasculature[d]	Normal	0
	Obscure with normal diameter	1
	Stretched or broad vessels	2
Surface or margin[e]	Smooth	0
	Irregular	1

The cirrhosis score was determined as follows.
[a]Liver parenchyma (hepatic parenchyma away from major vessel or bile duct were used in evaluation): 0 score for homogenous appearance of the parenchyma; 1 score for heterogenous with finely scattered hypoechoic and hyperechoic areas; 2 score for coarse liver with irregular or patchy pattern.
[b]Spleen size index (calculated as product of oblique and diagonal diameter): 0 score for spleen size index <1.7 cm^2; 1 score for larger spleen.
[c]Ascites: 0 score for absence of ascites; 1 score for presence of ascites.
[d]Vasculature: 0 score for smooth vessel wall; 1 score for obscured or blurred vessel with normal diameter; 2 score for irregular and narrowed vessel.
[e]Surface of margin: 0 score for smooth surface; 1 score for uneven surface or shallow, irregular nodular surface.

The housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as an internal control. Approximately 900 ng of total RNA extracted from liver tissue was reverse transcribed with MuLV Reverse Transcriptase using the GeneAmp RNA PCR Kit (Applied Biosystems, Foster, CA, USA) and oligo d(T)16 primers. Aliquots of the reverse transcriptase mix were used for PCR amplification of *α-SMA* (5′-TGGCTATTCCTTCGTGACTACTG-3′ and 5′-AAAGATGGCTGGAAGAGAGTCAC-3′), *collagen α1-I* (5′-GTTCGTGACCGTGACCTTGA-3′ and 5′-TTGGGGTTCGGGCTGATGTA-3′), and *GAPDH* (5′-ATCCCCAGAGCGTCATTCG-3′ and 5′-GAGAGAGCCCTGCCTGCC-3′). The transcription levels of each gene were normalized against those of GAPDH mRNA using a semiquantitative method as described previously [15, 24].

2.8. Statistical Analysis. The results were expressed as the mean ± standard deviation (SD). The differences between the DMN-treated group and the control group were evaluated using Student's *t*-test. Statistical significance was set at $P < 0.05$ (* or $^\#$).

3. Results

3.1. Correlation of Gross Liver Characteristics and HFU Image Findings In Vivo in Control and DMN-Treated Rats. In Figure 1, panel (A) presents the gross appearance of a normal liver, panel (B) presents the gross appearance of the 2-week DMN-treated rat liver, and panel C presents the gross appearance of 4-week DMN-treated rat liver. Panels (a), (b), and (c) show the ultrasonographic images corresponding to the photographs in panels (A), (B), and (C), respectively. In the normal rats (Figure 1(A)), the liver was a soft, pinkish-brown organ consisting of major four lobes (the median lobe, right lobe, left lobe, and caudate lobe). In the 2-week DMN-treated rats, the liver's surface was relatively darker than that in the normal rats, and the margin of the liver was coated with a yellowish material (Figure 1(B)). In the 4-week DMN-treated rats, the liver was smaller and relatively dark brown and was coated with yellowish material along the fissures and margins (Figure 1(C)). In the ultrasonographic images,

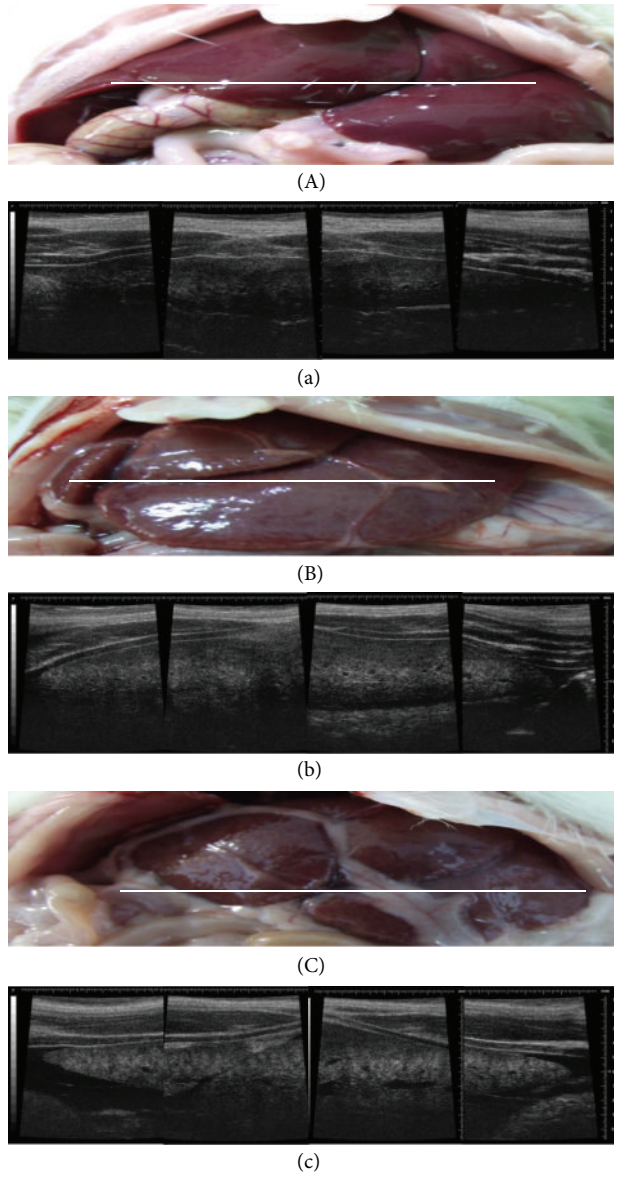

Figure 1: The gross appearance of a normal liver (A), 2-week DMN-treated liver (B), and 4-week DMN-treated liver (C). Panels (a), (b), and (c) are the ultrasonographic images (40 MHz) corresponding to panels (A), (B), and (C), respectively. The echogenicity of the DMN-treated rat livers was more hyperechoic than that of normal rat livers.

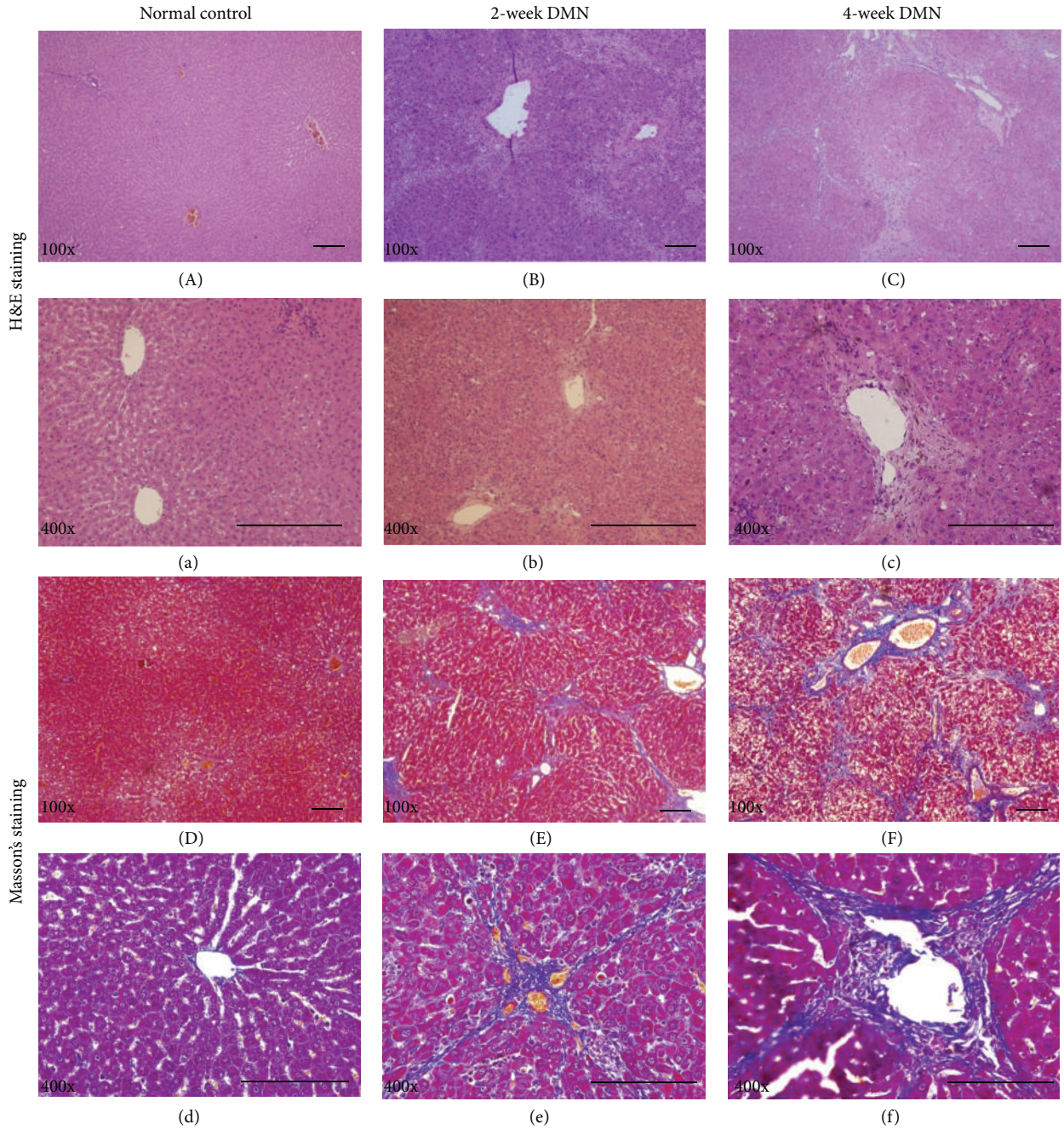

Figure 2: The normal lobular architecture with central veins and radiating hepatic cords was observed in normal control rats ((A), (a), (D), and (d)). Fatty degeneration, necrosis, the infiltration of inflammatory cells, and the apparent formation of fibrotic septa were present in the 2-week DMN-treated group ((B), (b), (E), and (e)) and 4-week DMN-treated group ((C), (c), (F), and (f)). Tissue stained with H&E and imaged at 100x ((A), (B), and (C)) or 400x ((a), (b), and (c)) magnifications. Tissue stained with Masson's trichrome and imaged at 100x ((D), (E), and (F)) or 400x ((d), (e), and (f)) magnifications.

the normal liver parenchyma had a uniform, sponge-like texture with low echogenicity (Figure 1(a)). Passing through the parenchyma were blood vessels, which were observed as branching tubular structures that could be traced toward the portal or hepatic vein. In the 2-week DMN-treated fibrotic rats, the ultrasonographic images of the liver were relatively hyperechoic (Figure 1(b)). The most hyperechoic pattern was observed for the 4-week DMN-treated rat livers (Figure 1(c)). In addition, ascites was also detected by ultrasound in the 4-week DMN-treated rat livers (Figure 1(c)).

3.2. Pathological Features in Normal Control and DMN-Treated Rats. To assess the reliability of HFU imaging, we performed hematoxylin and eosin (H&E) staining and Masson's trichrome staining to examine pathological alterations and collagen deposition (Figure 2). The normal lobular

architecture with central veins and radiating hepatic cords was observed in the livers of normal control rats (Figures 2(A), 2(a), 2(D), and 2(d)). Fatty degeneration, necrosis, the infiltration of inflammatory cells, and the apparent formation of fibrotic septa were present in the 2-week DMN-treated group (Figures 2(B), 2(b), 2(E), and 2(e)) and became more severer in the 4-week DMN-treated group (Figures 2(C), 2(c), 2(F), and 2(f)).

3.3. HFU Analysis of Associated Organs. The livers of either 2-week (Figures 3(E), 3(e), 3(F), and 3(f)) or 4-week (Figures 3(I), 3(i), 3(J), and 3(j)) DMN-treated rats were relatively smaller than those of the normal rats (Figures 3(A), 3(a), 3(B), and 3(b)). The ultrasonographic images of the spleens of DMN-treated rats showed increased echogenicity (Figures 3(G), 3(g), 3(K), and 3(k)). The kidneys were also hyperechogenic in DMN-treated rats (Figures 3(H), 3(h), 3(L), and 3(l)). Ascites was frequently detected in the DMN-treated rats.

3.4. Ultrasound Scoring to Evaluate YGJ Therapeutic Effect. In the modified scoring system, the normal control rats were scored as 0. After 2 weeks of treatment with DMN, the average score was 1.6 ± 0.9, and after 4 weeks of DMN treatment, it was increased to 6.2 ± 0.8. As shown in Table 2, we can see that the ultrasound score was significantly different in the 2-week DMN-treated rats ($P < 0.05$) and the 4-week DMN-treated rats ($P < 0.01$) compared to normal control rats. The average score of the 4-week DMN-treated and 2-week YGJ therapy group was significantly decreased (2.8 ± 0.8, $P < 0.05$) compared to the 4-week DMN-treated rats without YGJ therapy. To evaluate interobserver and intraobserver variability in the HFU imaging assessment of the severity of fibrotic liver, three experienced operators independently graded the hepatic images as normal, fibrosis, or cirrhosis. Weighted kappa statistics were used to analyze interobserver and intraobserver agreement as shown in Tables S1 and S2 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/302325. Results showed that the intraobserver agreement was 70–100% ($\kappa = 0.53$–1.0) and interobserver agreement was even higher from 93% to 100% ($\kappa = 0.85$–1.0). Therefore, this is a reliable sonographic assessment method for rat liver fibrosis and cirrhosis.

3.5. Physical and Biochemical Characteristics of the Normal, DMN-Treated, and DMN-Treated Combined YGJ Therapy Rats. As shown in Table 3, rats treated with DMN for either 2 weeks or 4 weeks were significantly different from the control rats in the terms of body weight, spleen index, hydroxyproline level, GOT level, and GPT level. However, rats treated with DMN and combined with 2 weeks YGJ therapy significantly decreased serum GOT and GPT levels and restored body weight compared to the DMN-treated without YGJ therapy group ($P < 0.05$). Moreover, the hepatic hydroxyproline concentration in the 4-week DMN-treated group increased 5.6-fold over the normal control group ($P < 0.001$) but significantly decreased in the YGJ therapeutic group ($P < 0.01$) compared to the DMN-treated alone group.

3.6. Effects of YGJ Herbal Therapy on Liver Histological Changes. Liver histopathological analyses consisted of tissue section staining with H&E and Masson's trichrome dye and combined with HFU imaging analysis as shown in Figure 4. Liver tissue from 4-week DMN-treated rats had more steatosis, fibrotic septa, necrotic hepatocytes, and incorporation of degenerated hepatocytes into pseudolobules as well as associated higher collagen content (Figures 4(g), 4(h), and 4(i)) than that of normal control rats (Figures 4(a), 4(b), and 4(c)) and 2-week DMN-treated rats (Figures 4(d), 4(e), and 4(f)). However, 4-week DMN-treated rats combined with YGJ herbal therapy for 2 weeks markedly alleviated the degree of liver fibrosis and significantly reduced the collagen deposition (Figures 4(j), 4(k), and 4(l)).

3.7. Effects of YGJ on Gene Expression of α-SMA and Collagen α1-I. Gene expression for *α-SMA* and *collagen α1-I,* which represent hepatic fibrosis factors, was analyzed using RT-PCR (Figure 5(a)). The mRNA levels of *α-SMA* and *collagen α1-I* were increased in either 2-week DMN-treated rats (1.5- and 1.7-fold) or 4-week (3.6- and 1.9-fold) DMN-treated rats, respectively, compared with the normal control group ($P < 0.05$). The YGJ herbal treatment significantly reduced the mRNA levels of *α-SMA* and *collagen α1-I* by 67% ($P < 0.01$) and 45% ($P < 0.05$), respectively compared with the 4-week DMN-treated alone group (Figure 5(b)).

4. Discussion

In the current study, we demonstrated that high-frequency ultrasound (HFU, 40 MHz) imaging is a useful tool for the *in vivo* analysis of liver tissue during chronic toxin exposure and the induction of liver fibrosis in rats. In addition, we developed an ultrasound scoring system to help assess the severity of liver fibrosis in rats. The mean HFU imaging score increased with increasing fibrosis stage. It is a useful tool to evaluate the therapeutic efficacy of the YGJ herbal medicine treatment.

Small animals have been used as models for the investigation of toxin- or virus-induced liver injury for decades [25–28]. To confirm the pathological findings of liver damage, the majority of studies have sacrificed animals in the experimental and control groups at different time points during the study. This type of study design has two drawbacks. First, it lacks continuity. For example, mouse A is sacrificed after 2 weeks of toxin exposure, and mouse B is sacrificed after four weeks of toxin exposure. In this situation, we usually regard the pathological changes exhibited by mouse B as the continuation of the changes observed in mouse A. However, even if the animals belong to the same strain, there may be some bias. Second, this type of study design requires many more animal sacrifices. If a study does not use imaging to assess animals, animals must be sacrificed at every observational and interventional time point.

Ultrasound has been used to analyze the livers of small animals for a long history [29, 30]. Initially, researchers found that diffuse liver disease appears ultrasonographically as a change in the liver's echogenicity relative to that of the

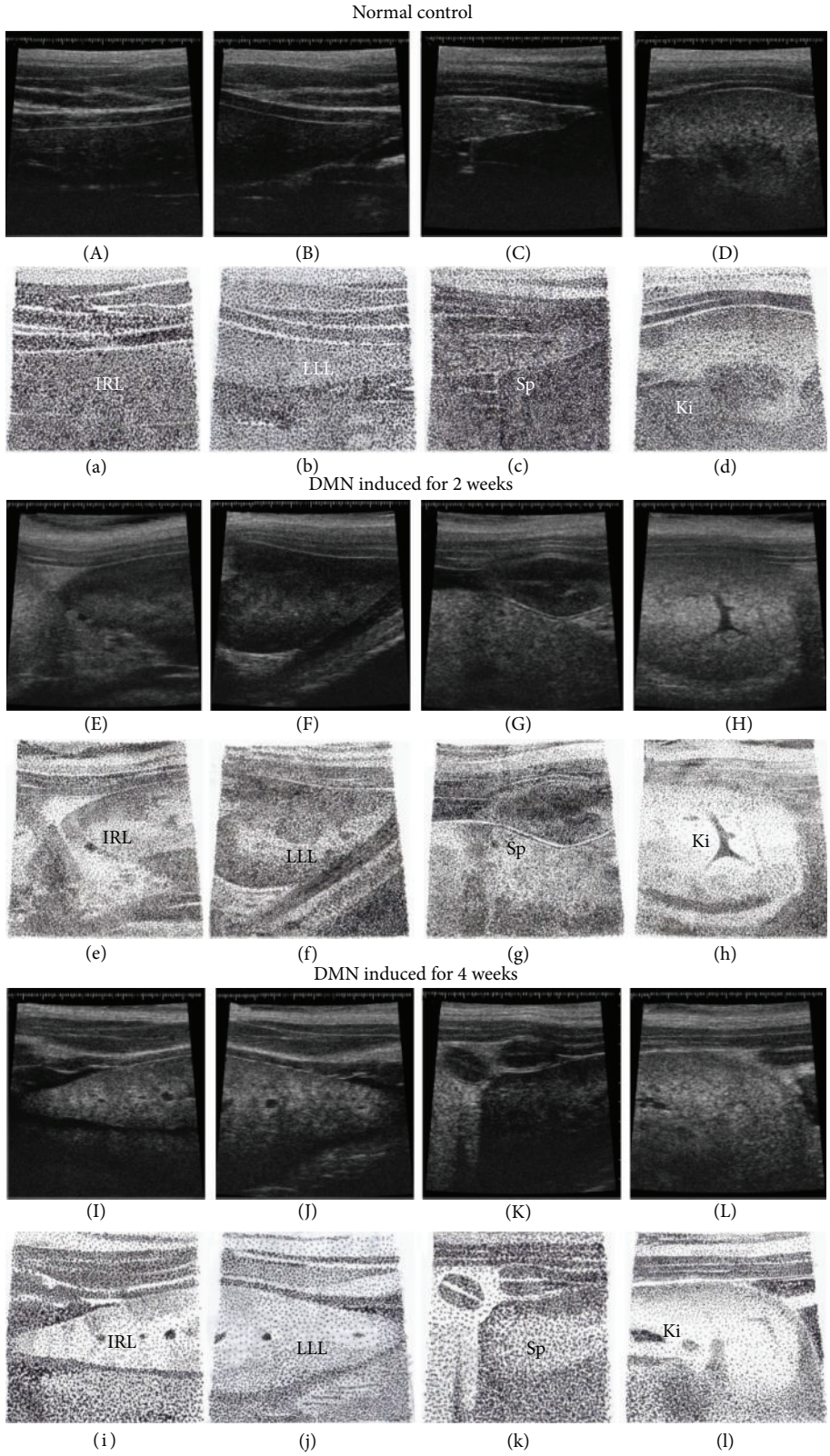

Figure 3: Ultrasonographic images (40 MHz) of normal control rats, 2-week DMN-treated rats, and 4-week DMN-treated rats. ((A), (B), (C), and (D)) The HFU images of the normal rat inferior right liver (IRL), lateral left liver (LLL), spleen (Sp), and left kidney (Ki), respectively. Panels (a), (b), (c), and (d) are the schematic diagrams of panels (A), (B), (C), and (D), respectively. ((E), (F), (G), and (H)) The 2-week DMN-treated rat inferior right liver, lateral left liver, spleen, and left kidney, respectively. Panels (e), (f), (g), and (h) are the schematic diagrams of panels (E), (F), (G), and (H), respectively. ((I), (J), (K), and (L)) The 4-week DMN-treated rat inferior right liver, lateral left liver, spleen, and left kidney, respectively. Panels (i), (j), (k), and (l) are the schematic diagrams of panels (I), (J), (K), and (L), respectively.

Table 2: Ultrasound characteristics (40 MHz) of fibrosis in normal liver, 2-week DMN-treated fibrotic liver, and 4-week DMN-treated fibrotic liver.

Treatment	Parenchymal echotexture	Spleen size	Ascites	Surface or margin	Vasculature	Sum	Average[†]
Normal control	0	0	0	0	0	0	0
	0	0	0	0	0	0	
	0	0	0	0	0	0	
	0	0	0	0	0	0	
	0	0	0	0	0	0	
DMN-induced fibrosis (2 wk)	1	0	0	0	0	1	$1.6 \pm 0.9^{*}$
	0	0	0	0	1	1	
	1	0	0	0	0	1	
	1	0	1	0	0	2	
	1	1	0	0	1	3	
DMN-induced fibrosis (4 wk)/PBS (2 wk)	2	1	1	1	2	7	$6.2 \pm 0.8^{**}$
	2	1	1	0	2	6	
	2	1	0	1	2	6	
	2	1	1	1	2	7	
	1	1	0	1	2	5	
DMN-induced fibrosis (4 wk)/YGJ (2 wk)	0	0	1	1	1	3	$2.8 \pm 0.8^{*,\#}$
	0	0	1	0	1	2	
	1	1	0	1	1	4	
	0	1	1	0	0	2	
	1	1	0	0	1	3	

[†]Each value represents the mean ± SEM of five rats.
$^{*}P < 0.05$, $^{**}P < 0.01$ versus normal control.
$^{\#}P < 0.05$ versus DMN-induced fibrosis/PBS group.

Table 3: Body weight, liver index, spleen index, hydroxyproline level, GOT level and GTP level in the normal control group, 2-week DMN-treated group, and 4-week DMN-treated group.

Treatment[†]	Normal control	DMN-induced fibrosis (2 wk)	DMN-induced fibrosis (4 wk)/PBS (2 wk)	DMN-induced fibrosis (4 wk)/YGJ (2 wk)
Body weight (g)	471.3 ± 42.9	$411.3 \pm 34.7^{*}$	$341.7 \pm 40.4^{**}$	$418.7 \pm 36.9^{*,\#}$
Liver index (%)	4.2 ± 0.3	4.1 ± 0.4	$3.5 \pm 0.7^{**}$	$4.3 \pm 0.8^{\#\#}$
Spleen index (%)	0.2 ± 0.0	$0.5 \pm 0.1^{**}$	$0.4 \pm 0.1^{*}$	$0.4 \pm 0.1^{*}$
Hydroxyproline (μg/g liver)	208.7 ± 19.5	$452.2 \pm 67.8^{**}$	$1187.1 \pm 220.1^{***}$	$403.5 \pm 61.3^{*,\#\#}$
GOT (U/L)	57.2 ± 1.7	$175 \pm 22.4^{***}$	$136.8 \pm 6.4^{**}$	$71.9 \pm 8.3^{*,\#}$
GPT (U/L)	39.0 ± 7.1	$83.4 \pm 11.0^{**}$	$97.8 \pm 10.5^{**}$	$52.0 \pm 7.7^{*,\#}$

[†]Each value represents the mean ± SEM of five rats.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus normal control.
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus DMN-induced fibrosis/PBS group.

renal cortex or spleen. To precisely assess the severity of liver disease, some quantitative methods and tools have been developed. Matsuhashi and colleagues found that the speed of the sound is useful for diagnosing fatty liver and cirrhosis [6]. Guimond et al. confirmed that quantitative ultrasonic tissue characterization can be used as a tool to continuously monitor chronic liver remodeling in mice [3]. Wang et al. suggested that stiffness measurements using acoustic radiation force can provide a quantitative assessment of the extent of fibrosis in the liver and can potentially be used for the diagnosis, management, and study of liver fibrosis [31]. Ho et al. suggested that ultrasound Nakagami imaging is a functional imaging tool to complement the use of conventional B-scanning in animal studies of liver fibrosis [32]. However, all studies mentioned above require the use of complicated or specific techniques or instruments. Due to technical advances, HFU, which refers to frequencies above 20 MHz, has become more readily available [7–10]. Fernández-Domínguez et al. reported that diagnostic HFU is an effective method for monitoring the progression of liver disease from steatosis to hepatocellular carcinoma in mice [33]. Similar to the results of their study, we found that HFU can be used as a noninvasive tool to monitor liver disease progression in rats.

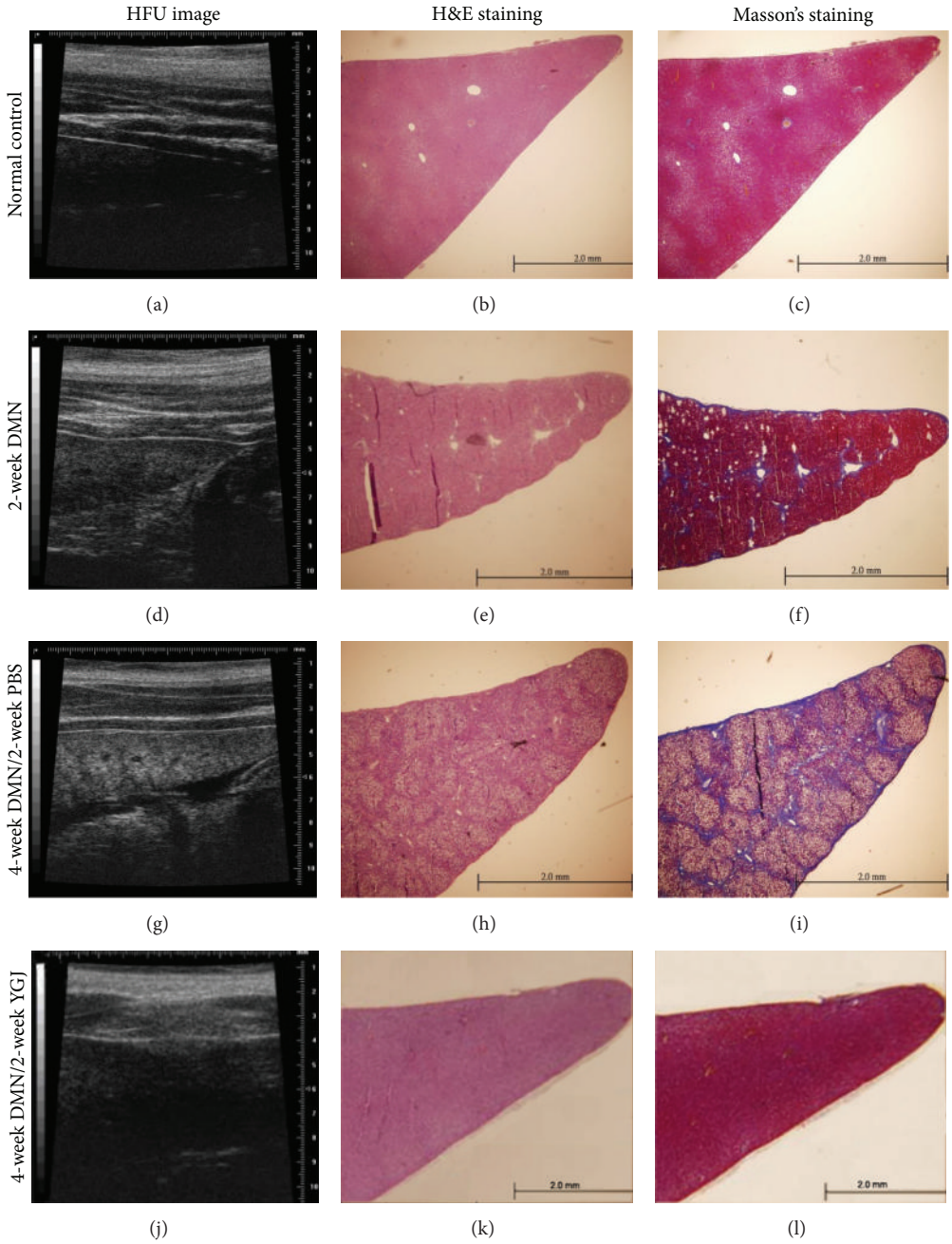

FIGURE 4: Pathological features for effects of the YGJ herbal therapy on DMN-induced liver fibrosis. ((a), (b), and (c)) The normal lateral left liver architecture with central veins and radiating hepatic cords was observed in normal control rats. ((d), (e), and (f)) Fatty degeneration, necrosis, infiltration of inflammatory cells, and apparent formation of fibrotic septa were present in the 2-week DMN-treated group. ((g), (h), and (i)) The more severer fibrotic liver architecture images were present in the 4-week DMN-treated group. ((j), (k), and (l)) The degree of liver fibrosis was significantly reduced in the YGJ-treated group. ((a), (d), (g), and (j)) Lateral left liver architectures of HFU (40 MHz) images. ((b), (e), (h), and (k)) Tissues stained with H&E, bar = 2.0 mm. ((c), (f), (i), and (l)) Tissues stained with Masson's trichrome, bar = 2.0 mm.

One study showed that both low-frequency (2–5 MHz) and high-frequency probes (5–12 MHz) were reliable and effective alternatives to the histological staging of chronic liver diseases [34]. However, our study using a 40 MHz probe showed that the images had a higher resolution than those obtained with 20 MHz probes and that it was easier to monitor the detailed changes in disease progression using the 40 MHz probe. Another advantage of the 40 MHz probe is that the rat's spleen can be easily detected. When using a 20 MHz probe, the acoustic waves are hampered by the rat's ribs.

The use of an ultrasound scoring system is a reliable method for evaluating the severity of diffuse liver disease in humans and small animals [5, 35, 36]. Yan et al. reported

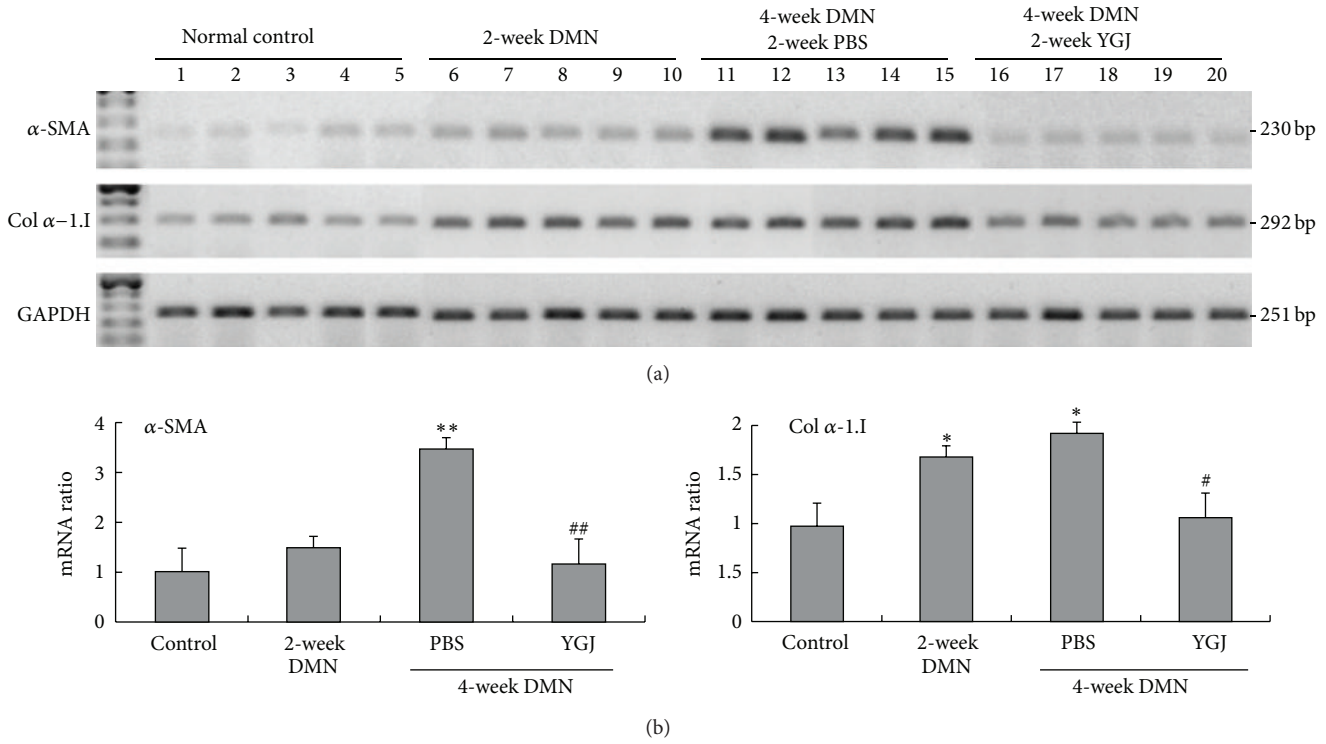

Figure 5: Fibrotic gene expression levels of *α-SMA* and Col *α-1.I* in normal control rats, DMN-induced fibrosis rats, and YGJ herb-treated rats. (a) Gene expression patterns were amplified by semiquantitative RT-PCR in four different groups including the normal control rats, 2-week DMN-treated rats, 4-week DMN-treated with 2-week PBS rats, and 4-week DMN-treated with 2-week YGJ rats ($n = 5$). (b) The expression levels of *α-SMA* and Col *α-1.I* were quantified using densitometry. Values were normalized against those for *GAPDH* mRNA expression as an internal calculation control. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the normal control group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with 4-week DMN-treated group.

that an ultrasound scoring system, especially when combined with computed tomography (CT) and serum fibrosis markers, had a higher value in the noninvasive quantitative diagnosis of hepatic fibrosis in longitudinal studies [5]. In our study, the scores were significantly different between the normal control, 2-week DMN-treated, and 4-week DMN-treated rats. To reduce the bias of the interpretation of the ultrasound images, operators have to practice using the HFU system for at least 3 weeks before initiating a study. In addition, ultrasound images were recorded and analyzed by two operators working based on consensus.

According to Chinese medicinal theory, liver disease is caused by an epidemic pathogen and dampness heat and would result in stasis of the blood [37]. When liver disease progresses to liver cirrhosis, the result is a blood deficiency. Serum activity of GOT and GPT are the most commonly used biochemical markers of liver injuries [38]. The present examination of the progress of liver injury induced by a repeated administration of DMN found that both serum GOT and GPT activities markedly increased. The YGJ herbal therapy clearly repressed DMN-induced GOT and GPT activities, indicating that the YGJ extract has potent hepatoprotective effects. Hepatic hydroxyproline content, *α-SMA*, and collagen *α-1.I* gene expression measurements also demonstrated that YGJ extract treatment significantly decreased the accumulation of collagen compared with the DMN-treated and PBS-administrated groups. The pathological changes of liver tissue obviously improved with YGJ extract treatment, indicating the inhibitory effect of the YGJ extract on hepatic fibrosis.

5. Conclusions

It is evident that HFU provides higher-resolution images for monitoring the progression of liver disease in rats. The use of HFU may reduce the number of invasive procedures required and reduces the number of sacrificed animals. In addition, the ultrasound scoring system based on HFU is useful for detecting, grading, and monitoring the progression of liver disease in rats, and the results of this system correlate well with pathological findings. We also demonstrated that the primary mechanism of YGJ therapeutic effect could be protection against hepatic injury via reduced serum levels of GOT and GPT as well as reduction of the activated HSCs via downregulation of *α-SMA* and collagen *α-1.I* mRNA expressions. It provides scientific evidence for the clinical use of YGJ extract in treating liver fibrosis and cirrhosis.

Conflict of Interests

The authors have declared that no competing interests exist.

Authors' Contribution

Wei Chen, Jiun-Yu Chen, Yu-Tang Tung, and Hsiao-Ling Chen contributed equally to this study.

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Research Article

Hepatoprotective Potential of Chestnut Bee Pollen on Carbon Tetrachloride-Induced Hepatic Damages in Rats

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Bee pollen has been used as an apitherapy agent for several centuries to treat burns, wounds, gastrointestinal disorders, and various other diseases. The aim of our study was to investigate the hepatoprotective effects of chestnut bee pollen against carbon tetrachloride (CCI_4)-induced liver damage. Total phenolic content, flavonoid, ferric reducing/antioxidant power, and DPPH radical activity measurements were used as antioxidant capacity determinants of the pollen. The study was conducted in rats as seven groups. Two different concentrations of chestnut bee pollens (200 and 400 mg/kg/day) were given orally and one group was administered with silibinin (50 mg/kg/day, i.p.) for seven days to the rats following the CCI_4 treatment. The protective effect of the bee pollen was monitored by aspartate transaminase (AST) and alanine transaminase (AST) activities, histopathological imaging, and antioxidant parameters from the blood and liver samples of the rats. The results were compared with the silibinin-treated and untreated groups. We detected that CCI_4 treatment induced liver damage and both the bee pollen and silibinin-treated groups reversed the damage; however, silibinin caused significant weight loss and mortality due, severe diarrhea in the rats. The chestnut pollen had showed 28.87 mg GAE/g DW of total phenolic substance, 8.07 mg QUE/g DW of total flavonoid, 92.71 mg Cyn-3-glu/kg DW of total anthocyanins, and 9 mg β-carotene/100 g DW of total carotenoid and substantial amount of antioxidant power according to FRAP and DPPH activity. The results demonstrated that the chestnut bee pollen protects the hepatocytes from the oxidative stress and promotes the healing of the liver damage induced by CCI_4 toxicity. Our findings suggest that chestnut bee pollen can be used as a safe alternative to the silibinin in the treatment of liver injuries.

1. Introduction

Apitherapy has been used in folk medicine since the early ages of human beings, and in the recent years, its application in the treatment of burns, wounds, gastrointestinal disorders, ulcers, and carcinogenesis has been increasing. Bee pollen is one of the richest and purest natural foods that way ever discovered; the tremendous nutritional and medicinal value of the pollen has been used for centuries. Bee pollen is a perfectly balanced food and is rich in amino acids, proteins, hormones, enzymes, carbohydrates, minerals, fats, a considerable amount of vitamins, phenolic

substances, phytochemicals, and significant quantities of antioxidant agents [1–3]. The chemical composition of the bee pollen depends on its botanical and geographical properties. Pollen contains about 1–5% total phenolic substances, which include different subtypes such as flavonoids, phenolic acids, anthocyanins, and tannins. They exhibit a wide range of biological activities including antioxidant, antimicrobial, anti-inflammatory, antiatherogenic, anticarcinogenic, and antithrombotic activities [1–4]. Phenolic compounds are considered to be beneficial for human health since they decrease the risk of degenerative diseases caused by oxidative stress. Many researchers have demonstrated that the phenolic compounds within the pollens inhibit the occurrence and development of numerous degenerative disorders [2].

Carbon tetrachloride (CCI_4) is a hepatotoxic agent that enhances the formation of free radicals, which cause lipid peroxidation of cellular and organelle membranes [5], thus one of the most widely used toxic agents to induce liver diseases in animal models. CCI_4 is metabolized by cytochrome p450 system to highly reactive trichloromethyl free radicals and reactive oxygen species, which initiate lipid peroxidation and necrosis. In addition, the toxic agent causes inflammatory response initiated by the activated hepatic macrophages, mainly Kupffer cells [6]. Many researchers have pointed out that CCI_4 exposure could cause significant biochemical disorders such as fatty liver, hepatitis, and cirrhosis in laboratory animals [7, 8].

It is recognized that hepatocyte damage is one of the serious pathological disorders for human. To measure aspartate transaminase (AST) and alanine transaminase (ALT) activities in the serum or plasma is the simplest method to diagnose hepatocyte injuries [3, 9–11]. Oxidative stress was shown as one of the major causes of liver injury [11]. Silibinin or silybin is the active component of silymarin and commonly used for the treatment of various liver damages. Silymarin is obtained through the extraction of milk thistle seeds (*Silybum marianum*), which is rich in flavonoids, and used as a hepatoprotective drug for several decades. It has been shown that the bee pollen, which contains many phenolic substances, has similar effect as silibinin in terms of hepatoprotection [1, 2, 12]. For this purpose, many natural extracts and honey bee products, such as honey and pollen, were used to treat hepatic disorders in laboratory animals [3, 4, 9, 10].

In this study, we determined the therapeutic effects of chestnut bee pollen on the CCI_4-induced liver damage in the rat model. We analyzed the chemical and antioxidant properties of the chestnut pollen and determined that pollen supplementation recovered the body weight, AST and ALT enzyme levels, malondialdehyde (MDA), and superoxide dismutase (SOD) levels as well as decreased the histological damage and apoptosis at the hepatocytes following the CCI_4 treatment. We detected that both the bee pollen- and silibinin-treated groups reversed the CCI_4-induced hepatic damage; however, silibinin caused significant weight loss and mortality due to severe diarrhea in the rats. Our results suggest that chestnut pollen can be used as a safe alternative for the treatment of liver damaging diseases.

2. Material and Methods

2.1. Reagents. CCI_4, silibinin, gallic acid, quercetin, ethyl alcohol, methanol, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,4,6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), cyanidin-3-O-glucoside, TBA, 1,1,3,3-tetramethoxypropane, β-carotene, nitroblue tetrazolium, xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). AST and ALT diagnostic kits were also purchased from Sigma. Olive oil was obtained from KOMİLİ Sızma Company (İzmir, Turkey).

2.2. Bee Pollen Samples. Pollen samples were obtained from the expert beekeepers of Zonguldak, Turkey (Western Black Sea area) in 2008 flowering season. The samples were dried at 40°C oven and palynological identification was done by Dr. Sibel Silici of Erciyes University, Turkey. Nine families of pollen pellets were found in the sample: Fabaceae (*Medicago* spp., *Trifolium* spp.), Fagaceae (*Castanea sativa* L.), Asteraceae (*Aster* spp., *Cirsium* spp., *Carduus* spp.), Apiaceae (*Apium* spp.), Caryophyllaceae (*Dianthus* spp.), Poaceae (*Zea may*), Rosaceae (*Malus* spp.), Myrtaceae (*Myrtus communis*), and Rhamnaceae (*Rhamnus cathartica*). Chestnut *sativa* was dominant (>45%) in the pollen mixture.

For the analysis of antioxidant potential of the pollen, the samples were prepared by mixing 1 g of dried powder of pollen sample with 10 mL methanol in a flask attached condenser, then sonicated in a sonicator apparatus (Elma Transsonic Digital, Germany). After 3 h sonication, the extract was used for antioxidant tests. Although we dissolved the pollen in sterile H_2O to feed the animals by gavage, it has been shown that the antioxidant potential of the pollen is revealed with better percentage when it is extracted using methanol [13, 14].

Chemical analysis of the bee pollen was performed according to the method described at AOAC [15], and the values were calculated as per dried pollen weight (DW). In addition, the following potential antioxidant properties were measured according to the references mentioned: total phenolic content [16], total flavonoids [17], and total anthocyanins [18], total carotenoids [17], total antioxidant activity according to the ferric reducing antioxidant power (FRAP) [19] and to free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) [20]. FRAP values were expressed as Trolox equivalent antioxidant power, and radical scavenging activity of DPPH was expressed as SC_{50}, which represents the concentration of the extract (mg/mL) required to inhibit the 50% of the free radical scavenging activity. The lower SC_{50} value indicates the higher antioxidant activity.

2.3. Animals and Experimental Procedure. Forty-nine Sprague-Dawley rats were studied, which were 12 weeks old and of 250–300 g approximate weight. Animals were fed with standard rat feed and allowed to drink water. Animals were kept in temperature controlled (20–25°C) cages with 12 h dark and 12 h light cycles. *Food* was withdrawn for 12 h before the experiments.

Animals were divided into seven groups (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/461478). The first group (G1–G3) was used as controls and other experimental groups. The control groups received 0.8 mL of saline (0.9% v/v) in water [21], olive oil (0.8% v/v) and ethanol (0.2% v/v) once per day for the entire period (7 days) by i.p. injection, respectively. The rats in G4 to G7 were administered i.p. with CCl_4 dissolved in olive oil at a dose of 0.85 mL/kg/day body weight. G5 was fed with silibinin dissolved in ethanol at a dose of 50 mg/kg/day. G6 and G7 were fed with pollen at 200 mg/kg and 400 mg/kg once per day by gavage, respectively. In this study, dried pollen samples were dissolved in deionized water. This study was approved by the Animal Care and Use Committee from the Faculty of Medicine in Karadeniz Technical University (KTU).

2.4. Determination of Liver Enzyme Activities and Proteins. Plasma was obtained from the whole blood samples of the treated rats through centrifugation at 2000 ×g for 10 min and stored at −20°C until analysis. AST and ALT activities were measured by a Roche Diagnostics Modular Analyzer using the manufacturer's commercial kits according to the instructions (Roche Diagnostics GmbH, D-68298, and Mannheim, Germany).

Reduction of nitroblue tetrazolium by xanthine-xanthine oxidase system was used to measure superoxide dismutase (SOD) activity in erythrocyte hemolysate of the rat blood samples. Formazan formation was examined at 560 nm using the spectrophotometer (Beckman-coulter, DU 530). The enzyme activity that causes 50% inhibition was regarded as one unit using bovine erythrocytes SOD as standard, and the results were read as U/g Hb [22].

MDA levels were measured with a colorimetric test with thiobarbituric acid (TBA) which is used to assess endogenous lipid [23]. Fresh tissue samples obtained from the treated rats were kept at −80°C until the analysis. Liver tissues were weighed and homogenized in ice-cold 1.15% KCl. The homogenate was centrifuged at 2000 ×g for 10 min. The breakdown product of 1,1,3,3-tetramethoxypropane was used as standard, and tissue MDA levels were calculated as nmol/mL plasma or g tissue. Total protein of the liver extracts was analyzed using Lowry et al. [24] method with bovine serum albumin as the standard. The values were achieved by interpolation on a calibration standard curve at 650 nm.

2.5. Histological Preparation and Analysis. For histological analysis, liver tissue samples were fixed immediately in 10% buffered formaldehyde, dehydrated with ethanol series, cleaned with xylene, embedded in paraffin, and sectioned as 5 μm. Tissue sections were stained with hematoxylin and eosin (H&E) then examined under a light microscope (Olympus BX-51; Olympus Optical Co, Ltd, Tokyo, Japan). All liver tissue slides were examined at high magnification and images were recorded by an independent histologist. Five high-power fields were selected by random sampling and the following criteria were followed in semiquantification of the liver injuries: hepatocyte degeneration, vascular congestion, sinusoidal dilatation, congestion in enlarged sinusoids, and fatty degeneration. Each specimen was marked using a scale of 0 to 3 (0 = none, 1: mild, 2: moderate, and 3: severe). The mean histologic score was calculated for each group.

2.6. TUNEL Analysis. For the detection of apoptotic cells within our groups (G1–G7), 4 μm thick serial sections were prepared from the paraffin-embedded liver samples. For TUNEL analysis, *In situ* Cell Death Detection Kit (Roche, Mannheim, Germany) was used in accordance with the manufacturer's instructions to detect the fragmented DNA associated with apoptotic cells and clearly visible nuclear fragments or sharp and condensed chromatic masses or crescent in the nuclei. The stained sections were evaluated under a light microscope (Olympus BX51 microscope, Tokyo, Japan) at ×400 magnification. One hundred cells were counted per liver slide in five microscopic fields. The percentage of TUNEL-positive apoptotic cells was calculated and this represented the apoptotic index (AI). An independent histologist examined the stained specimens in a blinded fashion [11].

2.7. Statistical Analysis. The results were presented as mean values and standard deviations. Data and regression analyses were performed via Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA). Data were tested using SPSS (version 9.0 for Windows 98, SPSS Inc.). Statistical analyses of the results were based on Kruskal-Wallis test and Pearson correlation analysis, which is a nonparametric test. The significance of the differences was statistically considered at the level of $P < 0.05$.

3. Results and Discussion

This study investigated the hepatoprotective potential of aquatic extracts of chestnut bee pollen on CCl_4-induced hepatic damages in rats. Before the treatments, some chemical and antioxidant properties of the pollen were investigated, which are summarized in Table 1. The main components of the pollen were protein, starch, fat, minerals, and water. The chestnut pollen has approximately 28.87 mg GAE/g of total phenolic compounds (TPC), 8.07 mg QUE/g of total flavonoids, 92.71 mg Cyn-3-glu/kg of total anthocyanins, and 29 mg β-carotene/100 g of total carotenoids. These compounds are well established for their responsibility of the antioxidant activities of the natural honeybee products [1, 25]. The chestnut pollen also demonstrated substantial antioxidant activity, which is measured through ferric reducing antioxidant power (FRAP) and DPPH radical scavenging activity (Table 1). Although there are other honeybee products with higher bioactivity, such as royal jelly and propolis, chestnut pollen was used in this study due to its water solubility and relatively higher antioxidant capacity. Cheng et al. [26] recently studied the antioxidant and hepatoprotective effects of *Schisandra chinensis* pollen extract (SCPE) on CCl_4-induced acute liver damage in mice and found that SCPE had strong antioxidant activities and significant protective effect against the acute hepatotoxicity induced by CCl_4, which was also supported by the evaluation of liver histopathology in

Table 1: Chemical and antioxidant properties of the investigated honeybee pollen of chestnut.

(a)

Chemical properties				
Moistures %	Ash %	Protein %	Starch %	Fat %
14.42 ± 0.42	2.23 ± 0.21	23.67 ± 2.08	6.83 ± 6.83	5.87 ± 2.39

(b)

Antioxidant properties					
Total phenolic content (mg GAE/g DW)	Total flavonoid (mg QUE/g DW)	Total anthocyanins (mg Cyn-3-glu/kg DW)	Total carotenoid (mg β-carotene/100 g DW)	FRAP (mM Trolox/g DW)	DPPH SC_{50} (mg/mL)
28.87 ± 2.48	8.07 ± 0.83	92.71 ± 25.21	29 ± 9.0	82.31 ± 2.41	0.62 ± 0.10

mice. Furthermore, in another recent study, Tohamy et al. [27] studied the antioxidant capacities of the water extracts of the Egyptian bee pollen (WEBP) on cisplatin (CDDP)-induced damages in multiple organs and found that WEBP was more potent in the recovery of hepatic and testicle damages.

The phenolic compounds of the pollens vary depending on the geographic characteristics and the flora of the region [1]. Recently, Ulusoy and Kolayli [13] studied the Anzer pollen from the Black Sea Region of Turkey and found that TPC of Anzer pollen ranged from 44.07 to 124.10 mg GAE/g, which is higher than the chestnut pollen. The TPC of the bee pollen was also studied by Mărghitaş et al. [28] using twelve types of pollen collected from Romania. In this study, the TPC ranged from 6.40 to 16.4 mg GAE/g dry matter of pollen, and Salix type of pollen showed the highest content of total phenolic and antioxidant activity. In another study, the phenolic content (polyphenolics, flavonols, flavones, and flavonones) and the antioxidant activity in six varieties of bee pollen from Sonoran Desert were studied [14]. They found that the phenolic content ranged between 15.91 and 34.85 mg GAE/g pollen, and a great variation was observed among the pollen samples. There was no previous study found about the phenolic content and antioxidant activity of chestnut pollen to make comparison with our results; however, there are a few studies about chestnut honey that contains crucial amounts of phenolic substances that are related to biological activities, such as antioxidant, antimicrobial, and anti-inflammatory features [29, 30].

We measured the initial weight of the 49 rats that we studied in 7 groups (G1–G7) and compared their weights at the end of the 7th day (Table 2; Supplementary Table 1). While the rats in the first two control groups of physiological saline (G2) and olive oil (G3) increased in weight (0.7%), the ethanol group (G3) decreased in weight (5.5%). Meanwhile, weight loss was detected in all of the CCl_4-injected groups (G4–G7); the untreated CCl_4 control group (G4) and silibinin-treated group (G5) showed the highest weight loss with −9.5% and −8.2%, respectively (Table 2). In addition, the significant weight loss in the silibinin group was due to severe diarrhea and caused mortality of 1 rat (out of 7) within the group (G5). Meanwhile, the weight loss was the least amount in the pollen-treated groups (G6-G7). The more the amount of pollen fed, the less the weight loss in the rats. This suggests that the bee pollen acted as an efficient nutrition supplement in the CCl_4-exposed rats, without any substantial side effect and mortality.

The activities of plasma AST and ALT enzymes were significantly elevated in rats treated with CCI_4 due to toxic effect (Table 3). Although the enzyme activities were not changed in the control groups (G1–G3), a significant increase in the CCI_4-treated groups (G4–G7) was observed. AST and ALT enzyme activities were markedly increased in all the CCI_4 administered groups, especially 4-5 times increased in the G4 (CCI_4-only group) than the control groups (G1–G3). In case of the intake of silibinin and the bee pollen were reduced both enzyme activities. ALT and AST enzymes are specific to liver damage; they are used routinely for the determination of liver injuries [31]. When the bee pollen was administered at higher doses of 400 mg/kg/day, AST and ALT levels were significantly decreased. In the present study, the silibinin-treated (50 mg/kg/day) rats (G5) showed better improvement than the low-dose pollen-treated (200 mg/kg/day) group (G6); however, as a critical side effect, severe diarrhea and weight loss were detected in the silibinin-treated group. Furthermore, the improvement of AST and ALT levels within the pollen-treated groups (G6 and G7) correlated with the concentration of the pollen given to the rats.

Among the apitherapy studies related to the prevention and protection of liver damage and toxicity, Türkez et al. [10] showed that propolis extracts inhibited the liver damage induced by $AlCl_3$ treatment in the mouse model. Similarly, Liu et al. (2012) [9] reported that quercetin, an antioxidant substance found in honeybee products, prevented liver injury from lead-induced oxidation. Kolankaya et al. [32] showed that the propolis of *Castanea sativa* showed a protective effect against alcohol-induced liver damage. El-Denshary et al. [33] indicated that dietary honey consumption reduced the hepatotoxicity against CCI_4-induced liver damage. Recently, there are some animal model studies that showed that many honeybee products exert curative potential for the protection of hepatocytes from oxidative damages [3, 10, 31, 32].

Malondialdehyde (MDA), a lipid peroxidation product, was measured in plasma, erythrocyte, and liver tissues of the treated animals to analyze the damage caused by oxidative stress induced via CCI_4 treatment. The amount of MDA in liver tissue serves as an indicator of lipid peroxidation,

Table 2: Experimental results of transaminase enzymes and antioxidant parameters in the plasma ($n = 7$). Data were given as mean ± SD.

Groups	Weight gain %[(1)]	Plasma				Erythrocytes		Liver	
		AST U/L	ALT U/L	MDA nmol/mL plasma	SOD U/mg protein	MDA nM/g Hb	SOD U/g Hb	MDA nmol/g tissue	SOD U/g liver
G1 (control)	+0.7 (±1.07)[b]	171.57 ± 21.74[a]	85.86 ± 14.31[a]	0.50 ± 0.02[a]	729.30 ± 30[c]	0.55 ± 0.3[a]	62.93 ± 31.5[bc]	4.85 ± 0.2[a]	540.10 ± 10.3[c]
G2 (control)	+0.7 (±1.07)[b]	207.14 ± 20.59[a]	86.57 ± 8.90[a]	0.51 ± 0.01[a]	750.10 ± 25[c]	0.53 ± 0.3[a]	80.93 ± 68.1[c]	5.82 ± 0.4[a]	520.20 ± 19.2[c]
G3 (control)	−5.4 (±0.946)[a]	145.50 ± 21.45[a]	44.50 ± 11.47[a]	0.53 ± 0.03[ab]	700.65 ± 45[c]	0.55 ± 0.1[a]	46.12 ± 19.5[ab]	5.95 ± 0.5[a]	515.15 ± 23.1[c]
G4 (CCl_4)	−9.5 (±0.905)[a]	892.57 ± 29.11[d]	474.86 ± 134.76[d]	0.57 ± 0.08[b]	260.25 ± 15[a]	0.96 ± 1.3[a]	15.42 ± 21.7[a]	22.62 ± 2.7[e]	260.50 ± 42.1[a]
G5 (silibinin)	−8.2 (±0.918)[a]	518.80 ± 122[b]	218.80 ± 73.25[b]	0.53 ± 0.02[ab]	305.90 ± 40[b]	0.67 ± 0.3[a]	15.26 ± 14.1[a]	8.30 ± 0.8[b]	290.60 ± 23.2[b]
G6 (low pollen)	−6.7 (±0.933)[a]	690.83 ± 149[c]	383.40 ± 93.02[c]	0.53 ± 0.03[ab]	265.30 ± 30[a]	0.49 ± 0.3[a]	14.74 ± 6.6[a]	14.82 ± 1.5[d]	270.20 ± 12.3[bc]
G7 (high pollen)	−1.6 (±0.984)[ab]	464.0 ± 69.02[b]	277.00 ± 49.62[b]	0.52 ± 0.01[a]	270.25 ± 20[ab]	0.59 ± 0.5[a]	17.72 ± 8.7[a]	10.94 ± 0.9[c]	310.10 ± 14.4[b]

[(1)]Body weight (B.W.) changes during the experiments (mean ± SD); a positive (+) value shows an increase of the body of the rat whereas a negative value (−) shows decrease of the body of the rat.

[a]Values are significantly different from G4, G5, G6, and G7 ($P < 0.05$).

[b]Values are significantly different from those of controls (G1–G3) ($P < 0.05$).

[c]Values are significantly different from those of silibinin (G5) ($P < 0.05$).

[d]Values are significantly different from G7 (400 mg pollen/kg B.W.) ($P < 0.05$).

Table 3: Histologic analysis and scoring of liver sections of the treated and untreated rats.

Groups	Hepatocyte degeneration	Vascular congestion	Sinusoidal dilatation	Congestion in enlarged sinusoids	Fatty degeneration	Apoptosis index (AI)
G1 (control)	0.43 ± 0.535^{a}	0.57 ± 0.535^{ab}	0.29 ± 0.488^{a}	0.29 ± 0.488^{a}	0.0 ± 0.0^{a}	3.57 ± 2.15^{a}
G2 (control)	0.43 ± 0.535^{a}	0.71 ± 0.488^{abc}	0.86 ± 0.378^{ab}	0.29 ± 0.488^{a}	0.0 ± 0.0^{a}	4.71 ± 1.70^{a}
G3 (control)	0.29 ± 0.488^{a}	0.43 ± 0.535^{a}	0.71 ± 0.488^{ab}	0.29 ± 0.488^{a}	0.0 ± 0.0^{a}	3.86 ± 1.68^{a}
G4 (CCI_4)	2.71 ± 0.488^{c}	0.71 ± 0.488^{abc}	1.57 ± 0.535^{c}	0.86 ± 0.378^{b}	3.00 ± 0.0^{c}	35.57 ± 6.35^{d}
G5 (silibinin)	2.00 ± 0.577^{b}	1.14 ± 0.69^{bcd}	1.29 ± 0.756^{bc}	0.71 ± 0.488^{ab}	1.86 ± 0.69^{b}	20.0 ± 3.16^{c}
G6 (low pollen)	2.14 ± 0.378^{b}	1.43 ± 0.535^{d}	1.29 ± 0.756^{bc}	0.86 ± 0.378^{b}	2.14 ± 0.69^{b}	21.57 ± 4.04^{c}
G7 (high pollen)	1.71 ± 0.488^{b}	1.29 ± 0.488^{cd}	1.29 ± 0.488^{bc}	0.71 ± 0.488^{ab}	1.71 ± 0.756^{b}	15.86 ± 3.29^{b}

The histological score was calculated using a scale from 0 to 3, 0: none, 1: mild, 2: moderate, and 3: severe.
[a]Values are significantly different from G4, G5, G6, and G7 ($P < 0.05$).
[b]Values are significantly different from those of controls (G1–G3) ($P < 0.05$).
[c]Values are significantly different from those of silibinin (G5) ($P < 0.05$).
[d]Values are significantly different from G7 (400 mg pollen/kg B.W.) ($P < 0.05$).

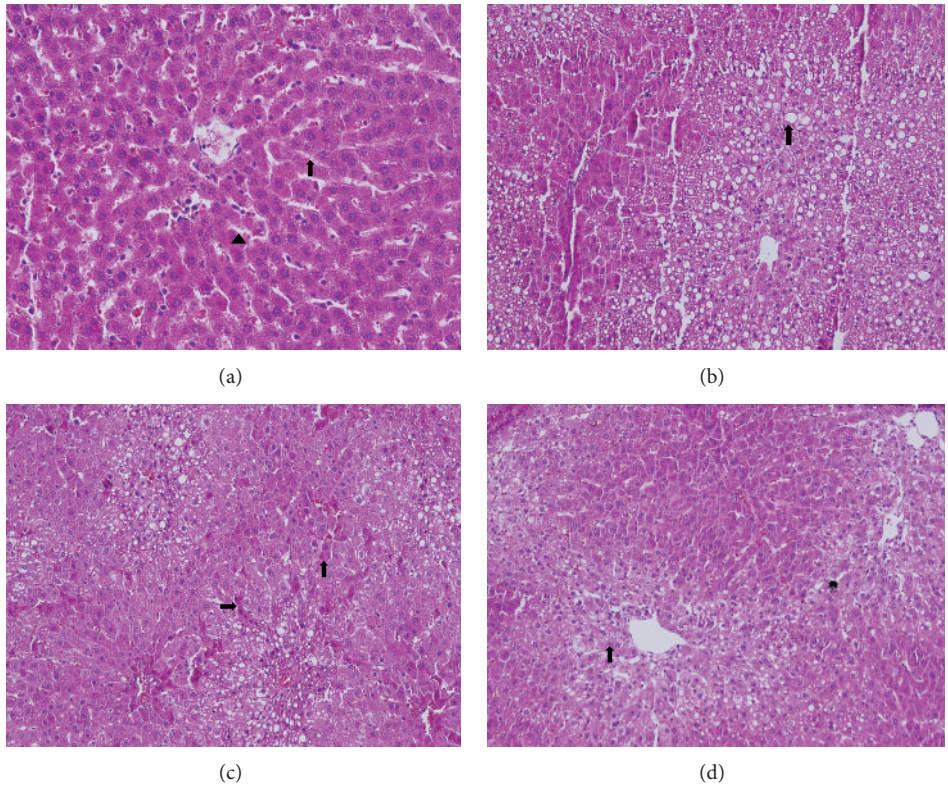

Figure 1: Histopathological analysis of treated and untreated liver sections. (a) Normal hepatocytes (↑) and sinusoids (▲) in the control group, untreated CCI_4 (H&E ×100). (b) Destroyed group with CCI_4 (G4), increased fatty degeneration (↑). (c) Pollen-treated group (G6) with 200 mg/kg, decreased fatty degeneration and regeneration in hepatocytes (↑). (d) Pollen-treated group (G7) with 400 mg/kg pollen, fatty degeneration markedly decreased (↑).

which is a well-known occurrence in the liver injury due to generation of reactive species [34]. Within the liver tissues, there was not a significant alteration detected in the MDA levels of the control groups (G1–G3), whereas the MDA level was significantly increased in the sick group (G4), which was almost 5 times more than G1–G3. A substantial decrease was observed in the MDA levels of the rats, which were exposed to silibinin and pollen groups (G5–G7) ($P < 0.05$). As a result of CCI_4 treatment, MDA levels were increased in the plasma, erythrocytes, and liver, which have demonstrated that CCI_4 caused lipid peroxidation. Liver plays a major role in the metabolism of xenobiotic; thus, it is vulnerable to many compounds, which are either toxic as themselves or produce metabolites that can cause liver damage [9, 34]. The efficiency

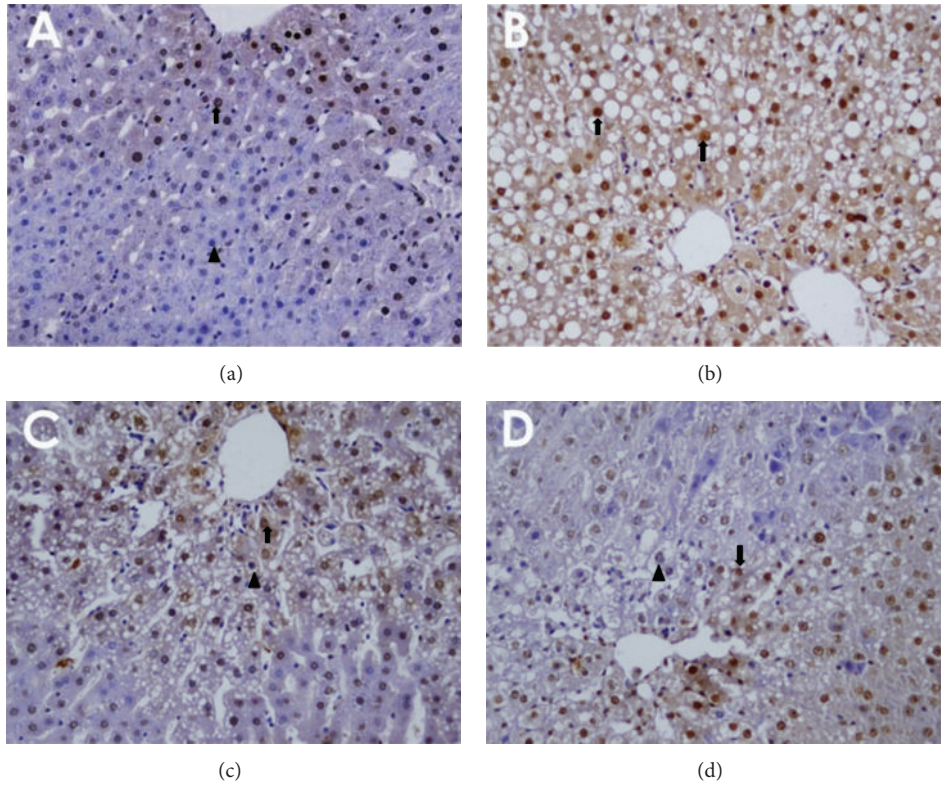

Figure 2: Results on apoptosis analysis (TUNEL) of CCI_4-induced apoptosis in primary rat hepatocytes, apoptotic hepatocytes (↑), and normal hepatocytes (▲) (×400). (a) Control group untreated with CCI_4 (G1), brown labeled apoptotic hepatocytes (↑), and blue labeled normal hepatocytes (▲). (b) Destroyed with CCI_4 (G4), apoptotic hepatocytes (↑). (c) Pollen-treated group (G6) with 200 mg/Kg. (d) Pollen-treated group (G7) with 400 mg/Kg.

of silibinin against oxidative stress in liver was reported in the previous studies [12]. Our results clearly showed that both the silibinin and the bee pollen are effective in the prevention of lipid peroxidation, as well as oxidative damage, which was induced by CCI_4. However silibinin caused mortality due to severe diarrhea in our rat model.

Superoxide dismutase (SOD) is an important antioxidant enzyme that protects the organism from the harmful effects of superoxide radicals formed as a result of oxidative stress [4]. In our study, there was no significant difference detected in SOD activities in plasma, liver, and erythrocyte among the control groups (G1–G3), whereas SOD activity was decreased in the CCI_4-exposed groups. The lowest SOD activity was detected in the rats exposed to CCI_4 (G4), and the activity slightly increased in the groups that were silibinin- and pollen-administered groups (G5–G7). SOD is physiologically synthesized in the liver cells similar to other antioxidant enzymes; thus, the protein synthesis is negatively affected as a result of the hepatocellular injury induced by CCI_4. However, SOD activity was increased in pollen- and silibinin-treated groups, possibly, owing to the healing of hepatocellular damage. Similar to our results, it has been shown that SOD activity was decreased notably in the mice treated with trichlorfon, which is an organopesticide, but significantly increased in the groups which were given pine honey [3]. The results of our present study are in agreement with the previous studies in terms of alterations in oxidative stress markers in response to apitherapy [3, 31].

Microscopic examination showed liver parenchyma and sinusoids of the hepatocytes were healthy in the control groups (G1–G3) (Figure 1(a)). Meanwhile, obvious damages were observed in the liver sections of the CCI_4-exposed group. Common degeneration and noticeable fatty vacuolization were observed in the hepatocytes from the sick group (G4). There was vascular congestion in the sinusoids (Figure 1(b)). A slight degree of vacuolar degeneration and fatty vacuolization were observed in the hepatocytes of G5 (silibinin), G6, and G7 (pollen-administered groups); however, the level of vacuolar degeneration of these groups was less than that of G4. And also, histological improvement of G7 (400 mg pollen/kg/day) was better than that of G6 (200 mg pollen/kg/day) (Figures 1(c) and 1(d)). The improvement was significantly relevant with the bee pollen amount given to the animals. Histological damage scoring and statistical evaluation of groups were shown in Table 3.

Hepatocyte apoptosis was analyzed using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay. The number of apoptotic nuclei was much higher in sick group (G4) than that of the control groups (Figures 2(a) and 2(b)); however, the number of apoptotic nuclei of G5–G7 (silibinin- and pollen-administered groups) was much reduced compared to the

sick group (G4) (Figures 2(c) and 2(d)). The apoptosis values in the liver and the results of the statistical analysis are shown in Table 3. Histopathological damage scoring and TUNEL analysis demonstrated that tissue damage in the liver was higher in the groups exposed to CCI_4 and found that the tissue damage was significantly reduced in the groups treated with pollen following CCI_4 exposure. The apoptosis index (AI) was significantly increased in the group that was exposed to CCI_4 without any treatment (G4) compared to the treatment groups (G5–G7) (Table 3). However, the AIs of these treatment groups were at least five times more than the control groups, which were not exposed to CCl_4 (G1–G3). This suggests that the initial exposure of the cells to CCI_4 massively increased the apoptosis of the hepatocytes; thus, the treatments of the rats with either silibinin or pollen could not completely reverse the devastating impact of CCI_4.

Experimental liver damage model is mostly achieved by exposing rats to CCI_4. It has been shown that lipid peroxidation increases and free oxygen radicals are released in the rats exposed to CCI_4 [7, 8]. Free oxygen radicals are known to be the mediators of tissue damage. Reactive oxygen radicals cause oxidative damage to cellular macromolecules such as nucleic acids, proteins, and lipids, which eventually lead to apoptosis. Free oxygen radicals cause lipid peroxidation, several metabolic disorders, and functional abnormalities through inducing membrane and DNA damages [35]. Lipid peroxidation is believed to be an important cause of destruction and damage to the cell membranes, which leads to changes in membrane fluidity and permeability. Moreover, lipid peroxidation also enhances the rate of protein degradation, which initiates the eventual lysis of the cells [11]. Our results indicated that the water pollen extracts inhibited the CCI_4-induced apoptotic cell death and hepatotoxicity. It has been reported that free oxygen radicals play important roles in CCI_4-induced cell injury [7]. When the amount of free oxygen radicals in the cell are increased, they overpower the defense systems and cause oxidative stress or cell injury, leading to development of various diseases. Thus, CCI_4-induced apoptosis is related to oxidative stress in hepatocytes and intracellular antioxidants may protect hepatocytes against cell apoptosis induced by CCI_4 [9].

4. Conclusion

CCI_4 is a hepatotoxic agent that enhances the formation of free radicals, which cause lipid peroxidation of cellular and organ damages. The chestnut bee pollen contains substantial nutrients and possesses many phenolic compounds, which are the factors of high antioxidant properties. Our study clearly shows that the chestnut bee pollen exerts highly beneficial biological activities in the protection of hepatocytes from oxidative stress and toxicity induced by CCI_4 exposure. Therefore, we conclude that the chestnut bee pollen could be safely included in the daily human diet as a food additive, which will enhance the inhibition of the oxidative stress. Chestnut bee pollen could be used as a suitable alternative to silibinin in the treatment of hepatocellular pathologies.

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Research Article

Zerumbone, a Natural Cyclic Sesquiterpene of *Zingiber zerumbet* Smith, Attenuates Nonalcoholic Fatty Liver Disease in Hamsters Fed on High-Fat Diet

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We investigated the effects of zerumbone, a natural cyclic sesquiterpene, on hepatic lipid metabolism in Syrian golden hamsters fed on high-fat diet (HFD). After being fed HFD for 2 weeks, hamsters were dosed orally with zerumbone (75, 150, and 300 mg kg^{-1}) once daily for 8 weeks. After treatment with zerumbone, the plasma levels of total cholesterol (TC) and triglycerides (TGs) and the contents of TC and TG in hepatic tissue as well as homeostasis model assessment of insulin resistance were lowered, especially in the zerumbone-treated group (300 mg kg^{-1}). Moreover, the histological evaluation of liver specimens demonstrated that the steatosis and inflammation in liver of zerumbone-treated groups were improved. Zerumbone exhibited the ability to decrease hepatic mRNA levels of sterol regulatory element-binding protein-1c and its lipogenic target genes, such as fatty acid synthase, acetyl-CoA carboxylase 1, and stearoyl-CoA desaturase 1. The hepatic mRNA expression of peroxisome proliferator-activated receptor α, together with its target genes including carnitine palmitoyl transferase-1, acyl-CoA oxidase, and acyl-CoA oxidase 1, was also upregulated by zerumbone. In conclusion, zerumbone improves insulin sensitivity, decreases lipogenesis, and increases lipid oxidation in the liver of HFD-fed hamsters, implying a potential application in the treatment of nonalcoholic fatty liver disease.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) represents a wide spectrum of diseases, ranging from simple fatty liver (hepatic steatosis) through steatosis with inflammation and necrosis to cirrhosis [1]. NAFLD, which is strongly associated with obesity, insulin resistance, and type 2 diabetes, is now well recognized as being part of the metabolic syndrome [2]. The metabolic pathways leading to the development of hepatic steatosis are multiple, including enhanced nonesterified fatty acid release from adipose tissue (lipolysis), increased *de novo* fatty acids (lipogenesis) and decreased β-oxidation. To date, caloric restriction and aerobic exercise are the effective treatments of NAFLD, but they are difficult to achieve for most NAFLD patients. Statins are the first-line agents to treat hyperlipidaemia, but there is a risk for liver injury in patients with NAFLD [3]. The most promising pharmacological treatment of NAFLD is peroxisome proliferator-activated receptor α (PPAR) α agonist, such as lipanthyl, that decreases lipid accumulation in hepatocytes [4], while the adverse effects may occur in some patients [5]. Recently, sterol regulatory element-binding protein-1c (SREBP-1c) has been highly evaluated as a potential target for the treatment of NAFLD, based on its advances to control lipogenic gene expression and regulate fatty acid and triglyceride (TG) homeostasis [6, 7]. Thus, inhibition of hepatic SREBP-1c signaling pathway could improve dyslipidemia and NAFLD. Until now, no specific pharmacological treatment for liver steatosis has been defined [8]. Therefore, the development of additional therapies for controlling lipid levels is warranted to attenuate hepatic steatosis.

Zingiber zerumbet Smith is one kind of plant growing mainly in Southeast Asia, which has been demonstrated to possess antinociceptive, anti-inflammatory, antiulcer, antihyperglycemic, and antiplatelet activities [9]. As a major compound extract, zerumbone ((2*E*,6*E*,10*E*)-2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one) is currently explored for its

potential broad use on cancers and leukemia, as well as virus infection [10–12]. Several studies have shown that zerumbone also produced a variety of pharmacological effects, including antioxidants, anti-inflammatory, and antibacterial [13, 14]. Our previous studies have indicated that *Z. zerumbet* might reduce the TG level in plasma and hepatic tissues in high-fat-diet-(HFD-) induced rats [15]; whether the effects of *Z. zerumbet* is mediated by zerumbone remained not clear.

The effects of dietary cholesterol and fat on plasma lipid profiles are similar in hamsters and humans, and fatty liver and mild diabetes were developed in hamsters after fed HFD [16]. The hamsters fed with HFD may thus be a good animal model for research on the treatment of diet-induced metabolic syndrome complicated by NAFLD [17]. Thus, the aim of this work is to assess the effects of zerumbone on preventing hepatic lipid accumulation in HFD-induced NAFLD hamsters. To gain further insights into the molecule mechanism by which zerumbone alters gene expression of hepatic lipids metabolism, the mRNA expression of SREBP-1c and its response genes were also determined. Besides the genes involved in fatty acid *de novo* synthesis, the genes involved in fatty acid β-oxidation, like PPARα and its target genes, were also measured in this study.

2. Materials

2.1. Animal Models and Treatment Protocols. Male Golden Syrian hamsters, 8-week old and weighing 90 ± 10 g, were obtained from the National Laboratory Animal Center (Taipei, Taiwan). They were maintained in a temperature-controlled room (25 ± 1°C) on a 12 h : 12 h light-dark cycle (lights on at 06:00 h) in in our animal center. Food and water were provided *ad libitum*. A regular chow diet (RCD; 10% kcal fat, no. D12450B, Research Diets, New Brunswick, NJ, USA) was used as the maintenance and control diet. A purified HFD with 45% kcal fat obtained primarily from lard (no. D12451, Research Diets) was used to induce a rapid increase in body weight and obesity [18]. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. These studies were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at Tajen University (approval number: IACUC 100-29; approval date: December 22, 2011).

After being fed a HFD for two weeks, hamsters were dosed by oral gavage once per day for eight weeks with zerumbone (≥98%; Sigma-Aldrich, St. Louis, MO, USA) doses of 75, 150, and 300 mg/kg in a volume of 1.5 mL kg^{-1} distilled water. Another group of HFD-fed hamsters was treated orally for eight weeks with 100 mg kg^{-1} per day lipanthyl (Laboratories Fournier SA, France). The dose of fenofibrate was based on study that has documented that long-term fenofibrate treatment could ameliorate hepatic insulin resistance and steatosis in high fructose-fed mice [4]. Another group of HFD- and RCD-fed rats were treated similarly, but the same volume of vehicle (distilled water) was used to prepare the tested compound solutions during the same treatment period. The water consumption, food intake, and body weight were measured once daily at the same time (09 : 00) on each day throughout the experiment. Food cups containing fresh food each time were weighed at the beginning and end of each 24 h period. Food intake was calculated by determining the difference in food cup weights, adjusting for any spillage that occurred. Water intake was calculated by measuring the difference in water bottle weights at the beginning and end of the daily change of water.

Eight weeks after treatment with zerumbone (total diet-fed period was 10 weeks), animals were weighed and anesthetized with ketamine after fasting for 12 hours. Blood samples were taken from the inferior vena cava in order to determine the plasma biomarkers. After collecting the blood, the liver was removed, rinsed with a physiological saline solution, and immediately stored at −70°C. The coefficient of hepatic weight was also calculated as liver weight (g) divided by body weight (100 g).

2.2. Determination of Metabolic Parameters and Insulin Sensitivity. Blood samples were centrifuged at 2,000 ×g for 10 minutes at 4°C. The plasma was then removed and placed into aliquots for the respective analytical determinations. Kits for determining plasma glucose (Cat. no. 10009582) concentration were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to quantify plasma insulin concentration (LINCO Research, Inc. St. Charles, MO, USA; Cat. no. EZRMI-13K). The diagnostic kits for determinations for plasma levels of total cholesterol (TC; Cat. no. 10007640) and TG (Cat. no. 10010303) were purchased from Cayman Chemical Company (Michigan, USA). The diagnostic kit for determinations for plasma levels of high density lipoprotein cholesterol (HDL-C) was purchased from Bio-Quant Diagnostics (CA, USA; Cat. no. BQ 019CR), low density lipoprotein cholesterol (LDL-C) was calculated by using Friedewald's equation [19]. Plasma free fatty acid (FFA) levels were determined using an FFA quantification kit obtained from Abcam plc (MA, USA; Cat. no. ab65341). All experimental assays were carried out according to the manufacturer's instruction. All samples were analyzed in triplicate.

Whole-body insulin sensitivity was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) by using the formula: (fasting serum glucose (mmol) times fasting serum insulin (mU mL^{-1}))/22.5 [20]. Because HOMA is negatively correlated with insulin sensitivity, low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA-IR values indicate insulin resistance.

2.3. Measurement of Hepatic Lipids. After removal from animals, sections of fresh liver samples were collected for determining the lipid content. Liver (1.25 g) was homogenized with chloroform/methanol (1 : 2, 3.75 mL), and then chloroform (1.25 mL) and distilled water (1.25 mL) were added to the homogenate and mixed well together. After centrifugation (1,500 ×g for 10 min), the lower clear organic phase solution was transferred into a new glass tube and

then lyophilized. The lyophilized powder was dissolved in chloroform/methanol (1 : 2) and stored at −20°C for less than 3 days [21]. Hepatic TC and TG levels in lipid extracts were analyzed with the diagnostic kits that were used in the plasma analysis.

2.4. Histological Staining and Oil Red O Staining. For histopathological analysis, the sections of liver were fixed in 10% formalin, dehydrated, embedded in paraffin, and stained with hematoxylin and eosin (H&E). For oil red O (ORO) staining, a stock solution of oil red O (0.5 g per 100 mL) in isopropanol was prepared, stored, and protected from light. Liver tissue was embedded in optimal cutting temperature gel. Air dried tissue sections of 5 μm were dipped in formalin, washed with ORO without counterstaining with hematoxylin. All slides were scanned at an absolute magnification of 200x using Image Pro Plus 7.0 software (Media Cybernetics) under a light microscopy (Olympus, BX51 microscope, Tokyo, Japan).

2.5. Analysis of mRNA Expression of Hepatic Genes. For analysis of gene expression, total RNA was extracted from 100 mg frozen liver samples using Trizol reagent (Invitrogen, USA). RNA was quantified by A260 and its integrity verified by agarose gel electrophoresis using ethidium bromide for visualization. For the RT reaction, 1 μg of total RNA per sample and 8.5 μg μL^{-1} random hexamer primers were heated at 65°C for 5 min and then quenched on ice. This mixture was combined with 500 μmol L^{-1} each of dATP, dTTP, dCTP, and dGTP, 10 mmol L^{-1} DTT, 20 mmol L^{-1} Tris-HCl (pH 8.4), 50 mmol L^{-1} KCl, 5 mmol L^{-1} $MgCl_2$, 40 units of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Boston, MA, USA), and 100 units SuperScript III reverse transcriptase (Invitrogen). Samples were subjected to DNase (Promega, Madison, WI, USA) treatment at 37°C for 20 min in a GeneAmp 9700 Thermal Cycler (Applied Biosystems, California, USA) and then held at 4°C. After aliquots were taken for immediate use in the PCR, the remainder of the cDNA was stored at −20°C. The mRNA expressions were measured by quantitative real-time RT-PCR in a fluorescent temperature Lightcycler 480 (Roche Diagnostics, Mannheim, Germany). The SREBP-1c primers sequences were as follows: forward, 5′-CGCTACCGTTCCTCTATCAA-3′; reverse, 5′-TTCGCAGGGTCAGGTTCTC-3′. The acetyl-CoA carboxylase 1 (ACC1) primers were as follows: forward, 5′-GGACAGAC1GATCGCAGAGAAAG-3′; reverse, 5′-TGGAGAGCCCCACACACA-3′. The fatty acid synthase (FAS) primers were as follows: forward, 5′-GGAACTGAACGGCATTACTCG-3′; reverse, 5′-CATGCCGTTATCAACTTGTCC-3′. The stearoyl-CoA desaturase (SCD)1 primers were as follows: forward, 5′-CCTTAACCCTGAGATCCCGTAGA-3′; reverse, 5′-AGCCCATAAAAGATTTC1GCAAA-3′. The PPARα primers sequences were as follows: forward, 5′-GAAGCAGATGACCTGGAAAGT-3′; reverse, 5′-AGCCTGGACAGCTCCCTAA-3′. The carnitine palmitoyl transferase (CPT)-1 primers sequences were as follows: forward, 5′-GCTTCCCCTTACTGGTTCC-3′; reverse, 5′-AACTGGCAGGCAATGAGACT-3′. The acyl-CoA oxidase (ACO) primers sequences were as follows: forward, 5′-ACTATATTTGGCCAATTTTGTG-3′; reverse, 5′-TGTGGCAGTGGTTTCCAAGCC-3′. The acyl-CoA oxidase 1 (ACOX1) primers sequences were as follows: forward, 5′-GTTGATCACGCACATCTTGGA-3′; reverse, 5′-TCGTTCAGAATCAAGTTCTCAATTTC-3′. The tumor necrosis factor (TNF)-α primers sequences were as follows: forward, 5′-ACACCATGAGCACGGAAAGC-3′; reverse, 5′-CCGCCACGAGCAGGAA-3′. The interleukin (IL)-1β primers sequences were as follows: forward, 5′-AATGGACAGAACATAAGCCAACA-3′; reverse, 5′-CCCAAGGCCACAGGGAT-3′. The β-actin primers sequences were as follows: forward, 5′-TCACCCACACTGTGCCCATCTA-3′; reverse, 5′-TTGCTGATCCACATCTGCTGG-3′. Primers were designed with Primer Express Software version 2.0 System (Applied Biosystems, Foster City, CA, USA). T PCR reaction was performed following the cycling protocol of 95°C for 5 min, followed by 45 PCR cycles with 95°C for 5 s, 58°C for 15 s and 72°C for 20 s. Dissociation curves were run after amplification to identify the specific PCR products. The mRNA expression levels were normalized by the β-actin mRNA levels and calculated according to the delta-delta Ct method [22].

2.6. Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed with one-way analysis of variance (ANOVA). Dunnett range post hoc comparisons were used to determine the source of significant differences, where appropriate. A P value $< .05$ was considered statistically significant.

3. Results

3.1. Effects of Treatments on the Body Weight, the Relative Liver Weights, and Feeding Behaviors of Hamsters. At the end of 8-week treatment, the body weight and relative liver weights in HFD-fed hamsters were significantly increased over those of RCD-fed group (Table 1). Zerumbone significantly suppressed body weight gain at high doses (300 mg kg^{-1} per day). The coefficient of hepatic weight *i* zerumbone-treated HFD-fed hamsters (300 mg kg^{-1} per day) was significantly lower than that of the vehicle-treated group. Similar results were seen in HFD-fed hamsters treated with lipanthyl (100 mg kg^{-1} per day, Table 1). No significant differences in daily food and water intake were observed among the groups over the experimental period (Table 1).

3.2. Effects of Treatments on Plasma Lipids Levels and Insulin Sensitivity of Hamsters. The HFD caused elevated concentrations of plasma TC, TG, and LDL-C. The moderate (150 mg kg^{-1} per day) and high doses (300 mg kg^{-1} per day) of zerumbone significantly reduced plasma total TC levels (14.9% and 17.1% reduction, resp.) compared with vehicle-treated, HFD-fed hamsters (Table 1). All doses of zerumbone decreased plasma TG levels in HFD-fed hamsters (Table 1). The low, moderate, and high doses of zerumbone significantly reduced plasma LDL-C levels (6.2%, 16.7%, and 22.5% reductions, resp.; Table 1). Plasma TC, TG, and LDL-C concentrations were reduced significantly by 20.6%,

Table 1: Summary of metabolic parameters in RCD- and HFD-fed hamsters receiving 8-week treatment.

	RCD fed	HFD fed				
	Vehicle	Vehicle	Zerumbone (mg kg^{-1} per day)			Lipanthyl (100 mg kg^{-1} per day)
			75	150	300	
Body weight (BW) (g)	124.37 ± 9.28^{d}	167.90 ± 7.31^{b}	161.84 ± 8.24^{b}	157.33 ± 6.43^{b}	150.61 ± 7.51^{a,c}	141.72 ± 8.23^{d}
Food intake (g day^{-1})	11.91 ± 3.26	12.08 ± 4.31	11.82 ± 4.06	11.97 ± 3.94	12.23 ± 3.23	12.16 ± 3.17
Water intake (mL day^{-1})	12.41 ± 2.13	13.12 ± 2.69	12.51 ± 2.24	12.78 ± 2.37	12.64 ± 2.83	12.23 ± 2.46
Liver weight (g 100 g^{-1} BW)	4.63 ± 0.22^{c}	5.38 ± 0.31^{a}	5.18 ± 0.29^{a}	4.93 ± 0.34	4.85 ± 0.26	4.72 ± 0.25^{c}
Plasma glucose (mmol L^{-1})	5.56 ± 0.26^{d}	8.87 ± 0.24^{b}	8.15 ± 0.19^{b}	7.92 ± 0.21^{b,c}	7.65 ± 0.23^{b,c}	6.75 ± 0.18^{a,c}
Plasma insulin (mU)	24.01 ± 0.19^{d}	46.25 ± 0.38^{b}	41.62 ± 0.42^{b,c}	35.66 ± 0.36^{b,c}	32.09 ± 0.27^{b,c}	29.73 ± 0.35^{a,d}
HOMA-IR	5.93 ± 0.16	18.23 ± 0.31^{b}	15.07 ± 0.29^{b,c}	12.55 ± 0.27^{b,c}	10.91 ± 0.24^{b,d}	8.92 ± 0.33^{a,d}
Plasma TC (mmol L^{-1})	3.98 ± 0.18^{d}	5.37 ± 0.23^{b}	5.13 ± 0.28^{b}	4.67 ± 0.22^{b,c}	4.45 ± 0.18^{a,d}	4.26 ± 0.21^{d}
Plasma TG (mmol L^{-1})	0.43 ± 0.03^{d}	1.33 ± 0.08^{b}	1.18 ± 0.09^{b}	1.01 ± 0.07^{b,c}	0.86 ± 0.06^{b,d}	0.67 ± 0.04^{a,d}
Plasma LDL (mmol L^{-1})	2.71 ± 0.17^{d}	4.25 ± 0.21^{b}	3.99 ± 0.26^{b}	3.54 ± 0.19^{b,c}	3.29 ± 0.25^{a,d}	3.05 ± 0.13^{a,d}
Plasma HDL (mmol L^{-1})	1.17 ± 0.11^{c}	0.85 ± 0.12^{a}	0.90 ± 0.13^{a}	0.93 ± 0.16^{a}	0.98 ± 0.14	1.05 ± 0.16
Plasma FFAs (mmol L^{-1})	0.63 ± 3.1^{d}	1.30 ± 4.7^{b}	1.08 ± 3.9^{b}	0.97 ± 3.5^{b,c}	0.84 ± 4.1^{a,d}	0.76 ± 3.8^{d}
Hepatic TC (μmol g^{-1} liver)	9.57 ± 0.38^{d}	18.68 ± 0.61^{b}	16.75 ± 0.64^{b,c}	15.07 ± 0.57^{b,c}	14.23 ± 0.45^{a,c}	12.10 ± 0.53^{a,d}
Hepatic TG (μmol g^{-1} liver)	8.06 ± 0.39^{d}	15.98 ± 0.61^{b}	14.84 ± 0.57^{b}	12.55 ± 0.41^{b,c}	10.36 ± 0.55^{a,c}	9.32 ± 0.49^{a,c}

Zerumbone or lipanthyl was dissolved in distilled water for oral administration at the desired doses in a volume of 1.5 mL kg^{-1} once a day into HFD-fed hamsters. The vehicle (distilled water) used to dissolve the tested medications was given at the same volume. Values (mean ± SD) were obtained from each group of 8 animals after 8 weeks of the experimental period. $^{a}P < .05$ and $^{b}P < .01$ compared to the values of vehicle-treated RCD-fed hamsters in each group, respectively. $^{c}P < .05$ and $^{d}P < .01$ compared to the values of vehicle-treated HFD-fed hamsters in each group, respectively.

49.6%, and 28.2%, respectively, in lipanthyl-treated HFD-fed hamsters compared with vehicle-treated HFD-fed hamsters (Table 1).

The plasma concentration of HDL-C in HFD-fed hamsters was reduced to 27.3% of the level in the RCD-fed group (Table 1). After 8 weeks of treatment with zerumbone (300 mg kg^{-1} per day) or lipanthyl, the plasma HDL-C concentration in HFD-fed hamsters was elevated to nearly that of the RCD-fed group (Table 1).

Plasma FFAs were significantly higher in vehicle-treated HFD-fed hamsters compared to RCD-fed rats (Table 1). The plasma FFA level was reduced by 35.4% in HFD-fed hamsters treated with zerumbone (100 mg kg^{-1} per day) compared with their vehicle-treated counterparts (Table 1). Lipanthyl treatment reduced FFA concentrations in HFD-fed hamsters by 41.5% relative to the level in vehicle-treated HFD-fed hamsters ($P < .05$; Table 1).

HFD-fed hamster was insulin resistant reflected by hyperinsulinemia as well as significantly increased value of HOME-IR (Table 1). Treatment HFD-fed hamster with zerumbone (300 mg kg^{-1} per day) produced a similar effect to lipanthyl on insulin resistance, evidenced by reduction of fasting serum insulin levels and improved HOME-IR (Table 1).

3.3. Effects of Treatments on Hepatic Steatosis. The hepatic TC level was significantly higher in HFD-fed hamsters than in hamsters from the RCD-fed group, which was reduced by 23.8% in HFD-fed hamsters treated with zerumbone (300 mg kg^{-1} per day; Table 1). Similarly, zerumbone treatment (300 mg kg^{-1} per day) also produced a significant reduction in hepatic TG concentration to 64.8% of that in vehicle-treated, HFD-fed hamsters (Table 1). Hepatic TC and TG levels were significantly reduced (by 35.2% and 41.7%, resp.) in lipanthyl-treated HFD-fed hamsters compared with vehicle-treated counterparts (Table 1).

The photomicrographs of the H&E stain showed that HFD feeding increased hepatic fat deposits, evidenced by the majority of the hepatocytes of HFD-fed hamsters that were distended by fat in comparison to the RCD-fed group (Figures 1(a) and 1(b)). The images of H&E stain also displayed macrovesicular steatosis in the hepatocytes of HFD-fed hamsters, as many single large droplets had displaced the nucleus and ballooning degeneration causing conspicuous swelling of the cell and cytoplasmic vacuolation (Figure 1(b)). The treatment of HFD-fed hamsters with zerumbone or lipanthyl reduced fat liver depots and less macrovesicular steatosis as revealed in vehicle-treated counterparts (Figures 1(c) and 1(d)).

ORO staining on frozen liver sections exhibited many lipid droplets in liver sections of HFD-fed hamsters (Figure 2(b)), whereas few lipid droplets were seen in the liver sections from the RCD-fed (Figure 2(a)) and lipanthyl-treated HFD-fed hamsters (Figure 2(d)). Similarly, there was a decrease in the lipid content of liver tissue in zerumbone-treated HFD-fed hamsters (300 mg kg^{-1} per day) compared with vehicle-treated counterparts (Figure 2(c)). Analysis of blindly scored ORO-stained sections showed a statistically significant increase in the lipid content of liver tissue of HFD-fed hamsters (Figure 2(e)). Levels of lipids were higher in the liver of HFD-fed hamsters (58.4 ± 4.5%) compared with

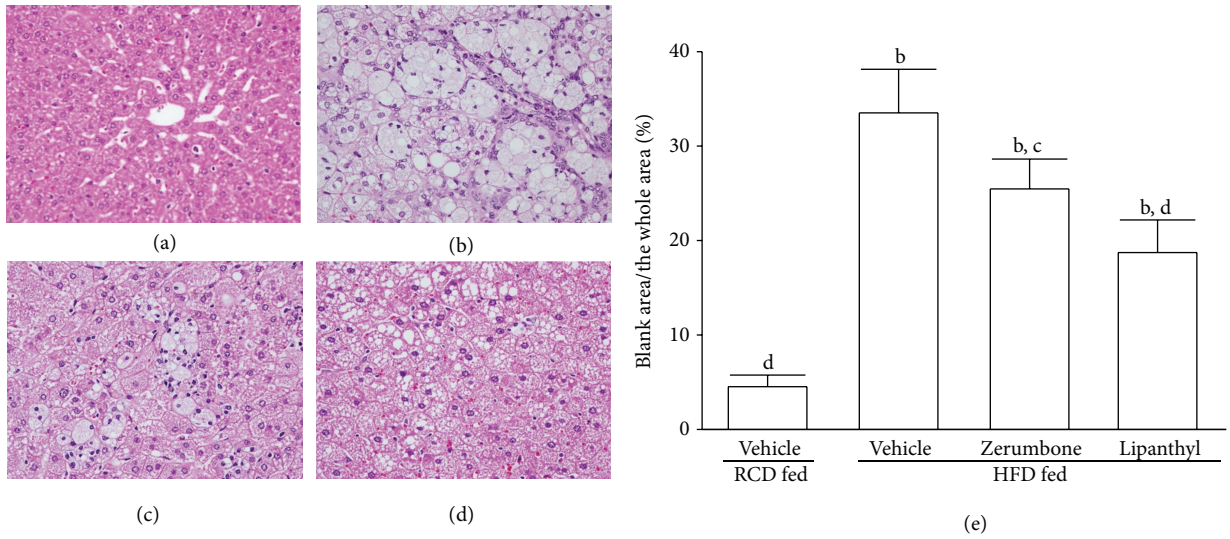

Figure 1: Representative images of H&E stain in livers from RCD- or HFD-fed hamsters receiving 8-week treatment. Photomicrographs are of tissues isolated from vehicle-treated RCD-fed hamsters (a), vehicle-treated HFD-fed hamsters (b), zerumbone-treated HFD-fed hamsters (300 mg kg^{-1} per day) (c), or lipanthyl-treated HFD-fed hamsters (100 mg kg^{-1} per day) (d). Photomicrographs were taken at a magnification of 200x. The quantification of the hepatic lipid droplets accumulation was presented as the percentage of the blank area (lipid droplets) relative to the whole area of the photomicrograph (e). Values (mean ± SD) were obtained from each group of 5 animals in each group. $^{b}P < .01$ compared to the values of vehicle-treated RCD-fed hamsters. $^{c}P < .05$ and $^{d}P < .01$ compared to the values of vehicle-treated HFD-fed hamsters in each group, respectively.

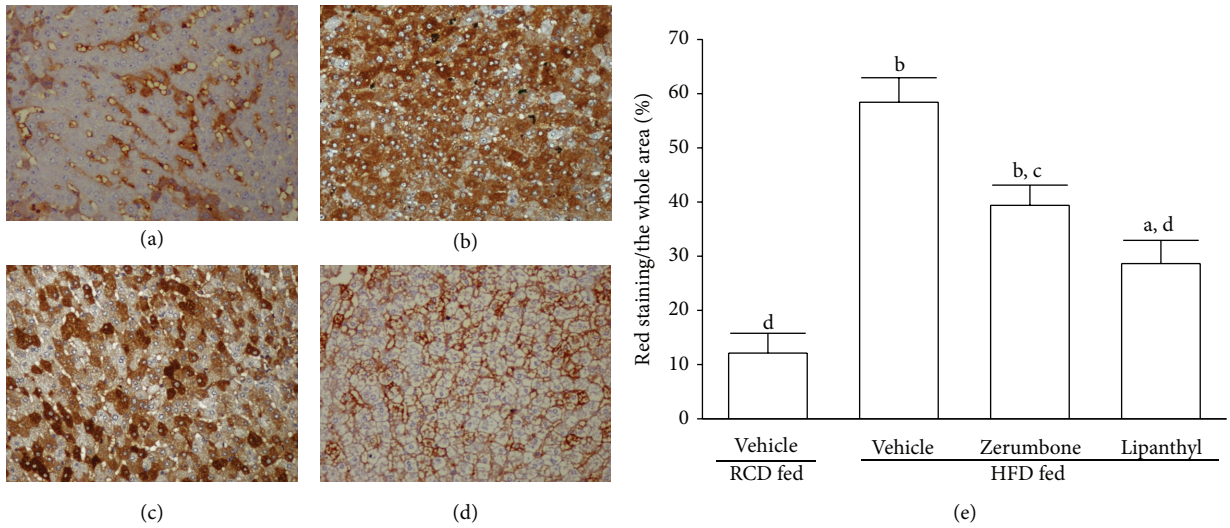

Figure 2: Representative images of ORO staining in livers from RCD- or HFD-fed hamsters receiving 8-week treatment. Photomicrographs are of tissues isolated from vehicle-treated RCD-fed hamsters (a), vehicle-treated HFD-fed hamsters (b), zerumbone-treated HFD-fed hamsters (300 mg kg^{-1} per day) (c), or lipanthyl-treated HFD-fed hamsters (100 mg kg^{-1} per day) (d). Photomicrographs were taken at a magnification of 200x. The quantification of the hepatic lipid droplets accumulation was presented as the percentage of the red staining area (lipid droplets) relative to the whole area of the photomicrograph (e). Values (mean ± SD) were obtained from each group of 5 animals in each group. $^{a}P < .05$ and $^{b}P < .01$ compared to the values of vehicle-treated RCD-fed hamsters in each group, respectively. $^{c}P < .05$ and $^{d}P < .01$ compared to the values of vehicle-treated HFD-fed hamsters in each group, respectively.

those in RCD-fed group (12.1 ± 3.6%). Hepatic lipids values were reduced significantly by 32.6%, and 50.7%, respectively, in zerumbone-treated HFD-fed hamsters (300 mg kg^{-1} per day) or lipanthyl-treated HFD-fed hamsters compared with vehicle-treated counterparts.

3.4. Effects of Treatments on Hepatic mRNA Expression of SREBP-1c and Its Lipogenic Target Genes in Hamsters. HFD feeding markedly increased the hepatic mRNA levels of SREBP-1c in hamsters to 2.2-fold relative to those in RCD-fed group (Figure 3(a)). Hepatic mRNA levels of SREBP-1c were

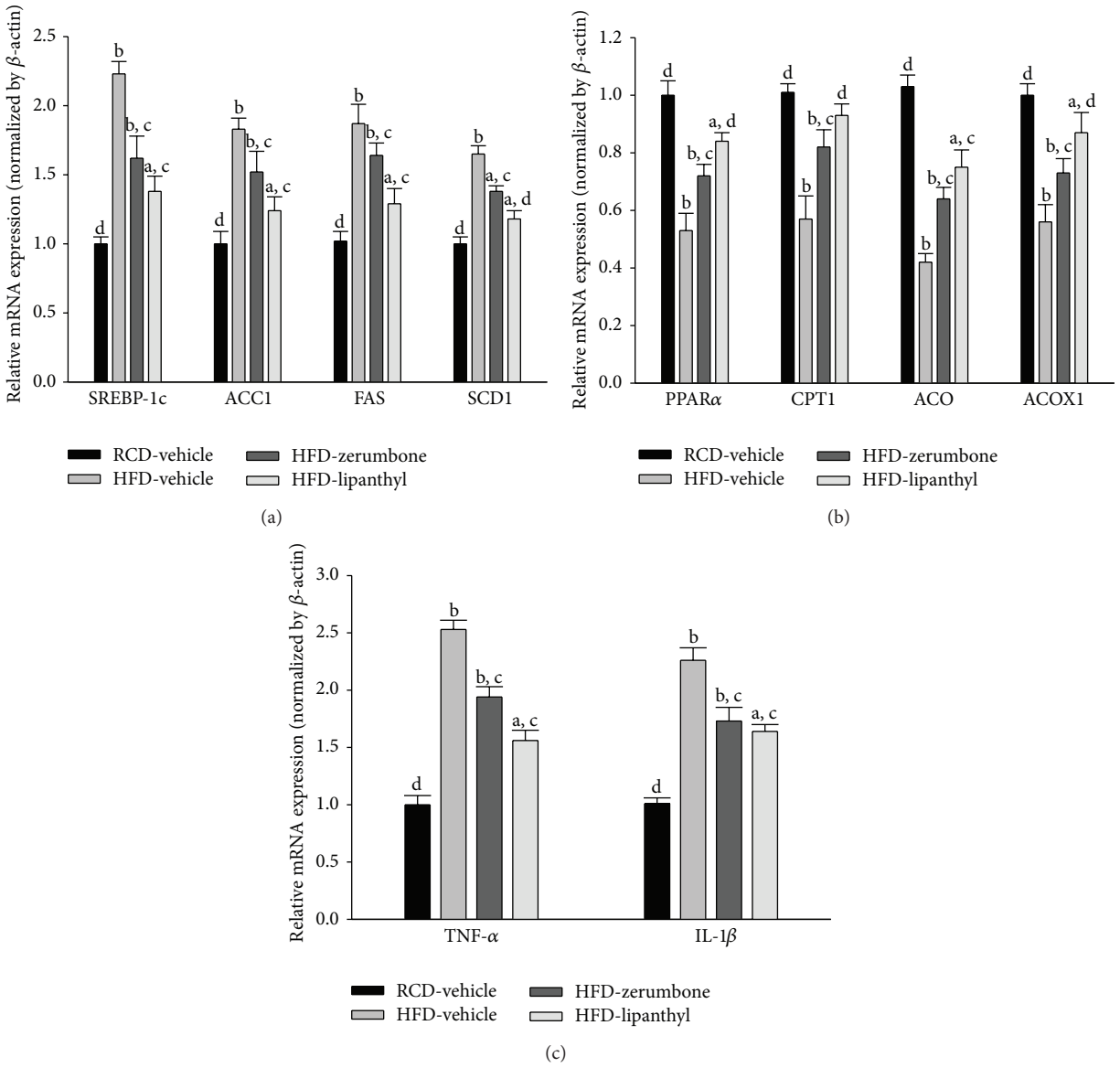

Figure 3: The hepatic mRNA levels of SREBP-1c and its lipogenic target genes (a), PPARα and its target genes responsible for fatty acid β-oxidation (b), and inflammatory cytokines (c) in RCD- or HFD-fed hamsters receiving 8-week treatment with zerumbone (300 mg kg^{-1} per day, HFD-zerumbone) or lipanthyl (100 mg kg^{-1} per day, HFD-lipanthyl). The mRNA expressions of the lipogenic genes were measured by RT-PCR and normalized to an internal control (β-actin). Animals not receiving any treatment were given the same volume of vehicle used to dissolve zerumbone. Similar results were obtained with an additional 4 replications. Data were expressed as the mean with SD ($n = 5$ per group) in each column. $^{a}P < .05$ and $^{b}P < .01$ compared to the values of vehicle-treated RCD-fed hamsters (RCD-vehicle). $^{c}P < .05$ and $^{d}P < .01$ compared to the values of vehicle-treated HFD-fed hamsters (HFD-vehicle).

significantly reduced (by 38.1%) in lipanthyl-treated HFD-fed hamsters compared with the vehicle-treated counterparts (Figure 3(a)). Zerumbone (300 mg kg^{-1} per day) suppressed the HFD-induced stimulation in hepatic mRNA levels of SREBP-1c to 72.6% relative to those in their vehicle-treated counterparts (Figure 3(a)).

HFD caused a 1.9-fold induction of hepatic ACC1 mRNA, a 2.0-fold induction of hepatic FAS mRNA, and a 1.7-fold induction of hepatic SCD1 mRNA relative to those in RCD-fed group. The HFD-induced mRNA levels of ACC1, FAS, and SCD1 in liver were significantly reversed after lipanthyl treatment (by 35.8, 37.9, and 28.5% decrease, resp.) compared to those of the vehicle-treated counterparts (Figure 3(a)). The hepatic mRNA levels of ACC1, FAS, and SCD1 were downregulated by zerumbone (300 mg kg^{-1} per day) treatment, with a decrease of 21.2, 21.1, and 16.4%, respectively, when compared with those observed in the vehicle-treated counterparts (Figure 3(a)).

3.5. Effects of Treatments on Hepatic mRNA Expression of PPARα and Its Target Genes Responsible for Fatty Acid β-Oxidation in Hamsters. The mRNA levels of PPARα in livers

of HFD-fed hamsters were lower to 53.1% of those from RCD-fed group (Figure 3(b)). Administration HFD-fed hamsters with lipanthyl or zerumbone (300 mg kg^{-1} per day) for 8 weeks significantly upregulated the hepatic PPARα mRNA levels to 1.4- and 1.6-fold relative to those in vehicle-treated counterparts, respectively (Figure 3(b)).

Compared with RCD-fed group, the hepatic mRNA levels of CPT-1, ACO, and ACOX1 in HFD-fed hamsters decreased obviously, which were up-regulated by lipanthyl treatment (155.3, 178.6, and 155.3% increases, resp.) (Figure 3(b)). The hepatic mRNA levels of CPT-1, ACO, and ACOX1 in HFD-fed hamsters receiving zerumbone (300 mg kg^{-1} per day) treatment were increased to 143.8, 152.4, and 130.4% relative to the expression levels in vehicle-treated counterparts, respectively (Figure 3(b)).

3.6. Effects of Treatments on Hepatic mRNA Expression of Inflammatory Cytokines in Hamsters. In HFD hamsters, TNF-α and IL-1β were significantly increased around 2.5- and 2.3-fold, respectively, as compared to that of RCD-fed group. Administration of HFD-fed hamsters with lipanthyl for 8 weeks significantly downregulated the hepatic TNF-α and IL-1β mRNA levels to 61.6 and 72.6% relative to those in vehicle-treated counterparts, respectively (Figure 3(c)). Zerumbone (300 mg kg^{-1} per day) treatment reversed HFD-induced increase of TNF-α and IL-1β mRNA expression to 76.6 and 76.5% of those observed in the vehicle-treated counterparts, respectively (Figure 3(c)).

4. Discussion

The pathogenic mechanisms of NAFLD are still under investigation; however, fat accumulation, mainly TG filtration within hepatocytes, is considered the first step in the development of NAFLD [1]. The clinical and animal studies demonstrated that levels of hepatic TG are positively correlated to visceral obesity and insulin-resistance [23]. Under insulin resistant status, FFAs from lipolysis of visceral tissue are increased with decreased oxidative capacity. The elevated FFAs in the blood stream will directly circulate into the portal vein where the liver deposits FFAs as TG in the hepatocytes and contributes to liver fibrosis [8]. Therefore, aggressive treatment of hyperlipidaemia plays a critical role in the overall management of patients with NAFLD [8]. HFD-induced animal model of NAFLD has been widely used to identify the pathogenesis and evaluate new treatment for NAFLD [17]. In our model, HFD-fed hamsters developed hepatic steatosis, visceral obesity, and hyperlipidaemia and increased FFA and HOMR-IR values, which mimics almost all of the clinical aspects of human NAFLD [24]. We observed that the increased levels of TG, TC, LDL-C, and FFA in the plasma were significantly suppressed, whereas the decreased plasma HDL-C levels were obviously elevated with zerumbone treatment in HFD-fed hamsters. We showed that zerumbone had effects on hypertriglyceridemia and attenuated the elevated plasma FFAs, indicating a potential application of zerumbone in treating fatty liver diseases.

TG is thought to be a surrogate marker of disrupted insulin signal. In other words, hepatic insulin resistance is associated with the accumulation of TG and fatty acid metabolites [25]. Zerumbone also presented similar effects to lipanthyl on hyperinsulinemia and improved HOME-IR, although its potency was much less than lipanthyl as the higher dose (300 mg kg^{-1} per day) of zerumbone than lipanthyl (100 mg kg^{-1} per day) was needed. The raised insulin sensitivity could also reflect less lipid accumulation in the liver indirectly.

Decrease of hepatic lipid accumulation is a rational target for NAFLD therapy since lipid loss should reduce many of the putative mediators of liver injury including insulin resistance, hepatic FFA supply and pro-inflammation [24]. Zerumbone treatment significantly decreased hepatic TG content. In addition, the relative liver weight in zerumbone treated hamsters were significantly lower than that of HFD-fed hamsters. Morphologically, the liver of HFD-fed hamsters showed abundant and large lipid droplets and obvious increase of liver derangement compared to that of RCD-fed hamsters. However, the liver of HFD-fed hamsters receiving zerumbone had fewer lipid droplets and more normal liver morphology, suggesting a beneficial effect of zerumbone on preventing lipid accumulation and reversal of disrupted structure of the liver. Zerumbone may also exert liver protective effect via inhibition of HFD-induced inflammation, since our results showed that zerumbone decreased gene expression of inflammatory cytokines resulting from lipid accumulation in liver [26].

To explore the possible mechanisms of zerumbone on decreasing hepatic lipids accumulation, we investigated the expression levels of several genes related to lipid metabolism including lipogenesis and β-oxidation. SREBP-1c has been shown to regulate the transcription of genes of lipogenic pathway [6]. Enzymes of the lipogenic pathway that are transcriptionally regulated include ACC1, FAS, and SCD1. ACC1 mediates the initial step of the fatty acid synthesis, and liver-specific ACC1 knockout mice show decreased hepatic triglyceride accumulation, suggesting that ACC1 plays a crucial role in the regulation of lipogenesis [27]. FAS catalyzes the last step in fatty acid biosynthesis, and thus, it is believed to be a major determinant of the maximal hepatic capacity to generate fatty acids by *de novo* lipogenesis [28]. SCD1 catalyzes the rate-limiting step in the production of monounsaturated fatty acids that are major components of tissue lipids [29]. Hence, it is suggested to the suppression SREBP-1c expression in liver may reduce the lipids accumulation. As a result of the low mRNA levels of SREBP-1c in livers of HFD-fed hamsters receiving zerumbone, there was a concomitant significant reduction in the mRNA expression of ACC1, FAS, and SCD1. Zerumbone is likely to have direct inhibitory effect on the SREBP-1c expression, which in turn influences the targeted lipogenic genes transcriptions, thereby reduces enzymes activity, resulting in a low rate of lipid synthesis. These results suggest that zerumbone can ameliorate HFD-induced hepatic steatosis via downregulation of lipid synthesis.

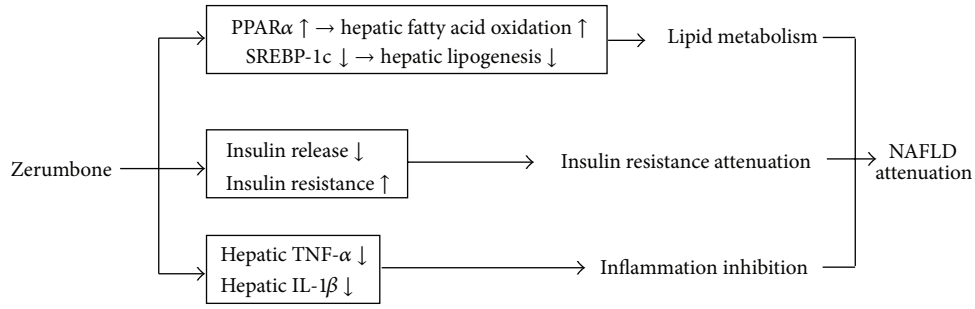

Figure 4: Possible mechanisms of zerumbone-mediated therapeutic activity in preventing and treating NAFLD.

The accumulation of hepatic lipids by HFD could also be the result of a decrease in fatty acid β-oxidation [30]. Fatty acid β-oxidation takes place in two cellular organelles: mitochondria and peroxisome [31]. CPT-1 was chosen as markers of mitochondria, because they are rate-limiting enzymes of fatty acid β-oxidation and used to reflect mitochondria oxidation activity [32]. ACO was chosen as marker because it is a point to control peroxisomal β-oxidation [33]. The results showed that zerumbone increased the hepatic CPT-1 and ACO mRNA in HFD-fed hamsters, suggesting an enhancement of fatty acid β-oxidation. Therefore, we proposed that zerumbone could promote the catabolism and utilization of fat through inducing an increased expression of several genes involved in fatty acid β-oxidation.

As we know, the β-oxidation of lipids in liver could be regulated by different transcription factors. PPARα is thought to be the principal regulator in the fatty acid oxidation [34]. The reduction of PPARα expression in liver is indicative of impaired β-oxidation of fatty acids which may further influence the imbalance of lipid metabolism toward lipid accumulation in the case of induced lipogenic transcription. ACOX1 is the first and a rate-limiting enzyme of the PPARα regulated and peroxisome proliferator-inducible fatty acid β-oxidation system [35]. To explore whether the effect of zerumbone on the attenuation of HFD-induced hepatic steatosis was related to PPARα activation, mRNA expression of PPARα and its target genes responsible for fatty acid β-oxidation were measured. Zerumbone markedly increased the HFD-induced low expression of hepatic PPARα mRNA. Similar to the tendency in the target genes expression involving in CPT and ACO, ACOX1 mRNA expression in liver of HFD-fed hamsters were upregulated by zerumbone treatment. The same trend among these genes suggested that zerumbone enhanced β-oxidation in liver via the pathway of PPARα-mediated gene transcription. Taken together, these results suggest that the effect of zerumbone on the treatment of hepatic steatosis may be partly associated with the enhancement of gene expression involved in lipid metabolism through PPARα activation.

In conclusion, our results show that zerumbone has a beneficial effect in inhibiting fat accumulation in liver, improves insulin resistance, inhibits inflammation, and possesses a repressive property on hepatic lipogenesis, which is associated with the inhibition of SREBP-1c and induction of PPARα, suggesting a potential application of zerumbone in treating fatty liver diseases. Possible mechanisms of zerumbone mediated therapeutic activity in preventing and treating NAFLD has been shown in Figure 4.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Identification of Target Genes Involved in the Antiproliferative Effect of Enzyme-Modified Ginseng Extract in HepG2 Hepatocarcinoma Cell

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Ginsenosides are ginseng saponins, which are the major biologically active components of *Panax ginseng*, often metabolized by intestinal bacteria into more effective forms. In this study, we found that the antiproliferative activity of ginseng increased after enzymatic processing of ginseng saponin (50% inhibitory concentration [IC_{50}], >30 μg/mL), which may be the result of the accumulation of minor saponins, such as Rh1, Rg3, compound K, and PPT constituents in ginseng saponin. Using the Agilent PrimeView Human Gene Expression Array, we found that the expression of several genes involved in apoptosis (*caspase-4, Annexin A2, HSPA9, AIFM1, UQCRC2*, and *caspase-7*) were increased in HepG2 human hepatocarcinoma cells after their treatment with enzyme-modified ginseng extract (EMGE). Furthermore, several genes implicated in cell cycle progression (*CDCA3, CDCA8, CABLES2, CDC25B, CNNM3*, and *CCNK*) showed decreased expression in HepG2 cells treated with EMGE. Finally, from flow cytometric analysis, we found that EMGE-treated HepG2 cells showed increased apoptotic sub-G1 population (24%), compared with that observed in DMSO-treated control cells (1.6%). Taken together, our results suggest that EMGE induces anticancer activity through the induction of apoptosis-related genes and cell cycle arrest via decreased expression of cell cycle regulatory genes.

1. Introduction

Panax ginseng (Korean ginseng) has been used as a traditional medicine for the treatment of cancer and has been shown to inhibit tumor cell proliferation and tumor growth, to induce differentiation and apoptosis, and to inhibit cancer cell invasion [1–4]. The major biologically active components of *Panax ginseng* are a series of saponin glycosides collectively known as the ginsenosides, a group of steroidal saponins. Over 50 ginsenosides have been isolated and identified from ginseng saponins to date [5].

Ginsenosides are characterized by a steroid-like skeleton consisting of 4 *trans*-rings, with modifications that depend on the type (e.g., glucose, maltose, and fructose) and number of sugar moieties, as well as the sites of attachment of the hydroxyl group (e.g., C-3, C-6, or C-20). Based on chemical structural characteristics, ginseng saponins can be divided into protopanaxadiol and protopanaxatriol groups, except for ginsenoside Ro, which is derived from an oleanolic group. In the protopanaxadiol group, sugars are attached to the β-OH at C-3 and another –OH at C-20, as found in Rb1, Rb2, Rc, Rd, Rg3, and Rh2. In the protopanaxatriol group, sugar residues are attached to the α-OH at C-6, with another –OH at C-20, examples being Re, Rg1, Rg2, Rh1, and Rf.

Recent studies have indicated that intestinal bacteria cleave the oligosaccharide connected to the aglycon stepwise from the terminal sugar to form the ginsenoside metabolite 20*S*-protopanaxadiol 20-*O*-β-D-glucopyranoside (compound K) after oral administration of ginseng extract in both humans and rats [6]. Wakabayashi et al. have revealed that the antitumor activities of ginsenoside following oral administration are attributable to the metabolites formed by colonic bacteria-mediated deglycosylation [7]. In addition, compound K is known to exhibit cytotoxicity by the induction of apoptosis and cell cycle arrest at the G_1 phase, by a caspase-dependent pathway via mitochondrial disruption in

tumor cells, and by reversing multidrug resistance in tumor cells [8–10]. The combined treatment of compound K and radiation enhances human lung cancer cell death [8, 11].

Various transformation methods, including mild acid hydrolysis, enzymatic conversion, and microbial conversion, have been tested for their ability to transform ginsenosides to enhance their physiological effectiveness. In this study, we investigated the antiproliferative effects and possible mechanism of action of EMGE that were transformed by enzyme treatment, by using the human hepatocarcinoma cell line HepG2.

2. Materials and Methods

2.1. Cell Culture. HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B (Invitrogen, Carlsbad, CA) in a 5% CO_2 atmosphere at 37°C. Confluent cells were detached with 0.25% trypsin-EDTA for 5 min, and aliquots were subcultured. Human hepatoma HepG2 cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea).

2.2. Preparation of Enzyme-Modified Ginseng Extract (EMGE). The root of regular ginseng (4 years old) was purchased from G&V, Korea. A total of 20 g of pulverized ginseng root powder were suspended in 380 mL of distilled water and then sterilized by heating at 121°C for 15 min. To reduce the complexity of components in ginseng root, the extract was fractionated via extraction with water, methanol, and butanol. Among the fractions, ginseng butanol extract contained the compounds with specific anticancer activity. In addition, the suspension was treated with aliquots of filter-sterilized commercial enzymes (laminarinase 100 mg, pectinase 100 mg) with an equimolar ratio (1 : 1, specific activity unit). For these treatments, the mixture was incubated at 40°C for 2 days and then evaporated to dryness at 60°C. These enzyme-modified ginseng powders were then suspended in 400 mL of 80% (v/v) methanol and subjected to ultrasonication for 5 min followed by filtration through Whatman No. 2 Filter Paper. The wet powder on the filter paper was then collected, suspended, treated in an ultrasound bath, and filtered in the same manner once again, after which the two filtrates were combined and evaporated to dryness at 50°C. The methanol extract dissolved in 200 mL of distilled water was washed with 200 mL of ethyl acetate in a separation funnel and then extracted with 200 mL of butanol. Next, the butanol extract was evaporated to dryness at 50°C and then dissolved to a concentration of 10% (w/v) in 70% ethanol.

2.3. HPLC Analysis of Ginsenosides. Standard ginsenosides including Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2, f1, Rd, Rg3(S), Rg3(R), PPT, Com K, Rh2, and PPD were obtained from ChromaDex Inc. (Irvine, CA) and analyzed using an Acquity UPLC system (Waters, Milford, MA) with an Acquity BEH C_{18} HPLC column. The mobile phase consisted of solution A (CH_3CN, HPLC-grade, JT Baker, NJ) and solution B (Milli-Q H_2O, Millipore, MA). The flow rate was set at 0.6 mL/min, and the injection of volume was fixed at 2 : 1. The separation of ginsenosides was performed using an isocratic gradient of 100% solution A for 27 min. The column temperature was maintained at room temperature during the separation, and the UV diode array detection was set at 203 nm. To confirm a reliable retention time of sample ginsenoside, the individual ginsenosides were identified and quantified based on comparison with the retention times of standard ginsenosides.

2.4. Cell Viability Assay. The effect of EMGE on cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, a yellow tetrazole) assay, which quantifies the number of viable cells. Cells were seeded (2×10^4 cells/well) in 24-well flat-bottomed plates (Nunc, EU). After incubation at 37°C for 24 h, the medium was replaced with EMGE at the appropriate concentrations. Control cells were treated with DMSO at a final concentration of less than 0.2%. After further 24 h incubation, cells were washed 3 times with Dulbecco's phosphate-buffered saline (DPBS), and 500 μL MTT solution (5 mg/mL in PBS) was added to each well. After incubating for 4 h, the medium was removed, and 200 μL of formazan (crystals dissolved in DMSO) was added to the cells. The absorbance of each well was read at 540 nm using a microplate reader.

2.5. RNA Extraction and cDNA Synthesis. Total RNA was extracted using the Easy-spin RNA Extraction kit (iNtRON, Seoul, Republic of Korea) according to the manufacturer's instructions. The purity of RNA was assessed by measuring the absorption at 260 and 280 nm (values of the ratio of A_{260}/A_{280} of 1.9–2.1 were considered acceptable) and by ethidium bromide staining of 18 S and 28 S RNA by gel electrophoresis. RNA concentrations were determined from the A_{260}. Two micrograms of total RNA were reverse-transcribed in a 20 μL reaction mixture containing 50 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 5 μM DTT, 40 units of RNaseOUT recombinant ribonuclease inhibitor (Invitrogen, Carlsbad, CA), 0.5 μM of random hexanucleotide primers, and 500 μM of dNTP mixture. The reverse transcription reaction was carried out at 50°C for 60 min. The reaction was terminated by heating the reaction mixture at 70°C for 15 min, and the cDNA was stored at −20°C.

2.6. Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Overexpression of the target genes was confirmed by quantitative real-time RT-PCR. All real-time PCR analyses were performed on an ABI Step One Real-time PCR system (Applied Biosystems). Each reaction contained 0.1 μM of each primer, 10 μL of 2x SYBR Green PCR master mix (including AmpliTaq Gold DNA polymerase with buffer, dNTPs mix, SYBR Green I dye, ROX dye, and 10 mm $MgCl_2$, Applied Biosystems), and 1 μL of the template cDNA in a 20 μL total reaction volume. The typical

Table 1: Primer sequences for real-time RT-PCR experiments.

Genes	Sense (5′-3′)	Antisense (5′-3′)
Caspase-4	GAAGGACAAACCCAAGGTCA	AAGAGGCCACTTCCAAGGAT
Cyp450	TGATAAGCACGTTGCAGGAG	CAAAGCAAATGGCACAGATG
Caspase-7	AGACCTCATCCGGTCAAGTG	TTATCAGACAGCGCAACAGC
Cyclin M	GAAGCGGAAGGAGGAGTTCT	CGGGCTGAATACATCCACTT
Cyclin K	GACATCTGCCACCAAATCCT	GCACTTGTGGTGTAGGCTGA
GAPDH	TGGAAGGACTCATGACCACA	TTCAGCTCAGGGATGACCTT

Table 2: HPLC analysis of EMGE.

	(mg/g)											
	Rg1	Re	Rf	Rh1	Rb1	Rc	Rb2	F1	Rd	Rg3	Com.K	Rh2
Control (ginseng)	9.2	7.2	2.5	—	7.7	4.7	2.3	2.0	0.6	—	—	—
EMGE	0.6	1.0	—	2.7	2.7	—	—	—	—	9.5	4.7	2.7

amplification program included activation of the enzyme at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, then annealing and extension at 60°C for 1 min. The C_T (cycle threshold) value for each gene was determined using the automated threshold analysis function of the ABI instrument and normalizing to $C_{T(\text{G3PDH})}$ to obtain dC_T (= $C_{T(\text{G3PDH})} - C_{T(\text{test})}$). The difference of n between the $2C_T$ or dC_T values indicates a 2^n-fold difference in the amount of the target sequence between the 2 cDNA samples being compared. The primers used in quantitative PCR are shown in Table 1.

2.7. Microarray Assay. Gene expression was analyzed with Agilent PrimeView Human Gene Expression Array containing 36,000 genes transcript and variants via Unigene annotation (Affymetrix, Santa Clara, CA), which are comprised of over 45,000 probe sets representing approximately 38,500 well-characterized human genes. For each gene, 11 pairs of oligonucleotide probes were synthesized *in situ* on the arrays. Biotinylated cRNA was prepared according to the standard Affymetrix protocol from 100 ng of total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). Following fragmentation, 12 μg of aRNA was hybridized for 16 h at 45°C on a GeneChip Human Genome Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GeneChip Scanner 3000 7G. The data were analyzed with Microarray Suite version 5.0 using the Affymetrix default analysis settings and global scaling as the normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalized and log transformed intensity values were then analyzed using GeneSpring GX 11.5.1 (Agilent Technologies, CA). Fold-change filters included the requirement that the genes be present in at least 200% of controls for genes with upregulated expression and less than 50% of controls for genes with downregulated expression. Hierarchical cluster analysis using GeneSpring GX 11.5.1 (Agilent technologies, CA) identified clustered groups as those that behaved similarly across experiments. The clustering algorithm was Euclidean distance, with average linkage.

2.8. Cell Count and Flow Cytometry Analysis. Cell counts were performed using a hemocytometer. Approximately 1×10^6 HepG2 cells were suspended in 100 μL of PBS, and 200 μL of 95% ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended in 250 μL of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μL of propidium iodide (50 μg/mL) for 30 min at room temperature. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) flow cytometry (FACScanner, Becton Dickinson) for relative DNA content based on red fluorescence.

3. Results

3.1. Chromatographic Analysis of Ginseng Saponin during the Enzyme Treatment. Ginseng saponins (ginsenosides) have been regarded as the principal components responsible for the pharmacological activities of ginseng, and a large number of ginsenoside derivatives have been identified in *Panax ginseng* [5]. Recently, cytotoxic effects of minor saponins, such as Rh1 and Rh2, on the growth of various cancer cells and also inhibitory effects of human intestinal bacterial saponin metabolites, such as compound K and protopanaxatriol (PPT), on the growth, invasion, and migration of tumor cells, have been reported [12–16]. High-performance liquid chromatography (HPLC) analysis was performed to determine the changes in chemical constituent in ginseng saponin following enzyme treatment. HPLC results showed the appearance of minor ginsenosides (Rh1, Rg3, and compound K), indicative of EMGE (Table 2).

3.2. Antiproliferative Effects of EMGE on HepG2 Hepatocarcinoma Cells. Ginsenosides have been shown to inhibit tumor cell proliferation and tumor growth and to induce differentiation and apoptosis [17]. To determine the antiproliferative effects of EMGE on HepG2 human hepatocarcinoma cells, we performed the MTT assay. EMGE inhibited HepG2 cell growth in a dose-dependent manner at 14.8, 22.2, 33.3, 50, and

Table 3: Genes with highly upregulated expression due to EMGE in HepG2.

Symbol	Gene description	Fold change	GenBank ID
CYP1A1	Cytochrome P450, family 1, subfamily A	20.0	NM000499
HGD	Homogentisate 1,2-dioxygenase	15.3	NM000187
AQP3	Aquaporin 3	14.5	NM004925
IL8	Interleukin 8	14.3	NM000584
ANXA2	Annexin A2	11.1	NM001002857
HSPA9	Heat shock 70 kDa protein 9	10.5	NM004134
CASP4	Caspase-4	10.4	NM001225
SMAD2	SMAD family member 2	9.8	NM001003652
MAPK6	Mitogen-activated protein kinase 6	9.3	NM002748
KLF6	Kruppel-like factor 6	9.0	NM001160124
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kda	8.3	NM001098209
AIFM1	Apoptosis-inducing factor, mitochondrion-associated, 1	7.8	NM001130846
PPHLN1	Periphilin 1	7.5	NM001143787
UQCRC2	Ubiquinol-cytochrome c reductase core protein II	7.4	NM003366
IL6ST	Interleukin 6 signal transducer	6.4	NM001190981
IFITM1	Interferon-induced transmembrane protein 1	5.1	NM003641
CASP7	Caspase-7	5.0	NM001227

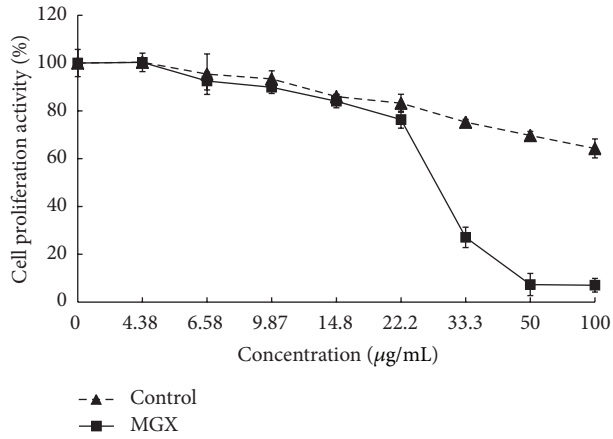

Figure 1: Antiproliferative effects of EMGE on human HepG2 hepatocarcinoma cells. Cell viability at the indicated concentrations of EMGE at 24 h was assessed by the MTT test. The treated cells were used for total RNA isolation followed by microarray analysis.

100 μg/mL at 24 h. The concentration at which 50% growth inhibition (IC_{50}) occurred was 30 μg/mL (Figure 1). However, exposure of HepG2 cells to 30 μg/mL nontreated ginseng saponin extract showed less growth inhibition (approximately 20%). Thus, these results suggest that enzymatic processing of ginseng saponin increased its antiproliferative effects on HepG2 cells.

3.3. Identification of Differentially Expressed Genes in HepG2 Human Hepatocarcinoma Cells Treated with EMGE. Although ginseng has been used as a traditional medicine for the treatment of cancer, the underlying mechanisms for the varying activities observed following exposure to ginseng are largely unclear. To identify relevant alterations of gene expression associated with treatment of EMGE, we analyzed the gene expression profiles of HepG2 cells by using Affymetrix Gene Chip arrays containing more than 38,500 genes. From the results of Affymetrix Gene Chip array analysis, we detected 31 differentially expressed genes exhibiting at least a 5-fold change following treatment with EMGE. Of these, 17 had significantly upregulated expression, and 14 had significantly downregulated expression when comparing treatment with the EMGE versus the control treatment (Tables 3 and 4).

Several genes involved in apoptosis (*caspase-4*, *Annexin A2*, *HSPA9*, *AIFM1*, *UQCRC2*, and *caspase-7*) were expressed at higher levels following treatment with EMGE (Table 3). The expression of genes involved in the regulation of cell signaling (*MAPK6*, *SMAD2*, *IL6ST*, *KLF6*, and *IFITM1*) also increased. Furthermore, several genes implicated in cell adhesion (*CTNNB1*), transport (*AQP3*, *IFITM1*), and the immune response (*IL8*) showed increased expression in HepG2 human hepatocarcinoma cells that were treated with EMGE.

In contrast, 14 genes showed reduced expression in HepG2 human hepatocarcinoma cells treated with EMGE. There was an overrepresentation of members of genes involved in cell cycle progression (*CDCA3*, *CDCA8*, *CABLES2*, *CDC25B*, *CNNM3*, and *CCNK*) and cellular metabolism (*CS*, *ALDH3B1*, and *NPRL3*). Table 4 lists the names of genes exhibiting a reduction in expression, where the difference was >5-fold. This group of genes that were expressed very differently may serve as an important target in EMGE-mediated anticancer activity in human hepatocarcinoma cells.

3.4. Real-Time RT-PCR Validation of Microarray Data. To confirm the results obtained using microarrays, we choose to determine if the expression of 5 target genes was regulated by EMGE in HepG2 cells by quantitative real-time RT-PCR analysis. These genes included 3 genes with highly upregulated expression (*cyp450*, *caspase-4*, and *caspase-7*)

Table 4: Genes with highly downregulated expression due to EMGE in HepG2.

Symbol	Gene description	Fold change	GenBank ID
CDCA3	Cell division cycle associated 3	−8.4	NM031299
CS	Citrate synthase	−8.0	NM004077
CDCA8	Cell division cycle associated 8	−7.7	NM018101
CABLES2	Cdk5 and Abl enzyme substrate 2	−7.4	NM031215
CDC25B	Cell division cycle 25 homolog B	−6.6	NM004358
CNNM3	Cyclin M3	−6.2	NM017623
ZNF362	Zinc finger protein 362	−5.8	NM152493
CCNK	Cyclin K	−5.8	NM001099402
RAD51AP1	RAD51 associated protein 1	−5.8	NM001130862
LEAP2	Liver expressed antimicrobial peptide 2	−5.3	NM052971
HEPACAM	Hepatocyte cell adhesion molecule	−5.1	NM152722
ALDH3B1	Aldehyde dehydrogenase 3 family, member B1	−5.0	NM000694
NPRL3	Nitrogen permease regulator-like 3	−5.0	NM001039476
DOT1L	DOT1-like, histone H3 methyltransferase	−5.0	NM032482

and 2 with highly downregulated expression (*Cyclin K* and *Cyclin M*). To accurately quantify the expression of these 5 genes, *GAPDH* expression was amplified and the mean data in each case was used to normalize the result.

As shown in Figures 2 and 3, the expression level of the 3 selected upregulated genes (*cyp450* (73-fold), *caspase-4* (6-fold), and *caspase-7* (3-fold)) significantly increased upon treatment with EMGE. Conversely, the expression of the 2 downregulated genes (*Cyclin M* (4-fold), *Cyclin K* (12-fold)) decreased. These results indicate that the real-time RT-PCR results are highly consistent with the microarray data.

3.5. Effect of EMGE on Cell Cycle Distribution and Apoptosis in HepG2 Hepatocarcinoma Cells. To determine whether EMGE has an effect on cell cycle arrest, apoptosis, or both in human HepG2 hepatocarcinoma cells, cells treated with DMSO or EMGE for 12 or 24 h were subjected to flow cytometric analysis after DNA staining. Histograms of flow cytometric data are shown in Figure 4. Control cells (DMSO-treated) progressed through the cell cycle normally. In contrast, EMGE-treated HepG2 cells showed increased apoptotic sub-G1 populations (24%) compared with that observed in DMSO-treated control cells (1.6%). These results suggest that EMGE induces antiproliferative effects in HepG2 cells, mainly through apoptotic mechanisms.

4. Discussion

We found that the enzymatic processing of ginseng saponin could increase the content of active constituents and enhance its anticancer activity, presumably because of the production of minor saponins, such as Rh1, Rg3, compound K, and PPT constituents in ginseng saponin. In this study, we used cDNA microarray analysis to detect target genes involved in the anticancer activity of EMGE. Microarray (DNA chip) is a 2D array of microscopic DNA spots for high-throughput expression screening. We found that several genes involved in apoptosis (*caspase-4*, *Annexin A2*, *HSPA9*, *AIFM1*, *UQCRC2*, and *caspase-7*) as well as several genes involved in cell signaling (*MAPK6*, *SMAD2*, *IL6ST*, *KLF6*, and *IFITM1*) were expressed at higher levels after treatment with EMGE. Furthermore, several genes implicated in cell cycle progression (*CDCA3*, *CDCA8*, *CABLES2*, *CDC25B*, *CNNM3*, and *CCNK*) and metabolism (*CS*, *ALDH3B1*, and *NPRL3*) exhibited decreased expression in HepG2 human hepatocarcinoma cells treated with EMGE. Thus, the apoptosis-related genes with upregulated expression and cell cycle-related genes with downregulated expression could be important target genes involved in the anticancer activity of EMGE.

In this study, *caspase-4* and *caspase-7*, which are components of the cell-intrinsic apoptosis machinery, were significantly induced by EMGE. *Caspase-4* has a functional role in endoplasmic-reticulum-stress- (ERS-) induced apoptosis in humans [18]. Upregulation of *caspase-4* has been demonstrated to mediate apoptosis in human bronchial epithelial cells [19], human neuroblastoma SKN-SH cells, and human esophageal squamous carcinoma EC109 [20]. Thus, the induction of apoptosis-related genes, such as *caspase-4* and *caspase-7*, in cells treated with EMGE may contribute to the antiproliferative activity of EMGE.

The cytochrome P450 1A1 (CYP1A1) is a member of the cytochrome P450 monooxygenase superfamily, which plays an important role in xenobiotic metabolism as well as in carcinogenesis [21]. In this study, CYP1A1 level was significantly increased after treatment with EMGE. The CYP1A1 enzymes mediate the bioactivation of carcinogens found both in the human diet and in the environment, including compounds such as polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene, benzo(a)pyrene, polyhalogenated aromatic hydrocarbons, and certain congeners of polyhalogenated biphenyls [22]. Our finding that the EMGE increased the expression of CYP1A1 is consistent with the previous results showing the induction of CYP1A1 expression by ginsenosides Rg1 and Rb1 [23].

Cell cycle progression is a tightly regulated process requiring regulatory factors such as CDCs and cyclins. Thus, lack of cell cycle machinery molecules often causes cell cycle arrest leading to cell growth inhibition, apoptosis, or both.

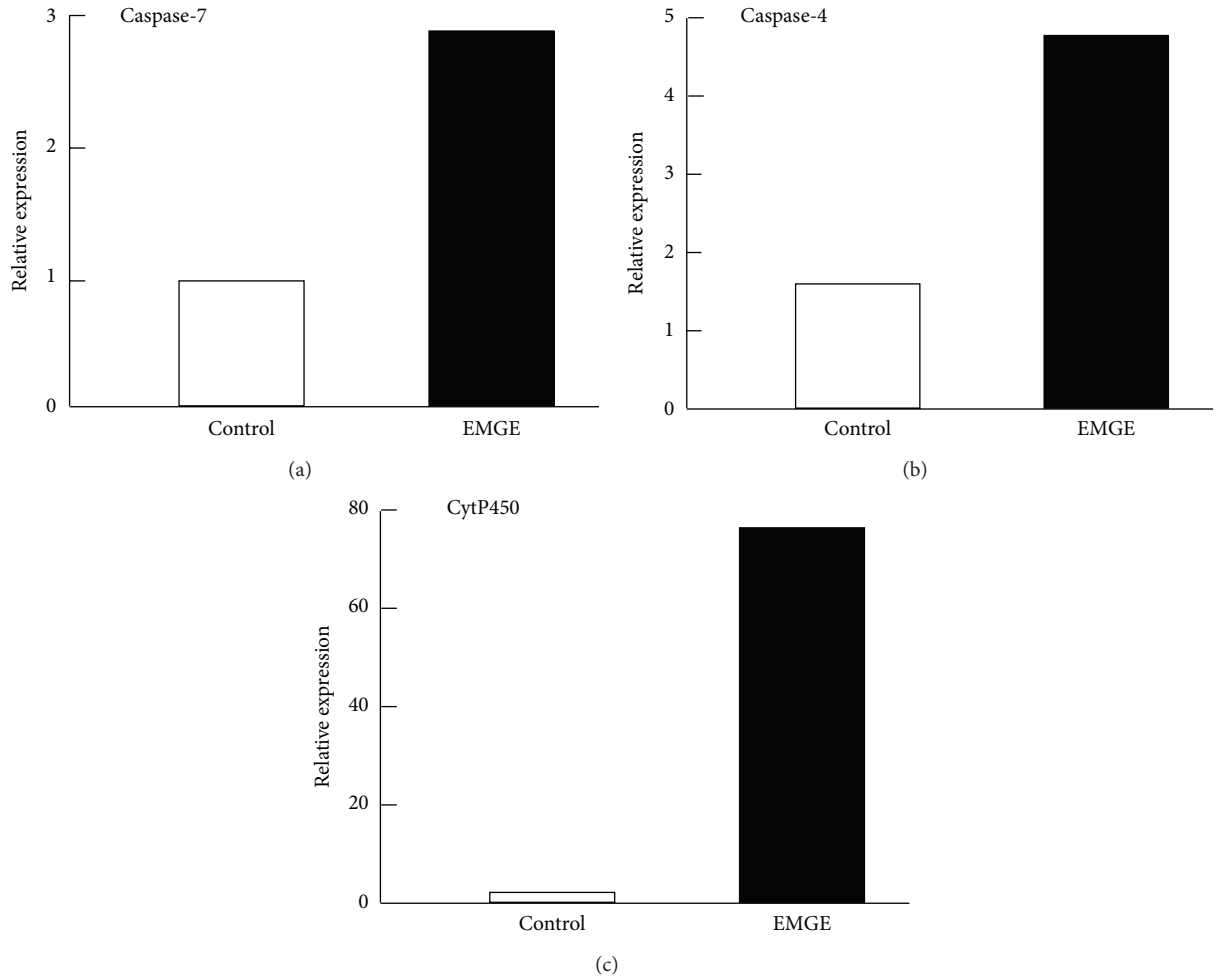

Figure 2: Quantitative real-time RT-PCR analysis of 3 genes with upregulated expression: *cyp450*, *caspase-4*, and *caspase-7*.

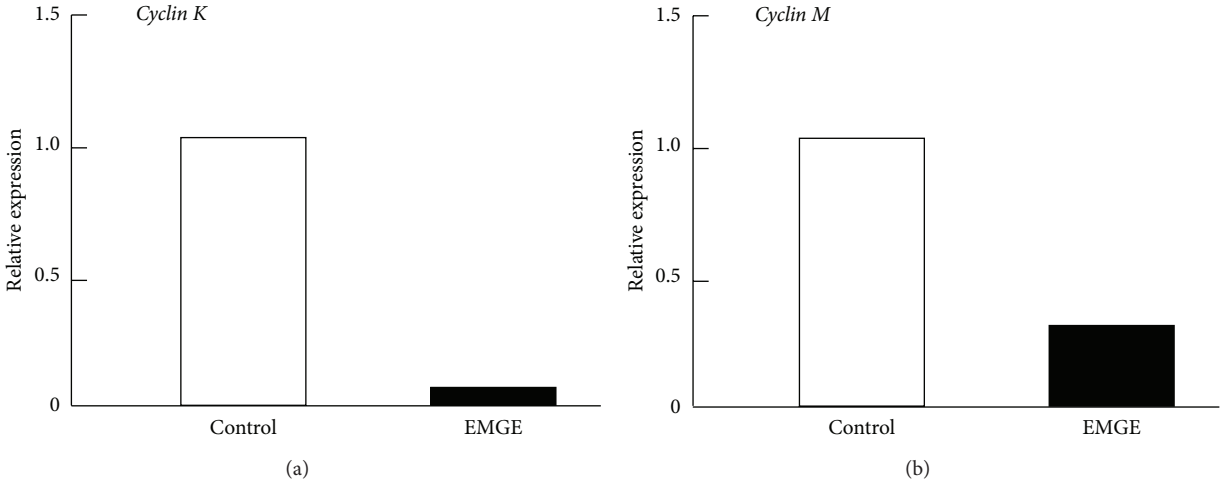

Figure 3: Quantitative real-time RT-PCR analysis of 2 downregulated genes: *Cyclin K* and *Cyclin M*.

Our results showing the decreased expression of several genes implicated in cell cycle regulation (*CDCA3*, *CDCA8*, *CABLES2*, *CDC25B*, *CNNM3*, and *CCNK*) in HepG2 cells treated with EMGE suggest that the induction of cell cycle arrest by EMGE may contribute to the antiproliferative activity of EMGE.

In summary, we found that the anticancer activity of ginseng was increased by enzymatic processing of ginseng

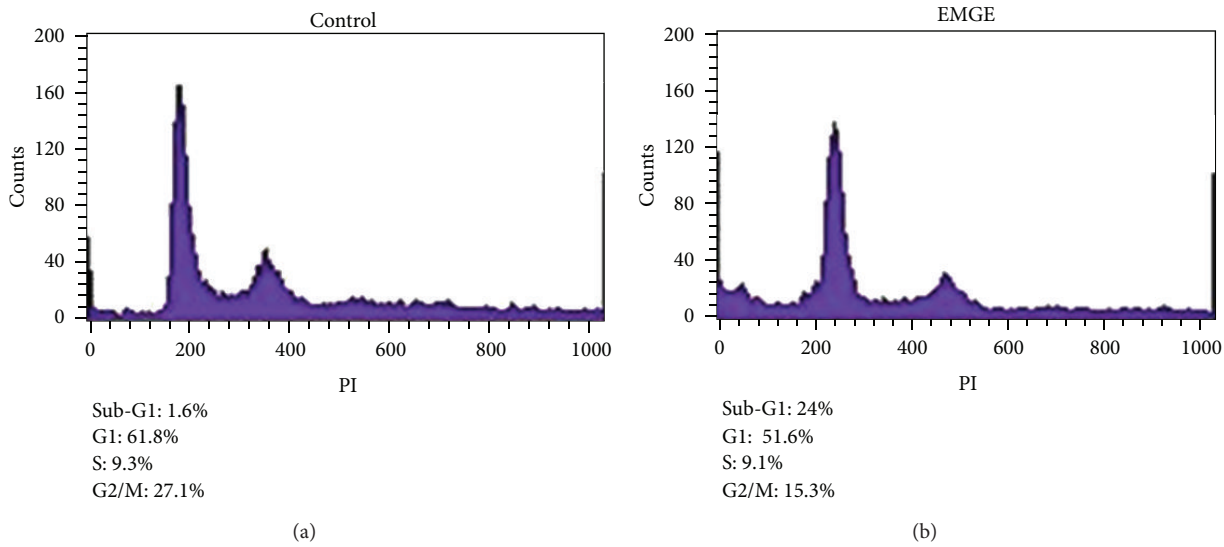

Figure 4: FACS analysis of PI-stained nuclei of HepG2 cells treated with EMGE. Cells were treated with DMSO (0.05%) or EMGE (50 μg/mL) for 24 h, and cell cycle analysis was conducted as described in Section 2. Data are representative of 3 independent experiments.

saponin, which may be the result of the relative accumulation of minor saponins, such as Rh1, Rg3, compound K, and PPT constituents in ginseng saponin. Moreover, the results of microarray analysis suggest that EMGE induces anticancer activity through the induction of apoptosis-related genes and the decreased expression of cell cycle regulatory genes. However, these results do not exclude the role of other genes that may be involved in anticancer activity.

Acknowledgments

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Review Article

A Current Update on the Rule of Alternative and Complementary Medicine in the Treatment of Liver Diseases

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There is a vast body of knowledge which is ever-increasing about the treatment of liver disease with alternative and complementary medicine for which hundreds of thousands of literatures have been documented. Liver disease is a general term. This term covers all the potential problems that cause the liver to fail to perform its specified operations. Liver disease has a variety of presentations and causes a great public health problem worldwide which threatens the wellness of billions of people. Incidences of many types of liver disease are currently rising. Although there is still a debate about the entity of alternative and complementary medicine, it is now widely used and it is improving. And it covers the shortages and compensates for the weaknesses of conventional methods in the treatment of liver diseases. Alternative and complementary medicine for liver diseases provides benefits by regulating immunity, controlling disease progression, improving quality of life, and prolonging survival. This paper reviews the increasing interest and growing research into alternative and complementary medicine for liver diseases, with a look at the rough classification, principle of management, evidence-based applications, and issues for prescription and perspectives.

1. Introduction

Liver disease is an expression for any damage that reduces the functioning of the liver. This general term covers all the potential problems that cause the liver to fail to perform its specified operations [1]. Liver diseases cause an enormous global public health problem which threatens the wellness of billions of people [2]. More seriously, incidences of many liver diseases are currently rising [3]. Increased immigration, fast and frequent travel, and globalization in the modern world lead to wide spread of virus hepatitis [4]. Obesity produces steatohepatitis which is evolving into a new and major health problem [5]. Heavy alcohol consumption, which is becoming a tendency in younger people, results in steatosis, alcoholic hepatitis, and cirrhosis [6]. Globally, the number of people chronically infected with hepatitis B virus (HBV) has reached 400 million and up to 2 billion people are with the evidence of exposure. Approximately 3% of the world population is infected by HCV or about 170 million people are HCV-infected [7, 8]. The liver is a very complex organ that is essential for living. It is complicated in both structure and function. The liver functions as a factory for products, a storehouse for supply, and a source of various substances for systemic operation and regulation of other organs and tissues [9]. These factors imply that the treatment of liver diseases is complicated and full of difficulties. Management of liver diseases should not be simplified, and one should concern all the aspects of the diseased parts, with the overall situation of the liver being considered [10, 11]. A comprehensive but pinpoint therapy is ideal. However, there are conflicts between the therapy with comprehensive approach and a pinpoint goal [12]. Alternative and complementary medicine for liver diseases has a very broad field that is different from Western or allopathic medicine [13]. This paper reviews the increasing interest and growing research into alternative and complementary medicine for liver diseases, with a look at the rough classification, principle of management, evidence-based applications, and issues for prescription and perspectives.

2. Overview of Liver Diseases

Appropriate and correct classification of liver diseases is the essence for directional and individualized treatment. However, clinically liver diseases are often insidious or present varied symptoms and signs, and this makes the classification of them become difficult and multiplex [39]. Based either on the evidence of their etiology or on the prominent clinical manifestations, liver diseases can be divided briefly into the following categories including alcoholic, autoimmune, cirrhotic, congenital, infectious, intoxicated, metabolic, and space-occupying liver diseases. Some clinical features overlap among the categories, and a disease entity can shift from one category to another [40]. Sometimes there is no clear-cut outline for a disease to fall into a certain category, and a patient may suffer at the same time several liver diseases in different categories [41]. The most well-known example is the gradual transformation from viral hepatitis to cirrhosis then to liver cancer [42].

Alcoholic liver diseases are predominate in Western countries, and they are caused by excessive consumption of alcoholic beverages. Alcohol abuse leads to liver disorders characterized by damage of liver cells and consequent liver steatosis and alcoholic cirrhosis [14]. Poor nutrition is often observed in patients of this category. The patients have malabsorption and maldigestion of nutrients and replace alcohol for normal food. The level of malnutrition is correlated to the severity of liver disease. Enteral nutrition can be taken as complementary and alternative medicine. It combats malnutrition and enhances clinical outcome, thus may be considered as frontline therapy [15].

Autoimmune liver diseases are diseases of unknown causes and are reported to be on the increase all over the world. They are concluded when a series of tests suggest that the body's immune system attacks the liver. This category of diseases includes autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis [16]. When the combination of biochemical, autoimmune, and histological studies implies the category and other categories are excluded, the diagnosis can be established. Standard therapy is the use of prednisone or prednisone with azathioprine. And budesonide may be an additional treatment option for patients without cirrhosis [17]. Curcumin is now used as a supplement in several countries, based on the favorable evidence from more than 65 human clinical trials [18]. Some herbs-induced liver injuries can be autoimmune like, and establishment of causality is necessary [43].

Cirrhosis is a slow process in which healthy liver tissue is replaced gradually with scar tissue. All the inflammatory, alcoholic, and autoimmune liver diseases can end in cirrhosis [44]. The etiology is different and with dynamic change in different areas in the world due to drug use, migration, and lifestyle change. Some cases are idiopathic, for which attention should be paid to a detailed history of drug and food intake to differentiate drug-induced chronic hepatitis, which falls into the category of intoxicated liver diseases [45]. Serious complications occur in patients with severe cirrhosis of the liver, including ascites, esophageal variceal hemorrhage, hepatic encephalopathy, and hepatorenal syndrome. Hemorrhagic ascites seems to be a symbol for poor outcome and remains a challenge to both conventional and alternative medicine [46].

Congenital liver diseases are inherited disorders that are present at birth, such as Alagille syndrome, alpha 1-antitrypsin deficiency, galactosemia, hematochromatosis, porphyria, tyrosinemia, type I glycogen storage disease, and Wilson's disease [47, 48]. Some of the ductus malformations are frequently associated with cardiac and other vascular anomalies, and a complex and combined surgery or hybrid operation is needed for treatment [25].

Infectious liver diseases are resulted from various infection, of the liver by different organisms and are more common in the developing countries. These include hepatitis viruses, bacteria, and parasites [28]. Geological difference exists. For example, high rates of hepatitis C virus infection are reported in Egypt while those of hepatitis B virus infection are reported in Asia. Diseases of this category present in the liver such as viral hepatitis, bacterial abscess, tuberculosis, hepatic schistosomiasis, fascioliasis, amebiasis, and echinococcosis. Specific probiotics are shown to have anti-inflammatory and selective antimicrobial effects as supplemental treatment for several acute and chronic infectious processes [30].

Intoxicated liver diseases are caused by toxicity from drug and food intake [32]. Substances toxic to the liver can also be inhaled or absorbed through the skin, such as carbon tetrachloride, formaldehyde, phosphate, and phenylamine [49]. This condition is often encountered in industrial or mining areas. Both acute and chronic intoxication by substances can lead to liver failure [33, 50]. Essential phospholipids from soybean accelerate the improvement or normalization of subjective symptoms in hepatic intoxication [34].

Metabolic liver diseases are caused by disorders in metabolism of amino acid, bile acid, carbohydrate, or copper. Nonalcoholic fatty liver disease is the most common liver disease in developed countries, being the hepatic manifestation of the metabolic syndrome [51]. Clinically, laboratory studies demonstrate that either the liver has no structural abnormalities but obvious functional disturbances or the liver cells are injured showing apoptosis, necrosis or cirrhosis [52]. Hepatomegaly can manifest presenting hepatic storage of lipid, glycogen, or other products. Weight reduction is recommended in obese patients with diet as the primary treatment [35].

Space-occupying liver diseases imply massive hepatic lesions that consist of abnormal or nonhepatic contents. These lesions replace the normal tissue in a part of the liver and are likely to grow or enlarge at site, and eventually they lead to damage to the liver and the whole body. This category comprises benign and malignant tumors, abscesses, cysts, and hydatidosis [53]. Complementary and alternative medicine is commonly used by hepatobiliary patients, more frequently by patients with unresectable malignant disease. The most common method is herbs or supplements, but prospective studies are needed to evaluate the outcomes [37].

3. Principles for Managing Liver Diseases

There are commonplace observations that we must make when any kinds of therapies are used to treat liver diseases [54]. The unique advantages of a particular therapy should also be noted [26]. Alternative and complementary medicine provides additional benefits to the patients with liver diseases who have been treated by conventional methods at the present time. Or it covers the shortages and compensates after the weaknesses of these methods. Choice of treatments must be evidence based and depends on the features of a certain category of liver diseases that the patient has [55].

Before treatment, evidence is to be collected to clarify that the diseased liver is altered in structure or function or both. Surgical and ablative therapies are indicated to liver diseases with structural abnormalities [56]. For all the patients with either structural or functional problems, medication is of paramount importance for a good outcome [57]. Special attention should be paid to nutritional and dietary therapies that are the constituents of complementary and alternative medicine.

In selected conditions without contraindications, the first choice is invasive management for those suffering from tumors, cholelithiasis, congenital deformity of bile ducts, localized purulent infection, liver trauma, secondary stricture or obstruction of bile ducts, portal hypertension, and the Budd-Chiari syndrome. To reduce perioperative mortality and morbidity rate, minimally invasive operation and careful postoperative management are advocated [38]. Drainage and ablation can be used to treat cysts larger than 5 cm in diameter, but caution is advised in the puncture process as fluid escaping from hydatid cyst leads to fatal allergic shock of the patient [29].

When the interventional and medical therapies failed to cure the disease and the life expectancy of the patient is between 6 and 12 months, a liver transplant is possible. Recipients of liver transplantation include those suffering from congenital liver diseases, malignancy, end-stage cirrhosis, and acute or sub-acute liver failure [58]. Transcatheter arterial chemoembolization and radiofrequency ablation may be used as a bridge therapy for unresectable liver cancer to become resectable or transplantable [21]. Transjugular intrahepatic portosystemic stent shunting is used to treat portal hypertension in patients awaiting liver transplant [22].

Medication for the liver is used to provide specific treatment for the underlying liver disease, to supplement substances that are not absorbed well due to poor liver function, and to prevent complications or relieve symptoms [59]. Drugs are used to work against infectious organisms including viruses, bacteria, and parasites [60]. Others are used to decrease serum aminotransferase, relieve jaundice, reduce intoxication, or protect the hepatocyte plasma membrane [19].

Alternative and complementary medicine for liver diseases provides benefits by regulating immunity, controlling disease progression, improving quality of life, and prolonging survival. Some of the current management decisions are based on consensus and opinions, but evidence is needed [40].

4. Alternative and Complementary Medicine

There is a long history in the treatment of liver diseases of using traditional Chinese medicine which is considered a complementary or an alternative medical system in most Western countries [61]. Herbs as complementary and alternative medicine (CAM) are effective in the treatment of liver diseases, such as antagonizing fibrosis, steatosis, and hepatitis viruses, and in protecting the liver cell [62]. A recent investigation from Saudi Arabia assessed the prevalence of and attitudes towards the use of alternative medicine in liver disease patients. The results demonstrated that over half of the patients confessed the use of alternative and complementary medicine and over two-thirds of the patients thought that many health benefits exist in alternative and complementary treatments. Most of these studies demonstrated surprisingly common usage and a generally positive attitude toward the use [13]. However, apart from its beneficial effects, there are also undesirable and unfavorable that effects resulted from CAM. First of all, we should be aware of common adverse interactions between herbal/dietary substances and prescription medications. A prospective study from Korea investigated the incidence of herbal medicine-induced adverse effects on liver functions and concluded that herbs are rather safe when used alone, but the risk of adverse reactions may increase when herbs and conventional drugs are used together [63].

Alternative medicine means the use of CAM in place of conventional medicine. This approach avoids the risk of adverse effects when herbs and conventional drugs are taken concurrently [63]. Nonetheless, there has long been a hot debate about its entity. Some authors think that the term "alternative medicine" is a misnomer and cannot be clearly differentiated from "conventional medicine" [64]. Others argue that this kind of therapy is irrational, absurd, and should be rejected [65]. Complementary medicine refers to the use of CAM together with conventional medicine; thus, the risk of adverse reactions may increase as explained afore [63]. Integrated medicine is the combination of methods from conventional medicine and CAM, based on certain high-quality evidence of safety and effectiveness. The highlight of this approach is its concentration on evidence on essential factor for therapeutic decision-making. A recent study evaluated the safety and effectiveness of treating infantile cytomegalovirus hepatitis with integrated Chinese and Western medicine. The total effective rate was 95.0% in the group with integrated medicine, significantly higher than that (77.5%) in the group with conventional medicine alone [66]. Another study conducted interviews with ten leading experts in the field of CAM and integrative medicine in Western countries and concluded that integrated medicine can aid in removing barriers and opening up medical practice and research toward new visionary health care delivery and is concerned with changing conventional medical practice [67].

CAM does exist. The term "nonconventional medicine" in itself convinces us of the differences between CAM and conventional medicine. There is no reason to worry about the withering away of conventional medicine, but it is necessary to integrate it with other less expensive, and more holistic,

more person-centered care [68]. Actually in 2007 alone, 83 million adults and 8.5 million children used CAM in the USA and paid about $34 billion in cash [69]. The widespread use of CAM in combination with Western drugs suggests that evidence-based research regarding the potential efficacy and safety of CAM methods is essential, especially when herbs are used in combination with Western drugs [70].

Extracted active components of several herbs have been shown on the molecular basis for pharmaceutical effects against liver diseases, including curcumin, emodin, and quercetin [18, 20]. In addition, clinical trials revealed that these phytochemicals act as chemosensitizers through regulating the key players of the death receptor pathway and inhibiting DNA repair and apoptosis, showing anti-ancer effects [71].

One of the 27 institutes and centers that make up the National Institutes of Health (NIH) within the Department of Health and Human Services of the federal government of the United States, the National Center for Complementary and Alternative Medicine (NCCAM) conducted a review of alternative and complementary treatments in 2002 [23]. The most promising herbal treatment for hepatitis C was silymarin, an extract of milk thistle, a plant that has been used for centuries to treat liver disease and jaundice [31]. NCCAM also studied other alternative treatments of liver diseases. These include licorice root, ginseng, Schisandra, thymus extract, and colloidal silver. They concluded that the former three might possibly have positive effects on the liver, while the latter two were found to be ineffective in treating liver diseases [72, 73].

Use of CAM has been considerably increased in patients with liver disease. Herbs are the most common and widely used, treatment in traditional Chinese medicine. The application is diverse in clinical practice. For the treatment of liver diseases, there are single herb, herb pairs, groups of herbs, and fixed formulas [55, 74, 75]. Forms of herbal drug include ointment, pellet, ball, powder, and fluid decoction [76]. More standardized treatments are extracts from single herbs and formula injections, such as the Yinzhihuang injection, the Kuhuang injection, and the Tanreqing injection [77]. Take the Kuhuang injection as an example. This injection is derived from the famous formula Yinchenhaotang by Zhongjing Zhang (150 to 219 AD), a Han Dynasty physician and one of the most eminent Chinese physicians [24]. The main ingredients are capillary artemisia, Chinese rhubarb, *Sophora flavescens*, indigo woad leaf, and herba bupleurl. At present, both injection and concoction forms of this formula are prescribed for treatment of jaundice in thousands of hospitals in China and believed to be very effective in jaundice relief, decreasing aminotransferase, and hepatoprotection [27].

However, both medical professionals and the patients should be more concerned about drug safety when herbal injection is used. Some patients who used herbal injection suffered adverse reactions, including death [77]. Here, things get very complicated. Quality of drugs and rational clinical administration are real problems at the present time. Falsified and substandard medicines are readily available in the private marketplace in low- and middle-income countries [78]. Insufficiency in detailed chemical analysis and research of related technologies on traditional Chinese medicine produces potential safety problems [77]. Even the standard and qualified herbal injections need pharmacovigilance and postmarketing safety monitoring. And there are problems that injections are misused and overused worldwide. Many injection treatments are unnecessary or could be given in an oral formulation. The behavior of patients and healthcare providers toward injection must be changed. Patients are inclined to injections because they think that they are stronger and faster cures. Doctors excessively prescribe injections because they believe that they please the patients well by so doing [79]. A special system should be set up to promote the rational use of herbal injections and supervise quality control of products and clinical application by acceptable worldwide guidelines [80, 81]. Table 1 demonstrates different treatments for various liver diseases provided by first-line clinical treatment in contrast to alternative treatment.

5. Issues with Prescription for Liver Diseases

There are a variety of liver medicines. Unfortunately, few of them are actually used successfully in humans [82]. The liver has a burden of various tasks for the support of the whole body such as detoxification, lipid, protein, and carbohydrate metabolism, as well as bile and urea production, and it is the primary port of entry for ingested drugs. Drugs for liver diseases make this burden even heavier and may impose additional problems with the diseased liver which is the mainstay to metabolize the drugs taken in [9]. Attention must be paid to relieve its workload when prescription is given [83]. Drugs with documented adverse effects on liver functions and structures must be borne in mind and avoided. When the patient has comorbidities and additional drugs have to be used to treat ailments of other systems, caution is to be taken to prevent that; the use of those drugs may cause liver dysfunction or damage, especially drugs that are frequently encountered and may cause active chronic hepatitis, acute hepatic fatty infiltration, chronic cholestasis, cholestatic jaundice, dose-dependent or independent acute hepatitis, liver cirrhosis or fibrosis, liver granuloma, neoplasms, and vasculitis [12, 39, 43].

Regrettably, such agents are numerous and their adverse effects to the liver are often not recognized or neglected. For example, long use or large doses of acetaminophen, a common agent for fever and pain relief, cause active chronic hepatitis which can be antagonized by small thorowax herb or liquorice root [36, 50]. Coincidental use of agents containing salicylic acids exacerbates liver damage leading to lethal liver cell injury [84]. These agents include Chinese wingnut leaf in decoction for killing parasites, poplar for hepatitis and other digestive diseases, levigation of catkin (willow seeds) for jaundice, and decoction of holly for diarrhea [85, 86]. Even a number of fruits that are regarded as having very safe edibility may create liver problems when they are used excessively. Dry kiwi fruit is used for anorexia, dyspepsia, and hiccough [87], and tomato has been used to treat hepatitis [88]. Both of them are used as raw materials to extract industrial salicylic acids [89]. Chinese waxberry has been grown in China at for least

Table 1: Treatments of various liver diseases provided by first line clinical treatment in contrast to alternative treatment.

Diseases/stages/conditions	First line treatment/conventional treatment	Complementary and alternative medicine	Mechanism
Alcoholic liver diseases	Nutritional therapy, maybe parenteral [14, 15]	Enteral nutritional supplementation [14, 15]	Combating malnutrition
Autoimmune liver diseases	Prednisone alone or with azathioprine [16, 17]	Curcumin [18–20]	Modulating biological activity of signaling molecules
Cirrhosis	Surgery/transjugular intrahepatic portosystemic shunts [21, 22]	Silymarin [23], Yinchenhao Tang, and Xaiocaihu Tang [24]	Hepatoprotective, directly affecting hepatitis virus
Congenital liver diseases	Combined surgery or hybrid operation [25], gene therapy [26]	Huangqi tang [24], the Kuhuang injection [27]	Hepatoprotective, relieving jaundice
Infectious liver diseases	Antimicrobial agents, invasive [7, 28, 29]	Probiotics, silymarin [30, 31]	Anti-inflammatory and selective antimicrobial effects
Intoxicated liver diseases	Chelation therapy, supportive care [32, 33]	Essential phospholipids from soybean [34]	Detoxification
Metabolic liver diseases	Diet, nutritional supplementation [35]	Glycyrrhizin and matrine [36]	Hepatoprotective and nonspecific anti-inflammatory effect
Space-occupying liver diseases (tumors)	Invasive (surgery, ablation, transplantation) [10, 37, 38]	Phytochemicals, curcumin, emodin, and quercetin [18, 20]	Death receptor pathway, inhibiting DNA repair and apoptosis

2000 years, and is used to treat digestive and inflammatory diseases. Recently, salicylic acids are identified and separated from this fruit, which accounts for the symptoms of nausea and vomiting resulted from excessive intake [90]. Another example is the damage to liver blood vessels caused by oral contraceptives [91], which can be made worse by coincidental intake of a large amount of vitamin A, contained in everyday consumption of animal viscera, eggs, milk products, and fruits [92].

Small amount or dosage is suggested for prescribing drugs for treatment of liver diseases. The essence of hepatoprotection is to reduce the liver's workload. Single agent, single remedy, or single drug prescriptions are encouraged [93]. When a compound recipe rather than a simple recipe must be used, the recommendation is the use of an empirical formula [94]. The simpler one is the better. Important origins of liver toxicity consist of adverse reaction to complementary medicines, over-the-counter medications, and prescription medications [12, 43, 95].

More words are provided here for reducing the liver's workload. Sometimes, we have to let the liver have more rest for reducing its workload and avoiding drug-drug reactions. No single potent drug is given to the patient but a prescription for easily digestible food. Glucuronolactone, vitamin C, and vitamin B complex can be used as supplements for the essential nutritional requirements for detoxifying functions of the liver [96, 97]. Adequate amounts of glucose and amino acids are needed for enriched nutrition for the liver. But too much glucose leads to diabetes, and to much fructose leads to fatty liver disease [5]. Inosine is necessary for cell repair and has been shown to have a therapeutic effect on leukocytopenia and thrombocytopenia [98]. Normal bowel movement is part of gastrointestinal well-being and beneficial for the gut microflora with the consequence of less absorption of toxins generated by intestinal bacteria [99].

In favor of the symbiotic relationship between the liver and intestinal tract, probiotic bacteria, for example, bifidobacteria, are better than antibiotic and prebiotic nutrition, say, nondigestible food ingredients [6]. As effects of drug-metabolizing enzyme systems on the pharmacological activity are more likely to occur in the intestine and liver, more attention should be paid to potential risk of nutrient-drug interactions to prevent undesired and harmful clinical consequences [100].

The liver depends for 80% of its blood supply on other organ systems [101]. So, bed rest is favorable for the nutrition and function of the liver by modifying its blood supply. The erect posture significantly decreases thee volume of blood flow to the liver up to 40% [102]. As the Chinese saying goes, "the liver stores blood." This means on daytime more blood flows to the extremities, while at night it shifts to the liver. In the supine position, there is abundant blood in the liver. Excessive exercise is hazardous because the reduction of hepatic blood flow goes up to 80–85% when exercising, and chronic hepatitis leads to slow liver blood flow and increased viscosity [96]. Blood supply of the liver can be improved by proper use of red-rooted salvia and ligustici chuanxiong [103, 104].

Consumption of meat and fat intake is also linked to liver disease. Meat, especially red meat, is assumed to enhance the risk of cancer [105]. It is reported that red meat and saturated fat may be associated with increased risk of chronic liver diseases and liver cancer, whereas white meat may be inversely associated with that risk [106]. Nonetheless, meat

consists of high concentrations and better bioavailability of folic acid, selenium, zinc, and other components that are asserted to be preventive candidates. Thus, a balanced diet is suggested that is rich in fruit and vegetables, containing meat and meat products in appropriate amount [105].

6. Recent Development

First do no harm. Beneficence, nonmaleficence, and self-government for the patient are substantial support for biomedical ethics that also adapt for the world of natural medicines including CAM for liver disease. Quality control of CAM methods and products is one of the greatest challenges that the professionals confront [62]. Hepatology has become an independent area with rapid expansion in some countries from a subspecialty of gastroenterology with doctors specializing as hepatologists [15]. A number of controlled prospective double-blind multicenter studies are ongoing with the newly discovered drugs with proven beneficial effects on animals [107, 108].

Structure and function of the liver are observed more precisely. The motion and deformation of the liver in real time within a region of interest can be measured by a real-time 3D image guidance system with a seeker needle including embedded electromagnetic tracking sensors [10]. This facility is useful in radiofrequency ablation and other percutaneous ablative procedures for treatment of space-occupying lesions. Tests are developed of various antibodies for autoimmune liver diseases that have overlapping features in clinical, histological, radiological, and serological manifestations, and currently randomized and controlled outcome data are insufficient to develop standardized diagnostic criteria [40].

Acceptable effects have been obtained on researches in treatment with alternative and complementary medicine of chronic liver diseases in the aspects of idiopathic hepatitis, nonalcoholic steatohepatitis, liver fibrosis, oxidative stress, and immune regulation. Adiponectin is shown to play a role in the suppression of the metabolic derangements that may result in nonalcoholic fatty liver disease. Leptin is revealed to play a key role in regulating energy intake and energy expenditure, including appetite and metabolism [5]. The combination use of adiponectin and leptin has been shown to completely reverse insulin resistance associated with obesity [109]. Herbs that increase adiponectin and/or leptin include Chinese dong quai root, goldenseal root, bacopa, green tea, and milk-vetch [104, 110, 111].

Trace elements serve critical roles in normal liver function and liver disease. However, excessive trace elements are commonly toxic to the liver. Selenium deficiency in hepatocytes leads to cell death by oxidative stress. Foods rich in selenium comprise nuts, sunflower seeds, shellfish, and liver [105]. A number of herbs are also high in selenium, for example, garlic, ginger, mustard seeds, fenugreek, and chervil [23, 112]. Other important trace elements with respect to liver diseases include iron, copper, zinc, calcium, magnesium, and manganese [48, 105].

Extensive and in-depth researches of CAM are needed to accumulate evidence of defined therapeutic effects. Although licorice root and ginseng have independent effects on liver cancer, the combinations of both extracts were found to have an antagonistic effect on cell viability and increased cultured Hep-G2 survival [113].

Systematic analyses are conducted of the therapeutic effects of the working mechanisms of some formulas using immunohistochemistry, biochemistry, metabolomics, and proteomics [114]. Chronobiology emerges with the current knowledge of circadian rhythms in Hepatology. A certain drug can be therapeutic and safe when taken at one time, but insufficient or poorly tolerated at another. Circadian rhythm abnormalities are associated with a variety of types of chronic liver diseases including hepatitis C, primary biliary cirrhosis, Wilson disease, and fibrosis and hepatic encephalopathy [115].

7. Conclusions

There is a vast body of knowledge ever increasing about the treatment of liver disease with alternative and complementary medicine for which hundreds of thousands of literatures have been documented. Liver disease has a variety of presentations and causes a great worldwide public health problem which threatens the wellness of billions of people. Incidences of many types of liver disease are currently rising. Although a debate exists about its entity, alternative and complementary medicine is widely used and it is improving, and it covers the shortages and compensates for the weaknesses of conventional methods in the treatment of liver disease. Alternative and complementary medicine for liver diseases provides benefits by regulating immunity, controlling disease progression, improving quality of life, and prolonging survival. Some of the current management decisions are based on consensus and opinions, but evidence is needed to confirm the effects of single herb, herb pairs, groups of herbs, and fixed formulas.

Drug safety is concerned especially when herbal injection is used. Even the standard and qualified herbal injections need pharmacovigilance and postmarketing safety monitoring. A special system should be set up to promote the rational use of herbal injections and supervise quality control by acceptable worldwide guidelines. When CAM is used to treat liver disease, a manner of single agent, simple method, and small amount is suggested to reduce the liver's workload. Attention should also be paid to the nutrition and blood supply of the liver, as well as drug-food interactions.

Recent development includes researches in quality control of CAM methods and products, emerging of specialists such as hepatologists, more precise observation of the structure and function of the liver, acceptable effects on several liver diseases, importance of trace elements, and hepatic chronobiology. Studies are encouraged such as controlled prospective double-blind multicenter studies with the newly discovered drugs with proven beneficial effects on animals.

New aspects that CAM can provide include meditation, support groups, and integrative medicine. More and more individuals are using CAM for treatment of liver diseases, and most of them believe that CAM has numerous health

benefits. The growing public interest in CAM reflects the fact that CAM is now widely accepted as an important ally in our fight against liver diseases. However, many widely used CAM treatments remain untested. For example, St. John's wort which has been used as a treatment of depression from liver cancer is now the number-one-selling nutritional supplement in the USA. But it has not been rigorously evaluated and found with untoward effects on HIV drugs, transplanted organs, and sunshine in recent studies. So it is urgent to collect compelling and rigorous data of CAM treatments. The public needs to be kept well informed to reject readily modalities that are found unsafe or ineffective.

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Research Article

Ethnomedicinal Practices for Treating Liver Disorders of Local Communities in the Southern Regions of Korea

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This study aims to analyze and record ethnomedicinal practices for treating liver disorders of residents living in local communities in the southern regions of Korea. Data was collected using participant observations and in-depth interviews, as the informants also become investigators themselves through attending informal meetings, open and group discussions, and overt observations with semistructured questionnaires. In this study, ethnomedicinal practices for liver ailments were recorded by 1,543 informants (362 men, 1,181 women) at 160 sites. The kinds of liver disorders treated by ethnomedicinal practices were liver cancer, liver cirrhosis, jaundice, hepatitis, fatigue recovery, hangovers, and liver-related ailments. The category with the highest degree of consensus from the informants was jaundice (0.95), and the lowest degree of consensus was for liver cancer and liver cirrhosis (0.61). According to fidelity levels, 28 species resulted in fidelity levels of 100%. The internetwork analysis was first applied for the interpretation of ethnomedicinal knowledge of a community, although it has been strictly used until now for social science in the analysis of social trends and phenomena through the interrelationship of specific components.

1. Introduction

After the agreement of the Nagoya Protocol, a stronger interest for sharing the ethnomedicinal knowledge of genetic resources and their benefits to the world has occurred [1]. The ethnomedicinal practices hold significant value among the traditional knowledge of the local communities [2]. Also, ethnomedicinal practices hold an important position in caring for health issues in developing countries [3, 4]. Particularly, the ethnomedicinal practices of regions known for traditional medicine include China, Tibet, and India, nations which have actively utilized these treatments for health concerns.

The ethnomedicinal practices of Korea have been continuously successful for over 3,000 years. Korean ethnomedicinal practices contain not only medicinal practice, which include single-medicine proscription, herb fomentation, herb fumigation, and herb ablution, but also nonmedicinal practices like acupuncture, moxibustion, Korean traditional therapeutic touch, Korean traditional saunas, and Korean traditional divination practice. These days, Korean ethnomedicinal practices have been restricted to oral transmission because government policy, after the Korean War, centered on conventional medicine, and the Japanese imperialism, which Korea endured from 1919 to 1945, attempted to annihilate its culture [5].

A scientific investigation of liver disease, the first of its kind, was conducted on indigenous communities in Uttarakhand (India) and was related to jaundice research [6]. In Korea, studies on disease within local communities have been conducted on three separate occasions by the authors: respiratory diseases [7], diseases related to digestion [8], and pain relief [9]; however, this research is the first one to focus strictly on liver disorders.

Our research on liver disorders contains an important meaning to first record and analyze the ethnomedicinal practices of local communities in East Asia, including Korea. Additionally, our research newly included the quantitative analysis method known as internetwork analysis (INA), which has allowed more information to be attained in regard to ethnomedicinal knowledge.

Generally, quantitative analysis for ethnomedicinal knowledge of local communities has solely relied on the

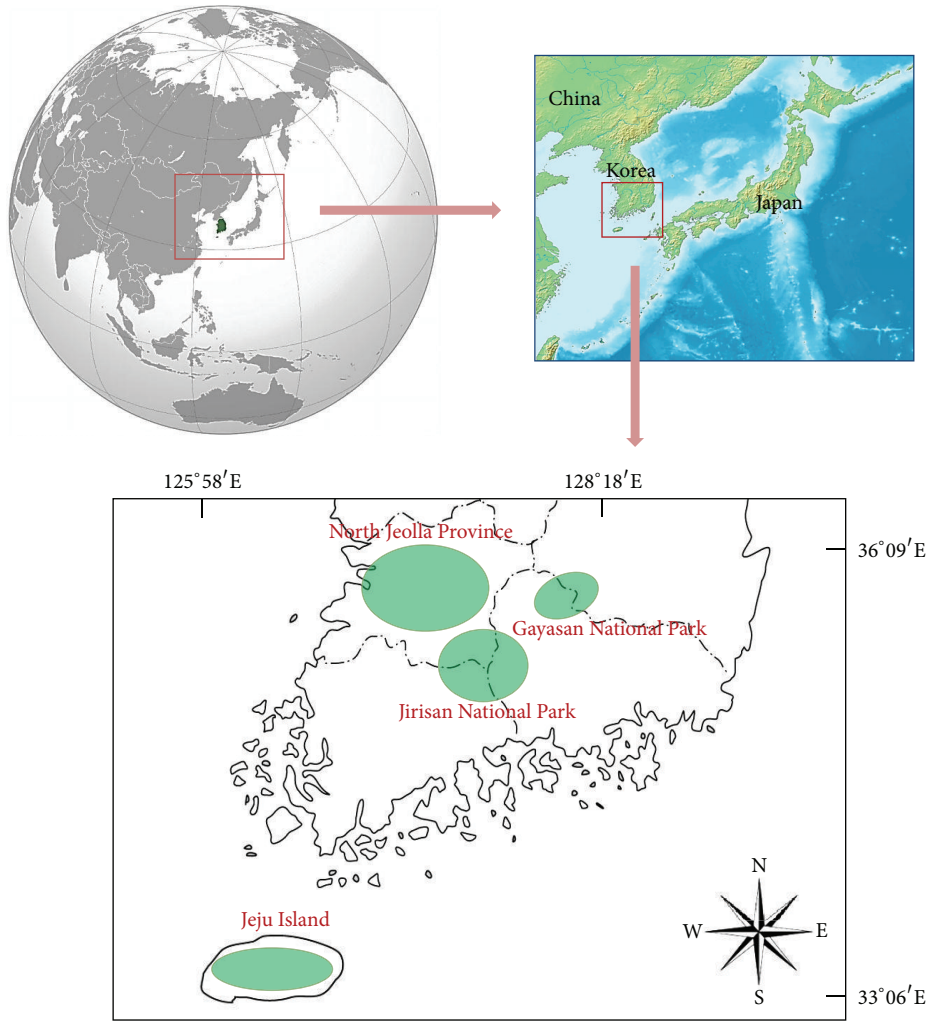

Figure 1: Investigation sites.

consensus of the informants and the recorded fidelity levels; therefore, a need exists to utilize the internetwork analysis method to consider the traditional ethnographical properties.

Particularly, a deeper analysis of ethnomedicinal practices in treating specific diseases within the local communities is necessary for obtaining more specific details regarding the internetwork of the components within ethnomedicinal knowledge.

Our research suggests that the applications gained from utilizing the internetwork analysis (INA) for ethnomedicinal practices on liver disorders within communities in Korea will result in further research incorporating INA.

2. The Research Area and Method

2.1. Natural and Social Environment of Research Area. The study area consists of the southern region of the Korean peninsula and its many islands, which lie between 33°06′N to 36°09′N latitude and 125°58′E to 128°18′E (Figure 1). The total population in 2012 of the study area was 5,914,270. The area measures approximately 485 km^2 and includes three provinces, 15 cities, and 27 counties in its administrative district [10]. The annual precipitation is around 1,000~1,850 mm in which the coastal area generally receives more rainfall than the inland regions. The annual average temperature of the inland regions is 13.8°C while Jeju Island records 16.2°C [11].

2.2. Research Methods. Field investigations were conducted from March 2009 to November 2012. Proper data was collected using participant observations and in-depth interviews, as the informants also become investigators themselves through attending informal meetings, open and group discussions, and overt observations with semistructured questionnaires [12, 13].

The content of the semi-structured questionnaires was composed of diverse information regarding medicinal species used to treat liver disorders, including local names, used parts, ailments, methods of preparation, manufacturing and administration, dosage, and the usable duration regarding each curable formula [13–15].

All specimens were collected during their flowering or fruiting seasons and were organized utilizing the normal specimen manufacturing method [15, 16]. The voucher

specimens were deposited for preservation in the herbarium of Jeonju University. The precise identification of species mentioned by the informants was performed in accordance with Lee [17], Lee [18], Ahn [19], Lee [20], and Park [21]. Scientific names were confirmed by the National Knowledge and Information System for Biological Species [22] of Korea.

2.3. Quantitative Analysis

2.3.1. Informant Consensus Factor (ICF). The ICF was used to analyze the agreement degree of the informants' knowledge about each category of ailments [23, 24]. The ICF was calculated using the following formula:

$$\mathrm{ICF} = \frac{(n_{\mathrm{ur}} - n_t)}{(n_{\mathrm{ur}} - 1)}, \quad (1)$$

where n_{ur} is the number of use reports of informants for a particular liver disorder and n_t is the number of species used by all informants for a particular liver disorder.

2.3.2. Fidelity Level (FL). The FL was employed to determine the most important species used for treating certain liver disorders by the local practitioners and the elderly people living in the study area [13, 16, 25]. The FL was calculated using the following formula:

$$\mathrm{FL}\,(\%) = N_p \times \frac{100}{N}, \quad (2)$$

where N_p is the number of informants that mentioned the specific species used to treat certain disorders and N is the total number of the informants who utilized the species as medicine for treating any given disorder.

2.3.3. Internetwork Analysis (INA). Internetwork analysis does not focus on the independent characteristics of an individual within the community, but it considers the results of the interrelationship among each individual of a community. Internetwork analysis has been applied within communities to various ethnographical problems, including ethnogenesis [26] and obesity [27–29]; however, prior to this research, the internetwork analysis had yet to be applied to ethnomedicinal knowledge, included with its ethnographical properties in the results.

Our research newly applied this method in order to attain more internetwork information from the treatment of ethnomedicinal practices on liver disorders within communities in Korea. The results of the internetwork analysis of disorders and medicinal species were analyzed using UCINET (Ver. 6.460) and NetDraw (Ver. 2.125) software [30, 31].

3. Results and Discussion

3.1. Demographic Characteristics of the Region. All 2,069 informants were randomly selected at the community halls, the senior welfare centers, and the traditional markets at 305 sites. Among them, ethnomedicinal practices for liver disorders were recorded by 1,543 informants (362 men, 1,181 women) at 160 sites (Figure 1). The average age of the informants was 75 years old, with a range in age from 36 to 94, with residents living more than 40 years in the study area. The ethnographical characteristics of the communities are summarized in Table 1.

Table 1: Demographic characteristics.

Gender	
Male	362 (23.5%)
Female	1,181 (76.5%)
Age	
30–39	9 (0.6%)
40–49	4 (0.3%)
50–59	73 (4.7%)
60–69	250 (16.2%)
70–79	707 (45.8%)
80–89	460 (29.8%)
90–99	40 (2.6%)
Educational attainment	
Never attended school	1,127 (73.0%)
Attended school for less than 6 years	125 (8.1%)
Attended school for 6 years	133 (8.6%)
Finished middle school	101 (6.5%)
Finished high school	57 (3.7%)

Linguistically, the inland communities represented varying properties between the eastern and western communities, the Jirisan axis (1,915 m). The pronunciation of the two local communities depicts dissimilar intonations, while the languages of the communities on Jeju Island possess numerous dialects different from the inland communities.

In regard to foods, the local communities in the eastern region widely used the seed powder of *Zanthoxylum piperitum* (L.) DC. and the leaves of the *Isodon japonicus* (Burm.) Hara, while local communities in the western region did not consume these foods. Also, the food traditions in communities on Jeju Island are quite diverse from foods of the inland communities in regard to the recipe and ingredients.

The local communities in the east are politically conservative, while local inland communities in the west are more progressive. However, the communities on Jeju Island display extreme exclusiveness because of their historical experiences.

In homes within the inland communities, men usually support their families financially, while women traditionally support their families on Jeju Island.

3.2. Analysis of Ethnomedicinal Practices. The kinds of liver disorders treated by ethnomedicinal practices were liver cancer, liver cirrhosis, jaundice, hepatitis, fatigue recovery, hangovers, and liver-related ailments (Table 2). Also, this study area is three times larger than previous research, while the seven types of liver disorders recorded in this study were less than previous research, which classified 14 types of respiratory system diseases, 29 types of digestive system diseases, and 23 types of pain relief treatment [7–9]. We believe that the communities of this study area possess

Table 2: Information about ethnomedicinal practices recorded from residents in the research area.

Liver disorders	Scientific name	Korean name	Number of mentions	Kind	Used part	Preparation	Application	FL
Fatigue recovery	*Plecoglossus altivelis* Temminck et Schlegel	Euneo	1	Animal	Whole part	Sashimi	Oral	100.0
	Vespa mandarinia (Cameron)	Jangsumalbeol	12	Animal	Hive, imago, larva	Dissolution	Oral	100.0
	Vespula flaviceps lewisii (Cameron)	Ttangbeol	18	Animal	Hive, imago, larva	Dissolution	Oral	100.0
Hangover	*Hovenia dulcis* Thunb.	Heotgaenamu	4	Plant	Fruit, leaf	Decoction	Oral	3.2
	Oenanthe javanica (Blume) DC.	Minari	4	Plant	Aerial part	Maceration	Oral	10.3
Hepatitis	*Ganoderma lucidum* (Leyss.: Fr.) Karst.	Yeongjibeoseot	1	Fungi	Whole part	Decoction	Oral	100.0
	Plantago asiatica L.	Jilgyeongi	2	Plant	Leaf	Decoction	Oral	100.0
	Protaetia brevitarsis seulensis (Kolbe)	Huinjeombagikkonmuji	10	Animal	Larva	Decoction	Oral	21.7
	Sedum sarmentosum Bunge	Dollamul	18	Plant	Whole part	Juice	Oral	22.5
	Sophora flavescens Solander ex Aiton	Gosam	7	Plant	Root	Pill	Oral	100.0
	Viola mandshurica W. Becker	Jebikkot	2	Plant	Whole part	Decoction	Oral	50.0
	Actinidia arguta (Siebold & Zucc.) Planch. ex Miq.	Darae	3	Plant	Sap	Raw	Oral	100.0
	Akebia quinata (Houtt.) Decne.	Eureumdeonggul	4	Plant	Stem	Decoction	Oral	100.0
Jaundice	*Artemisia capillaris* Thunb.	Sacheolssuk	344	Plant	Aerial part, leaf, stem, whole part	A sweet drink made from fermented rice, brewing, decoction, dried, grain syrup, infusion, juice, pill, simmer	Oral	75.6
	Artemisia gmelinii Weber ex Stechm.	Deowijigi	1	Plant	Whole part	A sweet drink made from fermented rice, brewing, infusion	Oral	100.0
	Artemisia princeps Pamp.	Ssuk	10	Plant	Leaf	Rubbing	Topical	83.3
	Aster scaber Thunb.	Chamchwi	8	Plant	Leaf	Parboiled	Oral	100.0
	Bombyx mori (Linne)	Nuenabang	1	Animal	Larva	Dried, powder, steam	Oral	100.0
	Caragana sinica (Buc'hoz) Rehder	Goldamcho	8	Plant	Root	A sweet drink made from fermented rice	Oral	100.0
	Catalpa ovata G. Don	Gaeodong	1	Plant	Root	Decoction	Oral	100.0
	Cucumis melo var. *makuwa* Makino	Chamoe	19	Plant	Fruit, peduncle	Dried, powder	Topical	100.0
	Equus caballus Linn.	Mal	8	Animal	Hide, meat	Infusion, raw	Oral	100.0
	Fallopia japonica (Houtt.) RonseDecr.	Hojanggeun	2	Plant	Root	Decoction	Oral	100.0
	Gloydius blomhoffii brevicaudus Stejneger	Salmosa	1	Animal	Whole part	Raw	Oral	100.0

Table 2: Continued.

Liver disorders	Scientific name	Korean name	Number of mentions	Kind	Used part	Preparation	Application	FL
Jaundice	*Gloydius saxatilis* Emelianov	Kkachisalmosa	1	Animal	Whole part	Raw	Oral	100.0
	Gloydius ussuriensis Emelianov	Soesalmosa	1	Animal	Whole part	Raw	Oral	100.0
	Hordeum vulgare var. *hexastichon* (L.) Asch.	Bori	31	Plant	Malt, seed	A sweet drink made from fermented rice, tea	Oral	66.0
	Incilaria bilineata (Benson)	Mindalpaengi	3	Animal	Whole part	Dried, dissolution, maceration, roast	Oral	75.0
	Leonurus japonicus Houtt.	Ingmocho	16	Plant	Aerial part	A sweet drink made from fermented rice, infusion, juice, pill, tea	Oral	80.0
	Oenanthe javanica (Blume) DC.	Minari	8	Plant	Aerial part, leaf, stem	Seasoned cooked vegetables, juice	Oral	20.5
	Oryza sativa L.	Byeo	10	Plant	Seed	A sweet drink made from fermented rice	Oral	100.0
	Pelodiscus sinensis Wiegmann	Jara	1	Animal	Whole part	Simmer	Oral	100.0
	Plantago asiatica L.	Jilgyeongi	2	Plant	Leaf	Decoction	Oral	100.0
	Protaetia brevitarsis seulensis (Kolbe)	Huinjeombagikkonmuji	3	Animal	Whole part	Dried, panbroiled, powder	Oral	6.5
	Sasa borealis (Hack.) Makino	Joritdae	3	Plant	Root	Decoction	Oral	100.0
	Viola mandshurica W. Becker	Jebikkot	2	Plant	Whole part	Decoction	Oral	50.0
Liver cancer	*Allomyrina dichotoma* (Linne)	Jangsupungdengi	3	Animal	Larva	Dried, infusion, pill, powder, steam	Oral	20.0
	Breea segeta (Willd.) Kitam. f. *segeta*	Jobaengi	2	Plant	Root	Decoction	Oral	100.0
	Bupleurum falcatum var. *scorzoneraefolium* (Willd.) Ledeb.	Chamsiho	2	Plant	Whole part	Decoction	Oral	100.0
	Cetonia pilifera (Motschulsky)	Kkonmuji	5	Animal	Larva, whole part	Dried, infusion, panbroiled, pill, powder, simmer	Oral	38.5
	Cirsium japonicum var. *maackii* (Maxim.) Matsum.	Eonggeongkwi	2	Plant	Root	Decoction	Oral	100.0
	Fomes fomentarius (L.: Fr.) Fr.	Malgupbeoseot	1	Fungi	Whole part	Infusion	Oral	20.0
	Formica yessensis Wheeler	Bulgaemi	1	Animal	Whole part	Decoction, simmer	Oral	100.0
	Gallus gallus domesticus Linn.	Dak	1	Animal	Whole part	Decoction, simmer	Oral	11.1
	Meles meles melanogenys Allen & Andrew.	Osori	1	Animal	Gall bladder	Dried, mixed in liquor	Oral	50.0
	Protaetia brevitarsis seulensis (Kolbe)	Huinjeombagikkonmuji	5	Animal	Larva, whole part	Dried, infusion, panbroiled, powder, pill, simmer	Oral	10.9
	Protaetia mandschuriensis (Schurhoff)	Manjujeombagikkonmuji	5	Animal	Larva, whole part	Dried, infusion, panbroiled, powder, pill, simmer	Oral	38.5
	Rhododendron mucronulatum Turcz. var. *mucronulatum*	Jindallae	2	Plant	Flower	Fermentation	Oral	100.0

Table 2: Continued.

Liver disorders	Scientific name	Korean name	Number of mentions	Kind	Used part	Preparation	Application	FL
Liver cirrhosis	*Scolopendra subspinipes mutilan* L. Koch	Jine	2	Animal	Whole part	Decoction, simmer	Oral	33.3
	Bupleurum falcatum L.	Siho	2	Plant	Root	Decoction	Oral	100.0
	Cetonia pilifera (Motschulsky)	Kkonmuji	4	Animal	Larva, whole part	Dried, panbroiled, powder, simmer	Oral	30.8
	Cryptotympana dubia (Haupt)	Malmaemi	2	Animal	Larva	Dried, powder, pill	Oral	100.0
	Fomes fomentarius (L.: Fr.) Fr.	Malgupbeoseot	1	Fungi	Whole part	Decoction	Oral	20.0
	Graptopsaltria nigrofuscata (Motschulsky)	Yujimaemi	3	Animal	Larva	Dried, powder, pill	Oral	100.0
	Leptosemia takanonis (Matsumura)	Soyosanmaemi	2	Animal	Larva	Dried, powder, pill	Oral	100.0
	Meimuna opalifera (Walker)	Aemaemi	2	Animal	Larva	Dried, powder, pill	Oral	100.0
	Oenanthe javanica (Blume) DC.	Minari	2	Plant	Aerial part	Juice	Oral	5.1
	Oncotympana fuscata (Distant)	Chammaemi	2	Animal	Larva	Dried, powder, pill	Oral	100.0
	Platypleura kaempferi (Fabricius)	Teolmaemi	2	Animal	Larva	Dried, powder, pill	Oral	100.0
	Protaetia brevitarsis seulensis (Kolbe)	Huinjeombagikkonmuji	4	Animal	Larva, whole part	Dried, panbroiled, powder, simmer	Oral	8.7
	Protaetia mandschuriensis (Schurhoff)	Manjujeombagikkonmuji	4	Animal	Larva, whole part	Dried, panbroiled, powder, simmer	Oral	30.8
	Scolopendra subspinipes mutilans L. Koch	Jine	2	Animal	Whole part	Dried, powder, pill	Oral	33.3
Liver-related ailments	*Acer tegmentosum* Maxim.	Sangyeoreumnamu	3	Plant	Stem	Decoction, tea	Oral	100.0
	Adenocaulon himalaicum Edgew.	Myeolgachi	2	Plant	Whole part	Juice	Oral	100.0
	Allomyrina dichotoma (Linne)	Jangsupungdengi	12	Animal	Larva, whole part	Dissolution, dried, extraction, infusion, panbroiled, powder, roast, simmer	Oral	80.0
	Artemisia capillaris Thunb.	Sacheolssuk	111	Plant	Aerial part, leaf, whole part	A sweet drink made from fermented rice, decoction, grain syrup, infusion, juice, pill, simmer	Oral	24.4
	Artemisia princeps Pamp.	Ssuk	2	Plant	Whole part	Juice	Oral	16.7
	Canis lupus familiaris Linn.	Gae	1	Animal	Whole part	Infusion	Oral	100.0
	Capsella bursapastoris (L.) L.W.Medicus	Naengi	24	Plant	Whole part	Juice, seasoned cooked vegetables, soup	Oral	100.0
	Cetonia pilifera (Motschulsky)	Kkonmuji	4	Animal	Larva	Dried, panbroiled, powder, simmer	Oral	30.8
	Citrus junos Siebold ex Tanaka	Yujanamu	6	Plant	Fruit	Decoction	Oral	100.0
	Citrus natsudaidai Hayata	Hagyul	6	Plant	Fruit	Maceration	Oral	100.0
	Citrus tenuissima Tanaka.	Dangyujanamu	10	Plant	Fruit	Decoction	Oral	100.0

Table 2: Continued.

Liver disorders	Scientific name	Korean name	Number of mentions	Kind	Used part	Preparation	Application	FL
Liver-related ailments	*Corbicula (Corbicula) japonica* Prime	Ilbonjaecheop	8	Animal	Whole part	Simmer	Oral	100.0
	Cudrania tricuspidata (Carr.) Bureau ex Lavallee	Kkujippongnamu	43	Plant	Bark, stem	A sweet drink made from fermented rice, decoction, infusion	Oral	100.0
	Dendranthema indicum (L.) DesMoul.	Gamguk	3	Plant	Flower	Decoction	Oral	100.0
	Dendropanax morbiferus H. Lev.	Hwangchillamu	4	Plant	Leaf, stem	Decoction	Oral	100.0
	Duchesnea indica (Andr.) Focke	Baemttalgi	2	Plant	Fruit	Decoction	Oral	100.0
	Eleutherococcus sessiliflorus (Rupr. & Maxim.) S. Y. Hu	Ogalpinamu	19	Plant	Fruit, stem	Extraction, infusion	Oral	100.0
	Equisetum hyemale L.	Soksae	2	Plant	Stem	Decoction	Oral	100.0
	Eucommia ulmoides Oliv.	Duchung	2	Plant	Stem	Decoction	Oral	100.0
	Felis catus Linn.	Goyangi	1	Animal	Whole part	Simmer	Oral	100.0
	Fomes fomentarius (L.: Fr.) Fr.	Malgupbeoseot	3	Fungi	Whole part	Decoction	Oral	60.0
	Gallus gallus domesticus Linn.	Dak	8	Animal	Whole part	Infusion	Oral	88.9
	Glycine max (L.) Merr.	Kong	2	Plant	Seed	Steeped in vinegar	Oral	100.0
	Hedera rhombea (Miq.) Bean	Songak	2	Plant	Fruit	Decoction	Oral	100.0
	Hordeum vulgare var. *hexastichon* (L.) Asch.	Bori	16	Plant	Malt	A sweet drink made from fermented rice, pill	Oral	34.0
	Houttuynia cordata Thunb.	Yangmomil	5	Plant	Whole part	Decoction, infusion	Oral	100.0
	Hovenia dulcis Thunb.	Heotgaenamu	121	Plant	Fruit, leaf, stem	Decoction, infusion, tea	Oral	96.8
	Incilaria bilineata (Benson)	Mindalpaengi	1	Animal	Whole part	Dissolution, dried, powder	Oral	25.0
	Ixeridium dentatum (Thunb. ex Mori) Tzvelev	Sseumbagwi	5	Plant	Whole part, young leaf	Juice	Oral	100.0
	Kalopanax septemlobus (Thunb.) Koidz.	Eumnamu	9	Plant	Stem	Decoction, infusion	Oral	100.0
	Leonurus japonicus Houtt.	Ingmocho	4	Plant	Aerial part	Infusion, pill	Oral	20.0
	Lycium chinense Mill.	Gugijanamu	8	Plant	Fruit	Infusion	Oral	100.0
	Meles meles melanogenys Allen & Andrew.	Osori	1	Animal	Gall bladder	Brewing	Oral	50.0
	Nyctereutes procyonoides Koreensis Mori.	Neoguri	2	Animal	Gall bladder	Brewing	Oral	100.0
	Oenanthe javanica (Blume) DC.	Minari	25	Plant	Aerial part, stem, whole part	Juice	Oral	64.1
	Orostachys japonica (Maxim.) A.Berger	Bawisol	2	Plant	Whole part	Decoction, juice	Oral	100.0
	Paeonia lactiflora Pall.	Jagyak	2	Plant	Root	Decoction, roast	Oral	100.0
	Phasianus colchicus Linn.	Kkwong	1	Animal	Whole part	Soup	Oral	100.0
	Phellinus linteus (Berk. et Curt.) Teng	Mokjiljinheukbeoseot	3	Fungi	Whole part	Decoction	Oral	100.0

Table 2: Continued.

Liver disorders	Scientific name	Korean name	Number of mentions	Kind	Used part	Preparation	Application	FL
	Pinus densiflora Siebold & Zucc.	Sonamu	4	Plant	Leaf	Dried, powder, steam	Oral	100.0
	Protaetia brevitarsis seulensis (Kolbe)	Huinjeombagikkonmuji	24	Animal	Larva, whole part	Decoction, dissolution, dried, extraction, panbroiled, powder, simmer	Oral	52.2
	Protaetia mandschuriensis (Schurhoff)	Manjujeombagikkonmuji	4	Animal	Larva	Dried, panbroiled, powder, simmer	Oral	30.8
	Prunus padus L. for. *padus*	Gwirungnamu	2	Plant	Stem	Decoction	Oral	100.0
	Pueraria lobata (Willd.) Ohwi	Chik	1	Plant	Root	Decoction	Oral	100.0
	Sanguisorba officinalis L.	Oipul	2	Plant	Root	Decoction	Oral	100.0
	Schisandra chinensis (Turcz.) Baill.	Omija	6	Plant	Fruit, root, stem	Brewing	Oral	100.0
	Scolopendra subspinipes mutilans L. Koch	Jine	2	Animal	Whole part	Dissolution, dried, powder	Oral	33.3
	Scutellaria baicalensis Georgi	Hwanggeum	1	Plant	Root	Decoction	Oral	100.0
	Sedum sarmentosum Bunge	Dollamul	62	Plant	Aerial part	Juice, maceration, powder, raw, seasoned cooked vegetables, watery plain kimchi	Oral	77.5
Liver-related ailments	*Semisulcospira coreana* (Martens)	Chamdaseulgi	11	Animal	Whole part	Clear soup with flour dumplings, infusion, juice, simmer	Oral	100.0
	Semisulcospira forticosta (Martens)	Jureumdaseulgi	23	Animal	Whole part	Clear soup with flour dumplings, infusion, juice, simmer	Oral	100.0
	Semisulcospira libertina (Gould)	Daseulgi	30	Animal	Body, whole part	Clear soup with flour dumplings, infusion, juice, soup, simmer	Oral	100.0
	Sorbus commixta Hedl.	Magamok	10	Plant	Fruit, leaf, stem	Decoction, tea	Oral	100.0
	Sus scrofa Linn.	Metdwaeji	6	Animal	Gall bladder	Dissolution, juice	Oral	100.0
	Taraxacum platycarpum Dahlst.	Mindeulle	192	Plant	Aerial part, leaf, whole part	A sweet drink made from fermented rice, decoction, dried, extraction, infusion, juice, kimchi, raw, seasoned cooked vegetables, simmer, tea	Oral	100.0
	Trachelospermum asiaticum (Siebold & Zucc.) Nakai var. *asiaticum*	Masakjul	4	Plant	Leaf, stem	Decoction	Oral	100.0
	Ulmus davidiana var. *japonica* (Rehder) Nakai	Neureumnamu	8	Plant	Bark	Decoction	Oral	100.0
	Ursus thibetanus ussuricus Heude	Bandalgaseumgom	1	Animal	Flesh	Infusion	Oral	100.0
	Zingiber mioga (Thunb.) Roscoe	Yangha	2	Plant	Whole part	Juice	Oral	100.0

relative health issues related to liver conditions compared to other health concerns.

The 254 ethnomedicinal practices recorded from the communities were classified into 55 families, 85 genera, and 94 species that included plants, animals, and fungi (Table 2). Among these species, plants totaled 150 ethnomedicinal practices based on 31 families, 52 genera, and 57 species while animals included 99 ethnomedicinal practices based on 21 families, 30 genera, and 34 species. Fungi recorded five ethnomedicinal practices based on three families, three genera, and three species. These usage patterns were different from Korean traditional medicine, in which plants are used relatively much more than animals. Research confirms that the communities have focused on direct nutritional supplements from the traditional medicine rather than seek after an actual cure for their liver disorders.

This supposition was confirmed by the fact that liver-related ailments and jaundice require a greater necessity for nutritional supplements than other diseases which are contained within many medicinal species and various ethnomedicinal practices.

Namely, the number of medicinal species and ethnomedicinal practices for liver-related ailments consisted of 59 species (62.7% of the total species) and 143 ethnomedicinal practices (56.3% of the total practices). Jaundice used 25 species (26.6% of the total species) and 50 ethnomedicinal practices (19.7% of the total practices).

Also, the number of informants who mentioned liver-related ailments and cases of jaundice occupied 58%, which totaled 32% of the whole, respectively (Table 2). As a result, the communities tended to use ethnomedicinal practices to care for their overall health instead of as a cure for a long-term condition.

For plants, 13 used parts were used in practice, while 9 used parts of animals and one used part of fungi were used in treatment. Preparations of the plants consisted of 24 kinds, with 19 preparations for animals and two preparations for fungi (Table 2). The usage recorded is similar to previous research for other diseases [7–9].

Among the medicinal species, the most often mentioned plants were *Artemisia capillaris* Thunb., *Taraxacum platycarpum* Dahlst., and *Hovenia dulcis* Thunb. (50.99% mentioned), while the common animals were *Protaetia brevitarsis seulensis* (Kolbe), *Semisulcospira libertina* (Gould), and *Semisulcospira forticosta* (Martens) (6.90% mentioned). The number of mentioned plants focused more on minor species than animal species. Through continued research, these species can certainly be developed into functional foods for particular liver disorders.

3.3. Quantitative Analysis

3.3.1. Informant Consensus Factor (ICF). The informant consensus factor ranges from 0 to 1, where the increasing values indicate a higher rate of informant consensus among the illness category. The category with the highest degree of consensus from the informants was jaundice (0.95), followed by liver-related ailments and fatigue recovery (0.93), hepatitis (0.87), and a hangover (0.86). The lowest degree of consensus was for liver cancer and liver cirrhosis (0.61). These results inform that ethnomedicinal practices have been applied more often to minor health issues related to the liver.

More often, people suffering from serious liver disorders have been treated in the hospital using conventional medicine or Korean traditional medicine. However, ethnomedicinal practices have been used to cure jaundice, liver-related ailments, and fatigue recovery.

3.3.2. Fidelity Level (FL). The FL is useful for identifying the informants' most preferred species in use for treating certain liver disorders.

This information reveals that the informants had a tendency to rely on one specific species for treating one specific ailment rather than for several different ailments. The FL values in this study varied from 1.0% to 100%.

Generally, a FL of 100% for a specific species indicates that all of the usereports mentioned the same species for a specific treatment [32]. This study determined 28 species of plants with a FL of 100%, even without considering species that were mentioned above five times (Table 2). Diseases containing a higher number of species assessed to a FL of 100% were liver-related ailments (43 species) and cases of jaundice (16 species).

Special attention was given to important species (N, N_p) with a FL above 100%, regarding the viewpoint of the number of times mentioned and the consensus level for the specific ailment, like *Taraxacum platycarpum* Dahlst. (192, 192), *Cudrania tricuspidata* (Carr.) Bureau ex Lavallee (43, 43), *Semisulcospira libertina* (Gould) (30, 30), *Capsella bursapastoris* (L.) L. W. Medicus (24, 24), and *Semisulcospira forticosta* (Martens) (10, 10) (Table 2). Through further clinical study, these species possess a much higher potential in being used in the development of new drugs for liver disorders.

3.3.3. Internetwork between Liver Disorders and Medicinal Species. INA has originally analyzed social phenomenon and trends through the internetwork of components [33]. We attempted to analyze the interrelationship between liver disorders and the medicinal species recorded in the communities.

Considering Figure 2, the people in the communities used only animals to care for fatigue recovery and liver cirrhosis, except for the use of *Bupleurum falcatum* L. (plant) and *Fomes fomentarius* (L. : Fr.) Fr. (fungus), while using plants and fungi as a cure for hepatitis and hangovers. Also, people used plants, animals, and fungi to treat jaundice, liver cancer, and other liver-related ailments.

Protaetia brevitarsis seulensis (Kolbe), used as a medicinal animal, was applied as treatment for five liver disorders, which included liver cancer, liver cirrhosis, jaundice, liver-related ailments, and hepatitis. *Oenanthe javanica* (Blume) DC., used as a medicinal plant, was applied as treatment for the four liver disorders of liver cirrhosis, jaundice, liver-related ailments, and hangovers. *Protaetia mandschuriensis* (Schurhoff) and *Cetonia pilifera* (Motschulsky) were used as medicinal animals and *Fomes fomentarius* (L. : Fr.) Fr. as a medicinal fungus in the treatment of three disorders,

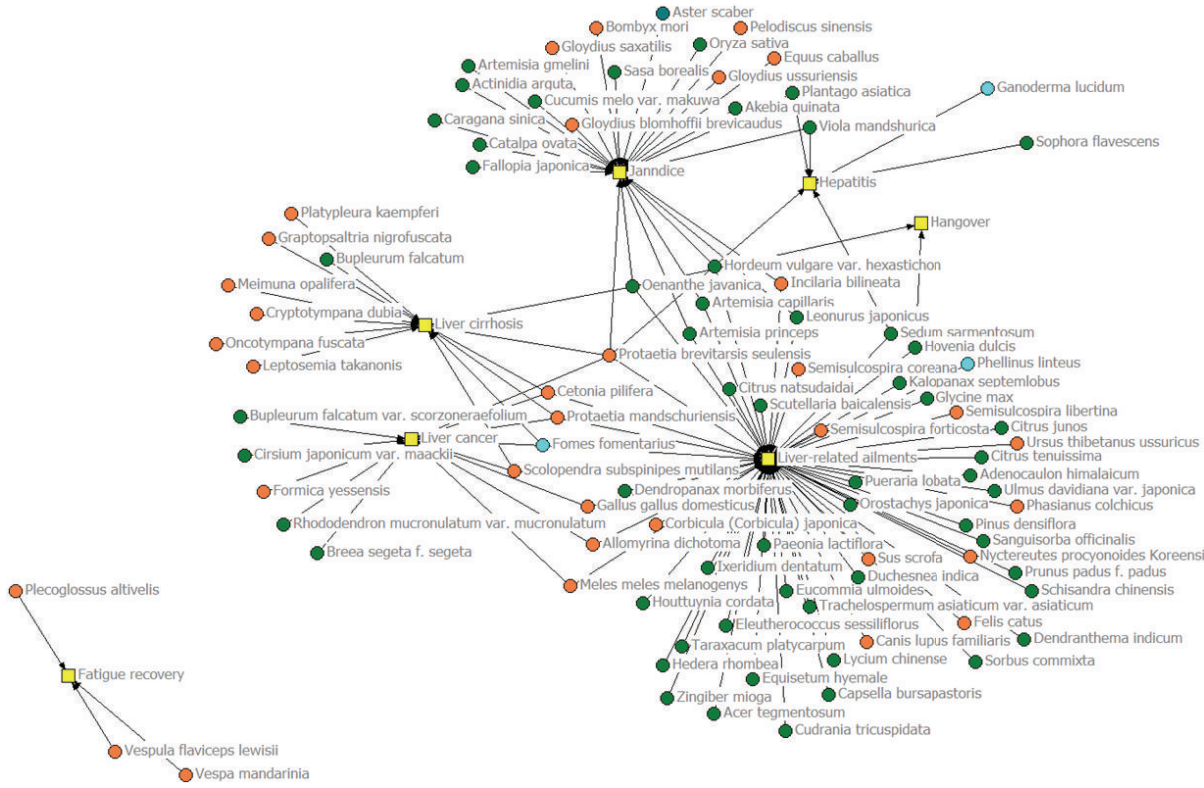

Figure 2: Internetwork analysis between medicinal species and diseases.

which included liver cancer, liver cirrhosis, and liver-related ailments. As further research is conducted, these species will certainly be developed as pharma foods used in treatment of liver disorders.

4. Conclusion

This research stands as the first study to record and analyze ethnomedicinal practices used as treatment for liver disorders within the communities in East Asia. After the 1950s, the National Health Care System legally admitted conventional medicine and Korean traditional medicine, which resulted in the near extinction of ethnomedicinal practice in Korea. Also, the fast westernization of local communities in Korea has accelerated the loss of ethnomedicinal practices.

From this research, recording 254 ethnomedicinal practices, as being used to treat seven liver disorders, was very inspiring. Particularly, the present usage of various bioorganisms displays evidence as to which ethnomedicinal practices are continuously transmitted in the communities. However, this present situation is not sustainable because the communities of these study areas consist of an aging society. It has become necessary for appropriate measures to be taken to conserve these ethnomedicinal practices.

Optimistically, the INA was first applied for the interpretation of ethnomedicinal knowledge of a community, although it has been strictly used until now for social science in the analysis of social trends and phenomena through the interrelationship of specific components.

The results of the INA application in this study provide various interpretations between liver disorders and medicinal species. Our research suggests an internetwork analysis as a new tool for various interpretations to ethnomedicinal knowledge within a local community. Through this study, we are confident that the useful value of INA has been proven and the three dimensional relationships of these components will extend beyond the existing understanding of ethnomedicinal knowledge within local communities around the world.

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Research Article

Meta-Analysis of the Clinical Value of Danshen Injection and Huangqi Injection in Liver Cirrhosis

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Objective. To evaluate the clinical value of Danshen injection and Huangqi injection for the treatment of liver cirrhosis. *Methods*. The Chinese Biomedical Literature Database (CBM), Chinese Scientific Journals Full-Text Database (VIP), Wanfang Database, China National Knowledge Infrastructure (CNKI), PubMed, and EMBASE database were searched to collect the literatures about the randomized controlled trials involving the treatment of liver cirrhosis with Danshen injection combined with Huangqi injection, and the data analyses were performed using RevMan 4.2 software. *Results*. A total of 11 studies involving 1086 patients (trials group: 554 cases, control group: 532 cases) were included in this study. Compared with those in control group, the meta-analysis showed-that the total effectiveness rate and the level of serum albumin increased, while serum total bilirubin, alanine transmninase, type III procollagen, hyaluronic acid, laminin, and type-IV collagen decreased in trials group. The Jadad score ranged from 1 to 2 and the funnel plot analysis suggests that publication bias may occur. *Conclusions*. Danshen injection combined with Huangqi injection may promote the curative efficacy of liver cirrhosis, which is a promising novel treatment approach. The exact outcome needs to perform rigorously designed, multicenter, and large randomized controlled trials.

1. Introduction

Liver fibrosis or cirrhosis is a common progressively pathological lesion of chronic liver diseases in response to various liver-damaging factors [1]. The main causes of liver fibrosis in industrialized countries include chronic HCV infection, alcohol abuse, and nonalcoholic steatohepatitis [2]. In China, cirrhosis is most commonly caused by hepatitis B and hepatitis C, because China has the largest number of hepatitis patients in the world. Of the 350 million to 400 million individuals worldwide infected with the hepatitis B virus (HBV), one-third reside in China, with 130 million carriers and 30 million chronically infected [3, 4]. Presently, the treatment for liver cirrhosis remained difficult, due to the lack of effective approaches. In recent years, many clinical trials using Danshen injection and Huangqi injection for the treatment of liver cirrhosis have been reported in China, suggesting that the new therapeutic approach may have potential value. However, there lacked a systematical review about the issue. Therefore, we conducted a meta-analysis of RCTs to assess the clinical value of Danshen injection and Huangqi injection for the treatment of liver cirrhosis.

2. Materials and Methods

2.1. Inclusion Criteria. The studies were randomized controlled trials (RCTs) and quasi-randomized controlled trials. Clinical diagnosis must meet the diagnostic criteria for cirrhosis of the liver (Chinese Commission of Infectious and Parasitic Diseases, Viral Hepatitis Prevention and Treatment Programs). The trials group added Danshen or compound

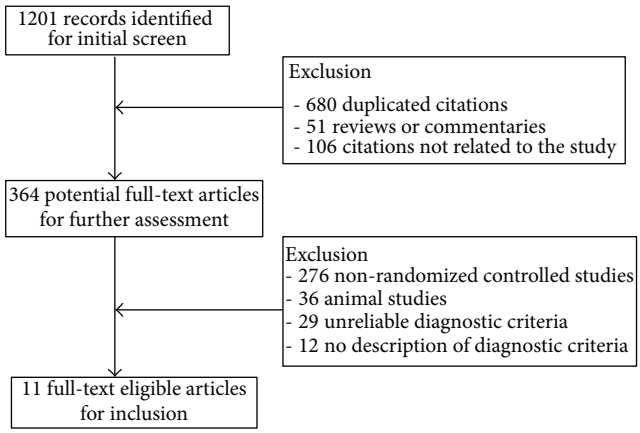

Figure 1: Flow diagram of the literature selection for this study.

Danshen injection and Huangqi injection apart from the drugs that were used by the control group. The outcome should include one or more indices as follows: (1) total efficacy rate; (2) serum indices of liver function: alanine aminotransferase (ALT), total bilirubin (TBIL), and albumin (ALB); (3) serum indices of liver fibrosis: hyaluronic acid (HA), type-IV collagen (VI-C), type-III procollagen (PC-III) and laminin (LN); (4) adverse events; E other data related to efficacy and outcome.

In this study, we had not set any restrictions on gender, race, and literature language.

2.2. Exclusion Criteria. Reviews, nonclinical studies, case observations, duplicated literatures, and noninjection formulae literatures were excluded.

2.3. Research Strategy and Data Extraction. Danshen, dan shen, huangqi, Beiqi, huang qi, salvia, salvia miltiorrhiza, astragalus, astragali, astragalus miltiorrhiza, Chinese traditional medicine herb, fibrosis, cirrhosis of liver, cirrhosis, liver cirrhosis, and liver fibrosis were selected as the search terms. The Chinese Biomedical Literature Database (CBM), China National Knowledge Infrastructure (CNKI), Chinese Scientific Journals Full-text Database (VIP), Wanfang Database, PubMed, and EMBASE database were searched by computer. Data extraction and quality assessment was independently performed by two researchers (C. Zhu and Y. Ling) and disagreements were resolved by consensus. The lack of information was supplemented by contact with the authors in charge of the clinical trials. Database retrieval process is shown in Figure 1. The methodological quality of trials was assessed by Jadad score. The randomization, concealment of allocation, blind methods, and loss of followup were considered as the scoring evidence.

2.4. Statistical Analysis. Statistical analysis was performed by Cochrane RevMan 4.2. Categorical variables were compared using relative risk (RR), and continuous variables were compared using weighted mean difference (WMD). Meanwhile, 95% confidence interval (CI) was calculated. Chi-square test was used for the heterogeneity of inclusion trials. The heterogeneity data adopted the random effect model was used, otherwise fixed effect model. A funnel plot was used for assessing the potential publication bias.

3. Results

3.1. Characteristics of Included Studies. Eleven articles [5–15] involving 1086 subjects (trials group: 554 cases; control group: 532 cases) were included in this study. Male patients with liver cirrhosis were dominated in the subjects. The general characteristics of the study were shown in Table 1, and interventions, treatments and outcomes were seen in Table 2.

3.2. The Quality Assessment. The design features clarified as randomization, double-blinding, withdrawals/dropouts, and allocation concealment were shown in Table 3. The Jadad scores ranged from 1 to 2. In this study, the curative effect assessment was performed by the clinicians and double-blinding design was not adopted. But, the measurements of serum indices of liver function and liver fibrosis were independently conducted by laboratory staff, and the double-blinding method was performed in all the included studies. However, the withdrawals/dropouts and allocation concealment were not conducted.

3.3. Meta-Analysis of Assessing the Total Effectiveness Rate. Four RCTs [5, 7, 8, 11] have evaluated the total efficacy rate. Heterogeneity analysis of the total efficacy rate was significant difference ($P < 0.05$), and a random effects model was used. The results showed the total efficacy rate in the trials group was higher than that in the control group (RR = 1.22, 95% CI (1.0, 1.48); $P < 0.05$) (Figure 2).

3.4. Meta-Analysis of the Serum Indices of Liver Function. The heterogeneity occurred in the data of ALT, TBIL, and ALB ($P < 0.05$); therefore, we adopted a random effects model. The results showed that the levels of ALT and TBIL in the trials group decreased significantly compared with the control group, while the ALB level increased. The WMDs with 95% CI of ALT, TBIL and ALB were −18.11 (−28.42, −7.81), −23.60 (−35.39, −11.81) and 4.26 (2.49, 6.04), respectively. Forest plots were shown in Figure 3.

3.5. Meta-Analysis of the Serum Indices of Liver Fibrosis. Heterogeneity analyses implied that PC-III, HA, and VI-C fit random effects model, while LN should adopt a fixed effects model. Meta-analysis showed that the WMDs of PC-III, HA, LN, and VI-C with 95% CI were −62.24 (−94.58, −29.90), −144.60 (−222.16, −67.04), −40.33 (−47.32, −33.33), and −39.98 (−52.17, −27.80), respectively, suggesting that the levels of serum PCIII, HA, LN, and VI-C in the trials group were lower than those in control group (Figures 4 and 5).

3.6. Liver Hemodynamics, HBeAg Seroconversion and HBV-DNA. Wu et al. [12] reported hemodynamic changes in liver as follows: after 4 weeks of treatment, portal vein diameter, the inner diameter, and blood flow velocity of the splenic

Table 1: Characteristics of the randomized controlled trials included in this study.

Author (reference)	Published year	Cases T/C	Age (years) Range, mean	Sex Male/female	History (years) Range, mean
Shen et al. [5]	1999	39/35	T: 39–72, 55.3 C: 40–71, 56.5	T: 26/13 C: 23/12	2–13, 5.7
Chen et al. [6]	2000	80/40	NR	NR	NR
Cai et al. [7]	2001	44/40	T: 8–51, 29 C: 12–50, 31	T: 35/9 C: 32/8	T: 0.8–11, 5.9 C: 0.7–11, 5.8
Zhou [8]	2002	60/60	T: 28–70, 49 C: 26–68, 47	T: 40/20 C: 45/15	NR
Liu et al. [9]	2002	56/44	35–67	78/2	3–25
Chen et al. [10]	2003	43/42	36–71, 54.72	NR	NR
Cai [11]	2004	66/122	T: 54.6 C: 58.7	T: 59/7 C: 107/15	NR
Wu et al. [12]	2006	37/31	35–65	51/17	>2
Yi and Li [13]	2007	45/43	T: 20–55, 37 C: 18–51, 33	T: 25/20 C: 25/18	T: 5–13 C: 5–10
Pen and Wang [14]	2010	52/44	T: 28–69, 36.8 C: 24–67, 35.6	T: 38/14 C: 28/16	NR
Liu et al. [15]	2010	32/31	25–62, 39.7	T: 26/6 C: 23/8	NR

T: trials group, C: control group. NR: no report.

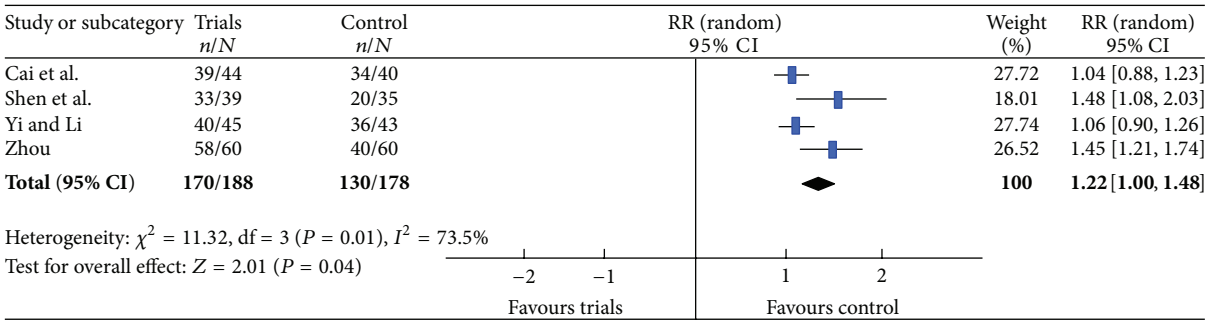

Figure 2: Meta-analysis of assessing the total efficacy rate.

vein in the trials group significantly decreased, while the flow velocity of portal vein blood significantly increased ($P < .05$). Liu et al [15] reported that no difference in the HBeAg seroconversion rate was found between the trials group and the control group ($P > .05$), while the copies of HBV-DNA in the trials group were significantly lower than those in the control group ($P < .05$).

3.7. Recurrence Rate. Cai et al. [7] reported that, when 3 months after treatment, 2 cases recurred in the trials group (38 cases received followup); while in the control group (20 cases received followup), 6 cases recurred. There was statistical difference ($P < .05$) between the two groups.

3.8. Adverse Effect. Amongst 11 literatures included in this study, only one [12] reported side effects and clarified that no side effects in clinical trials were observed.

3.9. Publication Bias. According to the data involving TBIL, the funnel plot was drawn using WMD as abscissa and SE (WMD) for the vertical axis, respectively. The plot was asymmetric (Figure 6), suggesting that the publication biases may occur in this study.

4. Discussion

Liver fibrosis results from chronic damage to the liver in conjunction with the accumulation of ECM proteins including collagen, which is a characteristic of most types of chronic liver diseases [16]. In contrast with the traditional view that cirrhosis is an irreversible disease, recent evidence indicates that even advanced fibrosis is reversible [17, 18]. However, up to now, there has been no standard Western medicine treatment for liver fibrosis. In traditional Chinese medicine (TCM), some Chinese herbs such as *Radix Salviae Miltiorrhizae* and *Radix Astragali Mongolici* were commonly

Table 2: The intervention and the outcome measures of the individual studies included in this study.

Study ID	The regimens of intervention		Time	Assessment of outcomes
	Trials group	Control group		
Shen et al., 1999 [5]	Compound Danshen injection (16 mL) and Huangqi injection (20 mL) were, respectively, added into 5%–10% glucose solution (250 mL), intravenously, qd.	Aspartic potassium, vitamin C, and B6 were added into 5% glucose, intravenously.	28 d	Total efficacy rate; serum indices of liver fibrosis and liver function.
Chen et al., 2000 [6]	Compound Danshen injection (20 mL) and Huangqi injection (20 mL) were, respectively, added into 5% glucose solution, intravenously, qd.	Used hepatoprotective treatment (mainly energy mixture and vitamins), qd.	90 d	Serum indices of liver fibrosis.
Cai et al., 2001 [7]	Compound Danshen injection (20 mL) and Huangqi injection (20 mL) were, respectively, added into 5% glucose solution, intravenously, qd.	Used silymarin orally, tid.	90 d	Total efficacy rate; serum indices of liver function.
Zhou, 2002 [8]	Compound Danshen injection (20 mL) and Huangqi injection (10 mL) were together added into 250 mL of 5% glucose solution, intravenously, qd.	Vitamin C, vitamin B6, inosine, and potassium chloride injection were added into 5% glucose solution intravenously, qd.	75 d	Total efficacy rate; serum indices of liver function.
Liu et al., 2002 [9]	Danshen injection (30 mL) and Huangqi injection (20 mL) were, respectively, added into 250 mL of 5% glucose solution, intravenously, qd.	Added ganlixin into the 5% glucose, intravenously.	90 d	Serum indices of liver function.
Chen et al., 2003 [10]	Danshen injection (30 mL) and Huangqi injection (16 mL) were, respectively, added into 250 mL of 5% glucose solution, intravenously, qd.	30 mL of ganlixin injection, intravenously, qd.	28 d	Serum indices of liver fibrosis and liver function.
Cai, 2004 [11]	Compound Danshen injection (30 mL) and Huangqi injection (10 mL) were, respectively, added into 250 mL of 5% glucose solution, intravenously, qd.	Energy mixture, branched-chain amino acids, albumin, and vitamin were used.	75 d	Serum indices of liver fibrosis and liver function.
Wu et al., 2006 [12]	Danshen injection (25 mL) and Huangqi injection (25 mL) were, respectively, added into 250 mL of 5% glucose solution, intravenously, qd.	Routine western medicine treatment.	84 d	Serum indices of liver function; liver hemodynamics.
Yi and Li, 2007 [13]	Danshen injection (20 mL) and Huangqi injection (20 mL) were, respectively, added into 250 mL of 5% glucose solution, intravenously, qd.	Used silymarin orally, 3 times a day.	90 d	Total efficacy rate; serum indices of liver function.
Pen and Wang, 2010 [14]	Danshen injection (30 mL) and Huangqi injection (30 mL) were, respectively, added into 250 mL of 5% glucose solution, intravenously, qd.	Hepatoprotective therapy using nosine and vitamins.	30 d	Serum indices of liver fibrosis and liver function.
Liu et al., 2010 [15]	Danshen injection (30 mL) and Huangqi injection (30 mL) were, respectively, added into 250 mL of 10% glucose solution, intravenously, qd.	Hepatoprotective and supportive therapy and lamivudine (100 mg) orally, qd.	90 d	Serum indices of liver fibrosis and liver function; HBeAg; HBV-DNA.

The regimens in the trials group included the drugs used in the control group. Compound Danshen injection: mainly contains Danshen and an added constituent (*Lignum Dalbergiae Odoriferae*).

Study or subcategory	Trials N	Trials Mean (SD)	Control N	Control Mean (SD)	WMD (random) 95% CI	Weight (%)	WMD (random) 95% CI
01 serum Tbil level							
Cai et al.	44	27.30 (19.50)	40	58.20 (22.60)		5.47	−30.90 [−39.97, −21.83]
Cai	66	28.00 (8.00)	122	52.00 (14.00)		7.21	−24.00 [−27.15, −20.85]
Chen et al.	43	25.10 (11.50)	42	30.50 (12.10)		6.75	−5.40 [−10.42, −0.38]
Liu et al.	32	20.50 (18.80)	31	38.50 (21.70)		5.15	−18.00 [−28.04, −7.96]
Shen et al.	39	16.05 (7.06)	35	20.56 (7.59)		7.16	−4.51 [−7.86, −1.16]
Yi and Li	45	28.30 (20.50)	43	68.20 (92.60)		1.61	−39.90 [−68.22, −11.58]
Subtotal (95% CI)	**269**		**313**			**33.35**	**−18.11 [−28.42, −7.81]**
Heterogeneity: $\chi^2 = 97.89$, df = 5 ($P = 0.00001$), $I^2 = 94.9\%$							
Test for overall effect: $Z = 3.45$ ($P = 0.0006$)							
02 serum ALT level							
Cai et al.	44	32.40 (27.70)	40	68.80 (40.80)		3.69	−36.40 [−51.46, −21.34]
Cai	66	79.00 (31.00)	122	88.00 (38.00)		5.14	−9.00 [−19.07, 1.07]
Chen et al.	43	36.00 (15.10)	42	46.40 (16.90)		6.21	−10.40 [−17.22, −3.58]
Liu et al.	32	31.50 (14.80)	31	56.50 (17.70)		5.8	−25.00 [−33.07, −16.93]
Yi and Li	45	33.40 (28.70)	43	78.80 (50.80)		3.16	−45.40 [−62.75, −28.05]
Subtotal (95% CI)	**230**		**278**			**24**	**−23.60 [−35.39, −11.81]**
Test for heterogeneity: $\chi^2 = 26$, df = 4 ($P < 0.00001$), $I^2 = 84.6\%$							
Test for overall effect: $Z = 3.92$ ($P < 0.00001$)							
03 serum ALB level							
Cai et al.	44	37.60 (12.40)	40	28.40 (10.40)		6.79	9.20 [4.32, 14.08]
Cai	66	36.10 (1.50)	122	30.10 (1.30)		7.53	6.00 [5.57, 6.43]
Chen et al.	43	38.10 (2.30)	42	34.20 (2.70)		7.49	3.90 [2.83, 4.97]
Liu et al.	32	32.90 (4.30)	31	31.80 (4.90)		7.36	1.10 [−1.18, 3.38]
Shen et al.	39	32.87 (4.57)	35	28.69 (3.90)		7.41	4.18 [2.25, 6.11]
Yi and Li	45	38.60 (13.40)	43	38.40 (20.40)		6.07	0.20 [−7.05, 7.45]
Subtotal (95% CI)	**269**		**313**			**42.65**	**4.26 [2.49, 6.04]**
Test for heterogeneity: $\chi^2 = 34.21$, df = 5 ($P < 0.00001$), $I^2 = 85.4\%$							
Test for overall effect: $Z = 4.71$ ($P < 0.00001$)							

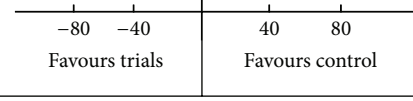

Figure 3: Meta-analysis of the serum indices of liver function (TBIL, ALT and ALB).

Study or subcategory	Trials N	Trials Mean (SD)	Control N	Control Mean (SD)	WMD (random) 95% CI	Weight (%)	WMD (random) 95% CI
01 serum PC-III level							
Cai	66	77.00 (30.00)	122	158.00 (32.00)		7.26	−81.00 [−90.20, −71.80]
Chen et al.	80	134.00 (21.00)	40	150.00 (26.00)		7.25	−16.00 [−25.28, −6.72]
Chen et al.	43	103.40 (96.10)	42	190.50 (95.40)		6.11	−87.10 [−127.81, −46.39]
Liu et al.	32	123.00 (76.00)	31	189.00 (98.00)		5.98	−66.00 [−109.40, −22.60]
Pen and Wang	52	128.80 (38.50)	44	186.90 (46.40)		7.08	−58.10 [−75.35, −40.85]
Shen et al.	39	135.00 (81.00)	35	212.00 (150.00)		5.33	−77.00 [−132.82, −21.18]
Subtotal (95% CI)	**312**		**314**			**39**	**−62.24 [−94.58, −29.90]**
Test for heterogeneity: $\chi^2 = 100.46$, df = 5 ($P < 0.00001$), $I^2 = 95.0\%$							
Test for overall effect: $Z = 3.77$ ($P = 0.0002$)							
02 serum HA level							
Cai	66	412.00 (84.00)	122	697.00 (113.00)		6.68	−285.00 [−313.51, −256.49]
Chen et al.	80	524.00 (118.00)	40	638.00 (146.00)		5.52	−114.00 [−166.11, −61.89]
Chen et al.	43	242.70 (172.50)	42	428.20 (184.50)		4.33	−185.50 [−261.47, −109.53]
Liu et al.	32	153.00 (76.00)	31	214.00 (105.00)		5.87	−61.00 [−106.38, −15.62]
Pen and Wang	52	172.80 (43.20)	44	304.80 (61.40)		6.94	−132.00 [−153.61, −110.39]
Shen et al.	39	440.00 (208.00)	35	514.00 (253.00)		3.11	−74.00 [−180.24, 32.24]
Subtotal (95% CI)	**312**		**314**			**32.45**	**−144.60 [−222.16, −67.04]**
Test for heterogeneity: $\chi^2 = 103.96$, df = 5 ($P < 0.00001$), $I^2 = 95.2\%$							
Test for overall effect: $Z = 3.65$ ($P = 0.0003$)							
03 serum VI-C level							
Cai	66	149.00 (29.00)	122	203.00 (31.00)		7.26	−54.00 [−62.90, −45.10]
Chen et al.	43	106.80 (25.20)	42	145.20 (26.70)		7.22	−38.40 [−49.44, −27.36]
Liu et al.	32	98.00 (42.00)	31	126.00 (48.00)		6.92	−28.00 [−50.30, −5.70]
Pen and Wang	52	90.30 (34.40)	44	122.40 (38.00)		7.15	−32.10 [−46.71, −17.49]
Subtotal (95% CI)	**193**		**239**			**28.55**	**−39.98 [−52.17, −27.80]**
Test for heterogeneity: $\chi^2 = 10.36$, df = 3 ($P = 0.02$), $I^2 = 71.0\%$							
Test for overall effect: $Z = 6.43$ ($P < 0.00001$)							

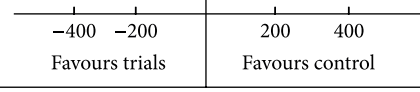

Figure 4: Meta-analysis of the serum indices of liver fibrosis (PCIII, HA and CIV).

Table 3: Quality of reports of 11 clinical trials using the Jadad assessment scale.

	Randomization	Double blinding	Withdrawals/dropouts	Allocation concealment	Scores
Curative effect assessment	Yes	No	No	No	1
Serum indices of liver function	Yes	Yes	No	No	2
Serum indices of liver fibrosis	Yes	Yes	No	No	2

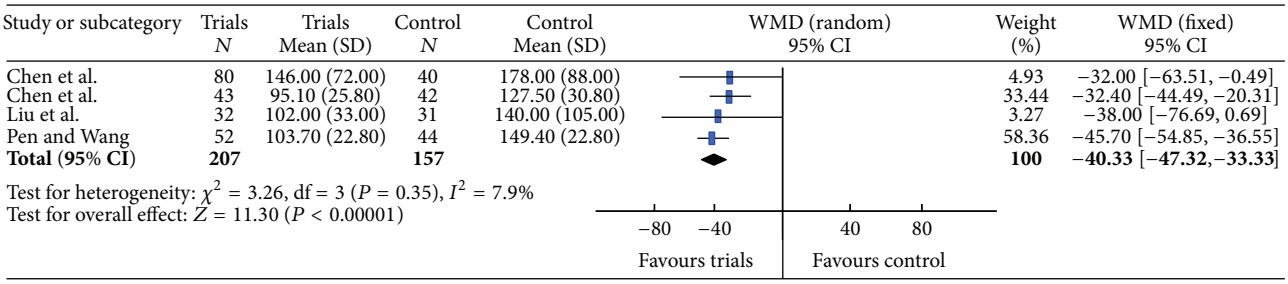

Figure 5: Meta-analysis of the indices of liver fibrosis (LN).

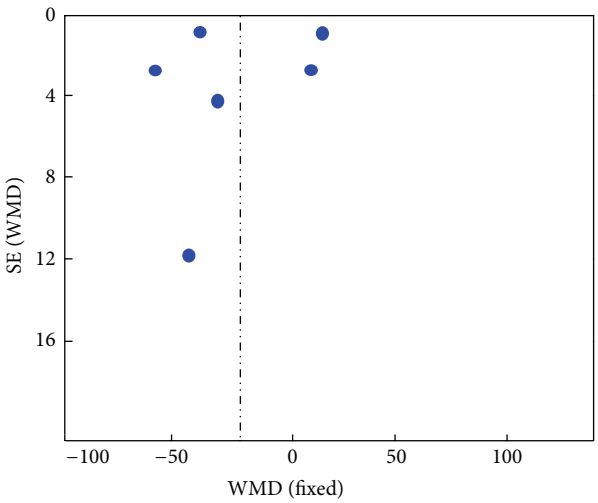

Figure 6: The funnel plot based on the data of TBIL.

used in cases of liver fibrosis and hepatitis. In recent years, the extract of their roots using ethanol was prepared as Danshen injection and Huangqi injection, respectively, which were often prescribed as the treatment for liver fibrosis. However, in China, Danshen injection was commonly used for coronary heart disease [19], myocarditis [20], stroke disease [21], myocardial infarction [22], hepatitis [23], epidemic hemorrhagic fever [24], acute disseminated intravascular coagulation syndrome so on [25]. Huangqi injection has been widely used in the treatment for chronic hepatitis [26], viral myocarditis [27], chronic heart failure [28], aplastic anemia [29], chronic nephritis [30], renal damage [31], nephrotic syndrome [32, 33], and diabetic nephropathy [34].

Radix Salviae Miltiorrhizae contains tanshinones, salvianolic acids, rosmarinic acid, lithospermic acid, protocatechualdehyde, caffeic acid, isoferulic acid, and so forth. It has been demonstrated that the ingredients can inhibit the proliferation and activation of stellate cells, and promote the repair and regeneration of liver cells, improve microcirculation in the liver, and increase hepatic parenchymal blood supply [35, 36]. Modern pharmacological studies have shown that, *Radix Astragali Mongolici* contains a series of cycloartane triterpene glycosides denoted astragalosides I–VII (saponins), which can inhibit the synthesis and the deposition of collagen, and promote the protein synthesis and the recovery of the cell function in liver [37–39].

In this study, Danshen injection and Huangqi injection combined with routine therapeutic regiment (the control) were used for the clinical treatment of cirrhosis. The treatment protocols were as follows: Danshen injection (10–30 mL) and Huangqi injection (10–30 mL) were respectively or together added into 250–500 mL of 5%–10% glucose solution for intravenous drip, qd, and the course of treatment ranges from 28 d to 90 d. Meta-analysis showed that the therapeutic effect in the trials group (using Danshen injection and Huangqi injection) was better than that in the control group (routine treatment). Serum ALT, ALB, and TBIL are the main markers of evaluating liver function, and the meta-analysis implied that using Danshen injection and Huangqi injection can decrease the serum levels of ALT, ALB, and TBIL, suggesting that the injections improve recovery of liver function. HA, LN, VI-C, and PC-III are the serum markers of the therapeutic evaluation of liver fibrosis; meta-analysis results showed that; the serum levels of HA, LN, VI-C, and PC-III in the trials group were lower compared with those in the control group. Thus, the treatment was believed to relieve liver fibrosis. In addition, individual study [12] showed that, after 4 weeks of treatment using Danshen injection and Huangqi injection, portal vein diameter, the inner diameter, and blood flow velocity of the splenic vein decreased, while the flow velocity of portal vein blood increased, indicating that the liver thermodynamics were bettered after the treatment using the injections. In addition, Liu et al. [15] reported

that the copies of HBV-DNA had significantly reduced after the treatment.

Based on the above results, we believed that using Danshen injection and Huangqi injection combined with the routine treatment may improve liver function recovery and reverse liver fibrosis. But, it must be noted that this meta-analysis had some limitations. Firstly, the quality of the methodological design of individual studies was not high. Secondly, the asymmetric funnel plot implied that publication biases may occur. Thirdly, the diversity of treatment dose and the small sample number and the lack of long-term follow-ups degraded the validity of the evidence of the clinical trials. In addition, the safety of the trials was reported insufficiently, and there only had one literature that mentioned side effect and none else clarified the safety observation. However, the previous study [40] reported that, using Huangqi injection in treating chronic hepatitis B patients showed flustered symptoms, and the symptoms disappeared after slowing drip rate. In addition, another study reported that the rarely moderate anaphylaxis in intravenous dripping of Danshen injection may occur [41].

5. Conclusions

Danshen injection combined with Huangqi injection may promote the curative efficacy of liver cirrhosis, which is a promising novel treatment approach. Considering that this systematic review had the limitations in some ways, rigorously designed multicenter, double-blind, randomized, and large-scale controlled trials are required.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Changtai Zhu, Hao Cao, and Xifa Zhou contributed equally to this work.

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Research Article

Hepatoprotective Effect of Fermented Soybean (Nutrient Enriched Soybean Tempeh) against Alcohol-Induced Liver Damage in Mice

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Recently, soybean tempeh has received great attention due to many advantages such as higher nutritional value, lower production cost, and shorter fermentation time. In this study, the *in vivo* hepatoprotective and antioxidant effects of nutrient enriched soybean tempeh (NESTE) were determined. NESTE fermentation process which involved anaerobic incubation was previously proclaimed to increase the content of amino acids and antioxidant properties remarkably. The evaluation of histological sections, serum biochemical markers (aspartate aminotransferase (AST), alanine aminotransferase (ALT), and cholesterol and triglycerides (TG)), liver immune response level (nitric oxide (NO)) and liver antioxidant level (superoxide dismutase (SOD), ferric reducing antioxidant power (FRAP), and malondialdehyde (MDA)) was conducted in order to compare the effects of nonfermented soybean extract (SBE) and fermented soybean extract (NESTE) on alcohol-induced liver damage in mice. Results demonstrated that 1000 mg/kg of NESTE can significantly reduce the levels of AST, ALT, cholesterol, TG, MDA, and NO. On the other hand, it also raised the level of SOD and FRAP. Furthermore, the histological examination on 1000 mg/kg NESTE treatment group showed that this extract was capable of recovering the damaged hepatocytes to their normal structures. Thus, it can be concluded that NESTE produced through fermentation process was able to enhance hepatoprotective and antioxidant effects *in vivo*.

1. Introduction

Liver is the largest human's organs after skin and it plays several vital functions including the metabolic, vascular, immunological, secretory, and excretory activities in the body [1]. Liver also provides a major function in the metabolism of fat, protein, and carbohydrate [1]. Numerous substances have been demonstrated to be toxic to the liver cells and alcohol was the most reported cause of liver disease worldwide [2].

Consumption of excessive alcohol can contribute to liver diseases which are mostly initiated by acute inflammation followed by the development of steatosis, alcoholic hepatitis, and eventually cirrhosis and fibrosis [3, 4]. To date, 90% of the heavy alcohol drinkers had developed fatty liver disease (steatosis) and 10% to 20% of these heavy drinkers are suffering from alcoholic hepatitis and cirrhosis [4]. Alcohol-induced liver damage is generally associated with the increasing level of free radicals which cause the development

of liver cell peroxidation and would eventually lead to the oxidative stress on the liver cells [5, 6]. Furthermore, the formation of oxidative stress was due to the decreased level of antioxidant defense as the level of free radicals increases [7, 8]. Immune response activity was also reported to be affected in alcohol-induced liver damage where increased level of nitric oxide had been reported [7]. Three different types of histopathological changes were commonly observed and reported on alcohol-induced liver damage, namely, steatosis (fatty liver), steatohepatitis (formerly known as alcoholic hepatitis), and cirrhosis [9]. All of these histopathological changes were linked to the prevalence of oxidative stress which was due to the increase level of reactive oxygen species (ROS) [7, 8]. One of the common histopathological observations for alcohol-induced liver damage was the development of steatosis whereby hepatocytes were occupied with lipid droplets [10].

Several modern medications and treatments are currently available for liver disease patients but, thus far, none of them could fully recover the liver from its pathological conditions [4]. Hence, the search for alternative medicines or supplements with hepatoprotective properties has gained more interest. For example, some studies have shown that soybean has potential bioactive substances that exhibit hepatoprotective properties [4, 11, 12]. Hodgson et al. [13] revealed that soybean might inhibit the oxidation of lipoprotein in serum. Yang et al. [14] have recently reported that soyasaponins-rich extract from soybean may also able to improve on the acute alcohol-induced hepatotoxicity in mice.

Fermented food, which is one of the alternative curative agents, has been claimed to contain increased level of bioactive constituents [15]. Fermentation processes were found to enhance the nutritional value and efficacy of the soybean [16]. As one of the fermented soybean products, Tempeh has been shown to contain several multifunctional bioactive substances. Furthermore, anaerobic fermented soybean tempeh had been reported to produce substantially higher amount of amino acids and antioxidants than conventional method which involves only aerobic fermentation [17–19]. To date, there was no or little information available on the effect of nutrient enriched soybean tempeh (NESTE) produced through anaerobic fermentation processes against alcohol-induced liver damage in mice model. The present study provides evidence that NESTE gave the best recovery effect against alcohol-induced liver injury in mice models as compared to unfermented soybean extract (SBE).

2. Materials and Methods

2.1. Materials. Soybeans originated from Canada were purchased from the local markets in Selangor, Malaysia. *Rhizopus 5351* inoculum was obtained from Malaysian Agricultural Research and Development Institute (MARDI) culture collection centre. Anaerocult A for microbiology and absolute ethanol were purchased from Merck (USA). Standardized milk thistle extract that contained 80% of silybin was obtained from Lipa Pharmaceutical Pty. Ltd., Australia.

2.2. Nutrient Enriched Soybean Tempeh Processes. About 1000 g of dehulled soybeans were soaked in cold water at room temperature for 18 h. The soaked soybeans were washed thoroughly and steamed for 40 min. After that, the steamed soybeans were cooled down to room temperature and subsequently mixed with *Rhizopus 5351* inoculum. The inoculated beans were packed into perforated polyethylene plastic bags and incubated aerobically for 30 h at 30°C. Anaerobic incubation was then conducted in order to obtain NESTE. This was carried out by transferring all the packed soybeans into containers containing *Anaerocult A for microbiology* and incubated for 20 h at 30°C. After that, NESTE was dried, ground, and extracted with deionized water. The water extracts of NESTE were then lyophilised using the VirTis BenchTop freeze dryer (SP industries, Inc., USA). Lastly, the lyophilised powder was stored at 4°C in an airtight amber container. The lyophilised extract of NESTE contained 0.338± 0.025 g/100 g DW (dried weight) of gamma-aminobutyric acid, 2.176 ± 0.006 g/100 g DW of total free amino acids, 42.64±1.59 μg/g extract of soluble phenolic acids, and 22.56± 0.31 mg GAE/g extract of total phenolic acids (manuscript submitted under review). Extract given to the mice was prepared fresh daily by dissolving the lyophilized powder of NESTE into distilled water before administration to the mice.

2.3. Animals. Inbred male Balb/c mice aged about 8 weeks old with an average weight of 18–22 g were obtained from the Animal House of Faculty of Veterinary, Universiti Putra Malaysia. The mice were placed in plastic cages at room temperature (22 ± 1°C) with 60% relative humidity and 12 h of dark/light cycle. The mice were fed with standard pellet diet and supplied with distilled water *ad libitum*. These animals were acclimatized for 7 days prior to the study. The liver protection study was conducted under the approval of Animal Care and Use Committee, Universiti Putra Malaysia (UPM) (UPM/FPV/PS/3.2.1.551/AUP-R2).

2.4. Experimental Design. The male Balb/c mice were randomly divided into seven groups with eight mice each. Ethanol and extract treatment of mice was performed according to previous study [20]. For liver damage induction, the mice from all groups except for the normal control (group I) were treated with 50% (v/v) ethanol at a dose of 2 g/kg via gavage for 7 d. After that, all mice were subjected to the treatments preorally once daily for 14 d via gavage as follows:

Group I: normal control group without ethanol induction receiving only phosphate buffer saline solution,

Group II: ethanol control group receiving phosphate buffer saline solution,

Group III: positive control group receiving 50 mg/kg of Silybin extract,

Group IV: treatment group receiving 200 mg/kg of soybean extract (SBE),

Group V: treatment group receiving 1000 mg/kg of SBE,

Group VI: treatment group receiving 200 mg/kg of NESTE,

Group VII: treatment group receiving 1000 mg/kg of NESTE.

At the end of the experimental period, all the mice were sacrificed after isoflurane anaesthesia. Blood was collected via cardiac puncture from each mouse and subjected to quantification of aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, and triglycerides (TG) concentrations in the serum. Then, the liver was excised from each mouse and weighed. The organs were meshed through 0.2 μm cell strainer (SPL Life Sciences, China) using syringe rubber plunger in cold phosphate-buffered saline solution. The liver homogenate was then used to determine the level of superoxide dismutase (SOD), malondialdehyde (MDA), ferric reducing antioxidant power (FRAP), and nitric oxide (NO). Mice liver was placed in 10% neutral buffered formaldehyde solution for histopathological examination.

2.5. Analyses of Liver Homogenate (MDA, SOD, NO, and FRAP Assay). Malondialdehyde (MDA) formation in the liver, which is a thiobarbituric acid-reactive substance, was measured by lipid peroxidation estimation according to Suhail et al. [21] with slight modification. The estimation of MDA level in liver homogenate was performed by mixing 100 μL of liver homogenate sample with 400 μL of phosphate-buffered saline, 12.5 μL of butylated hydroxytoluene (8.8 mg/mL absolute ethanol), and 250 μL of 30% w/v trichloroacetic acid. The mixed solution was then vortexed and put on ice for approximately 2 h. After that, the solution was centrifuged at 2000 g and 25°C for 15 min. 500 μL of supernatant was then mixed with 37.5 μL of 0.1 M EDTA and 125 μL of 1% v/v thiobarbituric acid in 0.1 M NaOH. The mixed solution was boiled for 15 min and cooled down to room temperature. The aliquot of 100 μL of solution was finally read *via* ELISA plate reader at 532 nm and 600 nm.

The activity of superoxide dismutase (SOD) was measured according to Ilouno et al. [22]. The assay was performed in 96 well plate by loading 10 μL of liver homogenate sample into each well with variable concentrations. The liver sample homogenate was then mixed with 290 μL of SOD reagent. The SOD reagent consisted of 0.015 M sodium cyanide, 1.5 mM nitroblue tetrazolium, 0.012 M riboflavin, and 0.067 M phosphate buffer (4: 2: 1: 51). The reaction solution was then read at 560 nm after 5 min of incubation.

The total antioxidant capacity in the liver was determined by ferric reducing antioxidant power (FRAP) assay according to Benzie and Strain [23] with some modifications. Ferum sulphate (Fe^{II}) calibration curve was constructed with concentrations range from 100 to 1000 μmol/L. In each 96 well plate, 20 μL of liver homogenate sample was loaded and added with 60 μL of distilled water. The sample solution was then mixed with the FRAP working solution which consists of 15 mL acetate buffer, 1.5 mL TPTZ solution, and 1.5 mL $FeCl_3 \cdot 6H_2O$. The reaction mixture was measured using ELISA plate reader at 593 nm for every 2 min up to 10 min.

Nitric oxide (NO) inhibition assay was conducted using Griess reagent kit for nitrite determination (Molecular Probes, Inc., USA).

2.6. Clinical Chemistry Analyses. All serum biochemical activity such as alanine aminotransferase (ALT), aspartate aminotransferase (ASP), cholesterol, and triglyceride (TG) was measured by using standard assay kits (Roche Diagnostics GmbH, USA) and analyzed using the 902 automatic analyzer (Hitachi-Boehringer Mannheim).

2.7. Histopathological Examination. The liver tissues were preserved in 10% buffered formaldehyde solution and then stained with hematoxylin and eosin (H and E) according to Luna [24]. The method was initially done by embedding the organs with paraffin wax prior to cutting process via microtome. The sliced organs which had been deparaffinised were then stained with hematoxyline and eosin. The stained organs were ultimately observed under a *Nikon Eclipse 90i* microscope (New York, USA) by Bright field optics with 100 times magnification.

2.8. Statistical Analysis. The significance value of all the data was analyzed using one-way analysis of variants (ANOVA) from SPSS 15 software. Duncan's multiple range test was used and P value less than 0.05 was considered significant.

3. Results

3.1. Concentrations of ALT, AST, Cholesterol, and Triglyceride in Mice Serum. Table 1 illustrates the level of biochemistry profile of different experimental groups. The levels of ALT and AST in ethanol control group were increased significantly as compared to other experimental groups. The high level of AST and ALT was due to the liver cell injuries induced by alcohols. The levels of AST and ALT in NESTE (200 mg/kg and 1000 mg/kg body weight) and SBE (200 mg/kg and 1000 mg/kg body weight) were significantly lower in values than the ethanol control group. Among all, NESTE at 1000 mg/kg body weight was the most effective concentration that restrained the level of AST and ALT.

The cholesterol and triglyceride (TG) level of untreated ethanol control group was significantly higher than the other experimental groups. In contrast, the levels of cholesterol and TG of the NESTE groups were significantly lower than the ethanol control group. Added to this, 1000 mg/kg of SBE and both NESTE concentrations were able to reduce the level of cholesterol and TG to near the normal value.

3.2. MDA, NO, SOD, and FRAP Level in Liver Homogenate. The measurement of MDA, NO, SOD, and FRAP level on liver homogenate was conducted based on the amount of liver total protein and the results were demonstrated in Table 2.

3.2.1. MDA Content on Liver Homogenate. The amount of MDA in ethanol control group was significantly increased as compared to other experimental groups. The increased amount of MDA in ethanol control group had shown the occurrence of substantial lipid peroxidation of liver cells which was due to the toxic effect of alcohol. Furthermore, all of the extract treatment groups were significantly different ($P < 0.05$) as compared to ethanol control group and the

Table 1: Serum biochemical parameter of liver protective effect of different experimental group.

Treatment	ALT (U/L)[1]	AST (U/L)[1]	Cholesterol (mmol/L)[1]	TG (mmol/L)[1]
Normal control (PBS)	14.09 ± 1.53[a]	98.16 ± 1.99[a]	3.14 ± 0.39[a]	1.48 ± 0.23[a]
Ethanol control (50% ETOH + PBS)	48.11 ± 1.78	367.30 ± 1.10	4.83 ± 0.3	3.87 ± 0.3
Positive control (50% ETOH + silybin (50 mg/kg))	26.72 ± 1.20[a]	171.70 ± 3.79[a]	4.11 ± 0.8[a]	2.53 ± 0.4[a]
50% ETOH + SBE (200 mg/kg)	35.14 ± 4.50[a]	183.28 ± 7.28[a]	3.26 ± 0.11[a]	2.16 ± 0.16[a]
50% ETOH + SBE (1000 mg/kg)	25.85 ± 6.49	174.49 ± 1.15	2.99 ± 0.29[a]	2.03 ± 0.01[a]
50% ETOH + NESTE (200 mg/kg)	23.28 ± 4.50[a]	168.39 ± 3.42[a]	3.22 ± 0.26[a]	2.88 ± 0.26[a]
50% ETOH + NESTE (1000 mg/kg)	18.50 ± 3.00[a]	157.62 ± 3.53[a]	3.08 ± 0.44[a]	1.94 ± 1.85[a]

[1]Values are expressed as mean ± S.E.M ($n = 8$), [a]values are significantly different from ethanol control ($P < 0.05$), ALT: alanine transaminase, AST: aspartate transaminase, TG: triglyceride.

Table 2: Liver protective effect on liver homogenate parameters of different experimental groups.

Treatment	MDA (nmol/g protein)[1]	NO (μM/mg protein)[1]	SOD (U/mg protein)[1]	FRAP (mM FeII/mg protein)[1]
Normal control (PBS)	3.20 ± 0.22[a]	10.13 ± 0.18[a]	17.17 ± 0.74[a]	9.42 ± 1.0 [a]
Ethanol control (50% ETOH + PBS)	7.25 ± 0.08	15.09 ± 0.86	12.46 ± 2.51	5.28 ± 0.01
Positive control (50% ETOH + silybin (50 mg/kg))	4.95 ± 0.08[a]	10.20 ± 3.08[a]	18.54 ± 0.37[a]	15.02 ± 0.07[a]
50% ETOH + SBE (200 mg/kg)	3.02 ± 0.25[a]	9.55 ± 0.35[a]	19.38 ± 0.10[a]	6.59 ± 0.03
50% ETOH + SBE (1000 mg/kg)	2.93 ± 0.34[a]	9.54 ± 0.04[a]	20.08 ± 1.58[a]	6.24 ± 0.04
50% ETOH + NESTE (200 mg/kg)	2.22 ± 0.17[a]	9.55 ± 0.58[a]	19.44 ± 0.67[a]	9.42 ± 0.02[a]
50% ETOH + NESTE (1000 mg/kg)	1.34 ± 0.23[a]	8.45 ± 0.27[a]	20.53 ± 0.24[a]	12.78 ± 0.10[a]

[1]Values are expressed as mean ± S.E.M ($n = 8$), [a]values are significantly different from ethanol control ($P < 0.05$), MDA: malondialdehyde, NO: nitric oxide, SOD: superoxide dismutase, FRAP: ferric reducing antioxidant power.

MDA content was normalized as compared to normal control and ethanol control groups. Both extracts of 1000 mg/kg SBE and NESTE had contributed to the lower amount of MDA as compared to normal control. Among all the treatment groups, 1000 mg/kg NESTE shows the lowest production of MDA (1.34 ± 0.23 nmol/g protein).

3.2.2. NO Level on Liver Homogenate. Nitric oxide (NO) level of the ethanol control group was significantly increased as compared to other experimental groups. This result confirms the sign of substantial liver cells injuries which was predicted to occur in the ethanol control. The extract treatment groups of SBE and NESTE at both concentrations also showed significant difference ($P < 0.05$) as compared to ethanol control groups where their NO level was lower and comparable to the normal and positive control. Nevertheless, high concentration of NESTE at 1000 mg/kg showed the lowest level of NO which was 8.45 ± 0.27 μM/mg protein.

3.2.3. SOD Level on Liver Homogenate. As compared to ethanol control group, all of the treatment groups showed significant difference ($P < 0.05$) and produced higher level of SOD. All of the extract treatment groups including the positive control group showed significantly higher SOD level as compared to the normal control. The highest SOD level was illustrated from both SBE and NESTE of 1000 mg/kg which were 20.08 ± 1.58 U/mg protein and 20.53 ± 0.24 U/mg protein, respectively.

3.2.4. FRAP Activity on Liver Homogenate. All of the treatment groups including normal and positive control groups were significantly different with ethanol control group. Based on the results, mice treated with 1000 mg/kg of NESTE showed a significantly higher level of FRAP as compared to ethanol control group which was 12.78 ± 0.10 mM Fe^{II}/mg protein while mice treated with 200 mg/kg of NESTE were shown to normalize the FRAP value. In contrary, both SBE-treated groups gave a lower level of FRAP value as compared to normal control group. These results indicated that 1000 mg/kg of NESTE could give a higher level of FRAP value even though this value was lower than the positive control group.

3.3. Histopathological Examination. Histopathological observation was based on the liver section from different experimental groups which had been stained with H and E staining and observed *via* a light microscope (Figure 1). Normal hepatocytes were observed in normal control group (1A) while, for the ethanol control (1B), the hepatocytes had developed fatty changes which were also known as microvesicular steatosis where the hepatocytes developed an open space around the nuclei. Positive control (silybin extract 50 mg/kg) (1C) showed the hepatocytes condition having

a recovery with minimal microvesicular steatosis. 1000 mg/kg of SBE and both NESTE concentrations showed a recovery effect of hepatocytes with minimal to no microvesicular steatosis.

4. Discussion

Alcoholic liver disease is associated with several reactions of free radicals such as reactive oxygen species (ROS) which then contribute to the augmentation of MDA and NO. Furthermore, the level of SOD and FRAP falls tremendously along with the rise of ROS. This condition as well could lead to the rise of serum biochemical markers such as AST, ALT, triglyceride, and cholesterol. Liver steatosis was normally found in alcoholic liver histology and it was marked as a primary indicator for alcoholic liver disease. NESTE was claimed to be enriched with amino acids and antioxidants might serve as a curable agent towards alcoholic liver disease where it could revert and lower the free radicals level.

In the present study, we had evaluated the hepatoprotective effect of NESTE and SBE against the ethanol-induced liver damage in mice. In this investigation, 1000 mg/kg of NESTE treatment had the ability to reduce the serum alanine transaminase (ALT) and aspartate transaminase (AST) level. Similar trend was observed for the serum cholesterol and triglycerides level where 1000 mg/kg of NESTE was able to reduce the level of these parameters in ethanol-induced mice. The rise of serum enzyme ALT and AST level had indicated that hepatocyte cells were injured where the leakage of cell membrane had contributed to the accumulation of these enzymes into the plasma [2, 25]. Therefore, prolonged treatment of 1000 mg/kg of NESTE administration could help to normalize the ALT and AST enzyme levels. In addition, significant reduction of triglycerides and cholesterol was in agreement with the previous studies where soybeans and soybean-based products have been revealed to have hypocholesterolaemic effect [4, 16].

Malondialdehyde (MDA) is known as a secondary metabolite that is produced from the lipid peroxidation of cell membrane and the amount of MDA is used as an indicator for lipid peroxidation of cell membrane which could cause cell damage [21]. In contrast, nitric oxide (NO) plays the role as an inflammatory indicator where it would accumulate as the hepatocytes injuries [26]. The levels of NO and MDA had reduced in both alcohol-induced liver damage treated with 1000 mg/kg of NESTE and SBE which suggested that both of these extracts had shown the protective and curative activities against liver injury *via* alcoholic intoxication. However, treatment with 1000 mg/kg of NESTE possessed the most reduction level of MDA and NO in alcohol-induced liver damage in mice. On the other hand, these results were in agreement with the previous investigations where soybean had been reported to reduce the MDA level on CCl_4-induced liver in rats [27]. Other previous study also showed that the fermented soybean milk which is one of soybean-based product could alleviate and protect the injured cells from the rise of NO level [28]. The findings from these studies were comparable to our investigation where alcohol-induced liver damage in mice treated with 1000 mg/kg of NESTE and SBE could reduce the level of MDA and NO.

The level of superoxide anions which is one of the reactive oxygen species (ROS) would arise as the cell injured or inflamed. Superoxide dismutase (SOD) enzyme was found naturally in most of the organ cells and basically protects the membrane cells from damage by harmful superoxide-free radicals [22, 29]. The decreased level of SOD indicates that the high risks of cell injuries and association with supplement or highly nutritious foods tend to increase or normalize the level of SOD. The level of SOD in the liver of alcohol-induced mice treated with both 1000 mg/kg of NESTE and SBE was observed to be increased. However, treatment of 1000 mg/kg of NESTE in alcohol-induced liver damage in mice showed the highest level of SOD as compared to 1000 mg/kg of SBE. In addition, conventional tempeh had been reported previously to have SOD enzyme [30], and this signified that NESTE could provide the elevated SOD enzyme to the injured liver cells which in turn could recover the liver cells to normal and eventually be able to produce the significant amount of SOD enzyme by itself as a protective action from the damage caused by toxic substance such as alcohol.

The bioavailability and antioxidant level in the alcohol-induced liver damage in mice treated with 1000 mg/kg of NESTE was increased demonstrated by the significant increase of FRAP value. This suggested that the extract may have a significant amount of antioxidant in order to recover the liver cell damage caused by alcohol intoxication. This result could also be correlated to the previous investigation where soybean tempeh was known to have substantial amount of antioxidant properties [4, 11, 12, 31, 32]. Our result was also comparable to the positive control (50 mg/kg silybin extract) since the FRAP value from positive control treatment was slightly higher than the treatment of 1000 mg/kg of NESTE.

Histological sections (Figure 1) present the comparative appearances of hepatocytes of the treated and untreated mice from alcohol intoxication. Figure 1(a) showed a normal control group with normal hepatocytes structure. Liver histology of ethanol control (Figure 1(b)) showed a clear damage with association of fatty liver degeneration which was also known as steatosis. The mechanism of steatosis was initially associated with the damage of hepatocytes structure such as mitochondria and reticulum endoplasmic. This damage inhibits the oxidation of fatty acid which would eventually accumulate in the hepatocytes [10]. The liver condition, as in Figure 1(b), was observed to be in a stage of microvesicular steatosis where the hepatocytes had loaded with excessive lipid droplets without dislocation of the nucleus to the periphery [10]. The severity of microvesicular steatosis could lead to the development of macrovesicular steatosis which has an opposite characteristic of microvesicular steatosis where the nucleus would dislocate and develop a single large vacuole of fat [10]. A minimum recovery from microvesicular steatosis was observed when treated with the positive control silybin extract (50 mg/kg) (Figure 1(c)), which is well-known to have substantial antioxidant and hepatoprotective effects

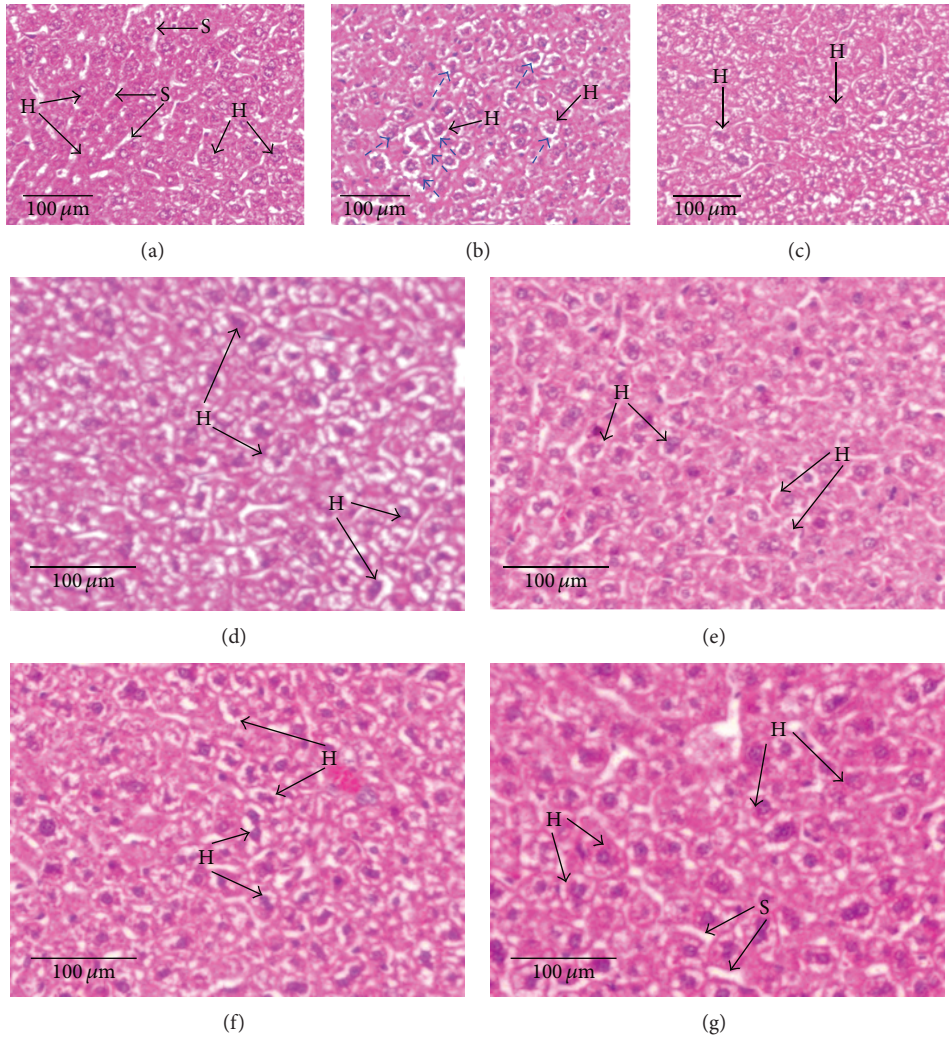

Figure 1: Histopathological microsection of liver of different experimental groups. The histopathological microsection of liver was stained with hematoxylin and eosin and captured via the light microscope with the magnification of 10 × 10. (a) Normal control with normal structure of liver cells, (b) ethanol control with a several-hepatocyte condition which has developed a microvesicular steatosis as indicated by the dashed line arrows, (c) positive control with a minimal condition of microvesicular steatosis, (d) 50% ETOH + 200 mg/kg SBE-treated group with the obvious development of microvesicular steatosis, (e) 50% ETOH + 1000 mg/kg SBE-treated group with recovery effect from microvesicular steatosis, (f) 50% ETOH + 200 mg/kg NESTE-treated group with some obvious development of microvesicular steatosis, and (g) 50% ETOH + 1000 mg/kg NESTE-treated group with recovery effect from microvesicular steatosis. H = hepatocyte, S = sinusoidal space, ⇢ hepatocyte developed with microvesicular steatosis.

[33]. The dissimilarity condition of hepatocytes between Figures 1(d) (200 mg/kg of SBE) and 1(e) (1000 mg/kg of SBE) was observed where hepatocytes condition in Figure 1(e) showed a recovery from microvesicular steatosis. This condition was also shown in Figures 1(f) (200 mg/kg of NESTE) and 1(g) (1000 mg/kg of NESTE) where the recovery from microvesicular steatosis was observed in Figure 1(g). As a result, the 1000 mg/kg of SBE and NESTE may have provided a recovery effect to hepatocytes from having a microvesicular steatosis. In addition, these histopathological findings could be linked to all of other results of our study where 1000 mg/kg of NESTE gave the best recovery effect against alcohol-induced liver cell injury among all other treatment groups.

5. Conclusion

Overall, the administration of 1000 mg/kg of NESTE demonstrated an effective amelioration against alcohol-induced liver. These results also showed that 1000 mg/kg of NESTE was not only comparable but was more effective than the positive control (silybin 50 mg/kg) in reverting the serum biochemical markers (AST and ALT), lipid profile (cholesterol and triglycerides), liver homogenate test (SOD, MDA, NO, and FRAP), and histological conditions back to normal. This effect was in agreement with the hypothesis that soybeans could ameliorate the liver cell damage and NESTE is one type of soybean-based products. Further investigation

such as combination of *in vitro* works would offer more understanding about the mechanism of amelioration of alcohol-induced liver damage *via* administration of NESTE.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

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Research Article

Bees' Honey Attenuation of Metanil-Yellow-Induced Hepatotoxicity in Rats

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The present study aims to investigate the protective effect of bees' honey against metanil-yellow-induced hepatotoxicity in rats. Rats were divided into 7 groups: control group; three groups treated with 50, 100, and 200 mg/kg metanil yellow, and three groups treated with metanil yellow plus 2.5 $mg \cdot kg^{-1} \cdot day^{-1}$ bees' honey for 8 weeks. The obtained data showed that the antioxidant/anti-inflammatory activity of bees' honey reduced the oxidative stress in the liver tissue and downregulated the inflammatory markers. In addition, the elevated levels of AGE and the activated NF-κB in the metanil-yellow-treated animals were significantly attenuated. Moreover, the levels of TNF-α and IL-1β were significantly attenuated as a result of bees' honey administration. Furthermore, the histopathological examination of the liver showed that bees' honey reduced fatty degeneration, cytoplasmic vacuolization, and necrosis in metanil-yellow-treated rats. In conclusion, the obtained data suggest that bees' honey has hepatoprotective effect on acute liver injuries induced by metanil-yellow *in vivo*, and the results suggested that the effect of bees' honey against metanil yellow-induced liver damage is related to its antioxidant/anti-inflammatory properties which attenuate the activation of NF-κB and its controlled genes like TNF-α and IL-1β.

1. Introduction

Metanil yellow is a highly-water-soluble dye. It belongs to the mono azo group of the dyes. Although the use of metanil yellow as a colorant agent is not permitted, it is still widely used as a colorant in many food industries. It is extensively used in the developing countries as a colorant in sweet meat, ice-creams, soft drinks, and beverages [1]. Because of its orange-yellow color, metanil yellow is also widely used in the coating of turmeric. It is extensively used in paper, leather, and many textile industries as a dye and colorant for the wool [1, 2]. Moreover, it is also used as a colorant for lacquers and cosmetic products. Furthermore, the dye is highly suitable for the preparation of colored water-fast inks [3] and can also be used analytically for the determination of trace amounts of Mo (VI) [4].

Like all azo dyes, the toxicity data showed that intraperitoneal and intratesticular administration or the oral feeding of metanil yellow in the laboratory animals like rats and guinea pig produces testicular lesions because of the damage of the seminiferous tubules and the decreased rate of spermatogenesis. Metanil yellow also results in alteration of the rat haematopoietic system and reduction of mucin secretion of the rat's intestinal cells [5]. In the case of oral administration, metanil yellow causes toxic methaemoglobinaemia [6] and cyanosis [7] in humans, while allergic dermatitis results from its direct contact with the skin [8]. In addition, metanil yellow has tumor-inducing effects and can also create intestinal [9] and enzymatic [10] disorders in the human body. Metanil yellow is not mutagenic but it can alter the expression of many genes [1].

The major metabolic pathway for detoxification of azo compounds proceeds via the reduction of the azo linkage leading to the formation of aromatic amines [1]. Reduction of azo compounds is catalyzed by liver microsomes [11], cytosolic enzymes, and colonic bacteria [12]. Reduction products of some azo compounds are found to possess toxic and mutagenic properties [13]. The two metabolites of metanil

yellow are p-aminodiphenylamine and metanillic acid, which are found to be toxic in rat gut mucosal epithelium [14].

Honey is a highly healthy material. Bees' honey is considered to be a balanced food source [15]. Although bees' honey is considered as a natural product, its chemical composition is very complex. Bees' honey contains about 180 compounds. The main constituents include enzymes, vitamins, amino acids, and minerals [16]. Previous studies showed that bees' honey was widely used in the folk medicine [17]. Cumulative data showed that bees' honey possesses a considerable anti-inflammatory, antioxidative, and antitumor activity. In addition, it is considered a potent radical scavenger especially for hydroxyl radicals. Moreover, it prevents the depletion of the antioxidant enzymes. Furthermore, it plays a key role in both normalizing of the kidney functions and protection of the liver from different toxic agents [15–17].

The present study aims to evaluate the hepatotoxic effects of metanil yellow as well as the hepatoprotective effects of bees' honey on rat liver. In addition, this study aims to investigate a possible pathway for the protective effect of bees' honey.

2. Materials and Methods

2.1. Animals. Adult male albino rats weighing 190–210 g were used in the present study. Animals were maintained under normal conditions and fed a normal diet with free access to water *ad libitum*. Rats were randomly divided into 7 groups, 10 rats each, as follows.

Group 1: this group of animals was healthy normal rats and serves as untreated control group.

Groups 2–4: animals of these groups were orally given metanil yellow at a dose of 50, 100, and 200 mg/kg body weight (lower than 1/20 of LD_{50}) [9].

Groups 5–7: animals of these groups were orally given Metanil yellow at a dose of 50, 100, and 200 mg/kg body weight (as in group 2–4) in addition to bees' honey 2.5 mg/kg body weight daily [17] for 8 weeks.

At the end of the experimental duration, the animals were weighed, anesthetized, and sacrificed. The liver was removed and washed with cold normal saline and divided into parts. The first part was used to prepare the liver homogenate, the second was used to prepare the nuclear extract for electrophoretic mobility shift assay (EMSA), and the third part was used for histopathological examinations.

2.2. Blood Collection for Estimation of Liver Functions. Before sacrificing the animals, blood was collected from retro-orbital plexus. The blood was incubated at 37°C for 10 min and centrifuged at 5,000 g for 10 min. The resulting serum was used for analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (t. protein), albumin, total bilirubin, gamma glutamyl transferase (GGT), and serum lactate dehydrogenase (LDH).

2.3. Assay of ALT, AST, ALP, T. Protein, Albumin, T. Bilirubin, and GGT. The levels of ALT, AST, ALP, t. protein, albumin, t. bilirubin, and GGT in the serum of all rats were determined by using the commercially available kits. The required kits were purchased from BioSystem (Barcelona, Spain). All analyses were performed according to the instructions of the manufacturer.

2.4. Determination of LDH. LDH was determined according to the method of King [18]. 100 μL of serum was added to 1.0 mL of buffered substrate (sodium pyruvate 37.5 mM in phosphate buffer 100 mM, pH 7.4). The mixture was incubated at 37°C for 15 minutes. After adding 200 μL of NAD^+ solution (10 mg/mL in phosphate buffer), the incubation was continued for further 15 minutes. The reaction was stopped by adding 100 μL of 2,4-dinitrophenylhydrazine (0.02% in concentrated HCl). The tubes were incubated again at 37°C for 15 min, then 7.0 mL of 0.4N NaOH was added, and the developed colour was measured using a spectrophotometer using phosphate buffer as blank.

2.5. Liver Homogenate Preparation. The liver of different groups was dissected and rinsed thoroughly with ice-cold normal saline. Moreover, it was smashed in a homogenization buffer containing a protease inhibitor tablet [19, 20]. The solution was sonicated in an ice bath for 30 sec followed by centrifugation at 13000 rpm for 4 min at 4°C. The supernatant was stored at −80°C and assayed for protein concentration using BCA kit (Pierce, Rockford, IL, USA) according to the instruction of the manufacturer [21]. Bovine serum albumin was used as a standard.

The liver homogenate was used for the determination of the level of lipid peroxidation (MDA), the concentration reduced glutathione (GSH), and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-px), glutathione-*S*-transferase (GST), and catalase. In addition the levels of advanced glycation end products (AGE), nitric oxide (NO), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α) were assayed in the liver homogenate. The second part of the liver from each group was stored in 10% neutral formalin solution and used for histopathological examinations.

2.6. Assay of the Oxidant/Antioxidant Markers of Liver Homogenate

2.6.1. Reduced GSH. Reduced GSH was measured by colorimetric end point assay using dithionitrobenzoic acid method described by Sayed [22]. GSH concentration was expressed as nmol/mg protein using GSH standard calibration curve.

2.6.2. Determination of GSH-Px Activity. The activity of GSH-Px in the liver homogenate was measured by the method of Paglia and Valentine [23] with slight modification. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (A340) providing a spectrophotometric means for monitoring GSH-px activity. The molar extinction coefficient for NADPH is 6220 M^{-1} cm^{-1} at 340 nm. To assay GSH-px, the tissue homogenate was added to a solution containing glutathione, glutathione reductase,

and NADPH. The enzyme reaction was initiated by adding the substrate, hydrogen peroxide, and the absorbance at 340 nm (A340) was recorded. The rate of decrease in the A340 was directly proportional to the GSH-px activity in the sample, 6.22×10^{-6}.

2.6.3. Determination of GST Activity. The activity of GST was assayed by using the electrophilic substrate 1-chloro-2,4-dinitrobenzene (CDNB) according to the procedure described by Moron et al. [24]. GST was estimated in 1 mL of incubation mixture containing 905 μL of 0.1 M phosphate buffer (pH 6.5), 20 μL of 20 mM CDNB reagent, 25 μL of 200 mM of reduced GSH, and 25 μL of Triton X100 (0.66%) and preincubated at 37°C for 5 minutes. The reaction was started by adding 25 μL of the liver homogenate. The activity of GST was determined by continuously monitoring the change in absorbance at 340 nm with the spectrophotometer for 3 minutes. The O.D. change/min was calculated, and GST activity was calculated by using the molar extinction coefficient [9.6 mM^{-1} cm^{-1}] of GST.

2.6.4. Assay of MDA. Malondialdehyde (MDA) was determined according to the method described by Mekheimer et al. and Sayed [25, 26] using 1,1′,3,3′- tetramethoxy propane as standard. In brief, 8.1% SDS was added to the tissue homogenate and incubated for 10 minutes at room temperature, followed by boiling with 20% acetic acid and 0.6% thiobarbituric acid for 60 minutes in a water bath. On cooling, a mixture of pyridine : butanol (1 : 15 v/v) was added and centrifuged at 12000 rpm for 5 min. Absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was expressed as nmol/mg protein.

2.6.5. Assay of SOD Activity. The activity of SOD was determined as the volume of homogenate that scavenges 50% of the superoxide anion which is generated from the photoillumination of riboflavin in the presence of EDTA (1 unit of SOD activity) [27] using a commercially available kit from BIO-Rad according to the instructions of the manufacturer.

2.6.6. Determination of Nitric Oxide (NO). The production of NO was determined indirectly by measuring the plasma level of nitrite by a calorimetric method according to Griess reaction [28]. Liver homogenate was diluted four times with water and deproteinized by adding 1/20 volume of $ZnSO_4$ (300 g/L) to a final concentration of 15 g/L. The mixture was centrifuged at 13,000 rpm for 5 min at room temperature. 10 μl of the supernatant was applied to a micro titer followed by 100 μl of Greiss reagent (1% sulfanilamide and 0.1% N-1-naphthyl ethylenediamine dihydrochloride in 2.5% polyphosphoric acid). The absorbance was measured at 540 nm with a Micro Reader (Hyperion, Inc., FL, USA) after 10 min of color development at room temperature. Sodium nitrate solution was used to obtain a standard curve.

2.7. Electrophoretic Mobility Shift Assay (EMSA). Part of the kidney from different groups was homogenized with 100 μL TOTEX buffer (100 mM HEPES-KOH, pH 7.9, 0.35 M NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 10 μg/mL leupeptin, 0.5 mM DTT, and 0.2 mM PMSF) for 30 seconds and incubated in ice bath for 30 minutes, mixed well, and centrifuged at 13000 rpm for 5 minutes. The supernatant which contained the total nuclear extract of the liver was transferred to a fresh tube and kept at −80°C for EMSA as discussed [29]. The nuclear extract of the liver was assayed for transcription factor binding activity using the NF-κBp65 consensus sequence: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabeled consensus oligonucleotides as previously described [29]. Quantification of activated NF-κB was performed by densitometric analysis of relative EMSA band intensities (R.I.) using ImageJ software using the band of the normal control as a reference band. EMSA experiments were performed at least three times.

2.8. Assay of AGE, TNF-α, and IL-1β. The levels of AGE, TNF-α, and IL-1β were determined using commercially available ELISA kits. The ELISA kits for TNF-α and IL-1β were obtained from R&D (Braunschweig, Germany) while the ELISA kit for AGE was obtained from Roche diagnostics (Mannheim, Germany). All ELISA assays were done according to the manufacturer's instructions.

2.9. Histopathological Examination. Liver tissues were collected after animal sacrifice, fixed in 10% neutral formalin, and embedded in paraffin. Sections of about 5 μm thick were prepared and stained using H&E as previously described by Fukuzawa et al. [30].

2.10. Statistical Analysis. All group values are expressed as the mean ± SD. Data were evaluated using SPSS 11.09 for Windows. An analysis of variance test was performed initially to test for differences in the treatment, a Tukey post-hoc test was performed to examine whether there were any significant differences between different treatment groups.

3. Results

3.1. Initial and Final Body Weights. The initial and final body weights of all rats in both control and treatment animals were presented in Table 1. There were no differences in the initial body weights of all groups. There was a significant decrease in the body weights of metanil-yellow-treated groups (groups 2–4) when compared to control group. Treatment of the rats with bees' honey resulted in a significant increase of the final body weight of rats in the groups 5–7.

3.2. Hepatic Biomarkers. As illustrated in Table 1, administration of metanil yellow induced a marked increase in the levels of AST, ALT, ALP, GGT, total bilirubin, and LDH and a significant decrease of serum albumin as compared to the control group. Both effects were found to be dose dependent. Administration of bees' honey significantly improved some of these hepatic biomarkers and normalized the others as indicated in Table 1.

Table 1: Body weight and biochemical parameters of liver function tests of rat. Group 1, control; groups 2–4, rats treated with 50, 100, and 200 mg metanil yellow/kg body weight; groups 5–7, rat groups treated with 50, 100, and 200 mg metanil yellow/kg body weight plus 2.5 g/kg bees' honey daily for 8 weeks, respectively.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Initial body weight, g	202 ± 15	200 ± 10	198 ± 14	205 ± 125	201 ± 16	200 ± 17	199 ± 16.5
Final body weight, g	253 ± 18	182 ± 12[a]	175 ± 10[a]	171 ± 14[a]	235 ± 12[b]	221 ± 11[c]	239 ± 19[d]
ALT, U/L	28.12 ± 1.12	95.32 ± 2.3[a]	102.43 ± 3.2[a]	110.12 ± 2.45[a]	35.2 ± 1.3	35.5 ± 3.4	34.9 ± 4.2
AST, U/L	36.78 ± 1.5	95.78 ± 1.34[a]	101.43 ± 2.5[a]	120.45 ± 4.3[a]	45.5 ± 5.3	51.3 ± 6.5	65.4 ± 5.2
ALP, U/L	194.34 ± 2.32	324.12 ± 16.8[a]	398.34 ± 17.9[a]	423 ± 23.5[a]	220.5 ± 10.2[b]	251 ± 15.6[c]	245 ± 16.2[d]
LDH, U/L	87.67 ± 2.12	150.56 ± 2.4[a]	201.45 ± 4.6[a]	234.76 ± 6.87[a]	102 ± 4.2[b]	124 ± 4.5[c]	136 ± 6.3[d]
GGT, U/L	72.36 ± 1.45	104.5 ± 2.43[a]	132.87 ± 3.9[a]	165.77 ± 5.4[a]	89 ± 3.5	95.3 ± 3.4[c]	99.5 ± 4.56[d]
T. protein, mg%	8.13 ± 0.76	6.15 ± 1.27	5.97 ± 1.21	5.65 ± 1.54	6.8 ± 2.1	6.2 ± 2.3	7.1 ± 3.1
Albumin, mg%	5.54 ± 0.98	3.1 ± 0.54[a]	2.86 ± 0.43[a]	2.43 ± 0.56[a]	4.95 ± 2.5[b]	4.8 ± 1.2[c]	4.75 ± 1.5[d]
T. bilirubin, mg%	0.8 ± 0.05	1.3 ± 0.23[a]	1.56 ± 0.56[a]	2.5 ± 0.93[a]	0.9 ± 0.01[b]	0.91 ± 0.02[c]	0.91 ± 0.02[d]

All values were expressed as mean ± S.D. of triplicate tests ($n = 10$).
[a] $P < 0.05$ versus group 1, [b] $P < 0.05$ versus group 2, [c] $P < 0.05$ versus group 3, [d] $P < 0.05$ versus group 4.

Table 2: Antioxidant parameters as well as AGE levels of rat liver homogenate. Group 1, control group; groups 2–4, rats treated with 50, 100, and 200 mg metanil yellow/kg body weight for 8 weeks, respectively; groups 5–7, rat groups treated with 50, 100, and 200 mg metanil yellow/kg body weight plus 2.5 g/kg bees' honey daily for 8 weeks, respectively.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
MDA, nmol/mg protein	0.42 ± 0.06	1.49 ± 0.19[a]	5.1 ± 0.85[a]	9.37 ± 1.8[a]	0.85 ± 0.02[b]	1.45 ± 0.35[a,c]	1.9 ± 0.92[a,d]
GSH, nmol/mg protein	101 ± 5.8	72.5 ± 1.6[a]	45.9 ± 4.6[a]	26.7 ± 3.8[a]	79.5 ± 2.1	78.5 ± 2.1[a,c]	80.2 ± 1.3[a,d]
GST, nmol/mg protein	18.6 ± 1.5	11.1 ± 1.3[a]	6.29 ± 0.39[a]	4.2 ± 0.94[a]	14.76 ± 0.86[b]	10.54 ± 0.45[a,c]	10.3 ± 0.87[a,d]
SOD, U/mg protein	15.8 ± 1.1	10.5 ± 2.5[a]	9.6 ± 2.1[a]	3.1 ± 1.2[a]	14.25 ± 1.1[b]	11.2 ± 1.3[a,c]	10.97 ± 1.5[a,d]
CAT, U/mg protein	5.95 ± 0.5	3.12 ± 0.2[a]	2.41 ± 0.31[a]	1.1 ± 0.11[a]	4.56 ± 0.54[b]	4.29 ± 0.23[c]	3.1 ±0.67[a,d]
GSH-Px, U/mg protein	4.95 ± 0.21	3.1 ± 0.31[a]	2.05 ± 0.12[a]	1.15 ± 0.10[a]	4.67 ± 0.65[b]	4.12 ± 0.32[c]	3.87 ± 0.56[d]
AGE, ng/mg protein	20.6 ± 4.5	26.2 ± 5.5[a]	36.5 ± 5.5[a]	48.7 ± 8.5[a]	22.2 ± 5.5[b]	28.2 ± 5.5[a,c]	32.2 ± 5.5[a,d]

All values were expressed as mean ± S.D. of triplicate tests ($n = 10$).
[a] $P < 0.05$ versus group 1, [b] $P < 0.05$ versus group 2, [c] $P < 0.05$ versus group 3, [d] $P < 0.05$ versus group 4.

3.3. Effect on GSH-px. Metanil yellow treatment caused a significant decrease in the level of GSH-px activity in liver tissue when compared with control group (Table 2). The decrease of GSH-px activity in the metanil-yellow-treated groups was dose dependent. The treatment of rats with bees' honey resulted in a marked increase of GSH-px activity when compared to the metanil-yellow-treated rats ($P < 0.05$).

3.4. Effect on the Activity of SOD. The activity of SOD in the tissue homogenates of all experimental rats was shown in Table 2. In the liver homogenate, treatment of rats with 50, 100, and 200 mg/kg metanil yellow caused a significant and a dose-dependent reduction of the SOD activity compared to the normal control group. Enhancement of SOD activity was observed as a result of bees' honey treatment, and the activity was found to be markedly increased.

3.5. Effect on the Activity of GST. Table 2 showed a significant and a dose-dependent decrease in the hepatic GST activity upon metanil yellow treatment as compared to the control group. A significant increase in GST activity was observed in the rat groups treated with bees' honey as compared to the metanil-yellow-treated groups ($P < 0.05$).

3.6. Effect on GSH. The effect of bees' honey on GSH levels for all experimental groups is shown in Table 2. Metanil yellow treatment caused a significant and a dose-dependent decrease of GSH levels in the liver homogenates compared to the normal control group. Rats treated with 50, 100, and 200 mg/kg metanil yellow had a lower GSH content than the normal control group. Administration of bees' honey significantly modulated these alterations, and the level of GSH rose.

3.7. Effect on Lipid Peroxidation (MDA). Lipid peroxidation results in the formation of free radicals and induction of oxidative stress. The degree of lipid peroxidation was measured as MDA in rat liver homogenate and the data were shown in Table 2. From the obtained data, it was found that bees' honey could significantly decrease the formation of malondialdehyde (MDA) in metanil-yellow-treated rats. After 50, 100, and 200 mg/kg metanil yellow administration, the liver MDA level significantly increased. However, the oral administration of bees' honey resulted in decreasing the level of MDA.

3.8. Effect on Catalase Activity. As a result of metanil yellow administration, the activity of catalase was markedly and dose-dependently reduced. Data in Table 2 showed that the activity of catalase was reduced in the metanil-yellow-treated groups. The orally administrated bees' honey increased these reduced activities.

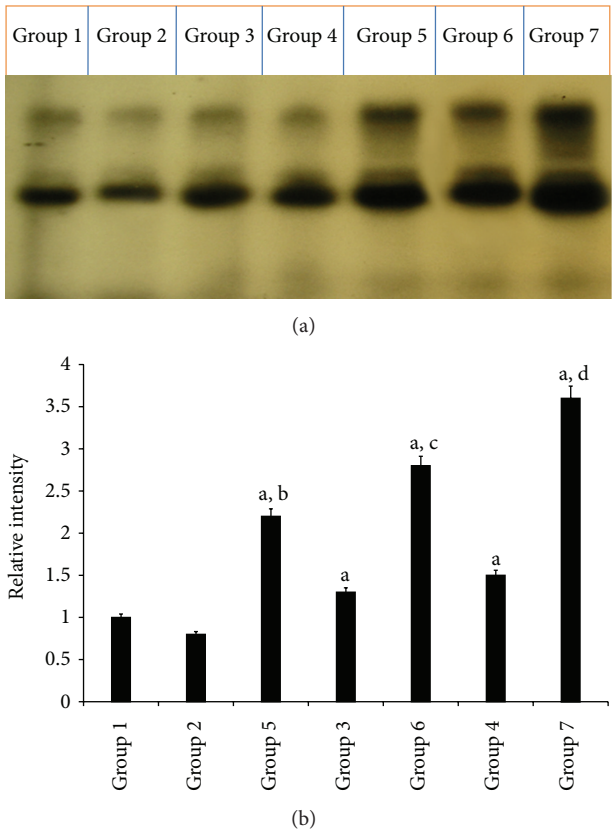

Figure 1: Binding activity of NF-κB-p65 (a). Group 1, untreated (normal control); groups 2–4, rats treated with 50, 100, 200 mg/kg metanil yellow, respectively; and groups 5–7, rats were treated with metanil yellow as in groups 2–4 plus bees' honey 2.5 mg·kg^{-1} d^{-1} for 8 weeks. Binding activity of NF-κB-p65 to its consensus sequence was assayed by EMSA of total protein extracts. Quantification of activated NF-κB-p65 was performed by densitometric analysis of relative EMSA band intensities (b) using the normal control band as a reference band. All values expressed as mean ± S.D. of triplicate tests ($n = 10$). [a]$P < 0.05$ versus group1, [b]$P < 0.05$ versus group 2, [c]$P < 0.05$ versus group 3, [d]$P < 0.05$ versus group 4.

3.9. Effect of Bees' Honey on AGE Levels and Activation of NF-κB. As a result of administration of metanil yellow, the levels of AGE were significantly increased indicating a high degree of oxidative stress and protein glycosylation. Bees honey feeding results in decreasing the elevated AGE as indicated in Table 2. Cumulative studies showed that the overproduction of AGE is a direct cause for the activation of the nuclear factor kaba B (NF-κB) [31, 32].

To test the effect of bees honey on the activation of NF-κB-p65 on the metanil-yellow-treated rats, the EMSA analysis was done. Data of Figures 1(a) and 1(b) showed that the administration of metanil yellow resulted in a significant and dose-dependent NF-κB-p65 activation, reaching a maximum at metanil yellow concentration of 200 mg/kg ($P < 0.05$) compared with the normal control rats. Administration of bees' honey resulted in a significant reduction of this activated NF-κB-p65.

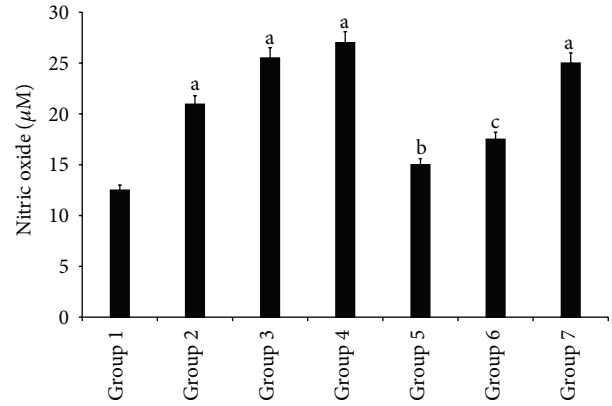

Figure 2: Effect of bees' honey on nitric oxide levels of rat liver homogenate treated with metanil yellow. Group 1, control group; groups 2–4, rats treated with 50, 100, and 200 mg metanil yellow/kg body weight for 8 weeks, respectively; groups 5–7, rat groups treated with 50, 100, and 200 mg metanil yellow/kg body weight plus 2.5 g/kg bees' honey daily for 8 weeks, respectively. All values were expressed as mean ± S.D. of triplicate tests ($n = 10$). [a]$P < 0.05$ versus group1, [b]$P < 0.05$ versus group 2, [c]$P < 0.05$ versus group 3, [d]$P < 0.05$ versus group 4.

3.10. Effect on NO, TNF-α, and IL-1β. The effect of metanil yellow as well as the effect of bees' honey on the levels of NO, TNF-α, and IL-1β in rats was studied. Data in Figure 2 showed that the level of NO was markedly and dose-dependently increased as a result of metanil yellow treatment. Administration of bees' honey reversed these effects and significantly reduced the elevated levels of NO in the groups 5–7 as shown in Figure 2.

In parallel, the levels of TNF-α and IL-1β were significantly and dose-dependently elevated in the metanil-yellow-treated rats (groups 2–4) as indicated in Figures 3 and 4. These elevated levels of TNF-α and IL-1β were significantly reduced as a result of bees' honey administration.

Furthermore, the histopathological findings in Figure 5 are in line with the obtained data above.

4. Discussion

Hepatic cells contain high concentrations of hepatic enzymes in the cytoplasm, and AST particularly exists in the mitochondria. Due to the damage caused to hepatic cells, the leakage of cytosol increases the levels of these hepatospecific enzymes in the serum. The elevated serum enzyme levels such as AST and ALT are indicative of cellular leakage and functional integrity of cell membrane in the liver [31]. The measurement of serum AST and ALT levels serves as a mean for the indirect assessment of the condition of the liver. In the present study, the capability of bees' honey in controlling metanil-yellow-induced hepatotoxicity was observed in that bees honey treated animals had decreased levels of hepatic enzyme in the serum when compared with the metanil yellow groups.

In the present study, we postulated that the hepatoprotective effect of bees' honey could be attributed to its

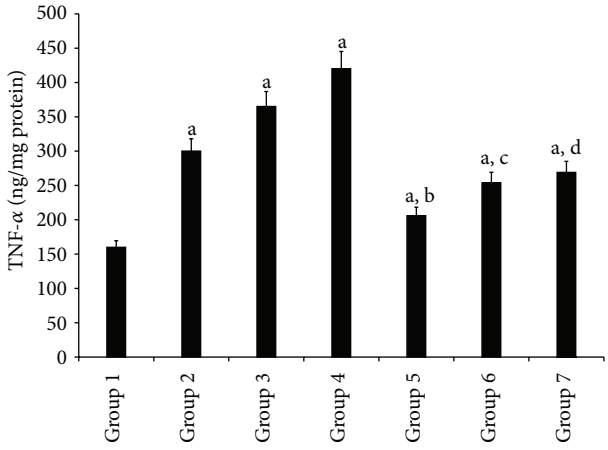

FIGURE 3: Effect of bees' honey on TNF-α level of rat liver treated with metanil yellow. Group 1, control group; groups 2–4, rats treated with 50, 100, and 200 mg metanil yellow/kg body weight for 8 weeks, respectively; groups 5–7, rat groups treated with 50, 100, and 200 mg metanil yellow/kg body weight plus 2.5 g/kg bees' honey daily for 8 weeks, respectively. All values were expressed as mean ± S.D. of triplicate tests ($n = 10$). $^{a}P < 0.05$ versus group1, $^{b}P < 0.05$ versus group 2, $^{c}P < 0.05$ versus group 3, $^{d}P < 0.05$ versus group 4.

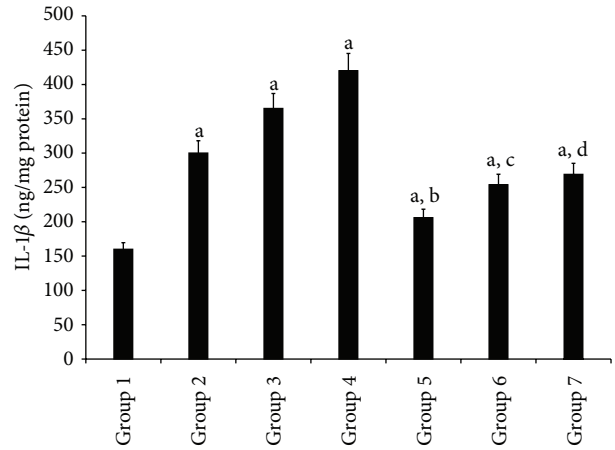

FIGURE 4: Effect of bees' honey on IL-1β levels of rat liver treated with metanil yellow. Group 1, control group; groups 2–4, rats treated with 50, 100, and 200 mg metanil yellow/kg body weight for 8 weeks, respectively; groups 5–7, rat groups treated with 50, 100, and 200 mg metanil yellow/kg body weight plus 2.5 g/kg bees honey daily for 8 weeks, respectively. All values were expressed as mean ± S.D. of triplicate tests ($n = 10$). $^{a}P < 0.05$ versus group1, $^{b}P < 0.05$ versus group 2, $^{c}P < 0.05$ versus group 3, $^{d}P < 0.05$ versus group 4.

antioxidant properties. We assayed MDA, a product of lipid peroxidation. MDA was increased in rat liver by induction of hepatotoxicity. However, we showed that bees' honey significantly reduced MDA formation. Hepatotoxicity not only initiates lipid peroxidation but also reduces tissue GSH-Px, GST, CAT, and SOD activities, and this depletion may result from oxidative modification of these proteins and our data are in line with previous data of Augustyniak et al. [32–34]. Normal cells have a number of mechanisms to defend themselves against the toxic effect of reactive oxygen species (ROS) and oxidative stress including free radical scavengers and chain reaction terminators such as SOD, CAT, and GSH-px systems. SOD removes superoxide radicals by converting them into hydrogen peroxide which is converted by CAT and GSH-px into water. Cellular injury arises when ROS production exceeds the cellular capacity of removal [35–37].

As a result of the increased levels of MDA and the reduced activities of the antioxidant enzymes, the metanil-yellow-treated rats undergo oxidative stress. The induced oxidative stress results in the overproduction of AGE seen in the metanil-yellow-treated rats. AGE combined with their receptors on the cell membrane leading to the activation of NF-κB and its controlled genes. Our data confirmed this hypothesis and are in line with previous studies [29, 31–34, 37]. The anti-inflammatory efficacy of bees' honey was confirmed by the data obtained from EMSA that showed that bees' honey feeding resulted in a significant reduction of NF-κB. This is a novel finding that correlates the beneficial effects of bees' honey with its effect on NF-κB.

In addition, the anti-inflammatory activity of bees' honey was evaluated *in vivo* by measuring the release of some inflammatory markers that are controlled by NF-κB like IL-1β, NO, and TNF-α. TNF-α is a proinflammatory cytokine that is elevated in acute and chronic diseases. Some phytochemicals have been shown to inhibit inflammation by blocking inflammatory pathways downstream of cytokine release and also by reducing macrophage production of proinflammatory factors [38]. TNF-α has been reported to play a key role in the pathogenesis of various liver diseases. Following its release from activated Kupffer cells, TNF-α worsens both oxidative stress and inflammatory responses in the liver [39]. In addition, TNF-α stimulates the release of cytokines from macrophages and induces phagocyte oxidative metabolism and NO production. Although several studies have showed that NO protects against liver injury using an NOS inhibitor, certain evidence indicates that excessive NO production by iNOS can lead to hepatic injury [40]. Recent reports also demonstrated that iNOS over reduction occurs in the liver of rats with acute liver injury, which suggested that iNOS may act as a mediator in the pathogenesis of hepatotoxicity in rats [41]. The obtained data are in line with previous work of many research groups [38–43] and are confirmed with the histopathological data in Figure 5.

These results have provided the evidence for the pharmacological effect of bees' honey in metanil-yellow-induced hepatotoxicity. Overall, bees' honey not only provided maximum conjugation with injurious free radicals and diminished their toxic properties, but also suppressed the inflammatory responses in metanil-yellow-induced liver injuries. The possible mechanism could be suggested that bees' honey is able to protect the liver against cellular oxidative damage and maintain of intracellular level of antioxidant enzymes which reduce the overproduction of AGE and attenuate the activation of NF-κB and its controlled genes as well as act as immunomodulator. However, further studies on the active compounds and their biochemical mechanisms

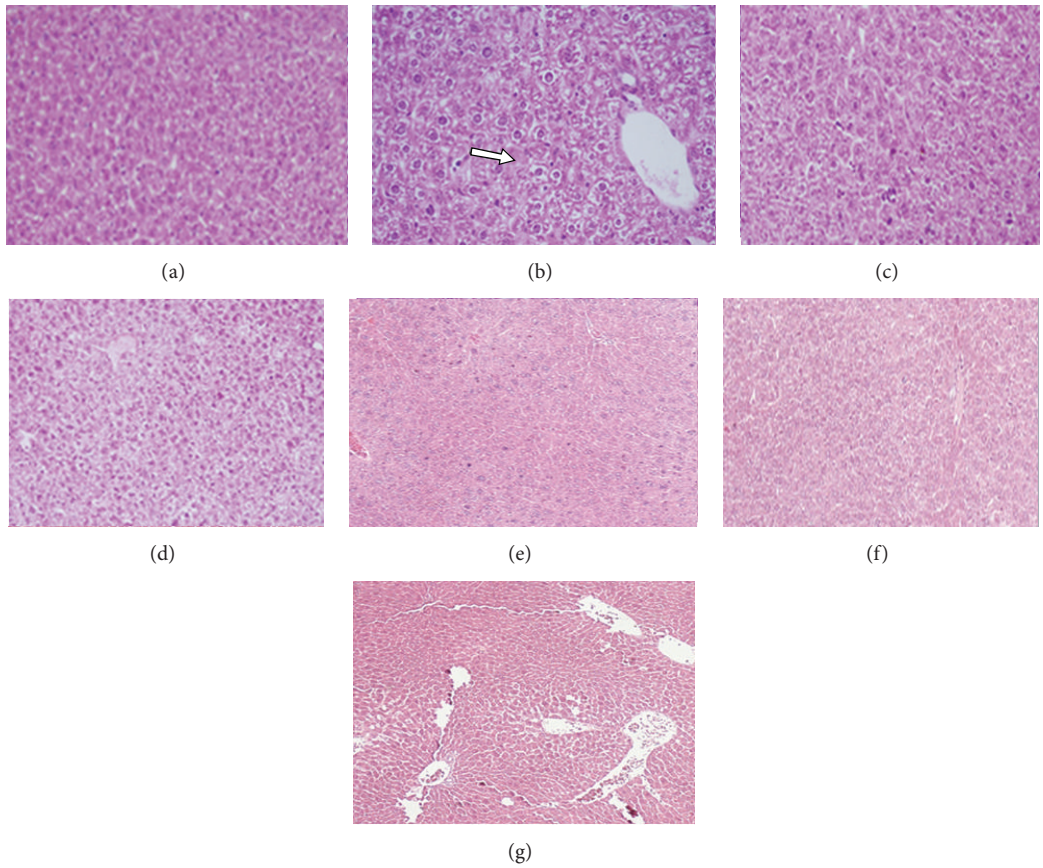

Figure 5: Histopathological findings of rat liver from different groups. Livers were removed, fixed, and embedded in paraffin. Sections were stained with hematoxylin-eosin (×200). (a) control group; (b)–(d) rats treated with 50, 100, and 200 mg metanil yellow/kg body weight for 8 weeks, respectively; (e)–(g) rat groups treated with 50, 100, and 200 mg metanil yellow/kg body weight plus 2.5 g/kg bees' honey daily for 8 weeks, respectively.

responsible for the hepatoprotective effect of bees' honey will be necessary.

Abbreviations

AGE: Advanced glycated end products
TNF: Tumor necrosis factor
IL: Interleukin.

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