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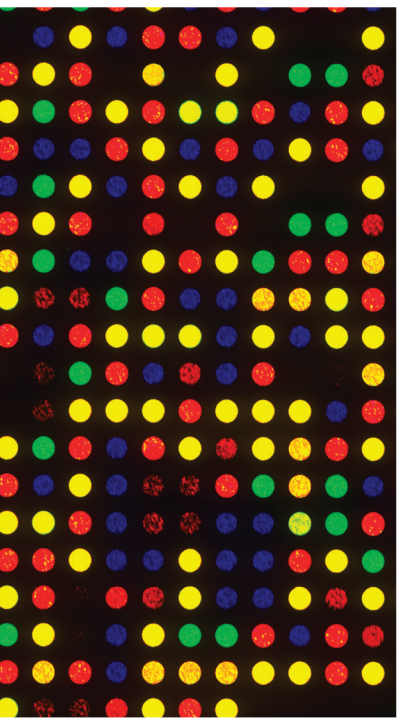
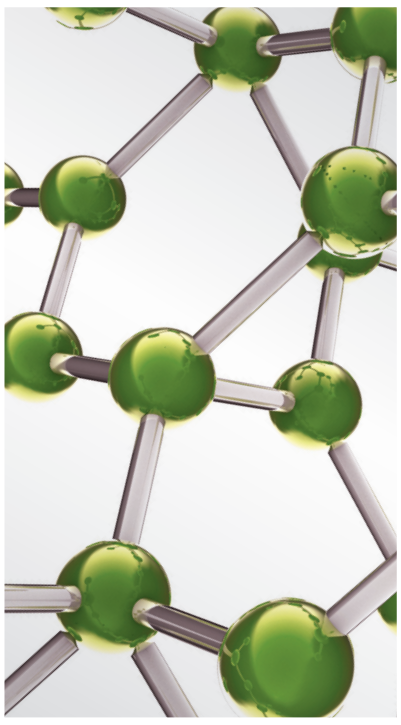
Natural Products for Liver Diseases: Basic, Clinical, and Translational Research

Guest Editors: Chang-Quan Ling, Jen-Hwey Chiu, Byeongsang Oh, and William C. S. Cho



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Editorial Board

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Editorial

Natural Products for Liver Diseases: Basic, Clinical, and Translational Research

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Liver diseases are important causes of human morbidity and mortality in the world. The incidence of liver diseases is rising due to the widespread of hepatitis and alcoholism. They may be caused by infection, injury, exposure to drugs or toxic compounds, autoimmunity, or genetic defect that leads to the deposition of harmful substances. Liver damage is likely to play a role in inflammation, scarring, obstructions, cirrhosis, liver failure, and even liver cancer.

The use of herbal medicines can be traced back several thousand years ago in ancient China. A large volume of evidences have shown that many natural products are available as chemoprotective agents against common liver diseases, such as hepatitis, cirrhosis, liver cancer, fatty liver diseases, and gallstones. This special issue aims to gather updated progress in this important area.

The papers in this special issue cover a wide range of topics, including a study of the anti-hepatitis B activity of 3,4-*O*-dicaffeoylquinic acid isolated from *Laggera alata* using the D-galactosamine-induced hepatocyte damage model and hepatitis B virus transgenic mice. *In vitro* results showed that the compound could markedly inhibit the production of HBsAg and HBeAg, whereas *in vivo* results indicated that the test compound significantly inhibited HBsAg production and increased heme oxygenase-1 expression in hepatitis B virus transgenic mice.

On the other hand, the study of *Graptopetalum paraguayense* showed cytotoxicity toward hepatic stellate cells. Microarray profiling indicated that the expression of most metabolism- and cell growth and/or maintenance-related genes recovered to near normal levels following *G. paraguayense* treatment as classified by gene ontology and laser scanning microscope analysis. A publicly accessible website was further established to present data of the identified genes, including 44 necroinflammation-related and 62 fibrosis-related genes. These might provide useful insight into the molecular mechanisms underlying liver damage and potential targets for the development of therapeutic drugs.

Another study demonstrated that the ethanolic extracts of *Cassia sophera* Linn. leaves showed hepatoprotective activity against carbon tetrachloride-induced hepatic damage in rats. Their study suggested that this possible activity might be due to the presence of flavonoids in the extracts.

Elephantopus scaber has been traditionally used as liver tonic. However, its protective effect on liver damage is still unclear. In the study of the total phenolic and flavonoid content of *E. scaber* ethanol extract, low concentration of *E. scaber* was able to reduce serum biochemical profiles and fat accumulation in the liver of alcohol-induced liver damage mice, while high concentration of *E. scaber* was able to revert the liver damage without possessing any oral acute toxicity on mice.

Chinese herbal medicines usually treat disease with a prescription of several herbs mixing together. It has been demonstrated that the antifibrotic properties of a Chinese herbal decoction Danggui Buxue Tang in a rat model of liver fibrosis were related to its ability to inhibit

angiogenesis. This antiangiogenic mechanism was associated with the improvement of oxidative stress, the expression and signaling regulation of angiogenic factors, and especially the modulation of hypoxia inducible factor-1α in fibrotic livers.

A number of anticancer herbal medicines have demonstrated anticancer effects [1]. Hepatocellular carcinoma (HCC) is always hard to treat; anoikis has been recognized as a potential target for anticancer therapy. *Polygonum cuspidatum* is a frequently used Chinese herb in the treatment of HCC. A study in this special issue showed an inhibitory effect of *P. cuspidatum* extract in HCC cells proliferation in a dose- and time-dependent manner. *P. cuspidatum* extract also inhibited the anchorage-independent growth of HCC cells in soft agar by inducing caspase-mediated anoikis in HCC cells which might relate to reactive oxygen species generation and focal adhesion kinase downregulation. Their study has provided new insight into the application of Chinese herbs for HCC treatment.

On the other hand, the root of *Actinidia valvata* has been widely used in the treatment of HCC. A report in this special issue demonstrated that the total saponin of *A. valvata* could effectively inhibit HCC growth and metastasis *in vivo*, inhibit the formation of microvessel, downregulate the expressions of vascular endothelial growth factor and basic fibroblast growth factor, and retrain angiogenesis of hepatoma.

In the omics era, a number of studies use omics technologies to study herbal medicines [2]. A ^{1}H nuclear magnetic resonance-based metabonomic approach was proposed to explore the biochemical characteristics of Yang deficiency syndrome in HCC based on serum metabolic profiling. The decreased intensities of metabolites (including low density lipoprotein/very-low density lipoprotein, isoleucine, lactate, lipids, choline, and glucose/sugars) in serum might be the distinctive metabolic variations of Yang deficiency syndrome patients with HCC. These metabolites might be potential biomarkers for the diagnosis of Yang deficiency syndrome in HCC.

Finally, a review article entitled "Herbal products: benefits, limits, and applications in chronic liver disease" described various examples of herbal products on pharmacokinetics, biological, and beneficial effects in metabolic, alcoholic, and viral hepatitis. The authors also pointed out that the lack of randomized, placebo-controlled clinical trials might be the main limitation of the applicability of complementary and alternative medicine.

We envision that this special issue will arise more interest in the field of hepatology, and more exciting investigations on liver diseases by herbal medicine will be conducted.

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William C. S. Cho

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Research Article

Effects of Lipid Regulation Using Raw and Processed Radix Polygoni Multiflori in Rats Fed a High-Fat Diet

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Raw and processed Radix Polygoni Multiflori have been used in the prevention and treatment of nonalcoholic fatty liver disease (NAFLD), hyperlipidemia, and related diseases in Asian counties for centuries. The lipid regulation ability of raw and processed Poligoni Multiflori Radix were compared in high-fat diet fed rats in this research. Total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-C) in blood and liver tissue were all significantly higher in model rats. However, triglyceride (TG) contents increased only in liver tissue, not in the blood samples. The rats fed the high-fat diets were considered the model of type IIa hyperlipidemia and early-stage nonalcoholic fatty liver disease. Both Radix Polygoni Multiflori (RPM) and Radix Polygoni Multiflori Praeparata (RPMP) revealed TC-lowing effects, and middling doses of RPMP displayed the most significant TC-lowing effects, as indicated by blood samples. Neither RPM nor RPMP was found to reduce LDL-C in rats' blood. Nevertheless, RPM showed dose-dependent TC- and TG-lowing effects in the liver tissue samples. In conclusion, RPM showed more pronounced effects on lipid regulation in liver samples in the treatment of early-stage NAFLD. RPMP, however, displayed better effects in regulating lipids in circulating blood for the treatment of hyperlipidemia.

1. Introduction

Fatty liver is a reversible condition in which large vacuoles of triglyceride fat accumulate in liver cells via the process of steatosis (abnormal retention of lipids within a cell). Considering the contribution that alcohol can make to this condition, fatty liver may be termed alcoholic steatosis or nonalcoholic fatty liver disease (NAFLD).

The development of nonalcoholic fatty liver disease comes from an imbalance between the influx and production of fatty acids and the use of fatty acids for oxidation or secretion. The progress of NAFLD is usually characterized by morphologic changes in the hepatocytes and hepatic triglyceride content (HTGC). However, early-stage NAFLD shows changes only in the fatty acid levels but no or few morphologic changes in hepatocytes [1]. In normal human livers, the mean values for total cholesterol (TC) and triglyceride (TG) are 3.9 and 19.5 mg/g wet weight, respectively. Hepatic steatosis (fatty liver) is a condition that is defined by fat accumulation within hepatocytes that exceeds 50 mg/g of the liver by weight [2, 3].

Fatty liver disease was recently recognized as a feature of the metabolic syndrome; fatty liver has evolved as a key player in the pathogenesis of hyperlipidemia. Hyperlipidemia is characterized by elevated TG, TC, and low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C) levels. Hyperlipidemia is a major risk factor for cardiovascular disease.

Because of the effectiveness and acceptable prices, the prevention and treatment of NAFLD and hyperlipidemia by traditional Chinese medicine attract more and more attention worldwide [4, 5]. Radix Polygoni Multiflori (RPM, heshouwu in Chinese) and Radix Polygoni Multiflori Praeparata (RPMP, zhiheshouwu in Chinese), originating from the root of *Polygonum multiflorum* Thunb., are used in the treatment of NAFLD and hyperlipidemia in oriental counties for centuries [6]. Although both RPM and RPMP have a history of use in the treatment of NAFLD and hyperlipidemia, RPMP is used more frequently in traditional Chinese medicine than RPM is. For example, the Pharmacopoeia of the People's Republic of China (2010 edition) lists three different prescription preparations containing RPMP for the treatment

(a) (b)

Figure 1: Photographs of raw (a) and processed (b) Radix Polygoni Multiflori.

of hyperlipidemia [6]: Xuezhining Wan, Xuezhiling Pian, and Shouwu Wan. It lists only one preparation, Zhengxin Jiangzhi Pian, that contains RPM. In the meantime, few studies have compared the effects of RPM and RPMP in the treatment of NAFLD, hyperlipidemia, and related diseases.

Our research group has performed previous studies including systematic comparisons of raw and processed RPM with respect to their antioxidative activities, adverse laxative effects, cytotoxicity, and *in vitro* lipid-regulation effects [7–9]. In our *in vitro* studies, raw RPM showed stronger abilities to regulate TG and TC than RPMP, indicating that RPM might be effective in the clinic treatment of NAFLD. However, *in vivo* results are required for corroboration. We compared the relative activities of raw and processed RPM in SD rats fed high-fat diets. Comprehensive studies of indexes of lipid metabolism in both the blood and liver tissue samples of the test animals were analyzed.

2. Materials and Methods

2.1. Chemicals. Simvastatin (Hangzhou MSD Pharmaceutical Co., Ltd., China) and fenofibrate (Laboratories Fournier S.A., France) were used as positive controls for lowering cholesterol and triglyceride levels, respectively. Lard oil was purchased from the Shuangliu Luxiao oil factory in Chengdu, Sichuan Province, China. Sodium chloride, methylthiouracilum (MTU), ether, and other reagents were of analytical grade.

2.2. Processing and Extraction of Radix Polygoni Multiflori. *Polygonum multiflorum* Thunb. plants were collected in June of 2008 by the authors in Luquan County within Yunnan Province and identified by Professor Ronghua Zhao of Yunnan University of Traditional Chinese Medicine. No specific permits were required for the described field studies. This location is not privately owned or protected in any way and the field studies did not involve endangered or protected species. Voucher specimens were deposited in the Herbarium of Pharmacognosy, Yunnan University of Traditional Chinese Medicine. RPMP was steamed by the authors from RPM with black soybean decoction according to the procedure recorded in Pharmacopoeia of the People's Republic of China (2010 edition) (Figure 1) [6].

The main components of RPM and RPMP were TSG (2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside), emodin, and physcion. The content of TSG was lower in RPMP than RPM. However, the contents of emodin and physcion were increased after processing [8, 9].

Extracts, 300 g RPM and 472 g RPMP, were decocted with water (10 times, 8 times, and then 6 times by volume) for three times, respectively. Extracts were combined, condensed, and lyophilized. The concentrations of RPM and RPMP were 0.6980 g/mL and 0.8580 g/mL, respectively. The recommended dosages of RPM and RPMP are 3–6 g and 6–12 g/per day according to the Pharmacopoeia of the People's Republic of China, 2010 edition [6]. We conversed the human dosage equivalently to rat dosage. The low, middle, and high dosages of RPM are 0.405, 0.810, and 1.62 g/kg body weight [10]. The low, middle, and high doses of RPMP are 0.810, 1.62, and 3.24 g/kg body weight.

2.3. Animals and Diets. SD rats of both sexes were provided by Experimental Animal Center of Yunnan University of Traditional Chinese Medicine. They were aged 8 weeks and weighed 245 ± 20 g. Rats of the same sex were housed six to a stainless steel cage containing sterile paddy husk as bedding in ventilated animal rooms. They were acclimated in the controlled environment (temperature 22 ± 1°C; 60 ± 10% humidity; and a 12 h/12 h light/dark cycle) with free access to water and a commercial laboratory complete food. All animal experiments were performed in compliance with the animal experimental ethics committee of Yunnan University of Traditional Chinese Medicine. All reasonable efforts were made to minimize the animals' suffering.

Diets designed to meet the nutritional requirements of rats were purchased from Suzhou Shuangshi Laboratory Animal Feed Science Co., Ltd., China. The high-fat diets contained 1% cholesterol, 10% lard, 0.2% methyl thiouracil, and 88.8% usual feed (moisture: ≤10%; protein: ≥20%; fat mix: ≥4%; calcium: 1.0–1.8%; phosphorus: 0.6–1.2; fiber: ≤5%; essential amino acids: ≥2%) and were prepared by the authors. The diet recipe was a classic formulae for the establishment of hyperlipidemia, non-alcoholic fatty liver disease (NAFLD), and related diseases recorded in Pharmacological Method (Third edition) [11].

One hundred and twenty SD rats of both sexes were randomly divided into 10 groups of twelve each (Table 1).

Table 1: Animal grouping and treatments in this research.

Groups	Diets	Treatment (from the 19th day of the experiment)	Dosage (g/kg body weight)
A	Normal diets	Physiological saline	1 mL per rat
B	High fat diets	Physiological saline	1 mL per rat
C	High fat diets	Water extraction of RPM	0.4050
D	High fat diets	Water extraction of RPM	0.8100
E	High fat diets	Water extraction of RPM	1.620
F	High fat diets	Water extraction of RPMP	0.8100
G	High fat diets	Water extraction of RPMP	1.620
H	High fat diets	Water extraction of RPMP	3.240
I	High fat diets	Simvastatin	0.001200
J	High fat diets	Fenofibrate	0.03300

The male and female rats were all six for each group. Group A received normal diets only and served as vehicle. Group B received high fat diets only and served as the model group. Other groups were fed high fat diets throughout the whole study periods. Eighteen days after the start of the study, the RPM group (Groups C, D, and E), RPMP group (Groups F, G, and H), and two positive control groups (simvastatin in Group I and fenofibrate in Group J) received corresponding treatments every day for another 24 days (Table 1). All rats were fasted for 2 h every day before administration of therapeutic agents.

2.4. Assessment of Total Cholesterol, Triglyceride, Lipoprotein, and Liver Marker Enzyme in Blood. Samples of blood 1.5–2 mL in volume were collected from the retro-orbital venous plexus once every 6 days throughout the study. Blood Samples were collected under ether anesthetic condition, two hours after administration of therapeutic agents in the morning. Serum was centrifuged at 16,000 rpm for 15 min and analyzed immediately. Levels of AST, ALT, TG, TC, LDL-C, and HDL-C in serum were determined by enzymatic colorimetric method using commercial standard enzymatic assay kits (Biosino Bio-technology & Sience Inc.) by AB-1020 automatic biochemical analyzer (Sunostik Medical Technology Co., Ltd.). TG and TC were tested on days 0, 6, 18, 24, 30, 36, and 42. LDL-C and HDL-C were tested on days 0, 6, 18, 24, 30, and 42. AST and ALT were tested only on days 0, 18, and 42 day. VLDL was analyzed using enzyme-linked immunosorbent assay kits from Rapidbio (U.S.). VLDL was tested only on days 0, 18, and 42. All bioassays were carried out in duplicate.

2.5. Assessment of Total Cholesterol, Triglyceride, Lipoprotein, and Liver Marker Enzyme in Liver Tissue. The rats were sacrificed by cervical dislocation two hours after the last administration of therapeutic agents in the morning. Tissue samples from their livers were immediately processed for biochemical analysis and morphologic observations. Single-gram tissue samples of liver were homogenized with 9 mL ice-cold 0.9% physiological saline. Homogenates of liver were centrifuged at 4000 rpm for 10 min at 4°C. Five milliliters of supernatant was maintained at −80°C until analysis. One hundred microliter samples of liver homogenate were diluted with 400 μL distilled water for determination [12]. AST, ALT, TG, TC, LDL-C, and HDL-C were tested in all liver tissue samples at the end of the study.

2.6. Morphologic Observations. For light microscopic observations, samples from liver were fixed in formalin fixative and processed routinely for embedding in paraffin. Tissue sections 5 μm in thickness were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

2.7. Statistical Analysis. All data in this study are expressed in the form of mean ± SD. The data were evaluated by one-way analysis of variance (ANOVA), and the differences between means assessed using Duncan's test with a significance level of $P < 0.05$, <0.01, and <0.001.

3. Results

3.1. General Condition of Rats. One hundred and twenty Sprague-Dawley rats of either sex were randomly divided into 10 groups of twelve in each (Table 1). The rats fed on normal diets showed about 43% weight gain in 42 days of experiment duration (Figure 2(a)). However, the body weights of rats fed high-fat diets were significantly lighter than those of rats fed normal diets groups beginning on day 18 day and persisted until the end of the experiment across all treatment groups (Figure 2(a)). We attributed the weight loss to the rats' displeasure with the taste of the high-fat diets.

Indexes of liver, kidney, and spleen were recorded after the rats were killed (Figures 2(b), 2(c), and 2(d)). Liver and spleen indexes showed no significant differences between the control group and the high-fat-diet group. However, kidney indexes in the high-fat diet group were slightly lower than those of the in control group. Slight decreases in the kidney and spleen indexes were observed in the middle-dose RPM (Group D). The liver and spleen indexes of rats in the simvastatin group also decreased. Indexes of liver lipid levels

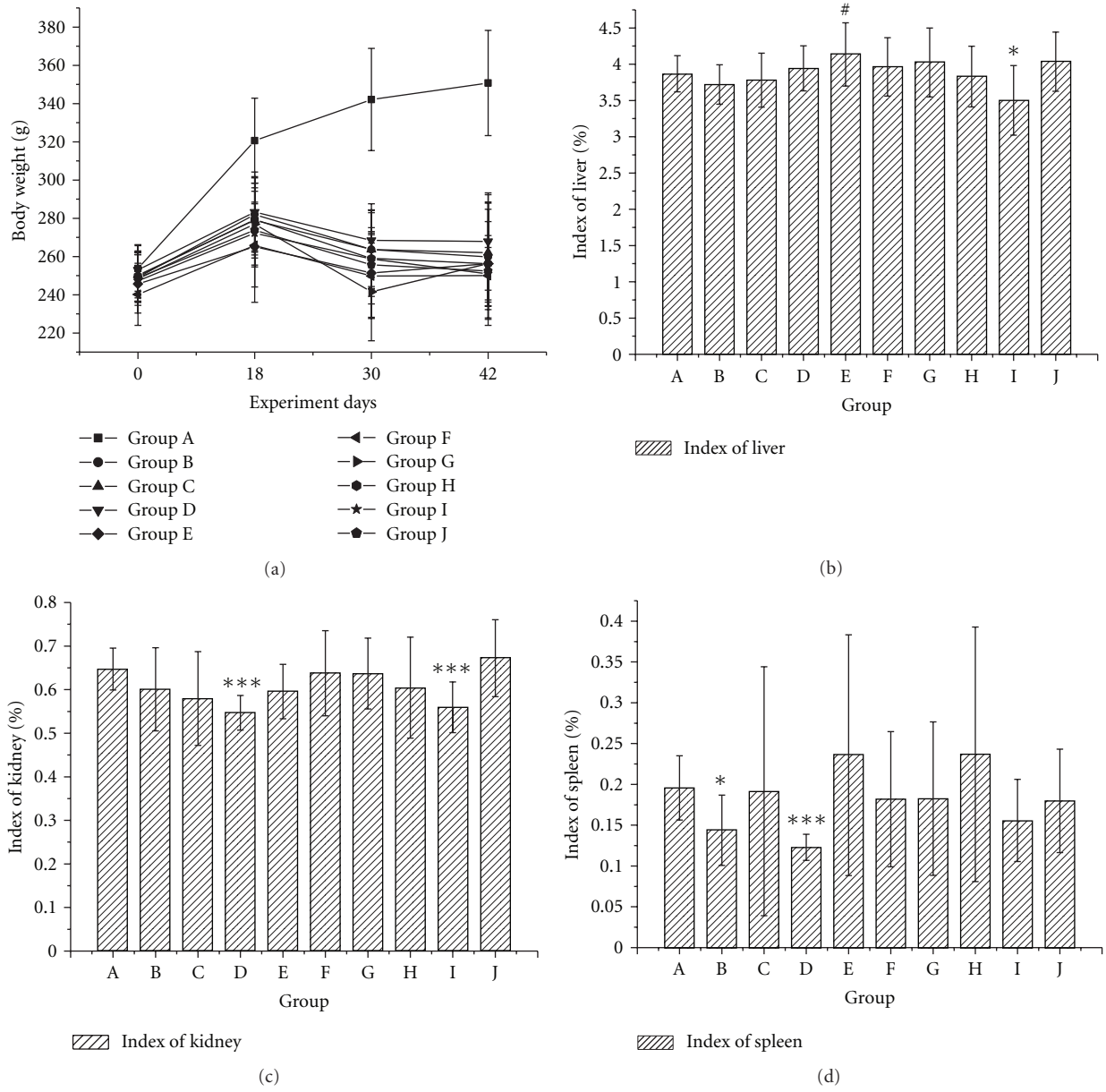


Figure 2: Body weight (a) and indexes of liver (b), kidney (c), and spleen (d) of rats in different groups. Note: The body weight was measured in every five days, however, only the data in 0th, 18th, 30th, and 42nd day were listed in this figure. The * indicates a significant difference compared with control group, $^{*}P < 0.05$ and $^{***}P < 0.001$. The # indicates a significant difference compared with model group, $^{\#}P < 0.05$.

were increased in the high-dosage RPM group (Group E). No serious pathological alterations were observed in this study, as determined by organ indexes.

3.2. Biochemical Indexes in Blood Sample of Model Group Rats. The TG contents in Group B were higher than those of Group A on day 6. After that, the TG contents dropped to a level lower than Group A (Table 2). The TC contents in Group B increased acutely from the beginning of the experiment (Table 3). The TC contents of Group B were increased to the highest level on day 24, 359.9 ± 61.90 mg/dL, almost 3 times than control group (Table 3). The amplitude of TC contents started to be decrease after day 24 but remained very high, at about 300 mg/dL.

LDL-C (Table 4) and HDL-C (Table 5) contents in Group B were higher than Group A from day 6 through the end of the study. Very low density lipoprotein (VLDL) content, as indicated by ELISA kits, showed no differences from the control group at the beginning of the study or on day 18 (Table 6). At the end of the experiment, the VLDL contents were even lower than those of control group.

Blood levels of aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) were evaluated on days 0, 18, and 42 (data not shown). No increases in AST or ALT

Table 2: Triglyceride (TG) content in blood samples.

Group	0D	6D	12D	18D	24D	30D	36D	42D
A	68.64 ± 17.17	122.1 ± 25.29	105.2 ± 37.06	97.09 ± 23.32	50.54 ± 13.77	60.36 ± 18.54	136.2 ± 44.40	94.54 ± 27.69
B	102.0 ± 25.19	196.8 ± 46.82***	28.80 ± 13.21***	51.08 ± 14.03***	35.40 ± 8.746**	34.89 ± 11.52**	80.00 ± 9.212**	66.00 ± 28.79*
C	76.63 ± 22.78#	157.6 ± 28.75**,#	23.09 ± 2.023***	44.27 ± 10.29***	37.27 ± 12.58*	50.82 ± 18.25#	101.4 ± 37.04	79.64 ± 20.25
D	76.45 ± 18.23#	175.4 ± 46.39**	23.00 ± 1.907***	36.91 ± 9.268***,#	34.67 ± 6.856**	47.88 ± 18.66	77.00 ± 14.70**	71.88 ± 19.09
E	94.33 ± 34.39*	148.4 ± 47.49#	22.42 ± 1.886***	31.60 ± 17.88***,##	37.20 ± 11.71*	41.00 ± 19.05*	84.22 ± 19.76**	72.56 ± 14.43*
F	57.36 ± 18.91###,†	104.1 ± 35.60###,††	55.00 ± 15.27***,###,†††	31.89 ± 8.978***,##,†	39.91 ± 14.88	41.78 ± 16.27*	72.44 ± 19.75***,†	78.11 ± 12.92
G	56.00 ± 15.42###,††	144.1 ± 70.24#	46.45 ± 9.903***,##,†††	28.00 ± 13.03***,###	22.45 ± 7.634***,##,††	47.10 ± 11.31#	78.00 ± 31.65**	71.14 ± 15.34
H	54.73 ± 15.94###,††	203.7 ± 63.82***,†	64.73 ± 15.97**,###,†††	29.45 ± 9.136***,###	26.18 ± 5.134***,##,†	52.09 ± 22.06#	101.1 ± 38.43	88.82 ± 20.89
I	47.08 ± 9.615**,###	139.4 ± 39.37##	44.00 ± 12.40***,#	21.91 ± 3.360***,###	27.09 ± 6.188***,#	34.18 ± 11.12***	93.91 ± 24.76*	86.18 ± 32.54
J	93.42 ± 37.48	119.3 ± 52.07###	34.08 ± 18.81***	31.64 ± 11.66***,###	41.45 ± 12.39	32.54 ± 4.034***	83.82 ± 26.78**	85.22 ± 22.16

Triglyceride (TG) contents were assayed by assay kits as described in the text.
Values are mean ± SD ($n = 12$) and expressed in mg/dL.
The * indicates a significant difference compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
The # indicates a significant difference compared with model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.
The † indicates a significant difference compared with PMR group (same dosage level, F compared with C, G compared to D, H compared to E), $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$.

Table 3: Total cholesterol (TC) contents in blood samples.

Group	0D	6D	12D	18D	24D	30D	36D	42D
A	79.06 ± 11.88	84.70 ± 20.02	66.45 ± 9.115	91.77 ± 11.27	122.9 ± 31.84	73.16 ± 12.32	61.99 ± 10.06	61.48 ± 10.08
B	84.12 ± 8.411	176.4 ± 36.61***	165.4 ± 27.44**	262.6 ± 45.02***	359.9 ± 61.90***	296.0 ± 80.12***	290.8 ± 66.87***	290.1 ± 46.93***
C	83.40 ± 18.30	159.2 ± 26.99***	184.6 ± 84.74***	261.7 ± 41.11***	360.2 ± 84.24***	294.5 ± 111.8***	328.8 ± 126.5***	239.7 ± 69.45***
D	85.43 ± 18.49	161.2 ± 56.09***	194.6 ± 26.80$^{***,\#}$	257.0 ± 32.58***	247.3 ± 54.32$^{***,\#\#\#}$	244.8 ± 41.50***	192.8 ± 33.40$^{***,\#\#}$	227.3 ± 39.93$^{***,\#}$
E	80.97 ± 8.531	178.7 ± 42.40***	187.5 ± 55.53***	255.1 ± 27.57***	247.8 ± 74.33$^{***,\#\#}$	221.8 ± 54.88$^{***,\#}$	198.2 ± 45.39$^{***,\#\#}$	227.6 ± 28.22$^{***,\#\#}$
F	89.7 ± 10.92*	143.4 ± 30.45$^{***,\#}$	168.0 ± 28.74***	291.8 ± 72.83***	232.1 ± 95.54$^{**,\#\#,\dagger\dagger}$	209.0 ± 57.89$^{***,\#}$	182.8 ± 47.29$^{***,\#\#,\dagger\dagger}$	212.3 ± 43.93$^{***,\#\#}$
G	88.61 ± 20.01	140.3 ± 26.14$^{***,\#}$	209.8 ± 54.84$^{***,\#}$	293.4 ± 47.51***,†	227.2 ± 57.10$^{***,\#\#\#}$	216.3 ± 47.35$^{***,\#}$	186.9 ± 104.9$^{***,\#}$	188.0 ± 50.93$^{***,\#\#\#}$
H	90.65 ± 26.83	244.4 ± 87.23$^{***,\#,\dagger}$	211.1 ± 33.37$^{***,\#\#}$	296.5 ± 73.34***	237.5 ± 44.76$^{***,\#\#\#}$	219.9 ± 58.06$^{***,\#}$	262.6 ± 60.32***,†	299.4 ± 70.86***,†
I	82.85 ± 7.949	183.6 ± 55.04***	227.7 ± 42.07$^{***,\#\#\#}$	274.7 ± 60.25***	235.5 ± 63.81$^{***,\#\#\#}$	238.4 ± 82.48***	239.5 ± 43.00***	263.3 ± 53.51***
J	78.25 ± 10.91	134.6 ± 17.74$^{***,\#\#}$	160.9 ± 22.66***	237.9 ± 32.93***	243.7 ± 40.02$^{***,\#\#\#}$	259.5 ± 61.93***	237.4 ± 91.13***	250.9 ± 34.42***

Total cholesterol (TC) contents were assayed by assay kits as described in the text.
Values were mean ± SD ($n = 12$) and expressed in mg/dL.
The * indicates a significant difference compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
The $^{\#}$ indicates a significant difference compared with model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.
The † indicates a significant difference compared with PMR group (same dosage level, F compared with C, G compared to D, H compared to E), $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$.

Table 4: Low density lipoprotein cholesterol (LDL-C) contents in blood samples.

LDL	0D	6D	18D	24D	30D	42D
A	22.33 ± 7.896	15.63 ± 4.228	28.45 ± 8.157	23.03 ± 3.350	16.94 ± 3.410	16.28 ± 3.567
B	26.23 ± 7.143	68.03 ± 24.60***	67.22 ± 13.45***	83.22 ± 21.49***	88.98 ± 32.26***	98.80 ± 35.21***
C	23.24 ± 6.831	51.57 ± 14.85***	70.48 ± 17.22***	89.36 ± 44.32***	114.1 ± 62.90***	92.35 ± 52.31***
D	18.56 ± 5.616	57.10 ± 17.34***	49.71 ± 14.07***,##	69.78 ± 20.54***	88.17 ± 19.97***	80.05 ± 29.15***
E	21.98 ± 3.974	47.85 ± 17.38***,#	51.16 ± 9.723***,##	60.05 ± 21.35***,#	98.18 ± 38.69***	74.29 ± 29.43***
F	19.76 ± 2.608	42.43 ± 16.61***,##	50.66 ± 12.67***,##,†	76.53 ± 43.37***	87.61 ± 48.40***	73.00 ± 32.86***
G	26.94 ± 6.340††	36.67 ± 13.89***,###,††	47.81 ± 14.72***,##	109.1 ± 44.91***,†	97.41 ± 40.59***	70.91 ± 63.17*
H	23.69 ± 3.758	57.83 ± 17.86***	42.15 ± 21.41##	125.0 ± 41.28***,##,†††	98.78 ± 49.10***	81.14 ± 37.11***
I	19.82 ± 8.215	41.70 ± 13.06***,##	58.39 ± 17.87***	125.3 ± 48.83***,#	83.46 ± 39.26***	61.13 ± 39.13**,#
J	18.98 ± 13.67	30.55 ± 11.18***,###	45.82 ± 8.996***,###	88.52 ± 19.35***	113.9 ± 53.11***	64.00 ± 35.28***

Low density lipoprotein cholesterol (LDL-C) contents were assayed by assay kits as described in the text.
Values were mean ± SD ($n = 12$) and expressed in mg/dL.
The * indicates a significant difference compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
The # indicates a significant difference compared with model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.
The † indicates a significant difference compared with PMR group (same dosage level, F compared with C, G compared to D, H compared to E), $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$.

Table 5: High density lipoprotein cholesterol (HDL-C) contents in blood samples.

HDL	0D	6D	18D	24D	30D	42D
A	59.94 ± 32.45	94.03 ± 29.81	64.19 ± 24.53	43.87 ± 8.363	56.49 ± 14.07	45.17 ± 10.13
B	87.52 ± 25.16*	231.0 ± 58.91***	82.43 ± 16.51*	103.2 ± 35.14***	120.6 ± 40.79***	159.5 ± 67.11***
C	70.63 ± 25.64	184.7 ± 53.03***	85.64 ± 21.35*	114.6 ± 65.55**	151.1 ± 43.02**	134.6 ± 51.15***
D	68.16 ± 18.51	203.2 ± 54.77***	146.9 ± 29.29***,###	91.30 ± 19.25***	151.5 ± 30.35***	154.2 ± 58.52***
E	84.37 ± 20.96*	175.0 ± 52.67***,#	159.2 ± 36.76***,###	104.7 ± 43.40***	164.3 ± 55.30***	162.7 ± 64.15***
F	49.46 ± 13.40†	142.6 ± 63.33*,##	143.8 ± 29.48***,###,†††	106.0 ± 47.44***	182.1 ± 79.76***	148.3 ± 50.91***
G	68.74 ± 13.56	157.5 ± 70.00**,#	124.7 ± 44.38***,##	101.6 ± 34.80***	193.7 ± 70.87***,#	120.1 ± 54.00***
H	65.84 ± 23.89	240.9 ± 59.83***,†	131.4 ± 68.25**,#	103.5 ± 34.80***	191.2 ± 74.25***,#	173.9 ± 63.37***
I	57.36 ± 10.28	167.1 ± 49.01***,##	65.42 ± 25.85	100.8 ± 41.64***	167.7 ± 68.80***	148.3 ± 86.91***
J	72.06 ± 29.60	148.3 ± 60.49*,##	113.4 ± 29.27***,##	126.1 ± 32.71***	189.2 ± 56.13***,##	147.5 ± 76.78***

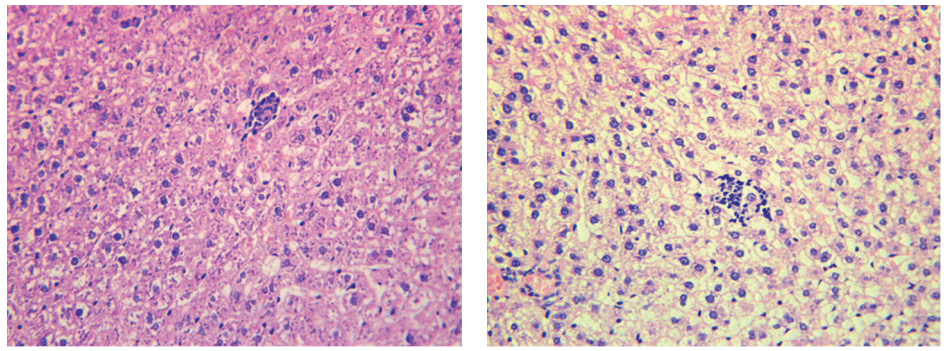
High density lipoprotein cholesterol (HDL-C) contents were assayed by assay kits as described in the text.
Values were mean ± SD ($n = 12$) and expressed in mg/dL.
The * indicates a significant difference compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
The # indicates a significant difference compared with model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.
The † indicates a significant difference compared with PMR group (same dosage level, F compared with C, G compared to D, H compared to E), $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$.

were observed, which indicated that liver damage did not occur during the research period.

3.3. Biochemical Indexes in Liver Tissues of Model-Group Rats. Levels of TG, TC, LDL-C, HDL-C, AST, and ALT contents in liver tissue were tested after execution at the end of the research (Table 7). TC, TG, and LDL-C levels in the high-fat diet group were significantly higher than in the control group, but other indexes showed no difference. The control and high-fat diet groups showed similar AST and ALT levels, indicating normal liver function. Morphologic observations were carried in every group. Livers in the high-fat diet groups were more paler than normal groups (figure not shown), probably due to the intracellular edema, However, no fatty deposits were observed in the liver biopsy slides of the model group (Figure 3). Actually, no fatty deposits were observed in normal diet group, high fat diet group, positive groups, RPM group, and RPMP groups (figure not shown). That is probably due to the detention of morphologic changes compared to the biochemistry changes. For these reasons, the model group rats were considered to have early-stage NAFLD.

According to the Fredrickson's classification of hyperlipidemia, type IIa hyperlipidemia is characterized by elevation of total cholesterol and LDL-C, while type IIb hyperlipidemia is characterized by elevation of total cholesterol, LDL-C, and VLDL-C. Judging from the lipid indexes listed above, the rats fed the high-fat diets were diagnosed with type IIa hyperlipidemia and early-stage non-alcoholic fatty liver disease.

3.4. Antihyperlipidemic Effects of RPM and RRPM. The TG levels in the blood samples were significantly higher in Group B after 6 days on the high-fat diet than those of control rats. TG did not continue to increase. TG levels were lower than those of control rats during the rest of the study. Considering the unremarkable increases in TG in the model group, we did



(a) (b)

Figure 3: Comparison of microscopic morphology in liver tissue between control group (a) and model group (b). Intracellular edema was widely distributed in liver tissue in model group; however, fatty drops were not observed in model group possibly due to the limited research time. Original magnification: 400x.

Table 6: Very low density lipoprotein (VLDL) contents in blood samples (μmol/L).

Group	0D	18D	42D
A	81.59 ± 12.18	106.9 ± 23.96	125.0 ± 13.12
B	95.15 ± 13.84	92.50 ± 11.50	89.00 ± 13.00**
C	97.48 ± 3.568*	81.80 ± 12.34	79.47 ± 23.60*
D	88.05 ± 13.22	74.28 ± 14.69	97.69 ± 13.09*
F	120.9 ± 4.996***,#,†††	98.86 ± 14.69	72.58 ± 5.376***
G	121.3 ± 13.33**,†	93.03 ± 12.43	96.84 ± 20.52
I	91.12 ± 30.99	76.08 ± 6.439*	104.9 ± 20.62

Very low density lipoprotein (VLDL) contents were assayed by assay kits as described in the text.
Values were mean ± SD ($n = 12$) and expressed in μmol/L.
The *indicates a significant difference compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
The #indicates a significant difference compared with model group, $^{\#}P < 0.05$.
The †indicates a significant difference compared with PMR group (same dosage level, F compared with C, G compared to D), $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$.

not conclude that there was a relationship between different treatments, dosages, and durations of high-fat diet.

The RPM, RPMP, and positive control treatments began on day 19 of the study. No TC melioration effects were observed in the low-dosage raw RPM group. Both the middle (Group D) and high dosage (Group E) RPM groups showed significant changes in TC regulation in blood samples. The most pronounced TC regulation effects of RPMP were observed in the middle-dosage RPMP group (Group G). Time-dependent TC regulation activities were observed in Group G. At the end of the experiment, the TC levels in Group G were reduced to 188.0 ± 50.93 mg/dL, significantly lower than that of model group ($P < 0.001$).

Similar TC-lowing effects were observed between Group I (simvastatin group), Group D, and Group G during the early stages of treatment (from day 19 through day 30). However, the rats given traditional Chinese medicines showed better results than those given simvastatin at the end of the study (Group D, E, F, and G).

Dosage relationships were observed between the LDL-C lowing activity in RPM groups, although no significant differences were observed across different groups (Table 4). Simvastatin (Group I) and fenofibrate (Group J) were found to remarkably downregulate the LDL-C content by the end of the study ($P < 0.05$).

HDL-C contents were generally higher in rats fed a high-fat diet than those fed a normal diet no matter what kinds of treatment were administered (Table 5).

3.5. Anti-Nonalcoholic Fatty Liver Disease Effects of RPM and PMPR. Liver tissue samples were homogenized and analyzed in order to evaluate the lipid regulation activities of raw and processed RPM in the liver. The RPM showed significant abilities to reduce levels of hepatic TG and TC. High doses of RPM were found to thoroughly control the progress of fatty accumulation in the liver. The TG and TC contents dropped to 153.6 ± 27.34 mg/dL and 57.18 ± 6.754 mg/dL, respectively, which were similar to the levels observed in the control group. Dose activity relationships were observed in the RPM groups. However, PRMP dosage was not found to be directly related to lipid regulation effects. Low and high doses of RPMP showed better effects than middle doses.

No remarkable increases in AST or ALT were observed in any of the treatment groups, indicating that liver function remained normal.

Simvastatin (Group I) and fenofibrate (Group J) showed excellent abilities to reduce TC levels, but simvastatin even showed more pronounced abilities to reduce TG than fenofibrate.

LDL-C contents were increased in RPM and RPMP treatment groups, especially the RPMP treated group. Simvastatin (Group I) and fenofibrate (Group J) were both found to downregulate LDL-C content.

4. Discussion

Hyperlipidemia and NAFLD are major risk factors for cardiovascular disease. Certain frequently used lipid-lowing drugs (fibrates, stains, and bile acid sequestraints) used for the treatment of hyperlipidemia and NAFLD have numerous

Table 7: Lipid indexes in the liver samples.

Group	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	AST (U/L)	ALT (U/L)
A	66.63 ± 4.093	147.22 ± 6.180	57.79 ± 10.96	10.74 ± 2.186	3286 ± 817.8	1048 ± 344.9
B	100.2 ± 19.22***	200.0 ± 32.56***	76.05 ± 25.82	28.36 ± 12.57**	2539 ± 308.4	1302 ± 519.2
C	105.8 ± 15.01***	197.8 ± 18.56***	79.92 ± 17.93**	28.79 ± 7.821***	2673 ± 428.4	1470 ± 424.5*
D	87.71 ± 17.19**	180.8 ± 15.94***	66.38 ± 17.49	24.49 ± 6.547***	2900 ± 541.4	1394 ± 309.6
E	57.18 ± 6.754**,###	153.6 ± 27.34#	84.69 ± 19.50**	24.86 ± 4.385***	1830 ± 288.3***,##	1040 ± 490.1
F	66.29 ± 28.08#,††	162.1 ± 39.88†	88.55 ± 35.98*	38.39 ± 18.53***	1907 ± 582.8**,#,††	1268 ± 737.5
G	89.48 ± 18.75**	205.8 ± 29.90***	139.6 ± 24.43***,##,†††	48.98 ± 12.02***,#,††	3661 ± 700.6##	1380 ± 693.9
H	69.82 ± 24.30#	165.0 ± 32.11	128.8 ± 49.55**,#	41.89 ± 16.49***,†	3579 ± 705.4##,†††	1607 ± 592.9*
I	43.67 ± 2.936***,###	150.8 ± 18.82##	83.79 ± 19.69**	9.990 ± 3.548##	3538 ± 304.4###	1971 ± 622.0**,#
J	44.66 ± 5.379***,###	195.8 ± 25.96***	73.20 ± 46.50	15.79 ± 9.505	4244 ± 545.0*,###	953.5 ± 167.7

All these biochemical indexes were assayed by assay kits as described in the text.
The * indicates a significant difference compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
The # indicates a significant difference compared with model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.
The † indicates a significant difference compared with PMR group (same dosage level, F compared with C, G compared to D, H compared to E), $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$.

side effects. For this, traditional herb drugs with remarkable effect and lower price seem to be the best option. The prevention and treatment of hyperlipidemia and NAFLD through traditional Chinese medicine have attracted more and more attention worldwide.

In our previous studies, RPM showed more pronounced effects on TG and TC regulation in *in vitro* assays. This contradicted the widespread use of RPMP in treatment of hyperlipidemia and NAFLD. In this study, lipid regulation activities of RPM and RPMP were compared in both blood and liver tissue samples from rats fed a high-fat diet. *In vivo* hyperlipidemia and early-stage non-alcoholic fatty liver disease were induced in rats by feeding them high-fat diets for 18 days. These high-fat diets were continued through the 24 days of the experiments. Increases in the TC, LDL-C, and HDL-C levels of the blood samples and the TC, TG, and LDL-C levels of the liver tissue indicated the formation of hyperlipidemia and early-stage non-alcoholic fatty liver disease.

The low, middle, and high doses of RPM and RPMP administered to test rats in this study were calculated using the corresponding dosages recommended for human clinical use in the Chinese Pharmacopeia, 2010 edition [6]. The recommended dosage of RPMP is two times higher than that of RPM in the *Pharmacopoeia of People's Republic of China* (2010 edition) [6]. Thus, the dosage of PRMR was two times high than that of RPM in the same dosage group in this research.

TC levels in the circulatory system increased after the rats were fed high-fat diets, and these increases could be inhibited by both RPM and RPMP treatments. Both low (0.8100 g/kg body weight) and middle (1.620 g/kg body weight) dosages of RPMP revealed better TC lowing activity than middle dosages of RPM (0.8100 g/kg body weight). Judging from these results, we recommend RPMP in the treatment of hyperlipidemia characterized by increased levels of TC.

Because TG levels did not show any regular pattern of increase or decrease in rats fed high-fat diets, the RPM and RPMP did not appear to have any TG-reducing effects. The increases in LDL-C levels in the blood of rats fed high-fat diets were not reversed by RPM or RPMP. This was probably because of the limited duration of the treatment. However, simvastatin and fenofibrate were both found to reduce LDL-C levels during the study.

Lipid accumulation in the liver is the major hallmark of NAFLD. The increasing trends of TC and TG in the liver tissue of the model group were reversed after administration of RPM and RPMP. Obviously, dose-dependent TC- and TG-reducing effects were observed in PMP groups. High doses of RPM were found to restore liver TC and TG levels to the normal values. However, no similar dose-dependent relationship was observed in RPMP-treated groups. Increases in LDL-C content were controlled by both simvastatin and fenofibrate. Although simvastatin showed significant TC- and TG-reducing effects, significant elevation of AST and ALT were observed.

5. Conclusion

The results of the present study demonstrated the lipid regulation properties of RPM and RPMP. Each of these compounds showed the most pronounced lipid regulation effects in different organs. RPM showed better effects in liver cells in the treatment of NAFLD. This is consistent with the results of our previous *in vitro* study, which was carried on steatotic human liver L-02 cells and showed that RPM extracts could regulate the lipid content within liver cell better than RPMP [9]. RPMP displayed better effects than RPM in lipid regulation in the circulatory system, indicating that RPMP would have more pronounced curative effects in the treatment of hyperlipidemia.

Abbreviations

AST: Aspartate aminotransaminase
ALT: Alanine aminotransaminase
HDL-C: High density lipoprotein cholesterol

HPLC-DAD: High-performance liquid chromatography coupled with diode array detector
LDL-C: Low density lipoprotein cholesterol
NAFLD: Non-alcoholic fatty liver disease
RPM: Radix Polygoni Multiflori
RPMP: Radix Polygoni Multiflori Praeparata
TC: Total cholesterol
TG: Triglyceride
TSG: 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside
VLDL: Very low density lipoprotein
VLDL-C: Very low density lipoprotein cholesterol.

Authors' Contribution

J. Yu and R. Zhao contributed equally to this work.

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Research Article

Hepatoprotective Activity of the Total Saponins from *Actinidia valvata* Dunn Root against Carbon Tetrachloride-Induced Liver Damage in Mice

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The protective activity of the total saponins from *Actinidia valvata* Dunn root (TSAV) was studied against carbon-tetrachloride-(CCl_4-) induced acute liver injury in mice. Mice were orally administered TSAV (50, 100, and 200 mg/kg) for five days and then given CCl_4. TSAV pretreatment significantly prevented the CCl_4-induced hepatic damage as indicated by the serum marker enzymes (AST, ALT, and ALP). Parallel to these changes, TSAV also prevented CCl_4-induced oxidative stress by inhibiting lipid peroxidation (MDA) and restoring the levels of antioxidant enzymes (SOD, CAT, GR, and GPX), GSH and GSSG. In addition, TSAV attenuated the serum TNF-α and IL-6 levels and inhibited the serum iNOS and NO levels. Liver histopathology indicated that TSAV alleviated CCl_4-induced inflammatory infiltration and focal necrosis. TSAV (200 mg/kg) also significantly decreased Bak, Bax mRNA and Fas, FasL, p53, and NF-κB p65 protein expressions and increased Bcl-2 mRNA and protein expressions. Meanwhile, TSAV significantly downregulated caspase-3 and caspase-8 activities and prevented CCl_4-induced hepatic cell apoptosis. In addition, TSAV exhibited antioxidant activity through scavenging hydroxyl and DPPH free radicals *in vitro*. These results indicated that TSAV could protect mice against CCl_4-induced acute liver damage possibly through antioxidant, anti-inflammatory activities and regulating apoptotic-related genes.

1. Introduction

The liver plays a critical role in regulating several important functions including synthesis, secretion, and xenobiotic metabolism [1]. Liver damage is a widespread pathology which can influence these physiochemical functions and be caused by viral hepatitis, alcoholism, or liver-toxic chemicals, one among them is carbon tetrachloride (CCl_4). It is metabolized by the P450 enzyme system to yield reactive metabolic products trichloromethyl free radicals, which can initiate the process of lipid peroxidation and contribute to the liver toxicity [2]. Herbal products and traditional Chinese medicine have been widely used for protection against chemical-induced toxicities because of their safety and efficacy. A number of studies have shown that herbal extracts possess antioxidant activity against CCl_4 hepatotoxicity through reducing oxidative stress and inhibiting lipid peroxidation [2, 3].

Actinidia valvata Dunn, affiliated to the genus *Actinidia*, is a shrub mainly growing in eastern China [4] and has a series of applications in traditional Chinese medicine and folk herb. The root of this plant, commonly known as Mao renshen in China, exhibited notable antiinflammatory and anti-tumoral activities and has been used for the treatment of hepatoma, lung carcinoma, and myeloma for a long time [5, 6]. Total saponins of *A. valvata* Dunn root (TSAV) are considered to be one important group of the pharmacologically active ingredients against inflammation and tumor [7, 8]. Recent studies have indicated that some triterpenoids isolated from TSAV showed anticancer activities *in vitro* [9, 10]. However, up to now, the report about hepatoprotective activity of TSAV against liver damage induced by CCl_4 was not found as far as we know.

The present study aimed to evaluate the hepatoprotective effects of TSAV against CCl_4-induced liver damage. The

activities of hepatic enzymes in mice were measured and the possible mechanisms of hepatoprotection were also investigated.

2. Materials and Methods

2.1. Plant Material. *Actinidia valvata* Dunn roots were collected in Changshan County, Zhejiang Province, China, in September 2006, and authenticated by Professor Zheng Hanchen (School of Pharmacy, Second Military Medical University, Shanghai, China). Voucher specimen (no. 20060929) was deposited at Department of TCM, Second Military Medical University.

2.2. Chemicals and Reagents. D101 macroporous resin was purchased from Haiguang Chemical Factory (Tianjin, China). CCl_4 and silymarin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diagnostic kits to measure ALT, AST, ALP, TNF-α, IL-6, iNOS, NO, MDA, SOD, CAT, GR, GPX, GSH, and GSSG were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China). DNA ladder extraction kit, caspase-3 and caspaes-8 activity assay kit, Trizol reagent, cell lysis buffer for western blot and IP were purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu Province, China). Two-Step IHC Detection Reagent was purchased from Zhongshan Goldenbridge Biotechnology Co. Ltd. (Beijing, China). Antibodies against NF-κB p65, Fas, FasL, and GAPDH were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and solvents were of the high commercially available grades.

2.3. Preparation of TSAV from the Medicinal Plant. TSAV was prepared as described by Zheng et al. [11], with minor modifications. The medicinal plant was pulverized into powders (20–40 mesh), and then 600 g of powders were refluxed with 3500 mL of 80% aqueous ethanol for 2 h. The extraction procedure was then repeated twice again. The solvents were evaporated to dryness under vacuum, and the residue was diluted in distilled water. After separating the precipitate by filtration, the remaining solutions were further separated on D101 macroporous resins and eluted with water, 40% aqueous ethanol and 90% aqueous ethanol to produce three fractions. The solutions eluted by water and 40% aqueous ethanol were discarded, and 90% aqueous ethanol solutions were collected and evaporated to dryness under reduced pressure at 50°C. Brown powders (24.34 g) were obtained containing total saponins.

The content of total saponins was determined with Vanillin-$HClO_4$ colorimetric method [12]. Quantification was done using external standard calibration, and 2α, 3α, 24-trihydroxyurs-12-en-28-oic acid was used as the reference standard to calculate the total saponins content. A series of standard of 2α, 3α, 24-trihydroxyurs-12-en-28-oic acid (ranged from 5 μg/mL–30 μg/mL) was prepared in 80% aqueous ethanol solution to obtain a liner curve with correlation coefficient of 0.9997 ($n = 6$). Absorbance was measured at 550 nm by UV-Vis spectrophotometer (TU-1901, Persee, Beijing, China). The total saponins content was 62.4% (w/w).

2.4. Free Radical Scavenging Activities of TSAV

2.4.1. DPPH Radical Scavenging Assay. The free radical scavenging activity was measured in terms of hydrogen donating or radical-scavenging ability using the stable DPPH radical. Different concentrations of test sample and ascorbic acid were prepared in 80% aqueous ethanol and 2 mL of the sample solution was mixed with 2 mL of 0.1 mg/mL ethanolic DPPH solution. The reaction mixture was shaken vigorously and incubated at 37°C for 30 min. Absorbance was measured at 517 nm using UV-Vis spectrophotometer. The percentage inhibition of the DPPH radical by the samples was calculated using the following equation: inhibition rate (%) = $[A_0 - (A_1 - A_2)]/A_0 \times 100\%$, where A_0 is the absorbance of the control, A_1 is the absorbance of the sample and A_2 is the absorbance of the sample, under identical conditions as A_1 with ethanol instead of DPPH solution. Ascorbic acid was used as a reference compound. IC_{50} value (the concentration required to scavenge 50% DPPH free radicals) was calculated. All determinations were performed in triplicate.

2.4.2. Hydroxyl Radical Scavenging Assay. Hydroxyl radical scavenging test was carried out by the described method [13]. The reaction solution was incubated at 37°C for 30 min. Absorbance was measured at 520 nm using UV-Vis spectrophotometer. The inhibition rate was calculated as follows: $[(A_1 - A_2)/(A_0 - A_2)] \times 100\%$, where A_0 is the absorbance of the control, A_1 is the absorbance of the sample, and A_2 is the absorbance of the blank sample.

2.5. Animals and Experiment Design. Kunming male mice (18–22 g) were obtained from the Sippr-Bk Lab. Animal Ltd. Co. (Shanghai, China) and fed with certified standard diet and tap water *ad libitum*. Temperature and humidity were regulated at 21–23°C and 50–60%, respectively. The study protocol was in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the United States National Institute of Health and was approved by the Animal Ethic Review Committees of Second Military Medical University.

After one week of acclimatization, the mice were randomly divided into six groups of ten each: (I) control group; (II) CCl_4 group; (III) low dose group of TASV; (IV) middle dose group of TSAV; (V) high dose group of TSAV; (VI) silymarin group. Groups I and II received 0.5% CMC-Na (200 mg/kg), Groups III, IV, and V received TSAV (50, 100, and 200 mg/kg, resp.) and Group VI received silymarin (200 mg/kg). Drugs and vehicle were administrated (i.g.) once daily for 5 consecutive days. Two hours after the final treatment, the mice in Groups II–VI were treated with 0.3% CCl_4 (10 mL/kg, i.p., dilution with olive oil), as described previously [14]. Twenty-four hours after administrating CCl_4, the blood was collected and mice were sacrificed. The collected blood was placed 45 min for clot formation and then centrifuged 4000 ×g for 10 min for separation of serum, which was used for the biochemical analyses. The livers were immediately rinsed with saline, blotted on filter paper, weighed and finally stored at −80°C pending biochemical analyses. One part of liver was cut and put into a flasket

containing 10% buffered formalin solution for the following histopathology analyses.

2.6. Serum Biochemical Parameters. The activities of ALT, AST, ALP, TNF-α, IL-6, iNOS, and NO in serum were measured by spectrophotometer using enzymatic kits according to the manufacturer's instructions.

2.7. Lipid Peroxidation and Antioxidant Enzyme Activities. Hepatic tissues were homogenized in frozen normal saline and centrifuged at 10000 ×g for 10 min. The supernatant was used for the measurement of SOD, CAT, GR, GPX, GSH, and GSSG, which were determined by following the instructions on the commercial kits. Lipid peroxidation was estimated according to MDA diagnostic kits.

2.8. Caspase-3 and Caspase-8 Activities. The activities of caspase-3 and caspase-8 were measured strictly according to the instructions of caspase-3 and caspase-8 activity assay kit. Briefly, the mixture of 80 μL detection buffer, 10 μL samples and 10 μL Ac-IETD-*p*NA was incubated at 37°C for 60 min, and OD_{405} was measured. The activities of them were calculated based on the standard curve.

2.9. Histology and Immunohistochemistry. Formalin-fixed tissue samples were embedded in paraffin and 5 μm sections were cut. Replicate sections were stained with haemotoxylin and eosin (HE) for observing the liver damage. Additional sections were used for the following immunohistochemistry test through two-step IHC detection. 3% H_2O_2 was used to block endogenous peroxidase activity for 10 min and normal goat serum to block nonspecific protein binding for 30 min. After microwave antigen repair, the sections were incubated at 4°C overnight with rabbit anti-Bcl-2 and anti-p53 antibody (1 : 100, dilution), followed by incubation at 37°C in PV6001 for 30 min, and were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate and counterstained by haemotoxylin. Image was taken by inverted digital image light microscopy (Nikon Eclipse TE2000-U, Nikon Instruments Co. Ltd., Tokyo, Japan). The tissues stained brown were considered as positive, and the IOD value was analyzed by Image-Pro Plus software (Media Cybernetics, MD, USA) to evaluate the protein expressions [15, 16].

2.10. Protein Extraction and Western Blot Analysis. The liver were washed twice with cold PBS and then lysed in appropriate volume of cold lysis buffer containing 1 mM PMSF lysates which were centrifuged at 12000 ×g for 15 min at 4°C. The total protein was obtained and protein content was determined by coomassie brilliant blue G. Western blot assays were performed as follows: protein (5 mg/mL) was denatured by mixing with an equal volume of 2 × sample loading buffer and then boiling at 100°C for 5 min [17]. An equal protein amount was loaded onto an SDS gel, separated electrophoresis, and transferred to a PVDF membrane. 10% polyacrylamide gel was used for all the electrophoresis. The transmembrane times for NF-κB p65, Fas, FasL, and GAPDH were 50, 45, 35, and 30 min, respectively. After the PVDF membrane was incubated with 10 mM TBS with 1.0% Tween 20 and 5% dehydrated skim milk to block nonspecific protein binding, the membrane was incubated with primary antibodies overnight at 4°C and incubated either rabbit anti-NF-κB p65 (1 : 600 dilution), rabbit anti-Fas (1 : 200 dilution), rabbit anti-FasL (1 : 500 dilution) or mouse anti-GAPDH (1 : 2000 dilution). Blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated goat anti-mouse IgG for 2 h at room temperature at a 1 : 2000 dilution. Detection was performed by an enhanced chemiluminescence method and photographed by BioSpectrum Gel Imaging System (UVP, Upland, CA, USA). The data were normalized by GAPDH (IOD of objective protein versus IOD of GAPDH protein).

Table 1: Primer sequences of three genes selected for RT-PCR with GAPDH as an internal control.

Gene name	Primers (5′–3′)
Bcl-2	F: ATGTGTGTGGAGAGCGTCAACC
	R: TGAGCAGAGTCTTCAGAGACAGCC
Bax	F: GGCAGCCCCGGCGCGGACCCCTCGGTCA
	R: TAACATCAGTTCCTCTCAGAGGAACCT
Bak	F: ACAAGGACCAGGTCCTCCTAGGC
	R: GCGATGCAATGGTGCAGTATGAT
GAPDH	F: ATGGTGAAGGTCGGTGTGAAC
	R: GTCTTCTGGGTGGCAGTGATG

F: forward primer; R: reverse primer.

2.11. Reverse Transcriptional Polymerase Chain Reaction (RT-PCR). Trizol reagent was used to prepare RNA of cells. 500 ng of total RNA was required in the following RT-PCR each time. Specific designed primers were shown in Table 1. RT-PCR was performed according to the protocol of Takara RNA PCR Kit and amplified in a TC-512 PCR system (Techne, Barloworld Scientific Ltd., UK). RNA samples were first reverse transcribed and immediately amplified by PCR. The amplification profile was performed by denaturation at 94°C for 1 min, annealing at 60°C, and extension at 72°C for 1 min, and 50 additional cycles were used for amplification. IOD values of the electrophoresis bands were analyzed by BioSpectrum Gel Imaging System.

2.12. TUNEL Assay. Apoptosis was detected by TUNEL staining using *in situ* apoptosis detection kit (Roche, Shanghai, China). Paraffin-embedded liver tissues were processed for TUNEL labeling. The images were obtained using fluorescence microscopy (Olympus, Tokyo, Japan).

2.13. DNA Ladder. DNA samples were extracted using DNA ladder extraction kit with spin column then were electrophoretically separated in 1% agarose gel and stained with ethidium bromide. The agarose gel was visualized and photographed under ultraviolet light by BioSpectrum Gel Imaging System.

2.14. Statistical Analysis. All data were expressed as mean ± standard deviation (SD) and significant difference between

Table 2: Effects of TSAV on serum enzyme activities with CCl_4-induced hepatic damage in mice.

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
I	$58.71^{a} \pm 17.02$	$24.10^{a} \pm 3.72$	$139.58^{a} \pm 15.53$
II	$1724.82^{d} \pm 478.62$	$4112.02^{d} \pm 1062.96$	$250.12^{c} \pm 14.99$
III	$1250.11^{c} \pm 334.93$	$2583.45^{c} \pm 426.52$	$217.23^{b} \pm 23.71$
IV	$1164.23^{c} \pm 259.20$	$2563.52^{c} \pm 401.25$	$194.98^{b} \pm 18.39$
V	$901.60^{b} \pm 231.51$	$2231.91^{b} \pm 407.22$	$151.08^{a} \pm 07.41$
VI	$1190.54^{c} \pm 262.20$	$2533.93^{c} \pm 446.91$	$166.78^{a} \pm 13.84$

Group I: control; Group II: CCl_4; Group III: TSAV 50 mg/kg + CCl_4; Group IV: TSAV 100 mg/kg + CCl_4; Group V: TSAV 200 mg/kg + CCl_4; Group VI: silymarin 200 mg/kg + CCl_4.
Values are given as mean ± SD (n = 10 mice/group); means with different alphabets differ significantly at $P < 0.05$ level.

Table 3: Effects of TSAV on liver lipid peroxidation and antioxidant enzymes with CCl_4-induced hepatic damage in mice.

Group	MDA[A]	SOD[B]	CAT[C]	GPX[D]	GR[E]	GSH[F]	GSSG[G]	GSH/GSSG
I	$0.82^{a} \pm 0.04$	$35.71^{b} \pm 3.60$	$382.72^{e} \pm 22.79$	$247.35^{d} \pm 42.21$	$7.56^{c} \pm 1.25$	$6.02^{d} \pm 0.55$	$0.39^{a} \pm 0.05$	$15.75^{f} \pm 3.12$
II	$1.81^{c} \pm 0.15$	$26.12^{a} \pm 0.82$	$218.86^{a} \pm 44.95$	$153.31^{a} \pm 26.34$	$5.12^{a} \pm 0.66$	$4.12^{a} \pm 0.79$	$0.78^{d} \pm 0.11$	$5.40^{a} \pm 1.28$
III	$1.69^{c} \pm 0.22$	$29.16^{a} \pm 2.50$	$241.06^{b} \pm 36.12$	$180.66^{b} \pm 24.72$	$5.87^{b} \pm 0.93$	$4.62^{b} \pm 0.87$	$0.68^{c} \pm 0.14$	$6.84^{b} \pm 0.98$
IV	$1.13^{b} \pm 0.11$	$35.32^{b} \pm 3.13$	$252.74^{b} \pm 32.43$	$207.92^{c} \pm 18.37$	$6.24^{b} \pm 1.02$	$4.88^{b} \pm 0.76$	$0.64^{c} \pm 0.05$	$7.65^{c} \pm 0.81$
V	$0.93^{a} \pm 0.08$	$42.87^{c} \pm 3.61$	$324.78^{d} \pm 25.19$	$236.84^{d} \pm 29.52$	$7.31^{c} \pm 1.21$	$5.64^{c} \pm 0.47$	$0.53^{b} \pm 0.09$	$10.96^{e} \pm 2.08$
VI	$1.25^{b} \pm 0.18$	$32.04^{b} \pm 2.05$	$289.45^{c} \pm 33.94$	$230.11^{d} \pm 21.26$	$7.24^{c} \pm 0.82$	$5.34^{c} \pm 0.38$	$0.58^{b} \pm 0.06$	$9.25^{d} \pm 1.10$

Group I: control; Group II: CCl_4; Group III: TSAV 50 mg/kg + CCl_4; Group IV: TSAV 100 mg/kg + CCl_4; Group V: TSAV 200 mg/kg + CCl_4; Group VI: silymarin 200 mg/kg + CCl_4.
[A]nmol/mg protein, [B]U/mg protein, [C]U/g protein, [D]U/mg protein, [E]U/g protein, [F,G]μmol/g protein. Values are given as mean ± SD (n = 10 mice/group); means with different alphabets differ significantly at $P < 0.05$ level.

the groups was statistically analyzed by one-way ANOVA. A difference was considered significant at $P < 0.05$.

3. Results

3.1. Antioxidant Activity of TSAV In Vitro. Free radical scavenging effect of TSAV was tested and the results are presented in Figure 1. TSAV in the range 15–250 μg/mL exhibited concentration-dependent DPPH scavenging activity (18.23–88.06% inhibition, IC_{50} 87.78 μg/mL), being less active than ascorbic acid (IC_{50} 12.08 μg/mL). Hydroxyl radical scavenging effect was 36.75%, 46.29%, and 56.87%, respectively, for 50, 75, and 100 μg/mL of TSAV. The IC_{50} value was 74.21 μg/mL and it was compared with ascorbic acid (IC_{50} 20.86 μg/mL) under same conditions. The present study showed that TSAV could effectively scavenge DPPH and hydroxyl radicals.

3.2. Histopathology. Histopathological profile of the control mice showed normal hepatocytes with well cytoplasm, prominent nucleus, nucleolus and central vein. There was no sign of inflammation or necrosis in these mice (Figure 2(a)). In mice treated with CCl_4 only, liver sections showed hepatocyte nuclear pyknosis, hepatic cord degeneration, inflammatory infiltration, and marked necrosis (Figure 2(b)). Pretreatment with TSAV at 50 and 100 mg/kg dose showed reduction of necrosed area and inflammatory infiltrates (Figures 2(c) and 2(d)). TSAV and silymarin at 200 mg/kg dose showed sparse inflammatory cell infiltration and greater reduction of nuclear pyknosis of hepatic cells (Figures 2(e) and 2(f)) as compared with 50 and 100 mg/kg dose. These results indicated that TSAV could ameliorate the severity of liver damage and protect liver from CCl_4-induced injury effectively.

3.3. Serum Enzymes. The effects of TSAV at three dose levels on serum marker enzymes in CCl_4-induced hepatic injury are shown in Table 2. Serum activities of AST, ALT, and ALP enzymes in CCl_4 group were significantly increased compared with the control group, which reflected the severity of liver injury. TSAV with different doses and silymarin pretreatments significantly reduced the levels of serum AST, ALT, and ALP as compared with the CCl_4 group. The effects of TSAV at high dose were found to more markedly reduce their activities than those of silymarin.

3.4. Lipid Peroxidation. The effects of different groups on the activities of MDA in the liver are shown in Table 3. CCl_4 increased the hepatic MDA concentration significantly which was inhibited by TSAV (middle and high dose) and silymarin pretreatment. TSAV at high dose was most effective in inhibiting the hepatic lipid peroxidation than that of silymarin.

3.5. Antioxidant Enzymes. Activities of the antioxidant enzymes of hepatic damage: SOD, CAT, GPX, and GR decreased significantly in CCl_4 group compared with the control group (Table 3). There were dramatic increases in their activities in TSAV (middle and high dose) and silymarin groups compared with the CCl_4 group, and antioxidant effects of TSAV pretreatments at high dose were better than those of silymarin.

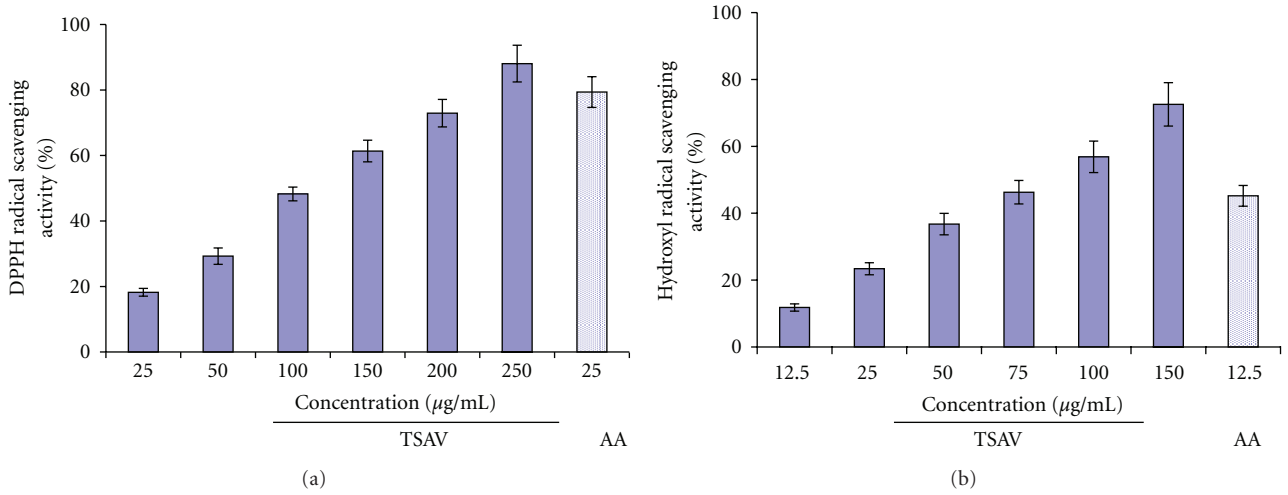

Figure 1: DPPH and hydroxyl radical scavenging activities of different concentrations of TSAV. Ascorbic acid (AA) was used as reference compound. The data are presented as means ± SD, $n = 3$.

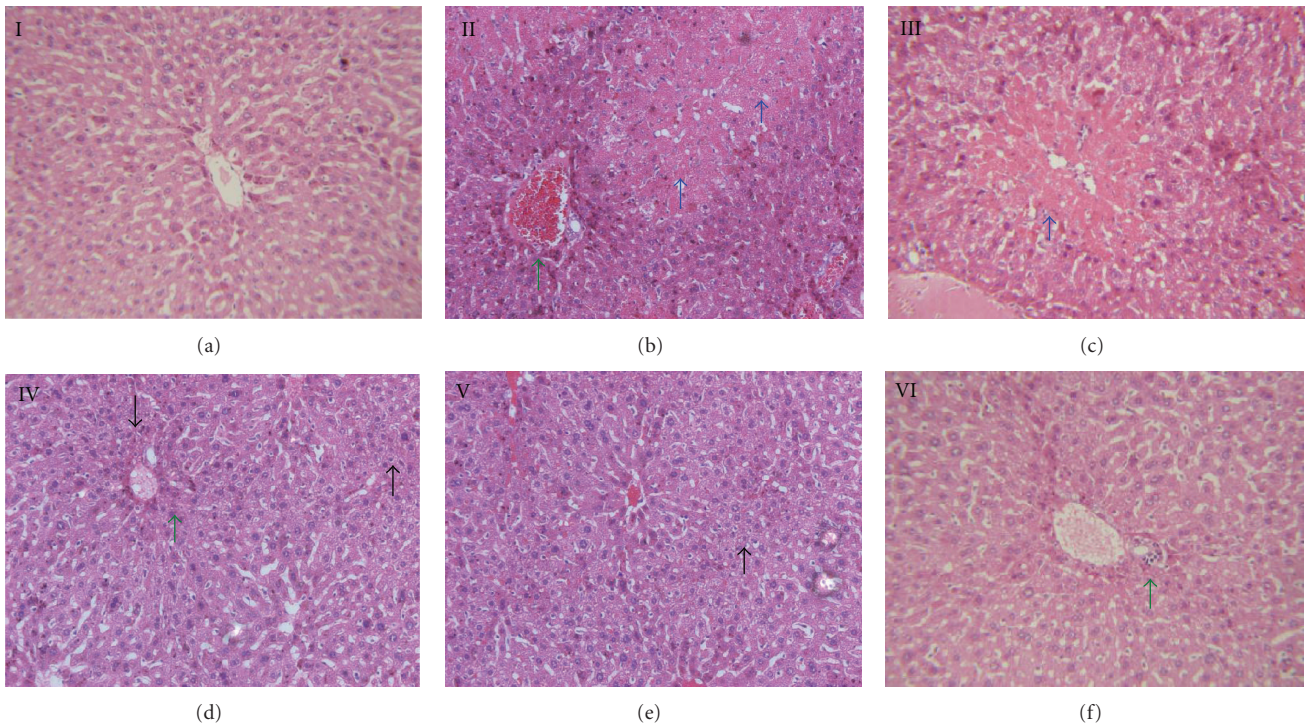

Figure 2: Protective effects of the TSAV pretreatment on CCl_4-induced liver damage. Histological examination was performed under a light microscope (original magnification: ×100) with HE staining on liver tissues. Group I: control; Group II: CCl_4; Group III: TSAV 50 mg/kg + CCl_4; Group IV: TSAV 100 mg/kg + CCl_4; Group V: TSAV 200 mg/kg + CCl_4; Group VI: silymarin 200 mg/kg + CCl_4. Blue, green, and black arrows indicate cell necrosis, inflammatory infiltration, and nuclear pyknosis, respectively.

3.6. Hepatic GSH and GSSG Levels. CCl_4 treatment caused a significant decrease of GSH and increase of GSSG compared with the control group. TSAV pretreatment prevented these changes more effectively than that of silymarin. GSH/GSSG ratio was significantly reduced in the CCl_4 group compared with the control group, while TSAV prevented this effect (Table 3).

3.7. Serum TNF-α, IL-6, iNOS, and NO Levels. CCl_4 treatment significantly increased the serum TNF-α, IL-6, iNOS, and NO levels in the mice liver. There were marked decreases of TNF-α level in high dose TSAV group and of NO level in the middle and high dose TSAV group, and there were dramatic decreases of iNOS level and IL-6 level in the three TSAV groups compared with the CCl_4 group (shown in

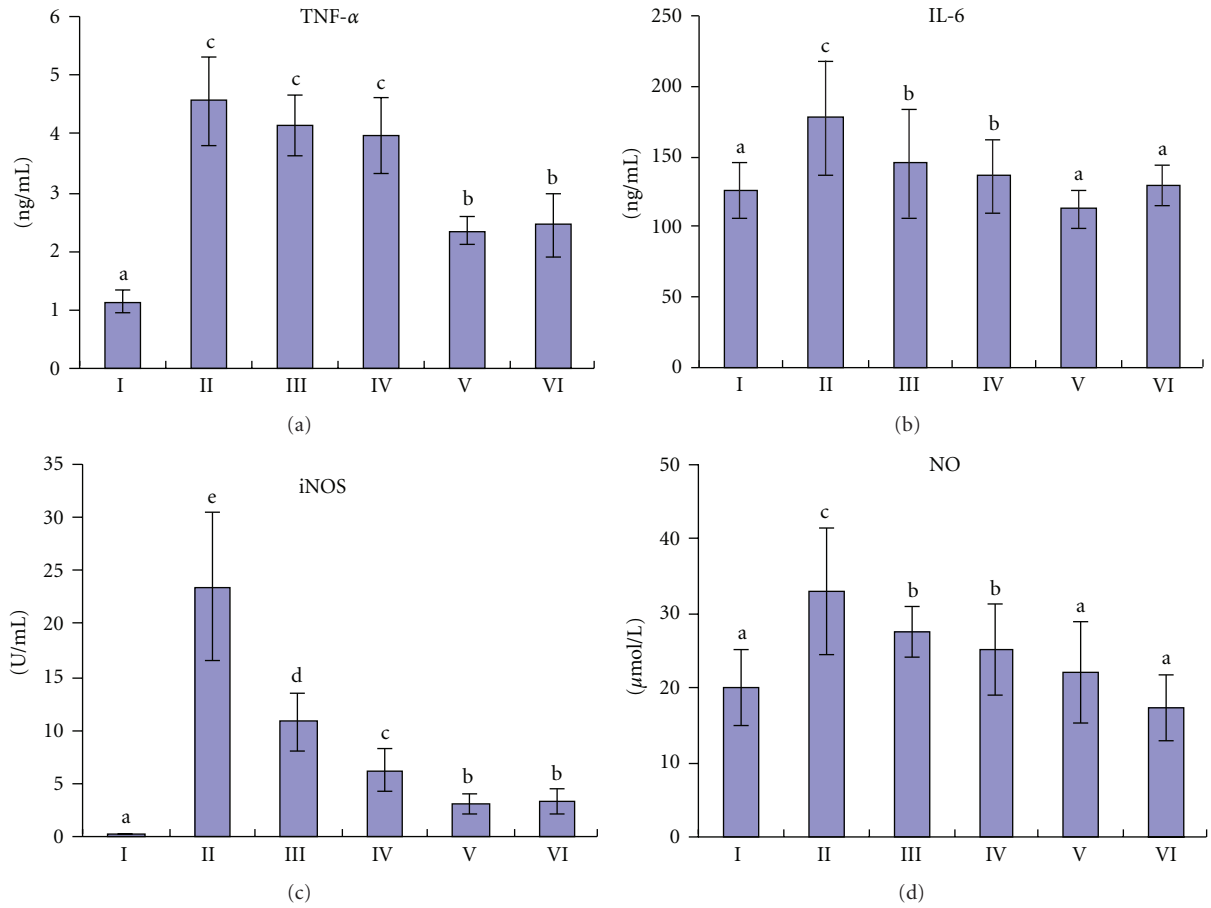

Figure 3: Effects of different concentrations of TSAV on the serum TNF-α, IL-6, iNOS, and NO activities. Group I: control; Group II: CCl_4; Group III: TSAV 50 mg/kg + CCl_4; Group IV: TSAV 100 mg/kg + CCl_4; Group V: TSAV 200 mg/kg + CCl_4; Group VI: silymarin 200 mg/kg + CCl_4. Each bar represents the mean ± SD, $n = 10$; bars with different alphabets differ significantly at $P < 0.05$ level.

Figure 3). Moreover, the effects of TSAV at high dose were found to more markedly reduce the levels of TNF-α and IL-6 than those of silymarin.

3.8. Caspase-3 and Caspase-8 Activities. The activities of caspase-3 and caspase-8 in the CCl_4 group were significantly increased. TSAV pretreatment at three doses significantly decreased their activities (shown in Figure 4). The activities of caspase-3 and caspase-8 in high dose of TSAV were reduced by 58.08% and 48.77% compared with the CCl_4 group.

3.9. TUNEL Assay and DNA Ladder. An *in situ* cell apoptosis detection kit was used to assess apoptosis in liver tissues. As shown in Figure 5, the number of TUNEL-positive apoptotic nuclei increased after CCl_4 treatment, and few TUNEL-positive cells were observed in the livers obtained from mice pretreated with TSAV (200 mg/kg) and silymarin. In addition, the effect of TSAV on DNA fragmentation was also investigated (Figure 6). The characteristic DNA ladder was found in the CCl_4 group; however, the DNA ladder was significantly attenuated in TSAV high-dosed group, which indicated that TSAV could inhibit hepatocyte apoptosis induced by CCl_4 in mice.

3.10. Western Blot Analysis. In order to evaluate the protective effect of TSAV against CCl_4-induced acute liver injury in terms of its influence on the Fas/FasL and NF-κB p65, TSAV at high dose was chosen to carry out the next experiments. As seen in Figures 7(a) and 7(b), the expressions of Fas and FasL protein were increased by nearly 2-folds and 5-folds in the liver of CCl_4-treated mice compared with the normal mice, while their expressions were significantly decreased in TSAV and silymarin mice. The expression of NF-κB p65 was increased by nearly 6-folds in CCl_4-treated mice and TSAV and silymarin partially prevented this effect (Figure 7(c)).

3.11. RT-PCR Analysis. The expressions of Bcl-2, Bak, and Bax mRNA are shown in Figures 7(d)–7(f). To exclude variations due to RNA quantity and quality, the data were adjusted to GAPDH expression. The levels of Bak and Bax were markedly increased, and the level of Bcl-2 was markedly decreased in the CCl_4 group compared with the control group, which indicated that the liver in mice was acutely injured. Their levels were significantly reversed in TSAV high-dosed group and silymarin group.

3.12. Immunohistochemistry Analysis. The immunohistochemistry was applied to analyze the expression of Bcl-2 and

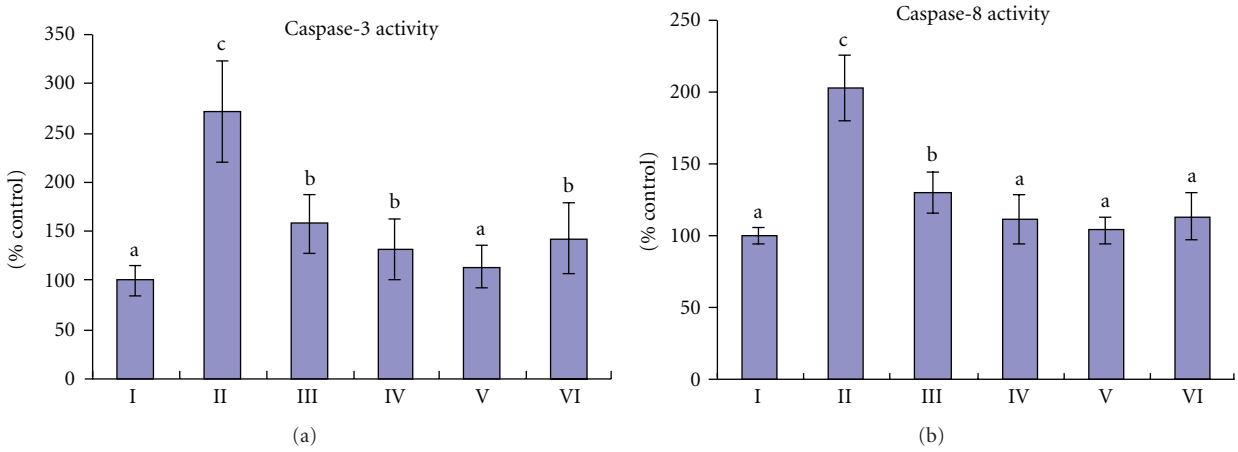

FIGURE 4: Effects of different concentrations of TSAV on the caspase-3 and caspase-8 activities. Group I: control; Group II: CCl_4; Group III: TSAV 50 mg/kg + CCl_4; Group IV: TSAV 100 mg/kg + CCl_4; Group V: TSAV 200 mg/kg + CCl_4; Group VI: silymarin 200 mg/kg + CCl_4. Each bar represents the mean ± SD, $n = 10$; bars with different alphabets differ significantly at $P < 0.05$ level.

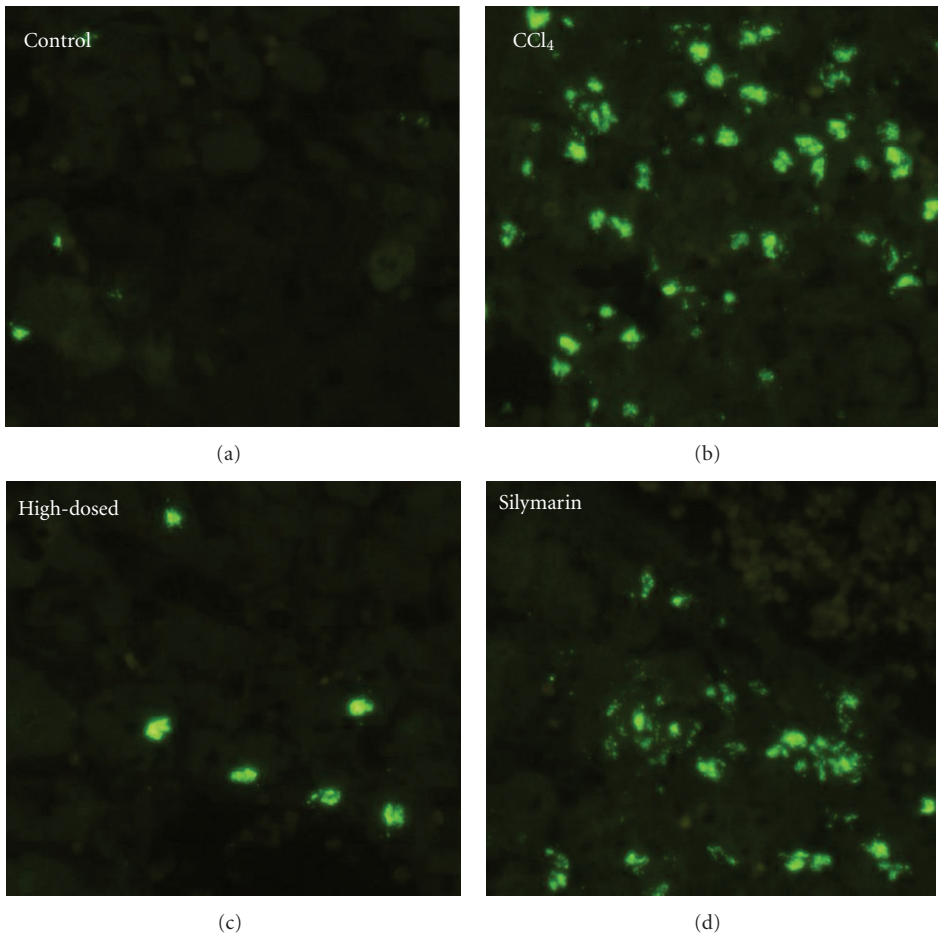

FIGURE 5: TUNEL stained histological examination was performed under fluorescence microscopy on liver tissues (original magnification: ×100). CCl_4: showing a large number of TUNEL-positive cells; high-dosed: TSAV 200 mg/kg + CCl_4, showing only a few TUNEL-positive cells; Silymarin: silymarin 200 mg/kg + CCl_4, showing a few TUNEL-positive cells.

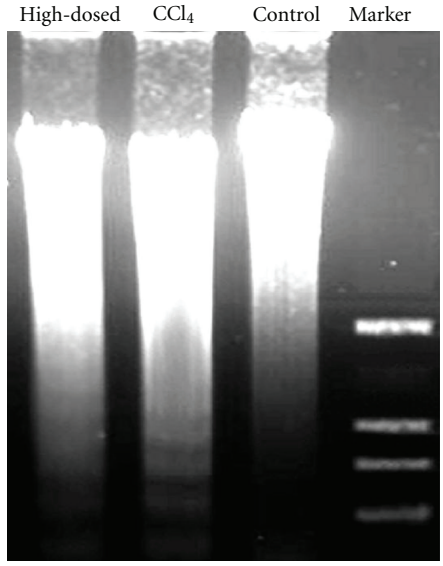

Figure 6: DNA laddering was electrophoresed on 1% agarose gels. High-dosed: TSAV 200 mg/kg + CCl_4.

p53 proteins. As shown in Figure 8, the expression of Bcl-2 in the CCl_4 group was 5 times lower compared with the control group. TSAV exhibited opposite effect, and Bcl-2 expression of TSAV at high dose was higher than that of the control group and silymarin group. The expression of p53 (Figure 9) in CCl_4 group was upregulated compared with the control group (8-folds), and the levels were significantly decreased in TSAV high-dosed group and silymarin group. TSAV at high dose was more effective in upregulating Bcl-2 protein and downregulating p53 protein than those of silymarin.

4. Discussion

A number of researches have focused on herbal and plant extracts that possess hepatoprotective activities in recent years [18, 19]. Saponins extracts of plants exert various pharmacological effects to control many diseases, including hepatoprotective activities [20, 21]. The present study is the first to evaluate the potential hepatoprotective effects of TSAV. Our data showed that TASV exhibited antioxidant, anti-inflammatory effects and regulating related apoptosis proteins against CCl_4-induced acute liver damage.

Serum hepatic enzymes such as AST, ALT, and ALP were employed in the evaluation of hepatic disorders. In CCl_4-induced acute liver damage models, the levels of ALT, AST, and ALP in serum were significantly suppressed by TSAV, and the inhibitory effects of TSAV (200 mg/kg) on levels of ALT, AST, and ALP in serum were better than those of silymarin (200 mg/kg). Histopathological lesions supported these biochemical results. As shown in Figure 2, hepatocyte and hepatic cord degeneration, focal necrosis, and inflammatory infiltration in mice livers were induced by CCl_4, which could be effectively ameliorated by TSAV pretreatment.

Lipid peroxide is a major parameter which can be included as a marker of oxidative damage. MDA is widely used as a parameter of lipid peroxidation [22]. In this study, increased liver MDA contents in CCl_4-treated group suggested that natural antioxidant defense mechanism to scavenge excessive free radicals has been compromised [23, 24]. TSAV at middle and high dose significantly inhibited the formation of MDA in the liver, and their inhibitory effects were better than those of silymarin.

Among the cellular antioxidant enzymes, SOD, CAT, GR, and GPX are important in terms of protecting the liver from CCl_4-induced damage [25]. SOD and CAT are easily inactivated by lipid peroxides or reactive oxygen species, as reported by this study in CCl_4-treated group, and there was a significant decrease in hepatic SOD and CAT levels. GR and GPX are glutathione-related enzymes, which can catalyze the synthesis of GSH to ease the burden of lipid peroxidation [25]. In the reaction catalyzed by GPX, GSH is oxidized to GSSG, which can then be reduced back to GSH by GR. In this study, CCl_4 administration to mice declined antioxidant capacity of the mice liver as evinced in decreased activity of the antioxidant enzymes. TSAV prevented the reduction in the antioxidant enzyme activities and consequent oxidative damage to the liver. GSH significantly decreased, while GSSG augmented in mice treated with CCl_4 in contrast with the control group, whereas TSAV reversed to normal levels these parameters. Therefore, the GSH/GSSG ratio decreased following CCl_4 administration, while TSAV and silymarin recovered this ratio partially.

TNF-α and IL-6 are pleiotropic cytokines associated with a variety of physiological and pathological conditions [26]. In our study, the serum TNF-α and IL-6 levels increased in CCl_4-treated group, which is in accordance with the finding of Simeonova et al. [27]. TNF-α seems to be responsible for regulating products that stimulate inflammation and fibrosis in CCl_4-induced hepatotoxicity [28]. TSAV pretreatment inhibited the increase of TNF-α and IL-6, suggesting TSAV attenuated CCl_4-induced inflammatory cascade in the liver. Considerable evidence suggested that TNF-α and IL-6 contribute to the pathogenesis of liver inflammatory diseases by activating the NF-κB signaling pathway [29]. We found

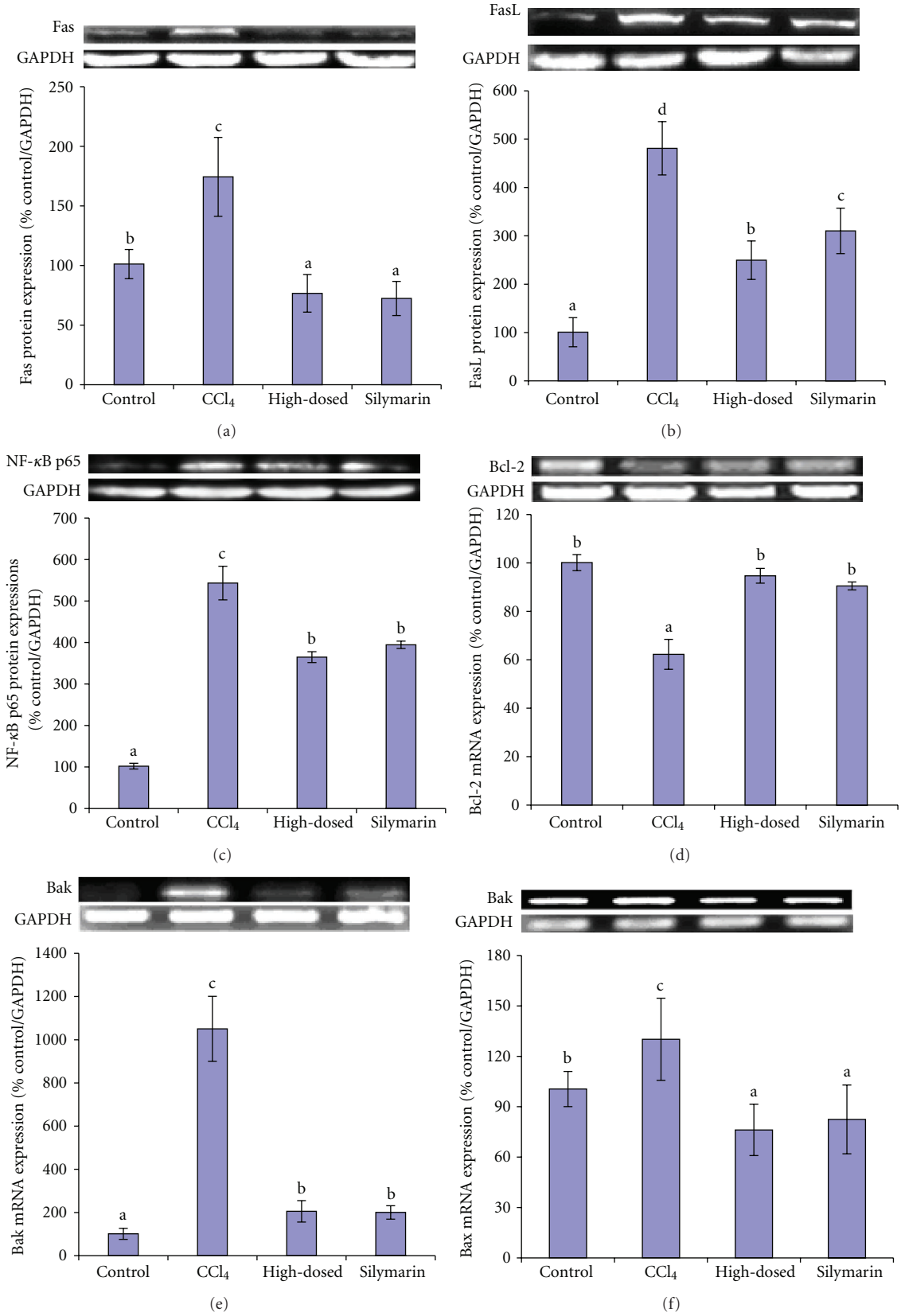

Figure 7: (a–c) Western blot analysis of Fas, FasL, and NF-κB p65 protein expressions in livers of the tested mice. (d–f) RT-PCR analysis of Bcl-2, Bak, and Bax mRNA expressions in livers of the tested mice. Values are given as mean ± SD ($n = 3$); bars with different alphabets differ significantly at $P < 0.05$ level.

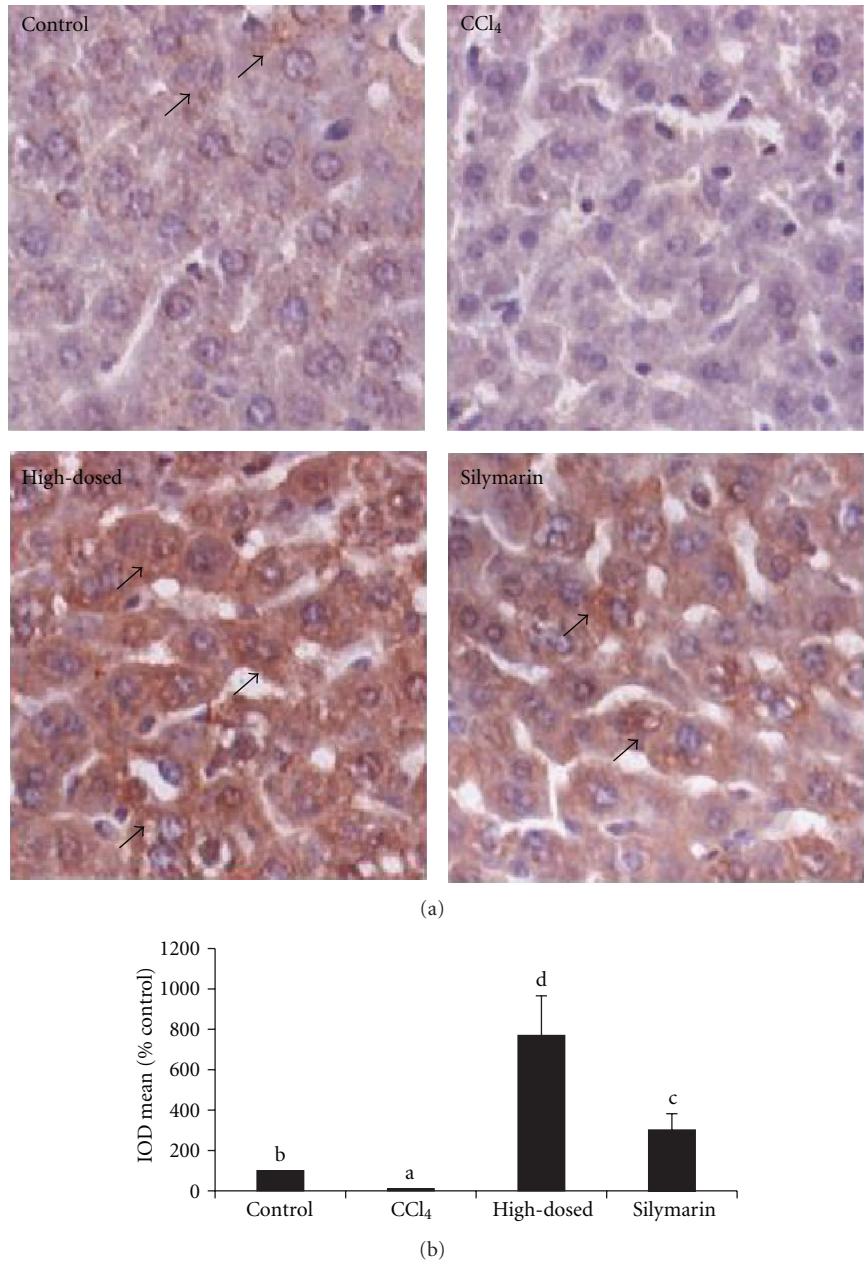

Figure 8: (a) Immunohistochemistry for Bcl-2 in livers of the tested mice. Solid arrows point to positive areas, which are characterized by intense brown nuclear staining. Original magnification: ×400. (b) The results of immunohistochemistry analysis for Bcl-2 in livers of the tested mice. Values are given as mean ± SD ($n = 3$); bars with different alphabets differ significantly at $P < 0.05$ level.

that NF-κB p65 was activated in the CC_4 group, and this activation was partially prevented by TSAV and silymarin administration, thus decreasing the inflammatory response.

Overproduction of iNOS is associated with inflammatory disorders and the pathophysiology of many disorders, including hepatocarcinoma and autoimmune diseases [30, 31]. TNF-α can induce iNOS, stimulate production of nitric oxide, and contribute to nitrosative stress [32]. In this study, increased iNOS level in the serum of CCl_4-treated mice indicated that enhanced production of NO and nitrosative stress, as a response to liver damage. Our findings suggested that iNOS mediated acute CCl_4-induced liver damage and its inhibition by TSAV exerted beneficial effects in the prevention of acute hepatic damage.

Caspase-3 and caspase-8 are two caspases proteins which play a central role in the execution-phase of cell apoptosis [33]. Our results showed that after CCl_4 treatment, considerable caspase-3 and caspase-8 were observed, which inferred that apoptotic effect of CCl_4 to hepatic cells could be related to the induction of caspase activation. TSAV pretreatment significantly decreased caspase-3 and caspase-8 activities, and TUNEL staining demonstrated that TSAV significantly decreased the number of TUNEL positive cells. In addition, TASV effectively inhibited the DNA ladder induced by CCl_4.

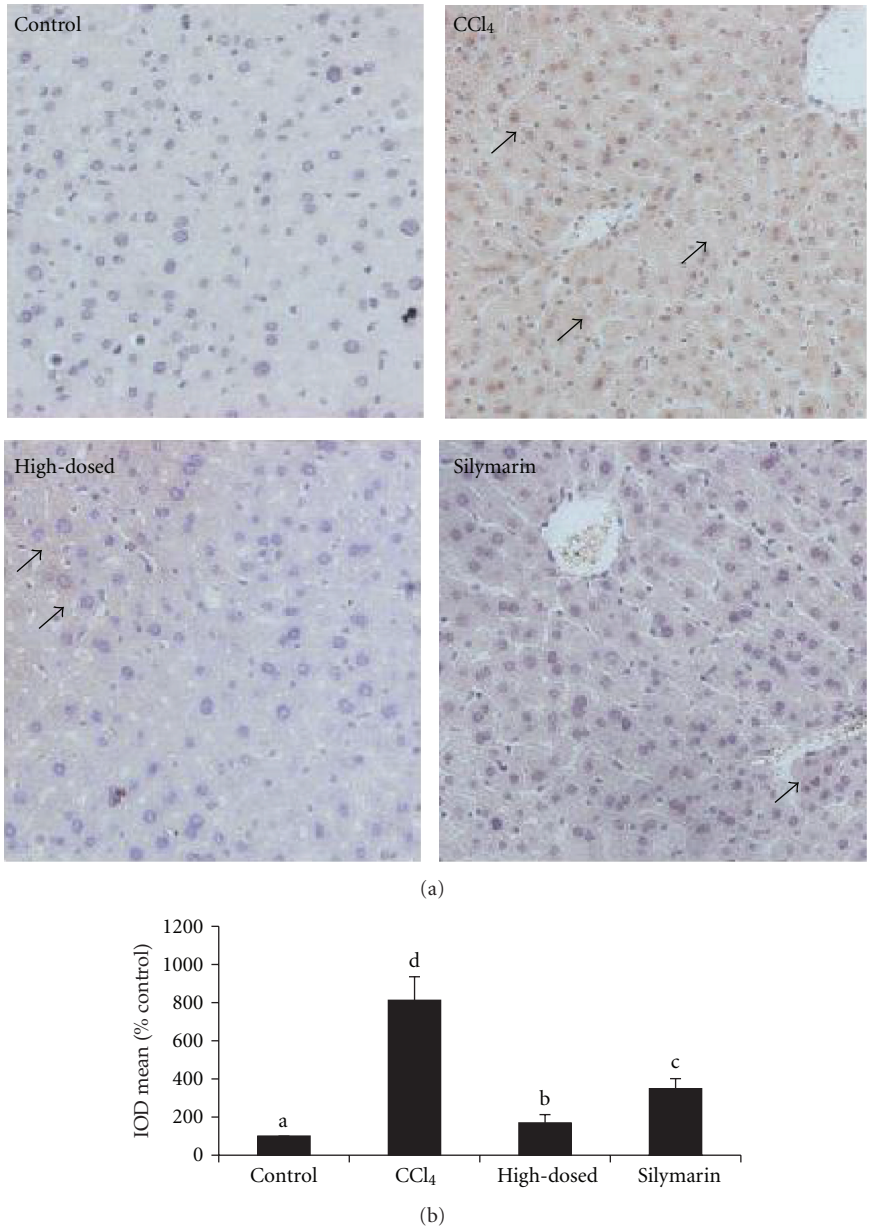

Figure 9: (a) Immunohistochemistry for p53 in livers of the tested mice. Solid arrows point to positive areas, which are characterized by intense brown nuclear staining. Original magnification: ×100. (b) The results of immunohistochemistry analysis for p53 in livers of the tested mice. Values are given as mean ± SD ($n = 3$); bars with different alphabets differ significantly at $P < 0.05$ level.

These results indicated that TSAV could restrain the activities of these two caspases and accordingly inhibit hepatic cells apoptosis.

Cell apoptosis involves at least two major pathways. One is the death receptor pathway. It is known that death receptor-ligand interactions, like the Fas/FasL interaction, are important initiators of apoptosis by the extrinsic pathway [34]. Researches indicated that the suppressions of Fas and FasL protein reduce hepatic cell death associated with liver injury [35, 36]. Moreover, activations of the caspase cascades by the Fas/FasL interaction have been demonstrated to be essential for apoptosis induction in liver injury [34]. In this study, high-dosed TSAV significantly decreased CCl_4-induced Fas and FasL upregulation, which inferred that the suppression of Fas and FasL protein expression and inhibition of caspase-3 and caspase-8 activities by TSAV played an important role in protecting against CCl_4-induced acute liver damage.

The other pathway of cell apoptosis is the mitochondrial pathway, which mainly involves the Bcl-2 family [35]. The release of proapoptotic factors from mitochondria is controlled by Bcl-2 and Bax, which are both members of the Bcl-2 family but have opposing effects on cell life and death [36]. In the present study, compared with CCl_4-treated

group, proapoptotic Bax and Bak mRNA were downregulated, whereas antiapoptotic Bcl-2 mRNA and protein were upregulated in high-dosed TSAV. In addition, Bcl-2 family proteins are known to be subject to regulation by p53 [37, 38], a tumor suppressor protein, and its expression in this study was significantly decreased in high-dosed TSAV pretreated group compared with the CCl_4 group. These results suggested that TSAV could exert its hepatoprotective effects through the interaction of these proteins.

5. Conclusions

In summary, this study demonstrated that TSAV has hepatoprotective effects against CCl_4-induced acute hepatic damage in mice, which suggested TSAV might be developed to be a potential hepatoprotective drug. Its effects could be associated with increase in antioxidant activities and inhibition of inflammatory mediators as well as regulating apoptosis-related genes. These results will benefit us to further study the exact mechanism of hepatoprotective actions by TSAV.

Acknowledgments

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Research Article

Metabolic Profiling Study of Yang Deficiency Syndrome in Hepatocellular Carcinoma by ^{1}H NMR and Pattern Recognition

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This study proposes a ^{1}H NMR-based metabonomic approach to explore the biochemical characteristics of Yang deficiency syndrome in hepatocellular carcinoma (HCC) based on serum metabolic profiling. Serum samples from 21 cases of Yang deficiency syndrome HCC patients (YDS-HCC) and 21 cases of non-Yang deficiency syndrome HCC patients (NYDS-HCC) were analyzed using ^{1}H NMR spectroscopy and partial least squares discriminant analysis (PLS-DA) was applied to visualize the variation patterns in metabolic profiling of sera from different groups. The differential metabolites were identified and the biochemical characteristics were analyzed. We found that the intensities of six metabolites (LDL/VLDL, isoleucine, lactate, lipids, choline, and glucose/sugars) in serum of Yang deficiency syndrome patients were lower than those of non-Yang deficiency syndrome patients. It implies that multiple metabolisms, mainly including lipid, amino acid, and energy metabolisms, are unbalanced or weakened in Yang deficiency syndrome patients with HCC. The decreased intensities of metabolites including LDL/VLDL, isoleucine, lactate, lipids, choline, and glucose/sugars in serum may be the distinctive metabolic variations of Yang deficiency syndrome patients with HCC. And these metabolites may be potential biomarkers for diagnosis of Yang deficiency syndrome in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a serious health problem worldwide. Chinese medicinal herbs have been widely used for treatment of HCC in China and some clinical trials have been done to investigate their effects [1, 2]. All these treatments were based on traditional Chinese medicine (TCM) diagnostics, and syndrome differentiation was the key. As known that TCM diagnostics is often based on observation of human symptoms rather than "microlevel" laboratory tests, therefore, TCM diagnostics often lacks standard and objective diagnostic methods for HCC in TCM practice. At present, many works have been done on diagnostic standards for TCM syndromes of HCC [3]. However, to our knowledge, no work has been done to explore the internal characteristics of TCM syndromes in HCC with a holistic view which is the basic nature of TCM theory.

Metabonomics, one of modern "-omics" approaches, provides a platform of systems biology to explore the metabolic pathway of biosystem by measuring the metabolic responses to pathophysiological stimuli or genetic modification [4]. It involves the global analysis of the entire metabolic profiling to elucidate the global functional status of the organism, which agrees well with the holistic view of TCM. In TCM theory, TCM syndromes are also the comprehensive responses of a certain stage in the disease process. Therefore, it is reasonable to believe that TCM theory is intrinsically correlated with metabonomics, so using metabonomics technologies is feasible for exploring the biochemical characteristics of TCM syndromes [5].

In our previous works, we established diagnostic principles for TCM basic syndromes of HCC [6, 7]. The patients with HCC are accordingly classified into eight types of basic syndromes, that is, Qi stasis, damp stasis, blood stasis, heat,

Table 1: Basic clinical feature of hepatocellular carcinoma patients with different syndrome.

Group	Number (n)	Gender (n)		Age ($\bar{x} \pm$ SD)	HCC clinical staging (n)						Child-Pugh grading (n)		
		Male	Female		Ia	Ib	IIa	IIb	IIIa	IIIb	A	B	C
YDS-HCC	21	18	3	55.76 ± 12.79	3	0	1	4	11	2	14	5	2
NYDS-HCC	21	19	2	58.10 ± 7.31	3	1	1	6	9	1	14	6	1

HCC: hepatocellular carcinoma; YDS: Yang deficiency syndrome; NYDS: non-Yang deficiency syndrome.

Qi deficiency, blood deficiency, Yin deficiency, and Yang deficiency. As one of these basic syndromes, Yang deficiency syndrome is a special syndrome which has typical clinical manifestations. The symptoms of Yang deficiency syndrome, mainly including intolerance of cold and cool limbs, are easy to be distinguished even for a junior physician, and the diagnosis of Yang deficiency syndrome is hardly controversial in TCM diagnostics. Therefore, in this study, we chose Yang deficiency syndrome patients with HCC as research subjects and tried to investigate the serum metabolic variations and discover the biochemical characteristics of Yang deficiency syndrome in HCC using a proton nuclear-magnetic-resonance- (^{1}HNMR-) based metabonomic approach, so as to provide evidences for objectification and standardization in diagnosis of TCM syndromes in HCC.

2. Materials and Methods

2.1. Patient Cohort and Classification. All experimental procedures were approved by the ethics and research committee of Changhai Hospital, Second Military Medical University, China. After obtaining informed consent, we collected a total of 42 serum samples from previously untreated HCC patients included in this study (see Table 1). Clinical diagnosis of the patients was performed using standard imaging and pathology criteria. Basic syndrome types of 42 patients with HCC were classified by TCM syndrome qualitative diagnosis criteria and quantitative diagnosis model for HCC patients into two groups, including Yang deficiency syndrome (YDS-HCC) group (21 cases) and non-Yang deficiency syndrome (NYDS-HCC) group (21 cases). Between two groups, the differences of gender, age, complication, and clinical stage were not significant ($P > 0.05$) (see Table 1).

2.2. Sample Preparation. To avoid the introduction of any analytical bias due to sample preparation, all samples were collected from the patients in the Department of TCM of Changhai Hospital and were processed consistently in the following manner. Venous blood samples were collected in the morning preprandially, after overnight fasting, using blood collection vacuum tubes with silicone coated interior and no additive (products of BD Company, USA, catalog no. 366431). About 2.0 mL blood was collected and then left clotted for 2 h at room temperature. After this, samples were centrifuged at 3500 rpm for 15 min. Serum was collected and immediately stored at −80°C until being used for metabonomic analysis. Preparation of serum samples for metabonomic analysis by ^{1}H-NMR was performed manually according to Beckonert et al. [8]. After being defrosted at room temperature, four hundred microliters of each sample were mixed with 50 mL D_2O for locking signal and with 50 mL phosphate buffer solution (0.2 M Na_2HPO_4/0.2 M NaH_2PO_4; pH 7.4) to minimize pH variations of the serum samples. Then, TPS (3-trimethylsilylpropanesulfonate sodium; 0.048% final concentration) was added as an internal standard. Finally, the samples were added to a 5 mm NMR tube and NMR acquisition was performed immediately.

2.3. NMR Acquisition. The NMR spectra were acquired using a Bruker Avance II 600 spectrometer (Bruker Biospin, Rheinstetten, Germany). The ^{1}H-NMR spectra of serum samples were collected at a constant temperature of 300 K (27°C) using the relaxation edited Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence to facilitate the detection of low molecular weight species and using a solvent presaturation pulse sequence to suppress the residual water resonance. Free induction decays (FIDs) were collected into 64 k data points, with a spectral width of 7289 Hz and an acquisition time of 2.04 s, giving a total pulse recycle delay of 3.04 s. The FID values were multiplied by an exponential weighting function equivalent to a line broadening of 0.3 Hz prior to Fourier transformation.

2.4. Data Processing and Statistical Analysis. All spectra were baseline corrected using multipoint baseline correction method, and chemical shifts were adjusted with reference to TPS. The chemical shift region δ4.60–5.06 was removed to eliminate any spurious effects of variability on the suppression of the water resonance. Peak detection and peak matching were performed using programs coded by Matlab 7.0 (The Mathworks Inc., Natick, MA, USA), and a data matrix containing all peak intensities was thus generated. The data were logarithm transformed and centered for partial least squares discriminant analysis (PLS-DA) using Matlab 7.0. In PLS-DA, the pathologic differences in patients can be visualized in score plot and metabolites responsible for the differences could be statistically identified in loading plot using Hotelling's T^2 test [9]. All results presented are described as mean ± SD. Statistical significance of the data was determined using the non parametric Mann-Whitney U test or t test, where appropriate, P values < 0.05 were considered to be significant. Metabolite assignments were performed using NMR metabolic profiling databases (http://www.ebi.ac.uk/nmrshiftdb or http://nmrshiftdb.org/), and if needed, with reference to any available assignments in the literature [10–12].

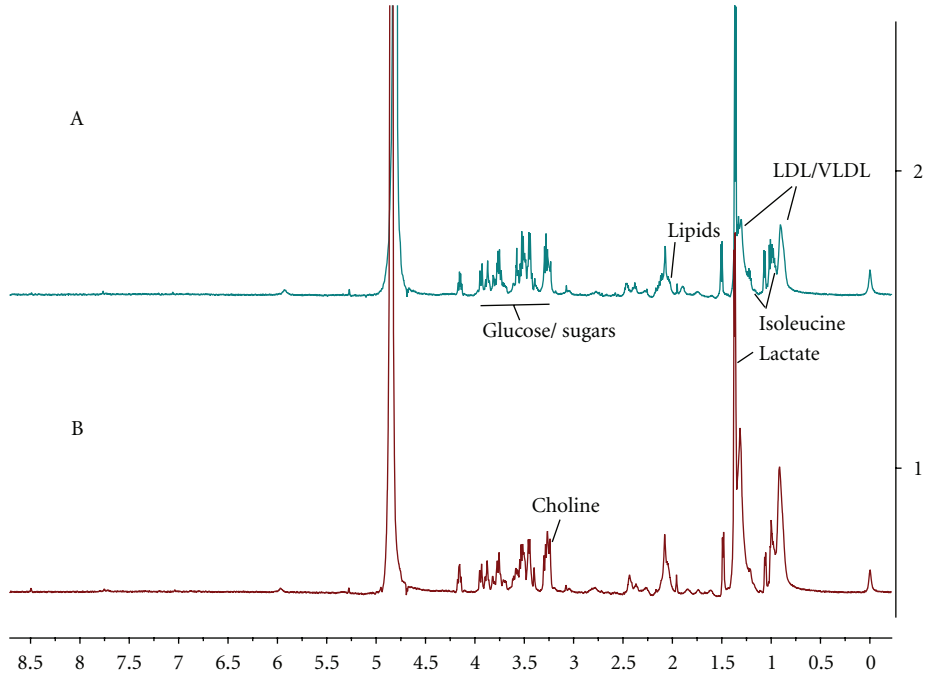

Figure 1: Representative ^{1}H NMR spectra of serum from YDS-HCC patients (A) and NYDS-HCC patients (B); LDL, low-density lipid; VLDL, very low-density lipid.

3. Results

Typical ^{1}H NMR spectra of the serum samples were shown in Figure 1. By data preprocessing, a total of 221 peaks were resolved for each serum sample. To determine whether any Yang deficiency syndrome-related variability existed within the data, PLS-DA was used. As shown in PLS-DA scores plot (Figure 2(a)), samples from different groups were well separated along the first PLS component, which indicates that NMR-based metabolic profiling could reveal characteristic pathological alterations in serum from YDS-HCC and NYDS-HCC patients.

As shown in PLS-DA loading plot (Figure 2(b)), eighteen important resonances responsible for the separation were selected. It displayed not only the differentiation between the two groups but also the specific compounds resulting in the separation. These resonances were structurally identified as low-density lipid/very low-density lipid (LDL/VLDL) (0.84 ppm, 0.92 ppm, 1.24 ppm, 1.28 ppm, 1.32 ppm), isoleucine (0.96 ppm), lactate (1.36 ppm, 1.40 ppm), lipids (2.00 ppm, 2.04 ppm), choline (3.24 ppm), and glucose/sugars (3.28 ppm, 3.40 ppm, 3.44 ppm, 3.48 ppm, 3.72 ppm, 3.84 ppm, 3.92 ppm). As shown in Table 2, aftere being confirmed by Mann-Whitney U test or t test, intensities of these six types of metabolites were changed markedly between different groups ($P < 0.05$ or $P < 0.01$). Differences in metabolite intensities mirrored those observed in the PLS-DA models with all the differential metabolite observed, including LDL/VLDL, isoleucine, lactate, lipids, choline, and glucose/sugars, being lower in YDS-HCC patients compared to NYDS-HCC patients.

4. Discussion

The serum metabolite profiling represents a collective "snapshot" of metabolic alterations in the biochemical pathways of the entire body induced by a plethora of factors, including disease, life habits (e.g., diet, smoking, and exercise), gender, drug intake, and environmental factors [10]. In TCM theory, syndrome also is a comprehensive reflection of physical status in one stage of disease. Different patients with the same disease might have different clinical signs and symptoms as well as TCM tongue condition and pulse condition. Yang deficiency syndrome is a special syndrome with typical clinical manifestations, mainly including intolerance of cold, cool limbs, pale tongue, and powerless pulse. To reveal the internal characteristics behind these special clinical manifestations in HCC patients is not only significant for objective diagnosis of TCM syndromes but also significant for understanding the heterogeneity of HCC patients.

Efforts to understand the biochemical characteristics of Yang deficiency syndrome in HCC or other diseases through analyzing various kinds of biological indicators have been made by a number of groups [13, 14]. However, to the best of our knowledge, this paper presents the first examination of serum metabolites in the context of Yang deficiency syndrome in HCC with a global view. We have examined the potential differentiating capacity of an ^{1}H-NMR-based metabonomic approach for HCC patients as classified by Yang deficiency status.

Supervised multivariate analysis in the form of PLS-DA enabled good separation of YDS-HCC and NYDS-HCC patients (see Figure 2(a)). PLS-DA statistical analysis

Table 2: Assignment and intensities of metabolites in serum responsible for PLS-DA separation between YDS-HCC and NYDS-HCC patients.

Metabolite	δ^1H (ppm)[a]	Assignment	Multiplicity[b]	Intensity[c] (10^3)		P value[d]
				YDS-HCC ($n = 21$)	NYDS-HCC ($n = 21$)	
LDL/VLDL	0.84, 0.92, 1.24, 1.28, 1.32	$CH_3(CH_2)_n/CH_3CH_2CH_2C=$	m	67.23 ± 32.64	115.66 ± 47.33	0.000
Isoleucine	0.96	δ-CH_3	t	8.27 ± 3.86	13.57 ± 6.20	0.002
Lactate	1.36, 1.40	CH_3	d	19.44 ± 15.19	53.44 ± 25.92	0.000
Lipids	2.00, 2.04	$CH_2C=C$	m	9.45 ± 3.73	13.89 ± 5.04	0.002
Choline	3.24	$N(CH_3)_3$	s	10.23 ± 5.53	16.05 ± 9.04	0.016
Glucose/sugars	3.28, 3.40, 3.44, 3.48, 3.72, 3.84, 3.92	Various	m	46.05 ± 27.74	68.39 ± 40.92	0.045

HCC: hepatocellular carcinoma; YDS: Yang deficiency syndrome; NYDS: non-Yang deficiency syndrome; PLS-DA: partial least square discriminatory analysis; LDL: low-density lipid; VLDL: very low-density lipid.
[a]Chemical shifts were reported with reference to 3-trimethylsilylpropanesulfonate (TPS) singlet resonance at 0.000 ppm.
[b]Multiplicity: s: singlet; d: doublet; t: triplet; m: multiplet.
[c]Data are expressed as mean ± SD.
[d]P value calculated by Mann-Whitney U test or t test.

indicated that the metabolic profiling of one particular YDS-HCC patient was more similar to that of YDS-HCC patients. And several Yang deficiency syndrome-related variations determined the distinction between YDS-HCC and NYDS-HCC (see Figure 2(b)). Examination of corresponding loading plots showed that the main metabolic variations in the data set were derived from various metabolic signals. The metabolites responsible for the separation of the YDS-HCC from the NYDS-HCC patients were structurally identified as LDL/VLDL, isoleucine, lactate, lipids, choline, and glucose/sugars. And the intensities of these six types of metabolites were lower markedly in YDS-HCC patients compared to NYDS-HCC patients (see Table 2). These six types of metabolites get involved in multiple metabolisms, including amino acid metabolism, lipid metabolism, glycometabolism, and energy metabolism. The results implied multiple Yang deficiency related biochemical pathways lose balance in HCC patients.

In ^{1}H-NMR serum profiling, lower intensities of LDL/VLDL, lipids, and choline in YDS-HCC than NYDS-HCC patients are correlated to lipid metabolism. LDL and VLDL play an important role in lipid metabolism as a transporter for cholesterol and triglyceride, respectively [15]. Lipids, the general term of fats and lipoids, are the best storage form of redundant energy and play the wide biological functions as different structural styles. Choline can increase fatty acid utilization and prevent abnormal accumulation of fat in liver. Choline is involved in the synthesis of creatine and indirectly involved in muscle energy metabolism [16]. Usually, lipid metabolism is abnormal in HCC due to the deficient abilities of both lipid catabolism and anabolism [17]. Lower intensities of LDL/VLDL, lipids, and choline in YDS-HCC patients implied the lower level of lipid metabolism in YDS-HCC patients. In TCM theory, Yang deficiency syndrome patients usually have a poor appetite and failed to utilize the food essence normally. Lipids and lipoproteins are composed by lots of materials from food. It is consistent with the results of lower level lipids and lipoproteins in Yang deficiency patients in our study. Yang deficiency can reduce the ability of transporting food essence to all the body. Lower intensities of lipoproteins, like LDL and VLDL, reflect the deficiency of transport capacity for nutrients. Intolerance of cold and cool limbs, the typical clinical manifestations of patients with Yang deficiency syndrome, is the outward appearance of deficient energy metabolism and fat reserves. Lower levels of lipids, lipoproteins, and choline reflect the decrease of ability to use and reserve the lipid energy substances. It is also coincident with the results of lower level of lipids, lipoproteins, and choline in Yang deficiency syndrome patients in our study. However, because of the poor analysis capabilities for lipids metabolites by the means of CPMG pulse sequence in ^{1}H-NMR spectra, it is necessary to analyze the relations between lipids metabolites and Yang deficiency syndrome by longitudinal eddy-delay (LED) pulse sequence in our further study.

In our examination, the differential metabolite involved in amino acid metabolism is isoleucine. Isoleucine, one of branched-chain amino acids, can synthesize protein for muscles and can transform into glucose for providing energy in the status of starvation or strenuous exercise. Branched-chain amino acids usually decrease in severe liver diseases. Supplement branched-chain amino acids can modify negative nitrogen balance and cachexia in HCC patients [18]. There is few research to explore the relation between Yang deficiency syndrome and amino acid metabolism. However, one research found that the serum concentration of branched-chain amino acids in the elderly was lower than the youth under the same nutritional intake. And the reduction of branched-chain amino acids was accompanied with decreasing immune status and lower level of hormone [19]. In TCM theory, Yang deficiency syndrome

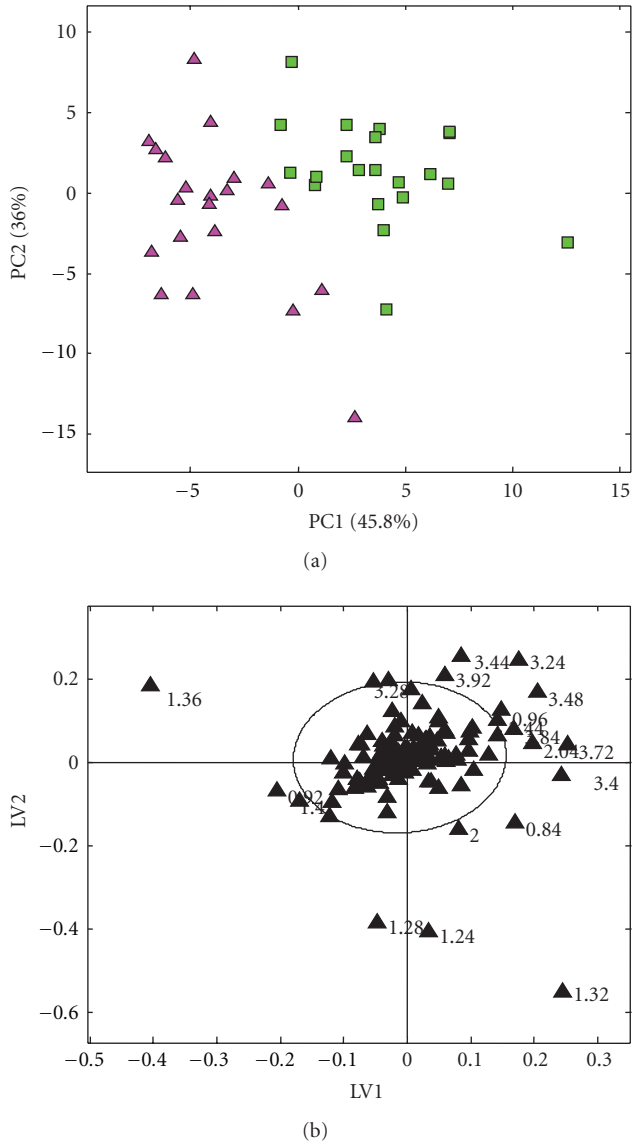

Figure 2: Results after PLS-DA using the ^{1}H NMR spectra of serum from HCC patients. (a) PLS-DA score plot shows a clear separation between Yang deficiency syndrome patients with HCC (YDS-HCC) (■) and non-Yang deficiency syndrome patients with HCC (NYDS-HCC) (▲). (b) Examination of the corresponding loading plot indicated that those variances out of the ellipse were responsible for the separation of YDS-HCC and NYDS-HCC patients marked with chemical shift (ppm).

is similar to natural aging. In other words, Yang deficiency syndrome usually means premature senility. Therefore, the level of branched-chain amino acids might partly reflect the pathological feature of Yang deficiency syndrome. And low level of isoleucine might be one of potential biochemical characteristics for Yang deficiency syndrome patients with HCC.

Glucose is the main source of energy for the human body and in a high-consuming status in tumors [20]. Lactate is an important intermediate of anaerobic glycolysis in glucose metabolism. Lactate can send back to liver or muscle and convert into glcogen. In normal condition, anaerobic glycolysis is intensive in organs or cells with high energy-consuming or lacking of mitochondria, such as adrenal medulla, neurons, and blood red cells and can keep a stable lactate concentration in blood [21]. Anaerobic glycolysis is also an important way of energy supply for tumor cells. Lactate is not only a metabolite of energy metabolism but also a kind of energy source for tumor cells [22]. In our examination, the intensities of both glucose/sugar and lactate decreased in Yang deficiency syndrome patients with HCC. It might mean that the energy metabolism of the organs or cells lacking of mitochondria or with high energy-consuming were weakened, but which one plays the leading role was not clear. On the other hand, glucose/sugar, lactate, lipids, and amino acids are all the energy source, and these reduced metabolites might imply high consume of energy in Yang deficiency syndrome patients with HCC.

In summary, this study provided an ^{1}H NMR-based metabonomic approach for exploring the biochemical characteristics of Yang deficiency syndrome based on the integral information about serum metabolites. Yang deficiency syndrome in HCC is correlated to the unbalance of multiple metabolisms. This metabonomic approach to study Yang deficiency syndrome in HCC may lead to better understand the heterogeneity of HCC. It is also beneficial to understand individualized treatment. The discussion regarding metabolic differences between patient groups in this study is far from exhausted due to few studies on Yang deficiency syndrome by tools of systems biology. We need to accumulate more data of TCM syndrome study by systems biology technology, so as to provide evidence of the objectivity of TCM syndrome.

5. Conclusions

In this work, a metabolic profiling study of Yang deficiency syndrome in HCC was performed using ^{1}H NMR and pattern recognition techniques, which has been proven to be an efficient approach to investigate the biochemical characteristics of Yang deficiency syndrome. With the application of PLS-DA, six types of metabolites responsible for the separation based on the status of Yang deficiency syndrome were structurally postulated. These metabolites included LDL/VLDL, isoleucine, lactate, lipids, choline, and glucose/sugars, which were mainly involved in lipid, amino acid, and energy metabolisms. The decreased intensities of these metabolites imply that multiple metabolisms, mainly including lipid, amino acid, and energy metabolisms, are unbalanced or weakened in Yang deficient syndrome patients with HCC. The results indicate that the NMR-based metabonomic approach is a promising technique for understanding the internal characteristics of TCM syndromes, and the potential biomarkers for diagnosis of TCM syndromes.

Authors' Contribution

X. Huang and Q. Chen contributed equally to this work and should be considered cofirst authors.

Acknowledgment

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Research Article

Polygonum cuspidatum Extract Induces Anoikis in Hepatocarcinoma Cells Associated with Generation of Reactive Oxygen Species and Downregulation of Focal Adhesion Kinase

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Anoikis has been recognized as a potential target for anticancer therapy. *Polygonum cuspidatum* (Huzhang) is a frequently used Chinese herb in hepatocarcinoma. In present study, we evaluated the effects of *Polygonum cuspidatum* extract (PCE) in hepatocarcinoma cells in suspension. The results showed that PCE inhibited the proliferation of hepatocarcinoma cells in suspension in a dose- and time-dependent manner. PCE also inhibited anchorage-independent growth of hepatocarcinoma cells in soft agar. PCE induced anoikis in human hepatocarcinoma Bel-7402 cells accompanied by caspase-3 and caspase-9 activation and poly(ADP-ribose) polymerase cleavage, which was completely abrogated by a pan caspase inhibitor, Z-VAD-FMK. In addition, PCE treatment induced intracellular reactive oxygen species (ROS) production in Bel-7402 cells. NAC, an ROS scavenger, partially attenuated PCE-induced anoikis and activation of caspase-3 and caspase-9. Furthermore, PCE inhibited expression of focal adhesion kinase (FAK) in Bel-7402 cells. Overexpression of FAK partially abrogated PCE-induced anoikis. These data suggest that PCE may inhibit suspension growth and induce caspase-mediated anoikis in hepatocarcinoma cells and may relate to ROS generation and FAK downregulation. The present study provides new insight into the application of Chinese herb for hepatocarcinoma treatment.

1. Introduction

Epithelial cells require attachment to the extracellular matrix to provide survival signal. Detachment from the extracellular matrix causes apoptosis, a process known as anoikis, or detachment-induced apoptosis. Anoikis is a Greek word that means homelessness, as apoptotic process; anoikis was first described by Frisch and Francis in 1994 [1]. Physiologically, anoikis played a critical role in organismal development and homeostasis [1]. Pathophysiologically, resistance to anoikis is acquired in epithelial cancer cells due to gene expression or activity abnormality, which allows cancer cells to survive in an anchorage-independent manner when deprived of extracellular matrix attachment during dissemination in blood or lymph, and associated with metastatic spread of cancer cells [2]. Anoikis has been suggested as a potential target for anticancer therapy [3, 4].

Hepatocarcinoma is one of the most frequent malignancies and remains the third leading cause of cancer death worldwide [5–7]. Huzhang (*Polygonum cuspidatum*) is a well-tolerated Chinese herb used for treating liver diseases with damp-heat and blood-stasis syndrome and has been regarded as an anticancer herb in modern traditional Chinese medicinal practice and frequently used in hepatocarcinoma. *Polygonum cuspidatum* has displayed anticancer effects in oral cancer and lung cancer cells [8, 9]. The active chemical ingredients of *Polygonum cuspidatum* include resveratrol, emodin, polydatin, and physcion

[10, 11]. Resveratrol and emodin have shown anticancer potential in various cancer cells, including hepatocarcinoma cells [12, 13]. Emodin may induce reactive oxygen species (ROS) and sensitize gastric carcinoma cells to anoikis [14]. We have shown that a *Polygonum cuspidatum* containing Chinese herbal formula may inhibit suspension growth and induce anoikis in hepatocarcinoma cells [15]. However, the effect of *Polygonum cuspidatum* against hepatocarcinoma cells in suspension remains unknown.

In present study, we evaluated anticancer potential of *Polygonum cuspidatum* against hepatocarcinoma cells in suspension growth. The results showed that *Polygonum cuspidatum* extract (PCE) inhibited suspension growth, activated caspases, and induced anoikis in hepatocarcinoma cells, and may relate to ROS generation and downregulation of focal adhesion kinase (FAK).

2. Materials and Methods

2.1. Chemicals and Reagents. CytoSelect 24-Well Anoikis Assay was provided by Cell Biolabs (San Diego, CA). Poly-HEMA was from Sigma-Aldrich (St. Louis, MO). Colorimetric CaspACE Assay System was the product of Promega (Madison, WI). Z-VAD-FMK, Caspase-8, and Caspase-9 Colorimetric Assay kits were from R&D Systems (Minneapolis, MN). Antibodies against poly(ADP-ribose) polymerase (PARP), FAK, Phospho-FAK, and GAPDH were obtained from Cell Signaling Technology (Danvers, MA). Cell Counting Kit-8 was purchased from Dojindo (Kumamoto, Japan). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and N-acetylcysteine (NAC) were provided by Beyotime Institute of Biotechnology (Jiangsu, China). Recombinant eukaryotic expression plasmid encoding full length of human FAK (re-FAK) and empty vector was obtained from Genechem (Shanghai, China). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Polydatin, Resveratrol Emodin, Physcion was purchased from Shanghai R&D Centre for standardization of Chinese Medicines (Shanghai, China), and the purity was higher than 98.0%.

2.2. Herbal Preparation. PCE was prepared as a lyophilized-dry powder of hot water extracts as described previously [15]. Authentic herb materials were provided by Longhua Hospital herb store. *Polygonum cuspidatum* (100 g) was soaked for 1 h and decocted twice with 8-fold volume of distilled water for 2 h. The decoction was filtered and centrifuged twice at 12000 rpm for 30 min to remove insoluble ingredients. The supernatants were mixed with an equal volume of ethanol and kept at 4°C overnight and centrifuged at 12000 rpm for 30 min to remove insoluble ingredients. The resultant supernatants were lyophilized, weighed, dissolved in RPMI1640 medium, and adjusted to a concentration of 400 mg/mL and were sequentially passed through 0.45 μm and 0.22 μm filters sterilization. The average yield of PCE obtained was 7.32%. Active compounds in PCE were detected by High Performance Liquid Chromatography. Chromatographic separations were carried out on a Merck C18 Hibar column (4.6 mm × 250 mm, 5 μm) as described [10]. The presence and proportion of the main constituents of PCE were identified as resveratrol (2.59%), emodin (7.84%), polydatin (5.10%), and physcion (5.50%).

2.3. Cell Culture. Human hepatocarcinoma cell line Bel-7402 and murine hepatocarcinoma cell line Hepa 1–6 were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Bel-7402 and Hepa 1–6 cells were grown in RPMI1640 medium with 10% FBS and 1% Pen-Strep and maintained at a 37°C in a humidified incubator with a 5% CO_2 atmosphere.

2.4. Anchorage-Independent Growth Assay. Cells in logarithmic growth phase were seeded into Poly-HEMA coated (10 mg/mL) 96-well plate (5×10^3 cells/well). After 24 h cells were exposed to various doses of PCE or equal volume of RPMI1640. At the end of treatment, the floated cells were collected, and cell viability was evaluated by using the Cell Counting Kit-8 assay according to the manufacturer's instructions. The cell survival rate was calculated as follows: cell survival rate (%) = (experimental OD value/control OD value) × 100%.

2.5. Soft Agar Colony Formation Assays. For the soft agar colony formation assays, 2×10^4 log-phase hepatocarcinoma cells were seeded and grown on a plate containing 1% base agar and 0.6% top agar and exposed to different concentrations of PCE or equal volume of RPMI1640 twice a week for 2 weeks and incubated at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Colonies were stained with crystal violet and counted under a dissecting microscope. The inhibition of colony formation was calculated as follows: inhibition of colony formation (%) = (1 − experimental colony number/control colony number) × 100%.

2.6. Anoikis Assay. Anoikis was detected by CytoSelect 24-Well Anoikis Assay according to the manufacturer's instructions. Briefly, log-phase hepatocarcinoma cells (3×10^4 cells/well) were inoculated in Poly-HEMA coated 24-well plate. On the second day, the cells were exposed to different dose of PCE or equal volume of RPMI1640 for 24 h. The floated PCE-treated or control cells were collected and stained with ethidium homodimer (EthD-1) at 37°C for 1 h. The presence of red EthD-1 fluorescence was monitored under a fluorescence microscope and quantitated with a fluorescence microplate reader at excitation wavelength of 525 nm and emission wavelength of 590 nm.

2.7. Flow Cytometric Assays. Cells were treated as indicated, collected, and stained with Annexin V-FITC and PI as recommended by the manufacturer (BD Biosciences). Apoptotic cells were detected in a FACScalibur flow cytometer (Becton Dickinson).

2.8. Caspase Activation Assay. After treatment for the indicated time with different concentration of PCE, caspase-3, 8, 9 activities were measured by the cleavage of the specific chromogenic substrate according to manufacturer's

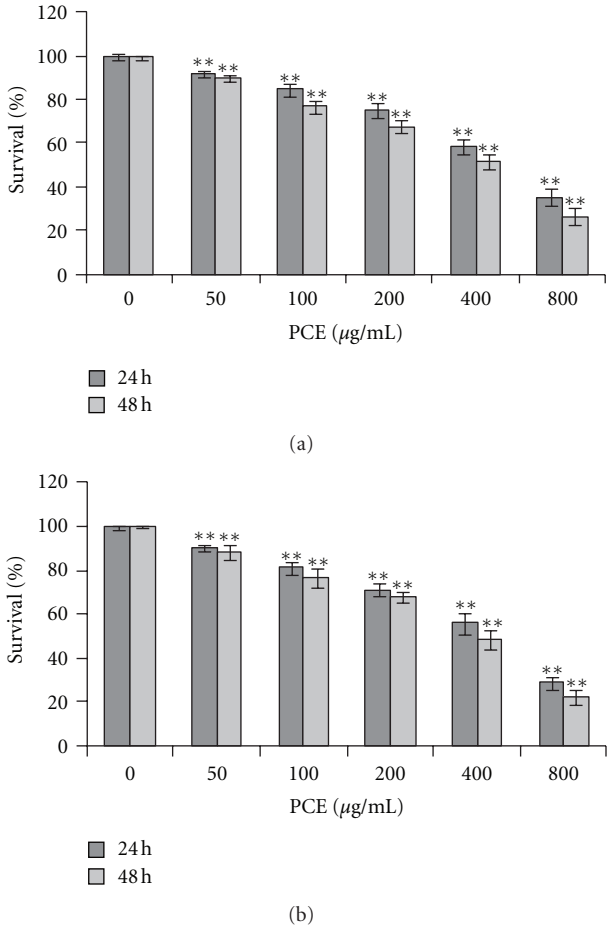

Figure 1: PCE inhibited proliferation of hepatocarcinoma cells in suspension. Human hepatocarcinoma Bel-7402 cells (a) and murine hepatocarcinoma Hepa 1–6 cells (b) were treated with different concentrations of PCE; cell viability was evaluated by CCK-8 assay. Data shown are representative of three independent experiments. $^{**}P < 0.01$, versus control group.

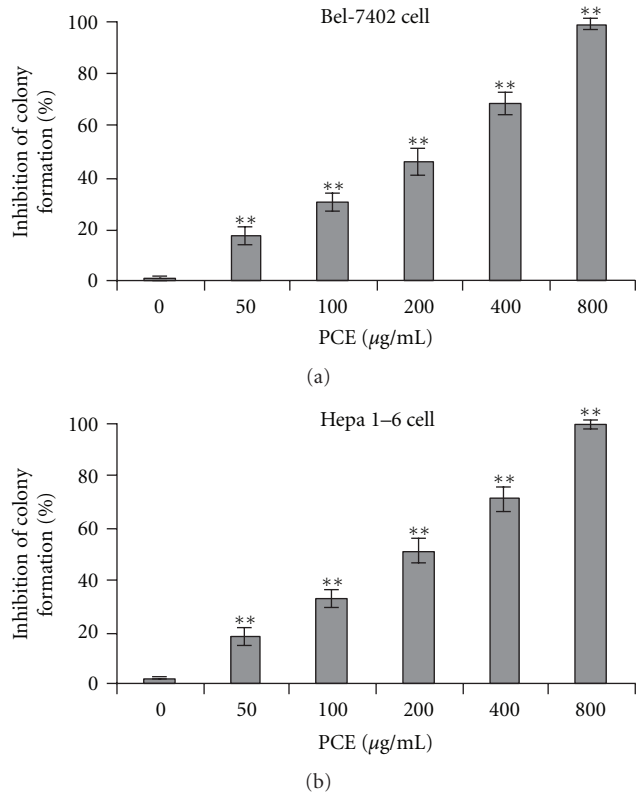

Figure 2: PCE inhibited colony formation of hepatocarcinoma cells in soft agar. Bel-7402 cells (a) and Hepa 1–6 cells (b) were treated with different dose of PCE twice a week for 2 weeks in the soft-agar colony formation assay. The colonies were stained and counted. Data shown are representative of three independent experiments (each conducted in triplicate). $^{**}P < 0.01$, versus control group.

instructions. For caspases inhibition, cells pretreated with Z-VAD-FMK (50 μmol/L, 2 h) were incubated with PCE for another 24 h.

2.9. Western Blot. Western blots were performed as described previously [15, 16]. Briefly, collected cells were lysed and subjected to 8–10% SDS-PAGE gel and transferred into a nitrocellulose membrane (Amersham). The transferred membrane was blocked with 5% nonfat milk, washed, and probed with antibodies against PARP (1 : 1000), FAK (1 : 1000), Phospho-FAK (1 : 1000), or GAPDH (1 : 2000). Blots were then washed and incubated with IRDye 700-conjugated (1 : 3000) or IRDye 800-conjugated (1 : 5000) secondary antibodies (Rockland Immunochemicals) and visualized in Odyssey Infrared Imaging System (LI-COR Biosciences).

2.10. Measurement of Intracellular ROS Levels. Intracellular ROS production was detected by DCFH-DA staining. DCFH-DA is cleaved intracellularly by nonspecific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound DCF [17]. Log-phase hepatocarcinoma cells (2×10^5 cells/well) were seeded in Poly-HEMA coated 6-well plate. On the second day, the cells were exposed to different dose of PCE or equal volume of RPMI1640 for 24 h and stained with DCFH-DA at 37°C for 20 minutes in the dark. The presence of DCF fluorescence was quantitated with a fluorescence microplate reader at excitation wavelength of 488 nm and emission wavelength of 525 nm. For ROS inhibition, cells were pretreated with NAC (50 mmol/L for 2 h), followed by desired PCE treatment.

2.11. Plasmid Transfection. For plasmid transfection, Bel-7402 cells were cultured on 6-well plate to 90–95% confluence, and 4.0 μg recombinant human FAK eukaryotic expression plasmid or control empty vector was introduced into the cells using Lipofectamine 2000 according to the manufacturer's recommendations. After 24 h of transfection, cells were subjected to anoikis assay, ROS detection, and western blot.

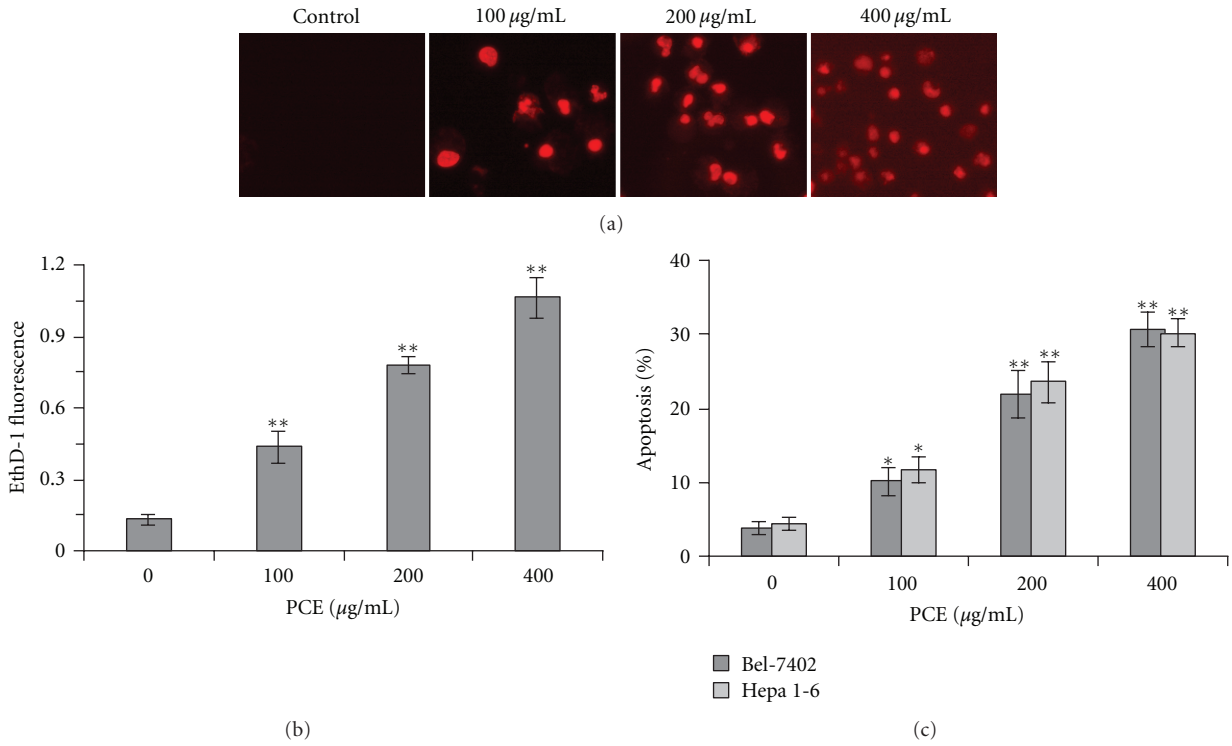

Figure 3: PCE induced anoikis in hepatocarcinoma cells. (a) Bel-7402 cells were treated with different dose of PCE for 24 h in Poly-HEMA coated plates, stained with EthD-1, and observed under fluorescence microscope (×100). (b) The fluorescence of EthD-1 absorbed by Bel-7402 cells was detected with fluorescence microplate reader and expressed as mean ± SD. (c) PCE-treated Bel-7402 cells and Hepa 1–6 cells were stained with Annexin V-FITC/PI and detected in FACScalibur flow cytometer. Data illustrated are from three separate experiments. $^{*}P < 0.05$, versus control group. $^{**}P < 0.01$, versus control group.

2.12. Statistical Analysis. Results are expressed as means ± standard deviation of at least two independent experiments, each conducted in triplicate. Differences between control and PCE treatment were analyzed by 1-way ANOVA. Differences were considered significant at $P < 0.05$.

3. Results

3.1. PCE Inhibited Proliferation of Hepatocarcinoma Cells in Suspension. The effects of PCE on cell growth of hepatocarcinoma cells in suspension were detected by using Poly-HEMA coated plate in which cell grew in an anchorage-independent manner [1, 18]. Human hepatocarcinoma cell line Bel-7402 and murine hepatocarcinoma cell line Hepa 1–6 from different species were used as model cells. The results showed that PCE, at a concentration of 50–800 μg/mL, significantly inhibited proliferation of Bel-7402 cells and Hepa 1–6 cells in suspension in a dose- and time-dependent manner (Figure 1) ($P < 0.05$).

3.2. PCE Inhibited Colony Formation of Hepatocarcinoma Cells in Soft Agar. Long-term effects of PCE on anchorage-independent cell growth were further investigated in soft agar colony formation assays. As shown in Figure 2, colony formation of Bel-7402 cells and Heap 1–6 cells was significantly inhibited by PCE treatment in a dose-dependent manner ($P < 0.01$). At higher dose (800 μg/mL) of PCE treatment, the inhibition of colony formation in Bel-7402 cells and Heap 1–6 cells was 98.60% and 99.07%, respectively.

3.3. PCE Induced Anoikis in Hepatocarcinoma Cells. EthD-1, a red fluorescent dye, was used to detect cell death in suspension culture. As shown in Figures 3(a) and 3(b), after PCE treatment, EthD-1 was absorbed by Bel-7402 cells yielding a red-fluorescent nuclear staining. In addition, the photodensity of the red fluorescence between PCE, in different dosage and control groups, showed a significant difference ($P < 0.01$). Flow cytometry analysis was used to further discriminate necrosis and apoptosis. As shown in Figure 3(c), PCE significantly induced apoptosis in Bel-7402 cells and Hepa 1–6 cells in suspension culture ($P < 0.05$). These results suggested PCE may induce anoikis in hepatocarcinoma cells.

3.4. PCE Activated Caspases in Bel-7402 Cells. Activation of caspases (cysteine aspartate-specific proteinase) has been recognized as hallmarks of apoptosis. Anoikis, a type of apoptosis in suspension, is also executed by caspases cascade [2, 19]. To determine whether caspases attributed to PCE-induced apoptosis in Bel-7402 cells during suspension growth, activities of caspase-3, 8, and 9 were detected. As

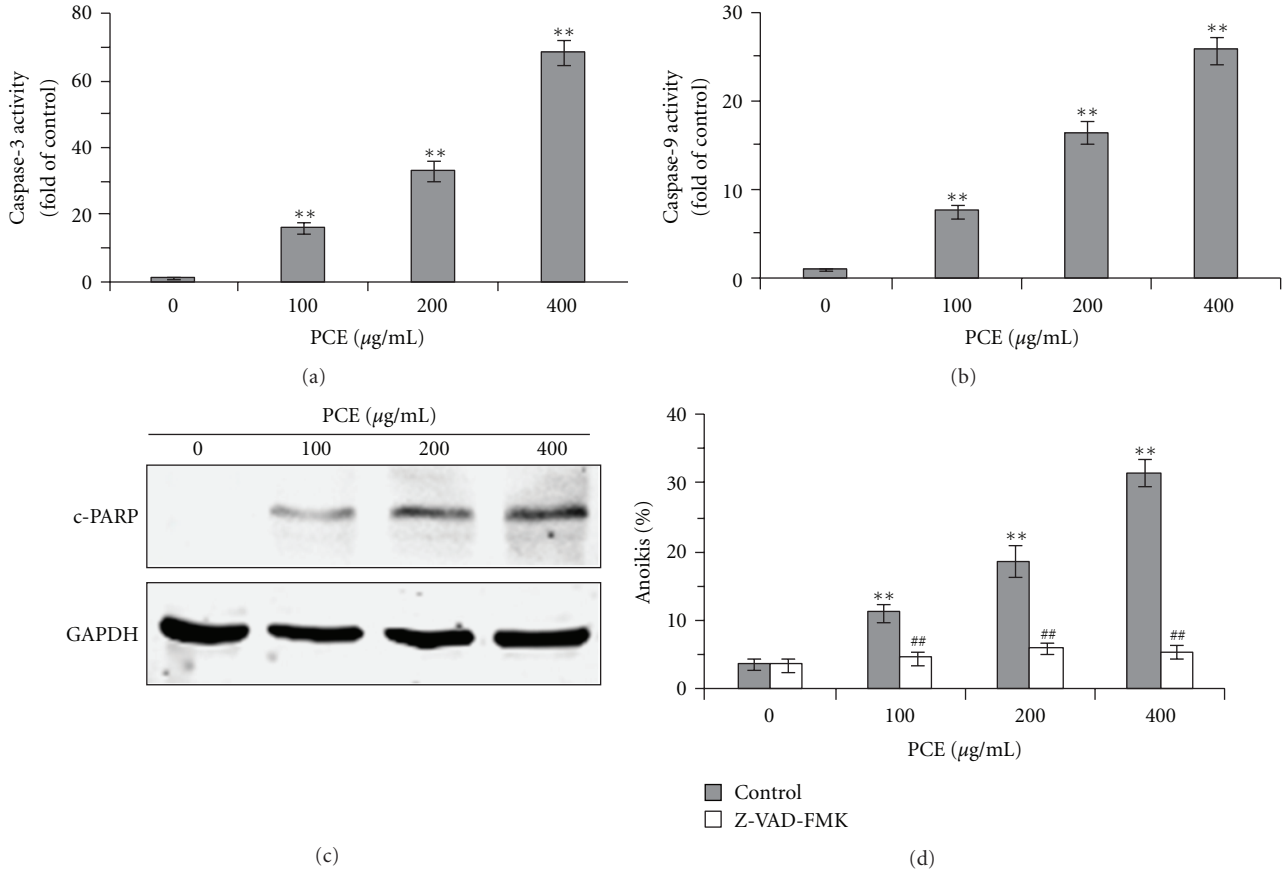

Figure 4: PCE-activated caspases in Bel-7402 cells. After 24 h PCE (100–400 μg/mL) treatment, caspase-3 (a) and caspase-9 (b) activity in suspension-cultured Bel-7402 cells were detected as described in Materials and Methods Section. Caspases activities were expressed as fold activation over control. (c) Cleaved PARP (c-PARP) was detected by Western blotting with specific antibody. GAPDH was used as a loading control. (d) Suspension-cultured Bel-7402 cells were pretreated with Z-VAD-FMK (50 μmol/L) for 2 h before treatment with PCE for 24 h, stained with Annexin V-FITC/PI, and analyzed by flow cytometry. Data presented are from three separate experiments. $^{**}P < 0.01$, versus control group; $^{\#\#}P < 0.01$, Z-VAD-FMK groups versus corresponding dose of PCE-treated (control) group.

shown in Figures 4(a) and 4(b), PCE activated caspase-3 and 9 in a dose-dependent manner and was compared with controls ($P < 0.01$). However, activity of caspase-8 was not significantly changed after PCE treatment (data not shown). In addition, PARP, one of the earliest substrates of caspase-3 during apoptosis [20], was also cleavaged after PCE treatment (Figure 4(c)). Furthermore, Z-VAD-FMK, a pan caspases inhibitor, significantly inhibited PCE-induced anoikis in Bel-7402 cells ($P < 0.01$) (Figure 4(d)), which indicated that the anoikis-inducing effect of PCE was dependent on caspases activation.

3.5. PCE Upregulated ROS Level in Bel-7402 Cells. It has been reported ROS is an important mediator of anoikis and related to caspases activation [21]. Natural products, such as emodin and curcumin, may sensitize cancer cell to anoikis through ROS generation [14, 22]. So we further tested the effects of PCE on ROS production. Using ROS sensitive fluorescent probe, we found PCE treatment induced intracellular ROS production in Bel-7402 cells in a dose-dependent manner and was compared with controls ($P < 0.05$) (Figure 5(a)). NAC, an ROS scavenger, partially but significantly abrogated PCE-induced anoikis in Bel-7402 cells ($P < 0.05$) (Figure 5(b)). Furthermore, PCE-induced activation of caspase-3 and caspase-9 was significantly reduced by NAC pretreatment ($P < 0.05$) (Figures 5(c) and 5(d)). These results suggested PCE inducing caspases activation and anoikis is associated with ROS generation.

3.6. PCE Downregulated FAK in Bel-7402 Cells. In cancer cell, detachment from the extracellular matrix may activate FAK and resistance to anoikis [23]. We also evaluated the effects of PCE on FAK expression. As shown in Figure 6(a), low level of expression and phosphorylation of FAK was detected in Bel-7402 cells. Upon detachment from adherence, expression and phosphorylation of FAK were upregulated. After PCE treatment, expression and phosphorylation of FAK were significantly decreased in dose-dependent manner. To examine whether downregulation of FAK contributes to PCE-induced anoikis, a recombinant eukaryotic expression plasmid encoding full length of human FAK (re-FAK) was transfected to Bel-7402 cells. As shown in Figure 6(b), FAK

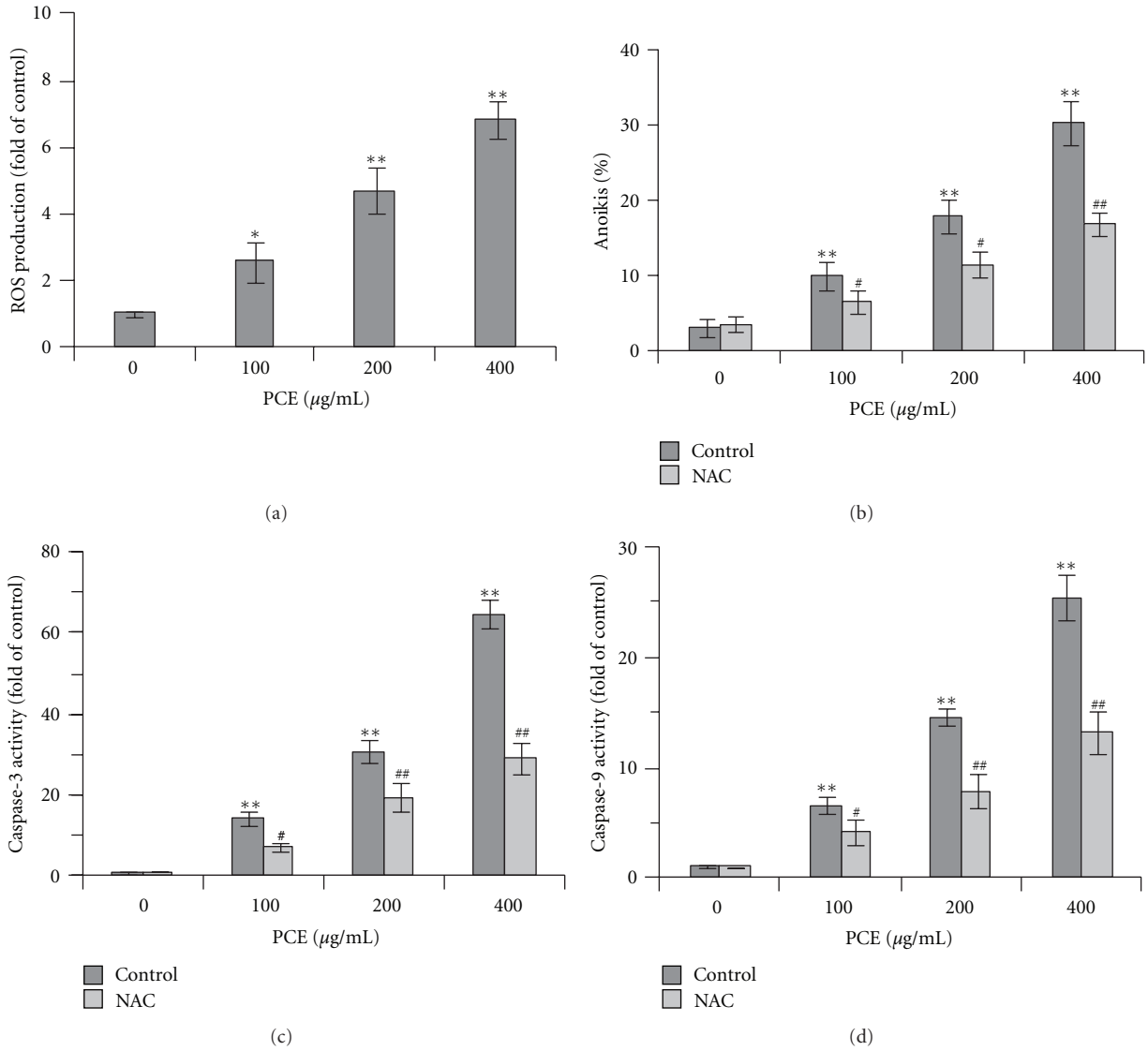

Figure 5: PCE upregulated ROS level in Bel-7402 cells. (a) After 24 h PCE (100–400 μg/mL) treatment, intracellular ROS production in suspension-cultured Bel-7402 cells was detected as described in Materials and Methods section. ROS level was expressed as fold activation over control. For ROS inhibition, suspension-cultured Bel-7402 cells were pretreated with NAC (50 mmol/L for 2 h), followed by PCE (100–400 μg/mL) treatment for 24 h, and subjected to apoptosis (b), caspase-3 (c), and caspase-9 (d) activity assay. Caspases activities were expressed as fold activation over control. Data shown are representative of three independent experiments. $^{*}P < 0.05$, versus control group; $^{**}P < 0.01$, versus control group; $^{\#}P < 0.05$, NAC groups versus corresponding dose of PCE-treated (control) group; $^{\#\#}P < 0.01$, NAC groups versus corresponding dose of PCE-treated (control) group.

was overexpressed in re-FAK transfected Bel-7402 cells. FAK over-expression partially but significantly abrogated PCE-induced anoikis and was compared with controls ($P < 0.05$). These observations suggested FAK downregulation contributed to PCE-induced anoikis.

3.7. The Relation between PCE-Induced ROS and FAK Downregulation. To elucidate the relation between PCE-induced ROS and FAK downregulation, FAK overexpressed Bel-7402 cells were treated with PCE and subjected to ROS detection. The result showed PCE-elicited ROS production was not changed upon FAK over-expression (Figure 7(a)). Furthermore, abrogation of ROS production by NAC pretreatment has no effect on PCE-induced FAK downregulation (Figure 7(b)). These observations suggested ROS production and FAK downregulation were independent events in PCE-induced anoikis in Bel-7402 cells.

4. Discussion

Poly-HEMA, a nontoxic polymer of 2-hydroxyethyl methacrylate, was extensively utilized to deprive anchorage *in vitro* because of its ability to reduce tissue culture plastic adhesivity

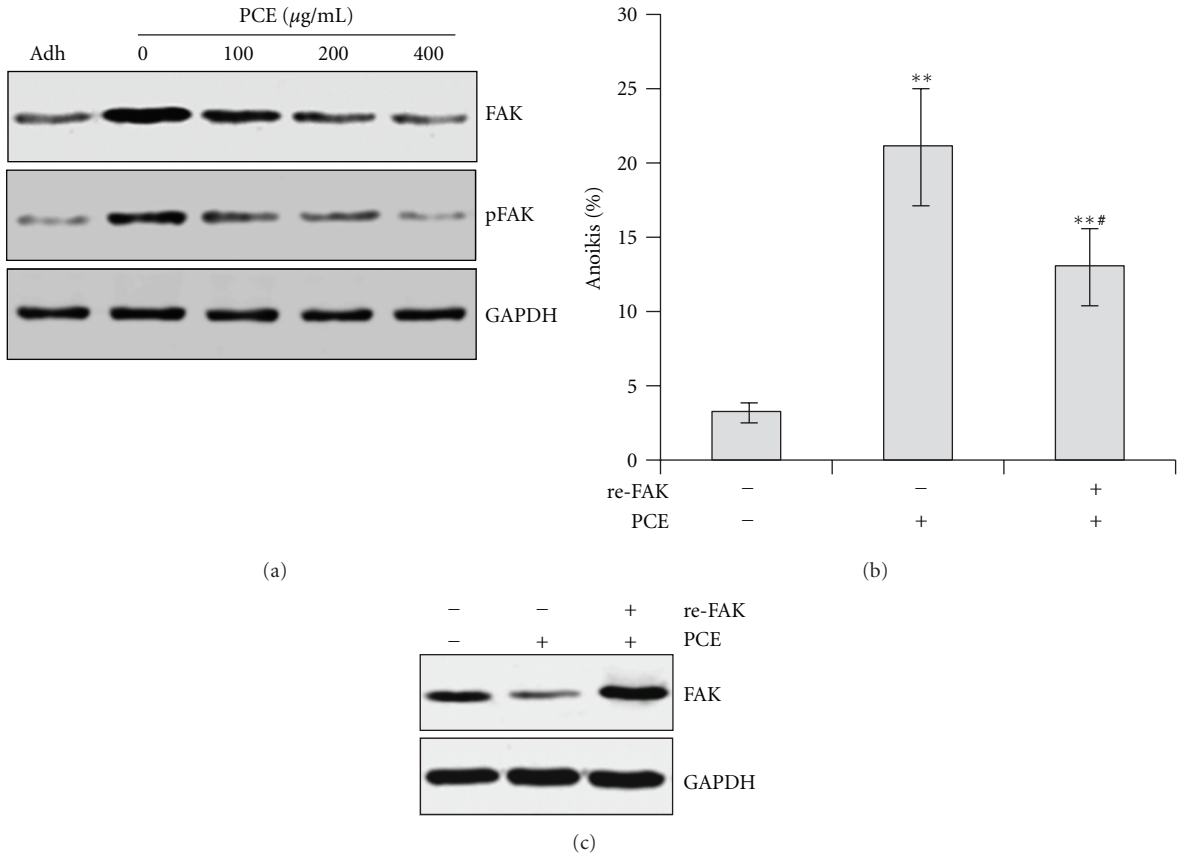

Figure 6: PCE inhibited FAK expression in Bel-7402 cells. (a) Adherent growth Bel-7402 cells (Adh) and PCE-treated suspension-cultured Bel-7402 cells were collected and subjected to western blots using antibody against FAK and Phospho-FAK (pFAK). (b) Bel-7402 cells were transfected with recombinant human FAK and empty vector and subjected to suspension-culture, PCE (200 μg/mL) treatment and anoikis assay. At the same time, FAK expression was determined by western blot (c). GAPDH was used as a loading control. $^{**}P < 0.01$, versus control group; $^{\#}P < 0.05$, re-FAK groups versus PCE-treated empty vector group.

[1, 18, 24]. Our present results showed that PCE can inhibit Bel-7402 cells and Heap 1–6 cells proliferation in the Poly-HEMA mimicked detachment from the extracellular matrix. Soft agar colony formation assay further demonstrated that PCE significantly inhibited colony forming capacity of Bel-7402 cells and Heap 1–6 cells in soft agar without adherence. These observations suggested PCE could inhibit Bel-7402 cells and Heap 1–6 cells growth in suspension.

EthD-1 is a high-affinity nucleic acid fluorescent dye, which can only penetrate cells once the membrane is damaged (a hallmark of dead cells) and produces bright red fluorescence upon binding to nucleic acids, and could be used to detect anoikis. In present study, EthD-1 staining indicated that partial Bel-7402 cells absorbed EthD-1 and emitted red fluorescence after PCE treatment. In addition, PI/Annexin V staining and flow cytometric assays further confirmed PCE may induce apoptosis in suspension-cultured hepatocarcinoma cells. These observations suggest that PCE may induce anoikis in Bel-7402 cells.

Similar to classic apoptosis, anoikis is also executed by intracellular caspases that are activated during the onset of apoptosis by extrinsic and intrinsic pathways [2, 4, 19]. The extrinsic pathway involves oligomerization of cell-surface death receptors by their ligands, resulting in recruitment and activation of caspase-8 followed by activation of executioner caspases-3. The intrinsic pathway involves the signals to mitochondria, which lead to the release of cytochrome c, Apaf-1, forming an apoptosome that activates the initiating protease caspase-9 which in turn activates executioner caspases-3, causing the cell to undergo apoptosis. The present study demonstrated that PCE activated caspase-3 and caspase-9 in Bel-7402 cells. In addition, PARP, one of the earliest substrates of caspase-3 during apoptosis [20], was also cleavaged after PCE treatment. Furthermore, blocking caspases activity completely abrogated PCE-induced anoikis. These results suggest that the anoikis-inducing effects of PCE were related to intrinsic apoptotic pathway.

Many natural products, such as casticin, curcumin, wogonin, Tanshinone IIA, and berberine, cause apoptosis through the mediation of ROS [25–29]. Emodin and curcumin may sensitize cancer cell to anoikis through ROS generation [14, 22]. The present results indicate that the anoikis induced by PCE in hepatocarcinoma cells is triggered by ROS-dependent activation of intrinsic apoptotic pathway.

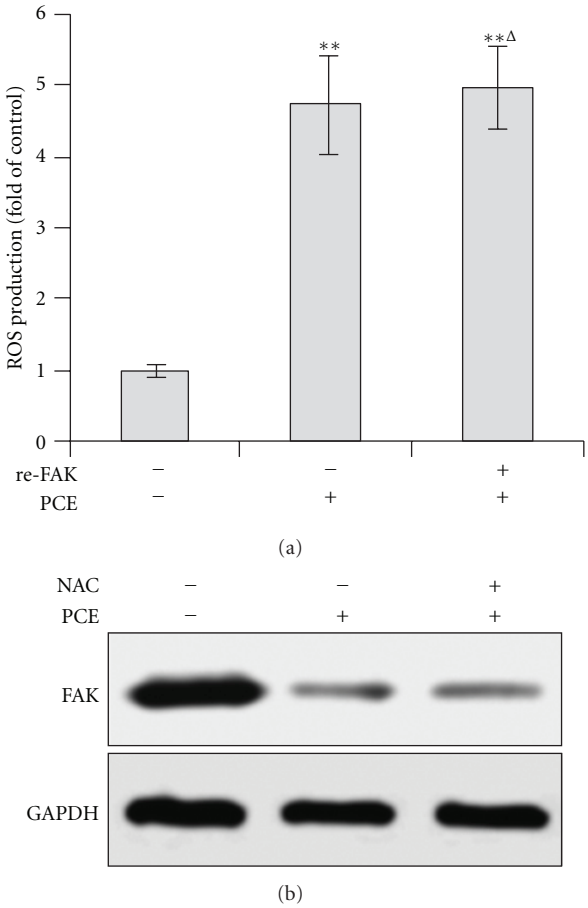

Figure 7: The relation between PCE-induced ROS and FAK downregulation. (a) Bel-7402 cells were transfected with recombinant human FAK and empty vector and subjected to suspension culture, PCE (200 μg/mL) treatment, and ROS assay. (b) NAC pretreated or untreated Bel-7402 cells were treated with PCE (200 μg/mL) and subjected to western blot. $^{**}P < 0.01$, versus control group; $^{\Delta}P > 0.05$, re-FAK group versus PCE-treated empty vector group.

This conclusion is based on the following observations: (1) PCE treatment caused a dose-dependent ROS production in Bel-7402 cells; (2) PCE-induced anoikis was significantly attenuated by ROS scavenger NAC; (3) PCE-induced activation of caspase-3 and caspase-9 was significantly reduced by NAC pretreatment. Since NAC pretreatment only partially abrogated PCE-induced anoikis in Bel-7402 cells, there were other mechanism suggested to participate in PCE-induced anoikis.

Epithelial cells require attachment to the extracellular matrix to provide survival signal. In cancer cell, detachment from the extracellular matrix may activate FAK and resistance to anoikis [23]. Downregulation of FAK may promote cancer cell anoikis [30, 31]. The present results demonstrated that PCE-induced anoikis in Bel-7402 cells in coincidence with FAK downregulation. In addition, FAK over-expression partially abrogated PCE induced anoikis. These observations suggested PCE-induced anoikis may be related to FAK. Nevertheless, further studies are needed to determine the upstream regulator and downstream effectors of FAK.

5. Conclusion

In conclusion, our findings demonstrated PCE may inhibit suspension growth, activate caspases, and induce anoikis in hepatocarcinoma cells and may relate to ROS generation and FAK downregulation. Since anoikis is associated with cancer metastasis and cell survival in blood or lymphatic circulation, the effects of PCE against hepatocarcinoma metastasis and hepatocarcinoma cells in blood or lymphatic circulation are worthy of further study. The present study provides new insight into the application of Chinese herb for hepatocarcinoma treatment that is worthy of further study.

Acknowledgments

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Review Article

Herbal Products: Benefits, Limits, and Applications in Chronic Liver Disease

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Complementary and alternative medicine soughts and encompasses a wide range of approaches; its use begun in ancient China at the time of Xia dynasty and in India during the Vedic period, but thanks to its long-lasting curative effect, easy availability, natural way of healing, and poor side-effects it is gaining importance throughout the world in clinical practice. We conducted a review describing the effects and the limits of using herbal products in chronic liver disease, focusing our attention on those most known, such as quercetin or curcumin. We tried to describe their pharmacokinetics, biological properties, and their beneficial effects (as antioxidant role) in metabolic, alcoholic, and viral hepatitis (considering that oxidative stress is the common pathway of chronic liver diseases of different etiology). The main limit of applicability of CAM comes from the lacking of randomized, placebo-controlled clinical trials giving a real proof of efficacy of those products, so that anecdotal success and personal experience are frequently the driving force for acceptance of CAM in the population.

1. Introduction

Complementary and alternative medicine (CAM) therapies sought and encompass a wide range of approaches, including two broad categories: exogenous chemicals such as herbal supplements, vitamins, or plant extract, and natural or self-therapies (NST) techniques including relaxation, meditation, prayer, hypnosis, biofeedback, or physical strengthening [1].

The use of herbal medicine began in ancient China at the time of Xia dynasty and in India during the Vedic period [2]. With the revolution of the natural sciences and evidence-based medicine, the divide between Western and Eastern medicines appeared to widen, with CAM reaching an increasing popularity in western countries through years (from 34% of the population in 1990 to 48% in 2004) [3].

The age-old system of herbal medicine is being revived by day-to-day practice for its long-lasting curative effect, easy availability, natural way of healing, and less side-effects, so that today herbal medicines are gaining importance and expanding throughout the world [4].

The widespread use of CAM is emphasized among people with chronic disease, since it promotes greater personal control over health decision, empowers people to manage their chronic condition, and helps to avoid dissatisfaction often associated with conventional health care [5].

CAM is believed to be safer and better than standard medical practice because they are "natural" or are based on a religious, philosophical or a strongly felt concept of "wellness" and health. Treatments with herbal medicine concentrate on reestablishing or reinforcing natural healing processes and wellness [6].

Despite increasing popularity, communication about the use of CAM between physicians and patients is limited: most physicians know little about CAM and patients avoid discussing CAM because they fear being received with indifference [7].

Moreover, physicians used to focus attention on potential toxicities, even though identification of toxicity from herbal preparations is often difficult, because patients generally self-medicate with these and may withhold this information.

Toxic hepatitis is the most common adverse reaction resulting from the use of CAM [8], often associated with the concomitant consumption of hepatotoxic ingredients such as acetaminophen and nonsteroidal anti-inflammatory agents or with hepatotoxicity of herbal ingredients themselves [9].

Physicians and health care providers need to become familiar with these products and to recognize potential interaction between conventional drugs and herbals, considering their actual diffusion [10].

Botanical medicines have been used traditionally by herbalists and indigenous healers worldwide for the prevention and treatment of liver disease. Clinical research in this century has confirmed the efficacy of several plants in the treatment of liver disease, so the fact that the patients with chronic liver disease seek primary or adjunctive herbal treatment is not surprising.

Particularly, silymarin (an extract of milk thistle) is the most popular product taken by subjects with liver disease and especially by those with hepatitis C virus infection [11]. Seeff et al. [12] found that 41% of outpatients with diagnosis of liver disease had used some form of CAM. Herbal products are often used to improve well-being and quality of life [13] and to ameliorate side effects in patients on antiviral treatment, as fatigue, irritability, and depression: lessening of these symptoms might permit a higher compliance and avoid the need to limit the dose and finally withdraw inter-feron.

A systematic review about the use of CAM in chronic hepatitis C has been conducted by Coon and Ernst [14], The authors cited fourteen randomized clinical trials considering the combined use of herbal products and interferon-alfa during antiviral treatment. Although difficulty in extrapolation and interpretation of results because of different methodological limits of the considered studies, the authors found that several herbal products and supplements (vitamin E, thymic extract, zinc, traditional Chinese medicine, Glycyrrhiza glabra, and oxymatrine) could exert potential virological and biochemical effects in the treatment of chronic hepatitis C infection, as a greater clearance of HCV-RNA and normalization of liver enzymes.

As shown in various studies [15, 16], the use of CAM could be predicted by social, cultural, and geographic factors: sex, age, higher education level, or marriage status of patients are associated with a different use of herbal products.

The aim of this study is to describe the potential role, benefits, and limits of some of known widespread herbal products in chronic liver disease. We conducted an updated research on Pubmed and Medline in order to refer to more recent articles about this issue.

2. Quercetin

2.1. Definition, Pharmacokinetics, and Biological Aspects. Quercetin is one of the major flavonoids, which represent a class of naturally occurring polyphenolic compounds, ubiquitously present in photosynthesising cells. The intake of flavones and flavonols is determined as 23-24 mg/day and quercetin, the main flavonol present in our diet, represents 70% of this intake. Quercetin is found in fruits (apple) and vegetables, especially onions [17].

Various ways of supplementing quercetin are possible, including a pure supplement or a diet intervention using a food component with a high quercetin content. Supplement usually contains only the aglycon form of quercetin, whereas a food component normally comprises high amounts of various quercetin derivatives that might have a better biological availability than the aglycon itself. Another advantage of a dietary supplementation versus a "conventional" supplement might be a better compliance, especially in long-term use [18].

The absorption of quercetin is considerably enhanced by its conjugation with a sugar group. After their facilitated uptake by means of carrier-mediated transport, quercetin glycosides often become hydrolysed by intracellular β-glucosidases. After absorption, quercetin becomes metabolized in various organs including the small intestine, colon, liver, and kidney. Metabolites formed in the small intestine and liver are mainly the result of phase II metabolism by biotransformation enzymes and, therefore, include the methylated, sulphated, and glucuronidated forms. Moreover, bacterial ring fission of the aglycon occurs in both small intestine and colon, resulting in the breakdown of the backbone structure of quercetin and the subsequent formation of smaller phenolics [19].

Quercetin appears to have many beneficial effects on human health, including cardiovascular protection, anticancer activity, antiulcer and antiallergy activity, cataract prevention, and anti-inflammatory effects. Quercetin has been shown to be an excellent *in vitro* antioxidant. Within the flavonoid family, it is the most potent scavenger of ROS (reactive oxygen species), including O_2 [20].

2.2. Quercetin and Alcoholic Liver Disease (ALD). The pathogenesis and progression of ALD are associated with free radical injury and oxidative stress, which could be partially attenuated by antioxidants and free radical scavengers. Lipid metabolism disorder and oxidative stress play an important role on the development and progression of ALD, and mitochondria compartment is presumed to be the main source and susceptible target of intracellular ROS. The hypothesis that quercetin could prevent ethanol-induced oxidative damage in hepatocytes has been investigated.

In animal studies, prophylaxis with quercetin-ameliorated ethanol-stimulated mitochondrial dysfunction manifested by decreased membrane potential and by induced permeability transition though suppressing glutathione depletion, enzymatic inactivation of manganese superoxide dismutase, and glutathione peroxidase, ROS overgeneration, and lipid peroxidation in mitochondria. Quercetin, thus, may protect rat, especially hepatic mitochondria, from chronic ethanol toxicity through its hypolipidemic effect and antioxidative role, highlighting a promising preventive strategy for ALD by naturally occurring phytochemicals [21, 22].

Quercetin tends to downregulate the ethanol-induced expression of glutathionine peroxidase 4 (GPX4). Furthermore, it tends to reduce the expression of SOD2 induced by ethanol, to downregulate the expression of Gadd45b at the presence of ethanol, which could permit to explain DNA demethylation associated with the upregulation of gene expression in experimental ALD [23].

Another study evaluated the effect of quercetin on the parameters classically associated with alcohol liver injury, as lactate dehydrogenase (LDH), aspartate transaminase (AST), malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) in order to address the alterations of cell damage and antioxidant state after quercetin intervention; the ethanol-intoxicated (100 mM for 8 h) rat primary hepatocytes were simultaneously treated, pretreated (2 h) and posttreated (2 h) with quercetin. The toxic insult of ethanol on hepatocytes was challenged by quercetin and biochemical parameters almost returned to the level of control group when hepatocytes were treated with quercetin at the dose of 50muM for 2–4 h before ethanol exposure [24].

A recent study elucidates also a neuroprotective effect of quercetin in alcohol-induced neuropathy through modulation of membrane-bound inorganic phosphate enzyme and inhibition of release of oxidoinflammatory mediators, such as malondialdehyde (MDA), myeloperoxidase (MPO) MPO, and nitric oxide (NO) [25].

In conclusion, pretreatment with quercetin provided protection against ethanol-induced oxidative stress in hepatocytes and may be used as a new natural drug for the prevention and/or treatment of ALD. Antioxidants significantly reduced the oxidative stress induced by ethanol intoxication, increased membrane integrity, and also increased organ regeneration [26].

2.3. Quercetin and Nonalcoholic Fatty Liver Disease (Nafld). Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States. It represents the hepatic manifestation of metabolic derangements, known as metabolic syndrome, with insulin resistance playing the major role. Nonalcoholic fatty liver disease includes a variety of histological conditions (ranging from liver steatosis and steatohepatitis, to fibrosis and hepatocarcinoma), all characterized by an increased accumulation/deposition of fat within the liver and associated with alterations in hepatic and systemic inflammatory state. Hepatocytes of a primary cell culture that are exposed to high glucose, insulin, and linoleic acid concentration respond with lipid accumulation, oxidative stress up to cell death.

Regarding the role of quercetin in NAFLD, it has been show that in mice fed with a Western diet chronic dietary intake of quercetin reduces liver fat accumulation and improves systemic parameters related to metabolic syndrome, probably mainly through decreasing oxidative stress and reducing expression of genes related to steatosis (as PPARα) [27].

Another study was aimed to examine the hypoglycemic and insulin-sensitizing capacity of onion peel extract (OPE, containing a high content of quercetin) in high fat diet/streptozotocin-induced diabetic rats and to elucidate the mechanism of its insulin-sensitizing effect. OPE might improve glucose response and insulin resistance associated with type-2 diabetes by alleviating metabolic dysregulation of free fatty acids, by suppressing oxidative stress, upregulating glucose uptake at peripheral tissues, and/or downregulating inflammatory gene expression in liver. Moreover, in most cases, OPE showed greater potency than pure quercetin equivalent. These findings provide a basis for the use of onion peel to improve insulin insensitivity in type-2 diabetes [28].

In hepatocytes from normal rats, the decrease in de novo fatty acid and TAG synthesis induced by quercetin represent a potential mechanism contributing to the reported hypotriacylglycerolemic effect of this agent [29].

The hepatic response to chronic noxious stimuli may lead to liver fibrosis and to preneoplastic cirrhotic liver. Fibrogenic cells activate in response to a variety of cytokines, growth factors, and inflammatory mediators. The involvement of members of the epidermal growth factor family in this process has been suggested. Amphiregulin is an epidermal growth factor receptor (EGFR) ligand, specifically induced upon liver injury. Recent study investigated the effects of quercetin on the amphiregulin/EGFR signal and on the activation of downstream pathways leading to cell growth: quercetin-ameliorated activation of survival pathways and downregulated the expression of genes related to inflammation and precancerous conditions. Suppression of amphiregulin/EGFR signals may contribute to this effect [30].

2.4. Quercetin and Hepatitis C Virus (HCV). Phytochemicals exert antiviral activity and may play a potential therapeutic role in hepatitis C virus (HCV) infection: these aspects were investigated by several studies.

Park et al. [31] investigated about the antiviral activity in HCV-infected patients of derivates of 7-*O*-arylmethylquercetins. Only five quercetin derivatives showed selective antiviral activity in HCV replicon cell-based assay.

Recent study studied the quercetin as a potential nontoxic anti-HCV agent in reducing viral production by inhibiting both NS3 and heat shock proteins (that are essential for HCV replication). It was found that quercetin inhibit NS3 activity in a specific dose-dependent manner *in vitro* catalysis assay. Moreover, as analyzed in the subgenomic HCV RNA replicon system, quercetin seemed to exert adjunctive effect: to inhibit HCV RNA replication and production in the HCV infectious cell culture system (HCVcc), as analyzed by the focus-forming unit reduction assay and HCV RNA real-time PCR. The inhibitory effect of quercetin was also obtained when using a model system in which NS3-engineered substrates were introduced in NS3-expressing cells, providing evidence that inhibition *in vivo* could be directed to NS3 and does not involve other HCV proteins. This work demonstrates that quercetin has a direct inhibitory effect on the HCV NS3 protease [32].

2.5. Quercetin and Hepatitis B Virus (HBV). Few recent paper discuss about the effects of quercetin on HBV replication and the importance of a glucoside in the structure of quercetin derivatives to exercise a biological action against HBV replication.

Thirteen flavone glucosides from the herb of Euphorbia humifusa were isolated. Among them, five compounds including apigenin-7-O-β-D-glucopyranoside (2), apigenin-7-O-(6′′-O-galloyl)-β-D-glucopyranoside (3), luteolin-7-O-β-D-glucopyranoside (7), luteolin-7-O-(6′′-O-trans-feruloyl)-β-D-glucopyranoside (8), and luteolin-7-O-(6′′-O-coumaroyl)-β-D-glucopyranoside (9) showed anti-HBV activity *in vitro*. Anti-HBV activity was closely related to the parent structure of these compounds (agigenin > luteolin > quercetin) as well as to the number of glucoside (flavone monoglucoside > flavone diglucoside). The structure of these agent also influences their cytotoxicity (flavone > flavone monoglucoside > flavone diglucoside). In addition, the substitution of acyl group on glucoside may be important to keep their anti-HBV activities (galloyl > feruloyl > coumaroyl) [33]. Quercetin did not show activities against HBeAg secretion: this limit might be due to the absence of the saccharide group in their structures [34].

3. Curcumin

3.1. Definition, Pharmacokinetics, and Biological Aspects. Curcumin is a low-molecular-weight polyphenol derived from the rhizomes of turmeric (curcuma longa). It represents a yellow pigment widely used as a coloring agent and spice in many foods. It has various beneficial pharmacological effects including antioxidant, anti-inflammatory, anticarcinogenic, hypocholesterolemic, antibacterial, antispasmodic, anticoagulant, and hepatoprotective activities [35].

Phase I clinical trials have shown that curcumin is safe even at high doses (12 g/day) in humans but exhibit poor bioavailability. Despite the promising biological effects of curcumin, low plasma and tissue levels of curcumin due to its poor oral bioavailability and absorption, rapid metabolism and systemic elimination in both rodents and humans [36] may be responsible for the unfavorable pharmacokinetic of this molecule. To improve the bioavailability of curcumin, numerous approaches have been undertaken. These approaches involve, first, the use of adjuvant like piperine that interferes with glucuronidation; second, the use of liposomal curcumin; third, curcumin nanoparticles; fourth, the use of curcumin phospholipid complex; fifth, the use of structural analogues of curcumin (e.g., EF-24) [35, 36].

3.2. Curcumin and Alcohol Liver Disease (ALD). Liver fi-brosis can be explained with an increased deposition of extracellular matrix (ECM). Chronic alcohol abuse is one of the main causes of liver fibrosis. Ingestion of polyunsaturated fatty acids (PUFAs) together with alcohol can aggravate the toxicity of alcohol. The degree of abnormal ECM degradation depends on the ratio of active matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). A recent work studied the influence of bis-desmethoxy curcumin analog (BDMC-A) on the expression of MMPs and TIMPs during alcohol and DeltaPUFA-induced liver toxicity. Administration of BDMC-A significantly decreased the levels of collagen and TIMPs and positively modulated the expression of MMPs. From this study, we can conclude that BDMC-A influences MMPs, TIMPs expression and that it acts as an efficient anti-fibrotic agent [37].

It has been demonstrated the potential protective effect of curcumin pretreatment against ethanol-induced hepatocytes oxidative damage, with emphasis on heme oxygenase-1 (HO-1) induction. Ethanol exposure resulted in a sustained malondialdehyde (MDA) elevation, glutathione (GSH) depletion, and evident release of cellular lactate dehydrogenase (LDH) and aspartate aminotransferase (AST), which was significantly ameliorated by curcumin pretreatment. In addition, dose- and time-dependent induction of HO-1 was involved in such hepatoprotective effects by curcumin. Curcumin exerts hepatoprotective properties against ethanol involving HO-1 induction, which provide new insights into the pharmacological targets of curcumin in the prevention of alcoholic liver disease [38].

To study the mechanism of curcumin-attenuated inflammation and liver pathology in early stage of alcoholic liver disease, female Sprague-Dawley rats were divided into four groups and treated with ethanol or curcumin via an intragastric tube for 4 weeks. A control group was treated with distilled water and an ethanol group was treated with ethanol (7.5 g/kg bw). Treatment groups were fed with ethanol supplemented with curcumin (400 or 1 200 mg/kg bw). The liver histopathology in ethanol group revealed mild-to-moderate steatosis and mild necroinflammation. Hepatic MDA, hepatocyte apoptosis, and NF-kappaB activation increased significantly in ethanol-treated group when compared with control. Curcumin treatments resulted in improving of liver pathology, decreasing the elevation of hepatic MDA, and inhibition of NF-kappaB activation. The 400 mg/kg bw of curcumin treatment also revealed a trend of decreased hepatocyte apoptosis. However, the results of SOD activity, PPARgamma protein expression showed no difference among the groups. In conclusion, curcumin improved liver histopathology in early stage of ethanol-induced liver injury by reduction of oxidative stress and inhibition of NF-kappaB activation [39].

3.3. Curcumin and Nafld. In animal study, it has been demonstrated the capacity of curcumin to improve liver histology of NAFLD. The NASH model was induced by high-fat diet combined with carbon tetrachloride. These rats were successively treated with curcumin and curcumin derivative. The results showed a remarkable reduction in serum ALT (U/L), AST (U/L) in rats treated with curcumin derivatives. The degrees of fibrosis were also significantly alleviated. The reduction of the gene transcriptions of TNF-alpha, NF-kappaB, and HMG-CoA was the mechanism proposed by wich curcumin exercise its beneficial effects in NASH.

The results of this study indicate, moreover, that the water-soluble curcumin derivative displays superior bioavailability to the parent curcumin, which can effectively improve the lipid metabolism and delay the progression of hepatic fibrosis in rats with steatohepatitis [40].

Interestingly, it also demonstrates that the activating effect of LDL can be reversed by curcumin. The hypocholesterolemic action of curcumin was reported by numerous studies: curcumin seems to reduce serum cholesterol concentrations by increasing hepatic expression of LDL receptors, by blocking LDL oxidation, increasing bile acid secretion, and faecal excretion of cholesterol, repressing the expression of genes involved in cholesterol biosynthesis and protecting against liver injury and fibrogenesis [41, 42]. Curcumin induces apoptosis and blocks proliferation of hepatic stellate cells (HSCs), and, via activation of PPARγ, inhibits extracellular matrix formation, downregulates the expression of LDL receptors, induces *SREBP-1c*,and increases the fat-storing capacity of HSC, and it may thereby restore this "protective" functionality, proving of therapeutic usage in preventing liver steatosis and fibrosis [43].

An Italian study tested whether the administration of curcumin limits fibrogenic evolution in a murine model of nonalcoholic steatohepatitis. They demonstrated that curcumin decreased the intrahepatic gene expression of monocyte chemoattractant protein-1, CD11b, procollagen type I, and tissue inhibitor of metalloprotease (TIMP)-1, together with protein levels of alpha-smooth muscle actin, a marker of fibrogenic cells. In addition, curcumin reduced the generation of reactive oxygen species in cultured HSCs and inhibited the secretion of TIMP-1 both in basal conditions and after the induction of oxidative stress. This study so proposed that curcumin administration effectively limits the development and progression of fibrosis in mice with experimental steatohepatitis and reduces TIMP-1 secretion and oxidative stress in cultured stellate cells [44].

High consumption of dietary fructose is an important contributory factor in the development of hepatic steatosis in insulin or leptin resistance. The effects of curcumin on fructose-induced hypertriglyceridemia and liver steatosis and its preventive mechanisms in rats have been investigated. Curcumin reduced serum insulin and leptin levels in fructose-fed rats and it protects against fructose-induced hypertriglyceridemia and hepatic steatosis by inhibition of PTP1B (hepatic protein tyrosine phosphatase 1B) and subsequent improvement of insulin and leptin sensitivity in the liver of rats. This PTP1B inhibitory property may represent a promising role for curcumin to treat fructose-induced hepatic steatosis induced by hepatic insulin and leptin resistance [45, 46].

3.4. Curcumin and HCV. Curcumin is known to exert antiviral activity against influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus [47, 48]. However, it remains to be determined whether curcumin can inhibit the replication of hepatitis C virus (HCV). A study showed that curcumin decreases HCV gene expression via suppression of the Akt-SREBP-1 activation, not by NF-kappaB pathway. The combination of curcumin and IFN-alpha exerted profound inhibitory effects on HCV replication. These results indicate that curcumin can suppress HCV replication *in vitro* and may be potentially useful as novel anti-HCV reagents [49].

3.5. Curcumin and HBV. Hepatitis B virus (HBV) infects the liver and uses its cell host for gene expression and propagation. Therefore, since targeting host factors is essential for HBV gene expression, it could represent a potential anti-viral strategy.

Curcumin treatment could complement the antiviral activity of the nucleotide/nucleoside analogues, which are considered as the gold standards for anti-HBV therapy. The combination of Lamivudine and curcumin treatments resulted in an enhanced suppression of HBV expression by up to 75%, as compared to nontreated cells. These results suggest that curcumin may work synergistically with the current anti-HBV nucleotide/nucleoside analogous, and that this combination may result in a better suppression of HBV. Moreover, curcumin inhibits HBV gene expression and replication, by downregulating PGC-1alpha, a starvation-induced protein that initiates the gluconeogenesis cascade and that has been shown to robustly coactivate HBV transcription [50, 51].

4. Silymarin and Its Derivates

4.1. Definition, Pharmacokinetics, and Biological Aspects. Silybum marianum, also known as milk thistle, is a member of Asteraceae family and is well recognized as a hepatoprotective herbal medicine. Silymarin is a lipophilic extract of the milk thistle seeds. It is composed of three isomers of flavonolignans (silybin, silydianin, and silychristin), and two flavonoids (taxifolin and quercetin).

Silymarin has revealed poor absorption, rapid metabolism, and ultimately poor oral bioavailability. For optimum silymarin bioavailability, issues of solubility, permeability, metabolism, and excretion must be addressed. An array of methods have been described in recent years that can improve its bioavailability, including complexation with β-cyclodextrins, solid dispersion method, formation of microparticles and nanoparticles, self-microemulsifying drug delivery systems, micelles, liposomes, and phytosomes [52].

Silymarin possesses various pharmacological activities, including hepatoprotective, antioxidant, anti-inflammatory, anticancer, and cardioprotective effects. *Silybum marianum* is the most well-researched plant in the treatment of liver diseases. Silymarin has been shown to have a variety of anti-inflammatory effects on liver, including mast cell stabilization, inhibition of neutrophil migration, and Kupffer cell inhibition [53]. Silymarin is commonly prescribed in cases of cirrhosis or viral hepatitis.

4.2. Sylimarin and Its Derivates in ALD. Hepatocyte models were proposed as a platform for screening of herbal

component against ethanol hepatotoxicity. Nanosilibinin, for the first time, found to perform significant protection against ethanol-induced hepatotoxicity while silibinin in normal particles could not inhibit such toxicity. This protection of nanosilibinin might be related to its high bioavailability compared to normal insoluble silibinin and could act as an antioxidative and antisteatosis agent against ethanol-induced hepatotoxicity [54].

The affect of silymarin on the levels of serum ALT and GGT in ethanol-induced hepatotoxicity in albino rats has also been tested. Eighteen male albino rats aged 6–8 weeks, weighing 150–200 gm, were divided into 3 groups of 6 rats each: group A as control, group B as rats taking ethanol at a dose of 0.6 mL (0.5 gm)/100 gm/day, and group C taking ethanol and silymarin at a dose of 0.5 gm/100 gm/day and 20 mg/100 gm/day, respectively, for 8 weeks. Silymarin tends to normalize liver function test in alcoholic liver disease [55].

Acute ethanol administration caused prominent hepatic microvesicular steatosis with mild necrosis and an elevation of serum ALT activity, induced a significant decrease in hepatic glutathione in conjunction with enhanced lipid peroxidation (oxidative stress) and increased hepatic TNF (necrosis factor-alpha) production. Supplementation with a standardized silymarin with its both antioxidant and anti-inflammation properties decreases TNF production and attenuated these adverse changes induced by acute ethanol administration. In view of its nontoxic nature, it may be developed as an effective therapeutic agent for alcohol-induced liver disease by its antioxidative stress and anti-inflammatory features [56].

4.3. Slymarin and Its Derivate in Nafld. Silymarin showed a significant hypocholesterolemic effect compared to the diet model with high fat-diet (HFD). Moreover, silymarin significantly reduces TG levels compared to HFD group.

The elevation of transaminases usually reflects necrosis of hepatocytes: with silymarin, ALT levels (a specific index of hepatic necrosis) were particularly reduced [57].

Assuming that oxidative stress leads to chronic liver damage, Loguercio et al. conducted a study about the antioxidant activity of silybin conjugated with vitamin E and phospholipids. Eighty-five patients were divided into 2 groups: those affected by nonalcoholic fatty liver disease (group A) and those with HCV-related chronic hepatitis associated with nonalcoholic fatty liver disease (group B), nonresponders to antiviral treatment. The treatment consisted of silybin/vitamin E/phospholipids. After treatment, group A showed a significant reduction in ultrasonographic scores for liver steatosis. Liver enzyme levels, hyperinsulinemia, and indexes of liver fibrosis showed an improvement in treated individuals. A significant correlation among indexes of fibrosis, body mass index, insulinemia, plasma levels of transforming growth factor-beta, tumor necrosis factor-alpha, degree of steatosis, and gamma-glutamyl transpeptidase was observed. Our data suggest that silybin conjugated with vitamin E and phospholipids could be used as a complementary approach in the treatment of patients with chronic liver damage [58].

4.4. Sylimarin and Its Derivates in HCV Infection. Silymarin and its purified flavonolignans have been recently demonstrated to inhibit hepatitis C virus (HCV) infection, both *in vitro* and *in vivo*.

Silymarin showed antiviral effects against hepatitis C virus cell culture (HCVcc) infection that included inhibition of virus entry, RNA and protein expression, and infectious virus production. Silymarin did not block HCVcc binding to cells but inhibited the entry of several viral pseudoparticles (pp), and fusion of HCVpp with liposomes. Silymarin also blocked cell-to-cell spread of virus [59].

Pegylated interferon (PEG-IFN) plus Ribavirin therapy is the current treatment for the patient with chronic hepatitis C. The main goal of therapy is to achieve a sustained virological response (SVR is defined as undetectable HCV-RNA in peripheral blood determined with the most sensitive polymerase chain reaction technique 24 weeks after the end of treatment). This goal is practically equivalent with eradication of HCV infection and cure of the underlying HCV-induced liver disease. This therapy is effective only in half of patients, because of important side-effects, resistance, and high cost related to therapy. Silymarin inhibits both HCV RNA (in a dose-dependent manner) and HCV core expression thanks to its direct effect against HCV 3a core or activation of JAK/STAT pathways, resulting in inhibition of HCV core gene, by phosphorylation of Stat1 on tyrosine and serine [60].

Silymarin but not silibinin inhibited genotype 2a NS5B RNA-dependent RNA polymerase (RdRp) activity at concentrations 5 to 10 times higher than required for anti-HCVcc effects. Furthermore, silymarin had inefficient activity on the genotype 1b. Although inhibition of *in vitro* NS5B polymerase activity is demonstrable, the mechanisms of silymarin's antiviral action appear to include blocking of virus entry and transmission by targeting the host cell [60, 61].

In another study, patients with chronic hepatitis C performing silymarin (650 mg/day) for 6 months improved serum HCV-RNA titer, serum aminotransferases (ALT, AST), hepatic fibrosis, and patient's quality of life. After the treatment, nine patients were found with negative HCV-RNA and statistically significant improvement in results of liver fibrosis markers was found only in fibrosis group.

So, for its antioxidant and anti-inflammatory actions, sylimarin could result useful in reducing hepatic inflammation in chronic liver disease, including HCV-related damage. It has been hypothesized that decreased hepatic inflammation-due to both direct and indirect effects of silymarin in decreasing viral replication has the potential to induce long-term benefit to the infected liver [62].

Since oxidative stress may play a pathogenetic role in chronic hepatitis C, and sustained virological response to antiviral therapy is limited in HCV1 genotype infection, a double-blind study was performed in HCV1 patients treated with pegylated interferon + ribavirin in order to assess the

efficacy of supplementation with the antioxidant flavonoid silymarin. In the silymarin group, a more rapid decrease in the malondialdehyde level as well as a marked decrease in superoxide dismutase and an increase in myeloperoxidase activity after twelve months of treatment were found. In particular, alanine aminotransferase normalized in 6/16 (versus control 9/16) cases and sustained virological response occurred in 3/16 (versus 7/16) patients [63].

4.5. Silymarin and Its Derivates in HBV Infection. Few recent data discuss about the role of silymarin in the hepatitis B. We only reported silymarin beneficial effects in early stages of liver pathogenesis, in preventing and delaying liver carcinogenesis.

This drug should be considered as a potential chemopreventive agent for HBV-related hepatocarcinogenesis [64].

5. Betaine

5.1. Definition, Pharmacokinetics, and Biological Aspects. Betaine is a naturally occurring dietary compound that is also synthesized *in vivo* from choline. *In vivo*, betaine acts as a methyl donor for the conversion of homocysteine to methionine and its also functions as an osmolyte.

5.2. Betaine and ALD. Chronic ethanol exposure has been shown both to decrease hepatic concentrations of *S*-adenosylmethionine (SAM) and plasma concentrations of folate in animal and human studies and to increase plasma concentrations of homocysteine and hepatic levels of *S*-adenosylhomocysteine (SAH) [65].

In the liver, betaine can transfer its methyl group to homocysteine in order to form methionine. This can result in decreased concentrations of homocysteine and increased concentrations of methionine in the liver, resulting in decreased hepatic concentrations of SAH, whereas the latter can increase hepatic SAM concentrations, which leads to an increased SAM : SAH. An elevated SAM : SAH can trigger a cascade of events leading to formation of proper VLDL, to export of triacylglycerol and attenuation of fatty liver. Increased hepatic concentrations of SAM can activate cystathionine-synthase and lead to upregulation of the *trans*-sulfuration pathway, to increase synthesis of glutathione and attenuate oxidative stress. Thus, betaine can ameliorate ALD by attenuating fatty liver, inflammation, and fibrosis [66].

The role of mitochondrial dysfunction in the pathogenesis of alcoholic liver disease has been long documented by multiple laboratories. The dietary supplementation with betaine protects against ethanol-induced loss in oxidative phosphorylation system proteins. Even if the exact mechanism for this protection at the organelle level is not known, betaine shows to preserve the function of the electron transport chain, to maintain the integrity of the liver, and to protect against the development of alcoholic liver injury by preventing NOS_2 induction and NO generation [67]. Moreover, specific changes are associated with the normalization of hepatic SAM : SAH ratio and maintenance of methylation potential in response to betaine supplementation during chronic ethanol ingestion.

Chronic alcohol administration increases gut-derived endotoxin in the portal circulation, activating Kupffer cells to produce several proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1.

Ethanol administration can also lead to the synthesis of Toll-like receptor 4 (TLR4) protein and its gene expression in Kupffer cells, indicating that TLR4 may play a major role in the development of alcohol-induced liver injury. The intragastric ethanol-fed rat model, which reproduces the pathological features of early alcohol-induced liver injury, was used to observe the changes of TLR4 expression and the effect of betaine in alcohol-induced liver injury animal models.

It has been suggested that betaine can prevent alcohol-induced liver injury effectively and improve liver function. The hepatoprotective mechanism of betaine is probably related to the inhibition of endotoxin/TLR4 signaling pathways. In rats with alcohol-induced liver injury, betaine feeding can decrease the levels of serum ALT, AST, endotoxin, TNF-α, IFN-γ, and IL-18, reduce the expressions of TLR4, and improve the degree of hepatic steatosis and inflammation in liver tissues.

In summary, the results of this study show that the expression of TLR4 increased significantly in ethanol-fed rats. Betaine administration can inhibit TLR4 expression, which may be the mechanism of protection from alcoholic liver injury exerted by betaine [68].

5.3. Betaine and NAFLD. The role of betaine in the treatment of NASH has been evaluated in human studies. Oral administration of betaine glucuronate in NASH patients for 8 weeks reduced both hepatic steatosis by 25% and hepatomegaly by 8%, and it significantly attenuated serum concentrations of AST, ALT, and glutamyl transferase. Similarly, a marked improvement in the degree of steatosis, necroinflammatory grade, and stage of fibrosis was obtained after treatment with betaine [69].

Nonalcoholic fatty liver disease (NAFLD) is a common liver disease, associated with insulin resistance. Betaine treatment would prevent or treat NAFLD in mice. Betaine reduces fasting glucose, insulin, triglycerides, and hepatic fat in mice submitted to a moderate high-fat diet (MHF). Betaine significantly improved insulin resistance and hepatic steatosis. Betaine treatment reversed the inhibition of hepatic insulin signaling in mHF and in insulin-resistant HepG2 cells, including normalization of insulin receptor substrate 1 (IRS1) phosphorylation and of signaling pathways for gluconeogenesis and glycogen synthesis. We can conclude that betaine treatment prevents and treats fatty liver in a moderate high-dietary-fat model of NAFL D in mice [70].

Moreover, betaine supplementation alleviated hepatic pathological changes, which were concomitant with attenuated insulin resistance (as shown by improved homeostasis model assessment of basal insulin resistance values and glucose tolerance test) and corrected abnormal adipokine productions (adiponectin, resistin, and leptin). Specifically,

betaine supplementation enhanced insulin sensitivity in adipose tissue as shown by improved extracellular signal-regulated kinases 1/2 and protein kinase B activations. In adipocytes, freshly isolated from mice fed a high-fat diet, pretreatment of betaine enhanced the insulin signaling pathway and improved adipokine productions. Further investigation using whole liver tissues revealed that betaine supplementation alleviated high-fat diet-induced endoplasmic reticulum stress response in adipose tissue as shown by attenuated glucose-regulated protein 78/C/EBP homologous protein (CHOP) protein abundance and c-Jun NH2-terminal kinase activation [71].

Song et al. showed that betaine significantly attenuated hepatic steatosis induced by high-sucrose diet (animal model), and this change was associated with increased activation of hepatic AMP-activated protein kinase (AMPK) and attenuated lipogenic capability (enzyme activities and gene expression) in the liver [72].

5.4. Betaine and HCV. The cause of failure of antiviral treatment with standard therapy, that is, pegylated interferon alpha (pegIFNalpha) combined with ribavirin, in half patients, is unknown, but viral interference with IFNalpha signal transduction through the Jak-STAT pathway could be considered. The expression of HCV proteins leads to an impairment of Jak-STAT signaling because of the inhibition of STAT1 methylation. Unmethylated STAT1 is less active since it can be bound and inactivated by its inhibitor, protein inhibitor of activated STAT1 (PIAS1). The treatment of cells with S-adenosyl-L-methionine (AdoMet) and betaine could restore STAT1 methylation and improve IFN alpha signaling. Furthermore, the antiviral effect of IFNalpha in cell culture could be significantly enhanced by the addition of AdoMet and betaine. S-adenosyl-L-methionine (SAMe) and betaine potentiate IFNα signaling in cultured cells expressing hepatitis C virus (HCV) proteins and enhance the inhibitory effect of IFNα on HCV replicons. SAMe and betaine were found to be safe when used with pegIFNα/ribavirin [73].

In conclusion, the addition of these drugs to antiviral standard therapy for patients with chronic hepatitis C could overcome the problem of drug resistance [74].

Homocysteine, a sulfuric amino acid involved in methionine metabolism, belongs to the group of intracellular thiols. Hyperhomocysteinemia is frequent in the Caucasian and its role in vascular pathology has been clearly established. In hepatology, experimental data in transgenic mice deficient in homocysteine metabolism enzymes have shown the presence of severe liver steatosis with occasional steatohepatitis. In chronic hepatitis C, preliminary data have shown that hyperhomocysteinemia is an independent risk factor for steatosis or even fibrosis. The physiopathological mechanism has now begun to be better understood. On one hand, there is a strong correlation between homocysteine and insulin resistance whatever its etiology. On the other hand, homocysteine has a direct effect on the liver, resulting in overexpression of SREBP-1 and favoring steatosis. It stimulates proinflammatory cytokine secretion such as NF kappa B increasing the risk of NASH. Finally, homocysteine could increase the risk of fibrosis by stimulating TIMP 1. Moreover, hepatitis C virus induces hypomethylation of STAT 1 and could decrease the antiviral activity of interferon. Results from *in vitro* studies have shown that the normalization of STAT 1 methylation by bringing betaine and S Adenosyl Methionine (which belongs to homocysteine cycle) restores the antiviral activity of interferon. Finally, treatment of hyperhomocysteinemia could have favorable consequences in steatohepatites and HCV infection [75, 76].

5.5. Betaine and HBV. Only few data exist about the role of betaine in histological or clinical effects of hepatitis-B-virus-infected patients.

6. Glycyrrhiza Glabra

6.1. Definition, Pharmacokinetics, and Biological Aspects. Glycyrrhiza glabra (licorice root), a perennial herb cultivated in temperate and subtropical regions of the world and native to Mediterranean region as well as to central and South-Western Asia, belongs to the Leguminosae family, genus Glycyrrhiza [77]. The aqueous extract of this plant contains Glycyrrizin (GL), a conjugate of two molecules of glucuronic acid and one of 18β-glycyrrhetic acid (GA) [78] and other substances as flavonoids, hydroxycoumarins and beta-sitosterols [79].

Stronger neominophagen C (SNMC) is a product used in Japan for the treatment of acute and chronic hepatitis. This solution is administered intravenously, 80–200 mg/day, for variable periods of time, and contains 0.2% glycyrrhizin, 0.1% cysteine, and 2% glycine in physiological solution. In the United States, glycyrrhizin is available in a multiplicity of nonstandardized oral formulations found over the country [80, 81].

GL injected i.v., is partially metabolized to 3-monoglucuronyl-glycyrrhetinic acid (3MGA) in the liver by lysosomal β-D-glucuronidase, and GL and 3MGA could be excreted with bile. The biliary-excreted GL and 3MGA are hydrolyzed by intestinal bacteria into GA, which is reabsorbed into the bloodstream. Orally administered GL is enzymatically hydrolyzed to GA by intestinal bacterial flora before absorption into the bloodstream. The circulated GA is further metabolized to 3MGA in the liver by UDP-glucuronyl transferase and then excreted with bile into the intestine [82]. The pharmacokinetic characteristics of i.v. administration of GL in patients with liver disease was studied in a Japanese and European report: SNMC has linear pharmacokinetics up to 200 mg, and steady state in achieved after two weeks of 200 mg doses administrated six times per week [83].

Potential interactions may occur with drugs metabolized by CYP450 3A4, although those have not been reported to date [80, 81].

Decreases of potassium, sodium retention, worsening of ascites, and hypertension are possible adverse effects due to the 11-hydroxy-steroid dehydrogenase inhibitory activity of GL and GA [84]. However, published data show no

increased rate of these side-effects during treatment although documentation of toxicity is poor in most reports [77].

The use of GL in acute and chronic hepatitis is due to its hepatoprotective, immunomodulatory, and anti-inflammatory effect. It reduced ischemia/reperfusion (I/R-) induced liver injury [85] and inhibited hight-mobility group box 1 (HMGB1), an inflammatory cytokine that acted in inflammation and organ damage in hepatic I/R-injury [85, 86].

Many studies shown that GL attenuated inflammatory responses due to decreased activation of nuclear factor kB (NF-kB) and mitogen-activated protein kinase (MAPK) pathways [87], moreover, inhibited the production of LPS-induced nitric oxide (NO) and tumor necrosis factor-α (TNF-α), prostaglandin E2, intracellular reactive oxygen species (ROS), proinflammatory interleukins as IL-4, IL-5, IL-6, IL-18, IL-1β [88–92], and increased the production of anti-infiammatory interleukins as IL-12 and IL-10 [92].

In animal studies, GL inhibits CD4+ T-cell and tumor necrosis factor- (TNF-) mediated cytotoxicity [93], activated NK cells and extrathymic T lymphocyte differentiation [94, 95], and promoted maturation of dendritic cells [92].

GL inhibited serum AST and ALT levels and histologically inhibited the infiltration of inflammatory cells and the spreading of degenerative areas of hepatocytes in an animal model of concanavalin A-induced liver injury [96].

6.2. Glycyrrizin in HCV and HBV Infection. Many studies showed GL have antiviral activity (reviewed in [97]). A mechanism proposed for explain this propriety is the membrane stabilizing effect, as demonstrated in rat hepatocytes incubated with antibody raised against rat liver cell membranes: rat hepatocytes released AST after incubation with antiliver cell antibody in the presence of complement, and the endogenous phospholipase A2 activity was increased, but glycyrrhizin suppressed phospholipase A2 activity and reduced transaminase level [98]. A more recent study confirms this propriety in HIV and Influenza A virus [99].

Despite a precedent review [100] evidenced that SNMC acts as an antiinflammatory or cytoprotective drug but does not have antiviral properties, a recent *in vitro* study found that GL inhibit HCV full-length viral particles and HCV core gene expression or function in a dose-dependent manner and had synergistic effect with interferon [101] and a European randomized trial showed biochemical effects of 26-week treatment with SNMC in patients with chronic hepatitis C [102].

Glycyrrhizin-modified glycosylation and blocked sialylation of hepatitis B surface antigen (HBsAg) [103]. An *in vitro* study, measuring the release of surface protein (HBsAg) and HBV-DNA in transfected HepG2 2.2.15 cells, showed that this compound had a moderate ability in reducing viral production [104].

Long-term clinical trials in Japan and The Netherlands demonstrate that interferon nonresponder patients with chronic hepatitis C and fibrosis stage 3 or 4 have a reduced incidence rate of HCC after glycyrrhizin therapy normalizes ALT levels [105, 106]. Other well-diagnosed studies are needed to better define the role of GL in HBV and HCV-related liver disease.

6.3. Glychyrrizin and Its Protective Role in Hepatic Fibrosis and Steatosis. 18 beta-glycyrrhetinic acid (18α-GL) can suppress the activation of hepatic stellate cells (HCSs) and induce their apoptosis by blocking the translocation of NF-kappaB into the nucleus, furthermore, it promoted the proliferation of hepatocytes in rats with CCl4-induced liver fibrosis [107]. GA inhibited type I collagen synthesis and progression of liver fibrosis probably through the suppression of collagen gene (COL1A2) promoter [108]. In transgenic mice expressing the HCV polyprotein fed an excess iron diet, SNMC prevented hepatic steatosis: this product attenuated ultrastructural alterations of mitochondria of the liver, activated mitochondrial β-oxidation with increased expression of carnitine palmitoyl transferase I and decreased the production of reactive oxygen species.

Wu et al. found that 18α-GL, the biologically active metabolite of GL, prevented FFA-induced lipid accumulation and cell apoptosis in *in vitro* HepG2 NAFLD models and also prevented high-fat-diet-induced hepatic lipotoxicity and liver injury *in vivo* rat NAFLD models. GA stabilized lysosomal membranes, inhibited cathepsin B expression and enzyme activity, inhibited mitochondrial cytochrome c release, and reduced FFA-induced oxidative stress [109].

6.4. Glycyrrizin and Anticancer Activity in Liver. A recent review revealed that triterpenoids, which are also found in Glycyzirra glabra extract, had antitumor activities. Triterpenoids could induce apoptosis in various cancer cells by activating various proapoptotic signaling cascades. The molecular mechanisms involved include inhibition of various oncogenic and antiapoptotic signaling pathways and suppression or nuclear translocation of transcription factors including NF-κB [110]. In a human hepatoma cell line, the expression of junB mRNA, a tumor suppressor gene, and JUNG protein is highly increased by GL treatment [111]. Zhao et al. studied the β-Cyclodextrin/glycyrrhizic acid functionalized quantum dots (β-CD/GA-functionalized QDs [112], and found that this drug has proapoptotic effects in hepatocarcinoma cells. β-CD/GA-functionalized QDs triggered G0/G1 phase arrest and induced apoptosis through an reactive oxygen species mediated mitochondrial dysfunction pathway.

7. Phyllantus spp.

7.1. Definition, Pharmacokinetics, and Biological Aspects. The genus Phyllantus (Euphorbiaceae) consist of about 6500 species in 300 genera, of which 200 are American, 100 African, 70 from Madagascar, and the remaining are Asian and Australian [113]. Many species are used in traditional medicine mainly in India and China to treat several diseases [114] and a morphological analysis of samples of Phyllanthus used in raw drug trade in southern India shown that 76% of the market samples contained P. amarus as the predominant

species (>95%) and other five different species, namely, *P. debilis, P. fraternus, P. urinaria, P. maderaspatensis*, and *P. kozhikodianus*, were found in the remaining 24% of the shops [115].

P. amarus has been widely studied because it is the most commonly used in the Indian Ayurvedic medicine in the treatment of gastrointestinal and genitourinary diseases. Its main active components are ligans, as phyllanthin and hypophyllanthin, and flavonoids, alkaloids, hydrolysable tannins, polyphenols, triterperens, sterols, and volatile oil [113].

Many animal studies evidenced the hepatoprotective activity of P. amarus. The extract enhanced liver and serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), acid phosphatase (ACP), glutathione-S transferase (GST). Furthermore lipid peroxidation level was significantly reduced in ethanol and CCl4-induced liver disease animal models [116–119]. The administration of P. amarus extract significantly decreased the levels of collagen and tissue inhibitors of matrix metalloproteinases (TIMPs) and positively modulated the expression of matrix metalloproteinases (MMPs) in rats with alcohol and thermally oxidized polyunsaturated fatty acid- (PUFA-) induced hepatic fibrosis [120]. In mice with aflatoxin B1-induced liver damage, P. amarus extract lowered down the content of thiobarbituric acid reactive substances (TBARSs) and enhanced the reduced glutathione level and the activities of antioxidant enzymes, glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT) [121].

In a recent animal study, it was found a synergistic effect between silymarin and P. amarus, especially with ethanoic extract of P. amarus, due to its higher concentration of phyllanthin in comparison to aqueous extract against CCl-induced nepatotoxicity [122].

The hepatoprotective action was studied also in other Phyllanthus species, as P. simplex [123], P. atropurpureus [124], P. acidus [125, 126], P. fraternus [127], P. emblica [128], P. urinaria, and P. maderaspatensis [129, 130], and significant antioxidant and anti-inflammatory activities were found especially in P. simplex extracts because of its high phenolic content [123]. Finally, the antioxidant activity was compared between P. virgatus and P. amarus and was found that the first had higher cytotoxicity, higher free radical scavenging activity, and more inhibition of peroxidation capacity [131].

7.2. Phyllanthus and Alcohol-Induced Liver Disease. Preclinical studies have shown that Emblica officinalis (P. emblica) protect against ethanol-induced hepatotoxicity (reviewed in [132]). Animal studies showed that the fruit extract improved plasma enzymes level, reduced lipid peroxidation, and restored the enzymatic and nonenzymatic antioxidants level in alcohol-induced liver disease, this action is probably due to tannoid, flavonoid and NO scavenging compounds present in the extract [133–135]. Phyllanthin restored the antioxidant capability of rat hepatocytes including level of total glutathione, and activities of superoxide dismutase (SOD) and glutathione reductase (GR) which were reduced by ethanol [136] and P. amarus-modified alcohol and thermally oxidized PUFA-induced fibrosis in rats because decreased the levels of collagen and TIMPs and positively modulated the expression of MMPs [120].

7.3. Phyllanthus in HCV and HBV Infection. P. amarus extract was used, in 1988, in a preliminary study involving 37 patients with chronic hepatitis B. 22 of 37 treated patients had cleared the virus 2-3 weeks after the end of the treatment period and only one of 23 placebo treated became HBsAg negative [137]. The mechanism of action appears to be related to the suppressive effect of Phyllanthus extract on HBsAg secretion and HBsAg mRNA expression [138] and the inhibition of hepatitis B virus polymerase activity [139]. A recent study isolated a polyphenolic compound, 1,2,4,6-tetra-O-galloyl-β-D-glucose (1246TGG), from P. emblica, and found that treatment with 1246TGG (6.25 μg/mL, 3.13 μg/mL), reduced both HBsAg and HBeAg levels in culture supernatant of HepG2.2.15 cells [140]. The role of Phyllanthus spp. in the treatment of chronic hepatitis B was studied in several reports that were evaluated in a recent Cochrane review. The authors included a total of 16 randomized trials but only one compared Phyllanthus with placebo and found no significant difference in HBeAg seroconversion after the end of treatment or followup. Fifteen trials compared Phyllanthus plus an antiviral drug like interferon alpha, lamivudine, adefovir dipivoxil, thymosin, vidarabine, or conventional treatment with the same antiviral drug alone and found that the combined treatment affect serum HBV DNA, serum HBeAg, and HBeAg seroconversion. The authors conclude that Phyllanthus in combination with an antiviral drug may be better than the same antiviral drug alone but clinical trials with large sample size and low risk of bias are needed to confirm these findings [141].

The metanoic extracts of P. amarus root and leaf are also recently studied for the treatment of chronic hepatitis C and the root extract showed significant inhibition of HCV-NS3 protease enzyme; whereas the leaf extract showed considerable inhibition of NS5B in the *in vitro* assays. Both extracts significantly inhibited replication of HCV monocistronic replicon RNA and HCV H77S viral RNA in HCV cell culture system. Furthermore, addition of root extract together with IFN-α showed additive effect in the inhibition of HCV RNA replication [142].

7.4. Phyllanthus, Prevention, and Treatment of Liver Cancer. P. emblica and P. urinaria are Phyllanthus species most studied for cancer treatment.

Water extract of P. urinaria induces apoptosis by DNA fragmentation and increased caspase-3 activity, reduces the viability of numerous cancer cells lines probably by telomerase suppression activity, and reduces the angiogenesis as suppressing MMP-2 secretion and inhibiting MMP-2 activity through zinc chelation [143].

P. emblica and some of its phytochemicals such as gallic acid, ellagic acid, pyrogallol, some norsesquiterpenoids, corilagin, geraniin, elaeocarpusin, and prodelphinidins B1

and B2 possess antineoplastic effects. It also possess other properties that are efficacious in the treatment and prevention of cancer as radiomodulatory, chemomodulatory, chemopreventive effects, free radical scavenging, antioxidant, anti-inflammatory, antimutagenic, and immunomodulatory activities [144].

In liver, P. emblica and P. urinaria inhibited HepG2 cell growth and five other cancer cell lines [145, 146]. Progallin A isolated from the acetic ether part of the leaves inhibited the proliferation of BEL-7404 cells, upregulated Bax, and downregulated Bcl-2 expression [147]. Defatted methanolic fruit extract of P. emblica suppressed carcinogen-induced response in rat liver with diethylnitrosamine-induced hepatocarcinoma [148].

8. LIV.52

Liv.52 is an Ayurvedic medicine that was used for 50 years in in the prevention and treatment of viral hepatitis, alcoholic liver disease, early cirrhosis, and a variety of conditions as protein energy malnutrition, loss of appetite, and others. It is composed by *Capparis spinosa, Cichorium intybus, Mandur bhamsa, Solarium nigrum, Terminalia arjuna, Cassia occidentalis, Achillea millefolium*, and *Tamarix gallica* [149].

The potential cytoprotective effect of Liv.52 was studied *in vitro* studies: it improved copper [150] and tert-butyl hydroperoxide (t-BHP) [151] toxicity in HepG2 cells by inhibition of lipid peroxidation, and increase of GSH content and antioxidant enzyme activity. Another recent study found that Liv.52 abrogated the ethanol-induced PPARγ suppression and ethanol-induced TNFα gene expression, it also upregulated PPARγ mRNA [152]. Pretreatment with low (2.6 mL/kg/day) and higher doses (5.2 mL/kg/day) of Liv.52 reversed paracetamol-induced liver toxicity in mice [153, 154].

Few randomized controlled clinical trials were made and results were conflicting [155], recently, a double-blind, placebo-controlled study reported that cirrhotic patients treated for 6 months with Liv.52 had significantly better Child-Pugh score, decreased ascites, and decreased serum ALT and AST levels compared with placebo group [156].

9. Complementary Alternative Medicine and Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is one of the most frequent cancers in the world and its incidence has been increasing recently in countries including the United States of America, western Europe, and eastern Asia [157]. Systemic chemotherapy plays a palliative role while yields unsatisfactory response rates, which is partly due to the poor selectivity and low uptake efficiency of chemotherapeutic drugs in tumor [158].

Since the prognosis of cirrhotic patients seems to be largely influenced by the development of HCC, every attempt should be performed to prevent HCC in such a high-risk group. Oka et al. reported in a randomized controlled trial that a kind of medicinal herb, "Sho-saiko-to" could significantly decrease hepatic carcinogenesis rate in patients wiith cirrhosis [159]. Moreover, a number of clinical and laboratory studies have been done in the past decades in order to provide the scientific basis for the effectiveness of traditional Chinese medicine against cancer. However, actually, there are a number of contradictory reports due to various factors, as inconsistency in treatment schemes, limited sampling sizes and lack of quality assurance of the herbal products well-designed randomized controlled trials (RCT). In general, most of the published clinical studies are trials without rigorous randomization or they involved single group pre-post, cohort, time series, or matched case-control studies [160]. Herbs are generally used in combination as "formulas," in the belief that in this way their benefits were enhanced and side effects reduced. Moreover, practitioners can adjust or customize the formulas to suit individual cancer patients. Through synergistic interactions between different effective ingredients, the herbal preparation can exert its effects in several ways: (i) they can protect the noncancerous cells and tissues in the body from the possible damage caused by chemo/radiotherapy; (ii) they can enhance the potency of chemo/radiotherapy; (iii) they can reduce inflammatory and infectious complications in the tissues surrounding the carcinoma; (iv) they can enhance immunity and body resistance; (v) they can improve general condition and quality of life; (vi) they can prolong the life span of the patients in the late stages of cancer [161].

The anticancer herbal drugs can be divided into three categories based on their target: (i) drugs that uniquely target topoisomerases (Topos) and perturb DNA replication; (ii) drugs that kill tumor cells through apoptotic pathways; (iii) drugs that alter signaling pathway(s) required for the maintenance of transforming phenotypes of the tumor cells. The cellular and animal studies have provided strong molecular evidences for the anticancer activities of the herbal medicine; however, several important questions remain to be answered. Specifically, three specific issues that will require focused attention: (i) more well-designed clinical trials to support the effectiveness and the safety of TCM in the management of cancers; (ii) new parameters based on the unique properties and theory of TCM to assess the clinical efficacy of TCM in clinical trials; (iii) new approaches to research, given the nature of TCM herbs as being fundamentally different from drugs. Undoubtedly, a clinical study of TCM treatment is more difficult and complicated than the study of single compound drugs. In addition, the effects, as well as the toxicity, of individual herbs or single compounds derived from the herb cannot completely reflect the benefits and toxicity of the herbal combination [162]. As a goal, to develop TCM into rational cancer therapy, more well-designed intensive clinical evaluations and translational laboratory studies are absolutely needed. Also, close collaboration between TCM and conventional Western medicine professions and a combination of TCM with modern multidisciplinary cutting-edge technologies, such as omic methodology on systems biology [163], would provide us with an attractive and effective strategy to achieve this goal.

Although there are many therapeutic strategies including chemotherapy to treat cancer, high systemic toxicity and drug resistance limit the success full outcomes in most cases. Accordingly, several new strategies are being developed to control and treat cancer. One approach could be a combination of and effective phytochemicals with chemotherapeutic agent, which, when combined, would enhance efficacy and reduce toxicity to tissues [164].

Several herbs and plants with different pharmacological properties are known to be rich of sources of chemical constituents that may have a potential for the prevention and the treatment of several human cancers.

9.1. Curcumin. Curcumin has been shown with chemopreventive and chemotherapeutic properties against tumors in animal models and clinical trials [165–167]. The anticancer effects of curcumin have been documented in many cancers; it induces apoptosis through the death receptor mediated pathway and mitochondrial dysfunction and also induces DNA damage response by cleaving caspase-3. In addition, curcumin induces cell cycle arrest by downregulating the protein expression on cdc2 and inhibits the proliferation of human hepatocellular carcinoma J5 cells in a time- and dose-dependent manner.

9.2. Glycyrrhizin. Glycyrrhizin has been shown to successfully prevent the occurrence of primary HCC in patients with HCV-related chronic liver disease by unknown mechanisms [168]. One of the principal roles of long-term administration of glycyrrhizin in decreasing the carcinogenesis rate seemed to be anti-inflammatory ones, which would retrieve an active carcinogenic process.

9.3. Quercetin. It has been shown that quercetin inhibits the growth of hepatoma cells in dose- and time-dependent manners. Particularly, in a recent study, quercetin treatment of hepatoma cells resulted in changes of cell cycle, reducing HCC progression [169].

Most of studies involving quercetin and HCC analyzed cotreatment with different chemotherapeutics.

A study showed that BB-102 (a recombinant adenovirus vector expressing the human p53, GM-CSF and B7-1 genes) and quercetin synergetically suppress HCC cell proliferation and induce HCC cell apoptosis, suggesting a possible use as a combined anticancer agent [170].

In a different study, the authors explored the effect of combination treatment of quercetin in combination with roscovitine in hepatoma cells. Results showed that roscovitine in combination with quercetin can be considered as a potential therapeutic target for treatment of HCC [171].

Furthermore, it has been demonstrate that reactive oxygen species production is involved in quercetin-induced apoptosis in human HCC cell lines so quercetin induces favorable changes in the antioxidant defense system of hepatoma cells that prevent or delay conditions which favor cellular oxidative stress [172].

Otherwise, quercetin, by inducing oxidative stress, potentiates the apoptotic action of 2-methoxyestradiol in human hepatoma cells [173].

9.4. Silymarin. The chemopreventive effect of silymarin on HCC has been established in several studies using *in vitro* and *in vivo* methods; it can exert a beneficial effect on the balance of cell survival and apoptosis by interfering cytokines. In addition, anti-inflammatory activity and inhibitory effect of silymarin on the development of metastases have also been detected. In some neoplastic diseases, silymarin can similarly be administered as adjuvant therapy.

9.5. Phyllanthus. Phyllantus Emblica exhibits a variety of pharmacological effects including antiinflammatory, antipyretic, antioxidant and anti-mutagenic effects [174]. The active principles of extracts of P. emblica have demonstrated anti-proliferative effects in several cancer cell lines both *in vivo* and *in vivo*, thanks to their ability to interfere with cell cycle regulation via the inhibition of cdc 25 phosphatase and partial inhibition of cdc 2 kinase activity [175].

A study examined the growth inhibitory effect of P. emblica on human hepatocellular carcinoma (HepG2) and its synergistic effect with doxorubicin and cisplatin: the effect of chemotherapeutic agents may be modified by combination of P. emblica and be synergistically enhanced in some cases [176]. Depending on the combination ratio, the doses for each drug for a given degree of effect in the combination may be reduced. The mechanism involved in this interaction between chemotherapeutic drugs and plant extracts remains unclear and should be further evaluated.

10. Herbal Products and Their Side Effects

Although research on complementary and alternative medicine (CAM) therapies is still limited, this systematic review has revealed sufficient evidence to conclude that CAM, particularly the herbal products examined can be effective for certain conditions. There are reliable evidences of potential therapeutic benefit. At the same time, the more limited state of knowledge regarding the side effects of this herbal products are studied in this issue.

These "natural products" have multiple pharmacological actions on various human physiological systems that would support the treatment of chronic disease like cancer. Moreover, the use of herbal medicines is safe compared with synthetic drugs. Further studies are required to determine the molecular mechanisms of their active ingredients.

The limitations of available clinical trials with regard to establishing safety are the same as those for establishing efficacy.

Several studies remark the importance of their protective effects for their principle antioxidants effects useful because it may help to prevent carcinogenicity-associated proliferative processes, but there are not recent publication about their toxicity or their side effects derived by their cronic or acute use. Anyway, if it presents, the side effects are poor

Table 1: Some herbal drugs associated with liver damage.

Herbal	Application	Toxicity (clinical presentation)
Atractylis gummifera	Antiemetic, diuretic, chewing gum	Acute hepatitis, FHF (fulminant hepatic failure)
Callilepsis laureola	Miscellaneous	Like Atractylis gummifera
Chaparra	Antioxidant, liver and health tonic, snake bites	Cholestasis, cholangitis, chronic hepatitis, cirrhosis
Greater Celandine	Dyspepsia, irritable bowel syndrome	Chronic (cholestatic) hepatitis, fibrosis
Germander Teucrium chamaedrys	Weight reduction	Acute and chronic hepatitis, fibrosis (subacute forms)
Kava	Anxiolytic, sleeping aid	Acute and chronic hepatitis, cholestasis, FHF
Pyrrolizidine alkaloids (PA)	Herbal tea, contamination of flour	Veno-occlusive disease
Sassafras	Herbal tea	Hepatocarcinogenesis (animals)
Valerian	Sedative	Mild hepatitis

(i.e., Glycyrrizin can induce hypokalemia, sodium retention, increase in body weight, and elevated blood pressure) [177].

Finally, hepatic damage from conventional drugs is widely acknowledged and most physicians are well aware of them. It is important to remember that acute and/or chronic liver damage occurred after ingestion of some Chinese herbs, herbals that contain pyrrolizidine alkaloids, germander, greater celandine, kava, atractylis gummifera, callilepsis laureola, senna alkaloids, chaparral, and many others. Several herbals have been identified as a cause of acute and chronic hepatitis, cholestasis, drug-induced autoimmunity, vascular lesions, and evenhepatic failure [79] (Table 1).

11. Conclusions

Oxidative stress is the common pathway of chronic liver diseases of different etiology (both viral and alcoholic). CAM seems to exert an antioxidant and antifibrotic effect on liver (even if histological proof of these actions is not provided in all studies), so its use alone or in association with etiologic and causal standard therapies is actually common.

For the majority of herbal products, proof of efficacy by randomized, placebo-controlled clinical trials is often lacking. Anecdotal success and personal experience are frequently the driving force for acceptance of CAM in the population [178].

In contrast to pharmaceuticals, CAM are usually distributed as "food supplements" and not evaluated formally for safety and efficacy; variations in methods of harvesting, preparing, and extracting the herb, which can result in dramatically different levels of certain alkaloids. The biologically active substances have been structurally defined and standardized for only a few of the herb: in most countries, their use is neither regulated nor controlled [179].

It has been clearly shown that herbal products can protect the liver from oxidative injury, promote virus elimination, block fibrogenesis, or inhibit tumor growth, but the active molecules must be isolated and tested in suitable culture and animal experiments and finally in randomized, placebo-controlled studies to enable rational clinical use of the agents [180].

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Research Article

Hepatoprotective Activity of *Elephantopus scaber* on Alcohol-Induced Liver Damage in Mice

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Elephantopus scaber has been traditionally used as liver tonic. However, the protective effect of *E. scaber* on ethanol-induced liver damage is still unclear. In this study, we have compared the *in vivo* hepatoprotective effect of *E. scaber* with *Phyllanthus niruri* on the ethanol-induced liver damage in mice. The total phenolic and total flavanoid content of *E. scaber* ethanol extract were determined in this study. Accelerating serum biochemical profiles (including AST, ALT, ALP, triglyceride, and total bilirubin) associated with fat drop and necrotic body in the liver section were observed in the mice treated with ethanol. Low concentration of *E. scaber* was able to reduce serum biochemical profiles and the fat accumulation in the liver. Furthermore, high concentration of *E. scaber* and positive control *P. niruri* were able to revert the liver damage, which is comparable to the normal control. Added to this, *E. scaber* did not possess any oral acute toxicity on mice. These results suggest the potential effect of this extract as a hepatoprotective agent towards-ethanol induced liver damage without any oral acute toxicity effect. These activities might be contributed, or at least in part, by its high total phenolic and flavonoid contents.

1. Introduction

Reactive oxygen species (ROS) are continuously generated during metabolic processes to regulate a number of physiological functions essential to the body [1]. ROS are prone to withdraw electron from biological macromolecules such as proteins, lipids, nucleic acids in order to gain stability in the biological system. When the production of ROS exceeds the capability of the body to detoxify these reactive intermediates, oxidative stress would be generated [2]. This may lead to drastic harm to the body such as membrane damage, mutations due to attenuation of DNA molecules, and disruption to various enzymatic activities in metabolism of the body [3–5].

Alcohol, a natural product that has been available for human consumption for thousands of years, is a common cause for ROS insult in the liver [6]. Despite the claim that small amount of alcohol consumption may be beneficial for preventing and reducing the mortality rate of coronary heart diseases and ischemic stroke, it should also be noted that alcohol is toxic to almost every organ of the body [7]. Metabolism of alcohol in liver generates excessive free radicals and increased peroxisomal oxidation of fatty acid, which would ultimately affect functionality of the antioxidant systems to eliminate ROS in the body [6]. Therefore, the mechanism to restore hepatic injuries caused by alcoholic oxidative stress is tightly regulated by the antioxidant status of a living system.

Many plants that portrayed good antioxidant activity are also associated with hepatoprotection potential. Some good examples include *Myristica malabarica* L. [8], *Calotropis gigantea* [9], *Acanthus ilicifolius* [10], *Momordica dioica* [11], and *Phyllanthus niruri* [12]. Members from the Elephantopus family, including *Elephantopus scaber* Linn., *Elephantopus mollis* Kunth., and *Elephantopus tomentosus* have also been shown to possess hepatoprotective activities in rats [13–15]. Additionally, the use of *E. scaber* liver protective purposes was also observed in folk medicinal practices. Back

in Brazil, root juice of *E. scaber* had already been consumed for years to heal liver troubles as well as hepatitis [16]. In China, a traditional herbal drink, which is made up of a few herbal products including *E. scaber,* had been claimed to provide protection of the liver against cancer, hemangioma, fatty accumulation, and cirrhosis as well as for anti-hepatitis B [17]. This formulation had also been named as "Yi-Gan-Yin," which means "drink that is beneficial to the liver" to relate to its function.

In Taiwan, a folk medicinal formulation (Teng-Khia-U) consisting of *E. scaber, Elephantopus mollis,* and *Pseudelephantopus spicatus,* which was originally devised for treating nephritis, edema, dampness, chest pain, pneumonia, and scabies, was shown to possess hepatoprotective activity against β-D-galactosamine-(D-Ga1N-) and acetaminophen-(APAP-) induced acute hepatic damages in rats [14]. *E. scaber* alone could also inhibit carbon-tetrachloride-(CCl4-)induced liver injury [18] and more recently, the plant was reported to show liver protection against lipopolysaccharide-induced liver injury in Sprague-Dawley Rats [19]. Various factors could be responsible for the liver protective ability of this plant. The antioxidant potential of *E. scaber*, which may be attributed to the phenolic and flavonoid content of the plant, may be among the underlying constituents that contributed to this bioactivity.

Here, we report for the first time evaluation on the hepatoprotection effect of the ethanolic leaf extract of *E. scaber* on ethanol-induced liver injury in mice models. In addition, the total phenolic content and total flavonoid content of the extract were also evaluated in this study. Likewise, the safety of consuming the herb was also a great concern since extracts or decoction of this plant has been applied widely. In this study, we had made an attempt to investigate the oral acute toxicity of this plant extract in mice. This would be crucial to justify the safety of consuming this plant despite its potential to protect the liver against toxic insults.

2. Materials and Methods

2.1. Preparation of E. scaber Ethanol Extract. The matured leaves of *E. scaber* were collected between 10.00 AM and 11.30 AM of October 18, 2009. The plant was identified and deposited with voucher specimen number FRI65693 in the Forest Research Institute Malaysia (FRIM), Kepong, Selangor. Ethanolic leaf extract of *E. scaber* was prepared as described previously [20]. Briefly, the leaves of *E. scaber* were powdered and extracted using absolute ethanol at room temperature. Extraction was repeated three times and the content of each extraction was mixed and filtered through grade 1 Whatman filter paper. The filtrate was then evaporated to dry under reduced pressure at <40°C using Aspirator A-3S (EYELA, Japan). We obtained a residue of about 8% yield of the initial dried leaves' weight, and this extract was stored at −20°C until use.

2.2. Determination of Total Phenolic Content (TPC). TPC of the ethanol extract of *E. scaber* was determined according to the method described by Singleton and Rossi (1965) with slight modification. Test samples were first prepared by dissolving the extract into methanol to yield a concentration of 500 μg/mL. Then, 100 μL of each of the sample was added with 500 μL of Folin and Ciocalteu's phenol reagent and 7.9 mL of distilled water. After 3 minutes, 1.5 mL of Na_2CO_3 (20% w/v) was added and the mixture was allowed to stand for 2 hours in dark with intermittent shaking. Absorbance reading was then taken at 765 nm using the μ Quant ELISA Reader (Bio-tek Instruments, USA). The measurement was carried out in triplicates and results were expressed as mg of gallic acid equivalents per g of extract (mg GAE/g of extract).

2.3. Determination of Total Flavonoid Content (TFC). TFC of E. scaber. Ethanol extract was determined using the aluminium chloride colorimetric assay [21] with slight modification. First, 250 μL of test sample at concentration of 500 μg/mL was mixed with 1 mL of distilled water and 75 μL of $NaNO_2$ (5% w/v). The mixture was let to stand for 5 minutes and added with 75 μL of $AlCl_3$ (10% w/v) subsequently. At sixth minute, the solution was added with 500 μL of NaOH (1 M) and the total volume was made up to 2.5 mL with distilled water. The solution was mixed well, and absorbance was measured against the sample's blank at 510 nm. The measurement was carried out in triplicates, and TFC was expressed as mg of catechin equivalents per g of extract (mg CE/g of extract).

2.4. Development of Liver Injury and Treatment Administration in Mice Model. Male ICR mice (8 weeks old) with body weight of 25 ± 3 g were used in this experiment. All animals were housed in prebedded plastic cages under controlled conditions of 22 ± 3°C, 55 ± 5% humidity and standard 12 hours of day/dark light cycles. The animals were provided access to standard pellets and tap water *ad libitum* and acclimatized for 2 weeks before starting the experiment. This work has been approved by Animal Care and Use Committee, Universiti Putra Malaysia (UPM), (Ref: UPM/FPV/PS/3.2.1.551/AUP-R2). Then, the mice were randomly divided into 6 groups with 8 mice each, and body weight of each of the mice was measured and recorded. Liver cell damage was induced in 5 groups of mice by feeding with 100 μL of ethanol (50% v/v) p.o. using intragastric tube for 7 consecutive days, while 1 group was fed with 100 μL of phosphate buffer saline to serve as the un-induced control. After 24 hours of the last ethanol dosing, treatment was given to all the mice through oral administration. Grouping and the treatments given to the mice is shown in Table 1. After receiving daily treatment for a total of 7 days, the mice were anesthetized with 2% isoflurane (Merck) and sacrificed by cervical dislocation at 24 hours after the last feed. Blood samples were collected immediately and stored in heparin-coated capillary tubes for analysis of liver enzyme profiling and biochemical analysis. Livers were excised from the animals, washed with normal saline, and then fixed in 10% buffered neutral formalin for histopathological examinations.

2.5. Biochemical Analysis. Blood samples collected from the animals were centrifuged at 3000 rpm for 15 minutes, and

Table 1: Induction and treatment given to 6 groups of mice for liver protection study.

Group	Preinduction with 50% (v/v) ethanol	Treatment through oral administration (7 days)	Amount of treatment given
Control	None	Phosphate buffer saline	1 x
Ethanol + PBS	7 days	Phosphate buffer saline	1 x
Ethanol + ESL	7 days	*E. scaber* ethanol extract	3 mg/kg BW
Ethanol + ESM	7 days	*E. scaber* ethanol extract	15 mg/kg BW
Ethanol + ESH	7 days	*E. scaber* ethanol extract	30 mg/kg BW
Ethanol + PN	7 days	*Phyllanthus niruri*	15 mg/kg BW

plasma from each sample was collected for analysis for various biochemical parameters including alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total billirubin (TB), total cholesterol, (TC) and triglyceride (TG). All the analyses were performed on the Hitachi 902 Automatic Analyzer using the adapted reagents from Roche (Germany).

2.6. Histopathological Evaluation. Tissues were dehydrated in ascending grades of ethanol (70% to 100%) and cleared in xylene with the use of TP1020 automated tissue processor (Leica, Germany) after fixing in 10% formalin for overnight. Then, the tissues were embedded in paraffin wax blocks on the EG 1140 H embedding station (Leica, Germany), section to 4 μm thick using Jung Multicut 2045 microtome (Leica, Germany) and mounted on glass slides. After that, deparaffination was carried out by immersing the slides in Xylol for 3 minutes and then in 100% ethanol for 1 minute, 96% ethanol for 1 minute, 80% ethanol for 1 minute, and 60% ethanol for 1 minute. Lastly, the slides were stained with haematoxylin (Sigma, USA) and eosin (Sigma, USA) and then mounted under coverslips using DPX mounting medium (BDH Laboratory, England). Histological changes in the tissue sections were examined and captured under the BX51 light microscope (Olympus, Japan).

2.7. Study on Acute Oral Toxicity in Mice. Acute Oral toxicity of *E. scaber* ethanol extract was performed according to the OECD guidelines 425 using female Balb/C mice aging 8–10 weeks old. All animals were housed in prebedded plastic cages under controlled conditions of 22 ± 3°C and standard 12 hours of day/dark light cycles. The animals were provided access to standard pellets and tap water *ad libitum* and acclimatized for 2 weeks before starting the experiment. The mice were fasted 4 hours prior to the experiment and then divided into groups of five. Body weight of each of the mice was measured and recorded after fasting. Then, animals in the treatment group were fed with single oral dose of *E. scaber* ethanol extract (5000 mg/kg BW) that was dissolved in PBS, while the control group received PBS solution only (10 mL/kg BW). The mortality, body weight, toxic symptom, and behavior of the animals were observed carefully at 15 and 30 minutes, 1, 2, 4, 12, 18, and 24 hours after oral administration and daily thereafter over a total period of 14 days.

2.8. Statistical Analysis. Results were expressed as mean ± standard error (SEM). Single comparison for differences between means was determined by Student's t-test, while multiple comparisons were evaluated using ANOVA test followed by Duncan test. A level of $P \leq 0.05$ was taken as statistically significant.

3. Results

3.1. Total Phenolic and Total Flavonoid Content in E. scaber. The presence of high flavonoids and phenolic compounds in plants had been identified to contribute to their antioxidant values [22]. Our results indicated that the total phenolic content of *E. scaber* was 193.05 ± 1.17 mg GAE/g of the extract. while the total flavonoid content was 120.87 ± 0.61 mg CE/g of extract.

3.2. Hepatoprotective Effect of E. scaber on Ethanol-Induced Liver Damage in Mice. Ethanol treatment resulted in a significant elevation of serum ALT, ALP, and AST levels in comparison to the control group (Table 2). However, the amount of these enzymes reduced after treatment by various concentrations of *E. scaber* as well as by *P. niruri* in general. The decrease of serum ALT concentration after treatment with ESH was greatest among the three concentrations of the extract, but ESL and ESM were capable of restoring the amount of ALT to near normal value. Overall, the reduced ALT level after treatment by all concentrations of *E. scaber* showed no significant difference from the normal control, while the level was significantly ($P < 0.05$) lower than the control group after treatment by *P. niruri*. Similarly, the level of serum ALP was also restored to normal by most of the treatments although differences between all groups were not statistically significant. Only treatment by ESL caused a slight increase in the ALP level, which was not significantly different from the normal control group. Treatment with ESH resulted in a reduction to ALP level that was closest to the normal control group. On the other hand, the effect of ethanol on AST level in mice was much more apparent than the other two liver enzyme markers. A marked increase (2.5 fold) was observed upon induction by ethanol. In spite of this, treatment with either *E. scaber* or *P. niruri* successfully reduced the AST concentrations in mice to a level that were not statistically different from the control group. Comparing between the 3 concentrations of *E. scaber*, the level of AST was most significantly reduced by ESH and the value was nearest to the normal control. Reduction by treatment with *P. niruri* was greater than reduction by all concentrations of

Table 2: Effect of *E. scaber* on activities of serum marker enzymes, triglyceride, and total bilirubin content in ethanol-induced mice.

Group	ALT (U/L)	ALP (U/L)	AST (U/L)	TG (mmol/L)	TB (mmol/L)
Control	81.9 ± 17.8$^{\#}$	84.0 ± 10.9$^{\#}$	187.4 ± 26.3$^{\#}$	1.07 ± 0.35	177.6 ± 21.2
Ethanol + PBS	172.2 ± 2.9*	118.0 ± 3.0*	902.8 ± 16.7*	1.53 ± 0.45	228.0 ± 19.2
Ethanol + ESL	83.8 ± 2.2*	119.0 ± 11.0$^{\#}$	434.8 ± 55.6*	1.54 ± 0.38	198.4 ± 40.9
Ethanol + ESM	82.8 ± 18.9$^{\#}$	99.7 ± 17.0	389.4 ± 14.6$^{\#}$	1.20 ± 0.19	189.7 ± 45.3
Ethanol + ESH	69.7 ± 5.6$^{\#}$	90.7 ± 13.3	348.2 ± 6.3$^{\#}$	0.70 ± 0.09$^{\#}$	136.3 ± 42.8
Ethanol + PN	48.55 ± 2.9$^{*,\#}$	146.0 ± 3.0*	229.8 ± 16.7$^{\#}$	1.12 ± 0.45	180.9 ± 19.2

Value represents mean values ± SEM of 8 mice. *Statistical significance ($P < 0.05$) compared to mice in the untreated control group, $^{\#}$Statistical significance ($P < 0.05$) compared to mice in ethanol-induced group.

E. scaber but the AST level was even lower than the normal control group.

On the other hand, differences in the triglyceride (TG) and total bilirubin (TB) content between the control and the treatment groups were not statistically significant. Seven days of preinduction with ethanol resulted in a slight increase of TG and TB content as compared to the normal control group (Table 2). Following treatment for 7 consecutive days by *E. scaber* or *P. niruri* extracts, both serum TG and TB content in the mice decreased. ESH resulted in the greatest reduction of both TG and TB concentration. However, the final TG level after treatment by ESM and the final TB level after treatment by ESL were found to be closest to the normal control group.

3.3. Effect of E. scaber on Alcohol-Induced Lesion in Mice Liver. Liver sections excised from mice from the control group, which did not receive pretreatment with alcohol, showed normal structure of the hepatocytes that were polyhedral in shape (Figure 1(a)). The cells exhibited well-preserved cell lining with well-defined cytoplasm and nucleus (Figure 1(b)). In contrast, pretreatment of 50% ethanol resulted in damage to the liver. Infiltration of mononuclear cells was detected in the liver section (Figure 1(c)), and normal structure of the hepatocytes was found to be distorted where the cells were rendered with undefined lining and loss of nucleus (Figure 1(d)). Besides, the presence of small foci of necrotic cells and the occurrence of steatosis or accumulation of fatty droplets was detected in many regions of the liver sections.

After treatment by *P. niruri* for 7 days, liver damage of the mice was recovered, as shown in Figure 1(e). When viewed under a higher magnification (Figure 1(f)), it was noted that recovery of the cells had not been completed where a small number of necrotic cells as well as very few lipid droplets were still observable in the liver sections. However, the abnormalities that were detected in the ethanol control group reduced significantly after treatment. Similarly, the administration of ESL after the alcohol induction also showed that regeneration of the hepatocytes had taken place but the lining of the cells had not been recovered completely (Figure 1(h)). The presence of mild monocytes infiltration and a few fat droplets were still detectable in the liver section. Likewise, normalization of hepatocytes into normal structure also occurred when the concentration of *E. scaber* was elevated. However, the recovery was more significant in these two groups where undefined lining of the cells were reverted to normal and distinct nucleus could be seen (Figures 1(i) and 1(k)). In addition, the group treated by ESM showed a greater reduction of monocyte infiltrate), and the lipid droplets were absent in comparison to the low dosage group (Figure 1(j)). The mice treated by ESH showed normal morphology of the hepatocytes, along with normal appearance of the centrilobular vein, peripheral vein and hepatic artery (Figure 1(l)), resembling the tissue architecture of liver section from the untreated control group.

3.4. Oral Acute Toxicology Study in Mice. Acute oral toxicity is defined as the adverse effects that can be observed after oral administration of a test substance at either single dose or multiple doses, which is given within 24 hours [23]. Therefore, median lethal oral dose (LD_{50}), which is defined as the statistically derived single dose of a substance that can kill 50% of animals when administered orally, can be determined form this assay. In this study, the acute oral toxicity assay was carried out following OECD guideline no. 423 (2011). No mortality was observed after 14 days of treatment with a limit dose of 5000 mg/kg BW of *E. scaber*. All the treated mice could tolerate with the extract given with no signs of abnormalities or gross lesions in necropsy findings (Table 3). No statistically significant differences in body weight and weight gain were noted between the treated group and the untreated control group (Table 4). Thus, LD_{50} of *E. scaber* could not be determined from this study and the extract could be regarded as nontoxic for oral consumption up to a concentration of 5000 mg/kg BW in mice.

4. Discussion

The liver plays a pivotal role in the biological system that is responsible for the metabolism and clearance of drugs and xenobiotics, including ROS [24]. Liver has become the central organ for detoxification as the liver cells (hepatocytes), the main components that make up the organ, contain majority of enzymes that are responsible for drug metabolism of the entire body [25]. However, when the amount of drugs or xenobiotics that is encountered has exceed the maximum metabolic capability of the liver; damaging effect of the toxins may lead to various liver ailments. Overconsumption of alcohol had been associated

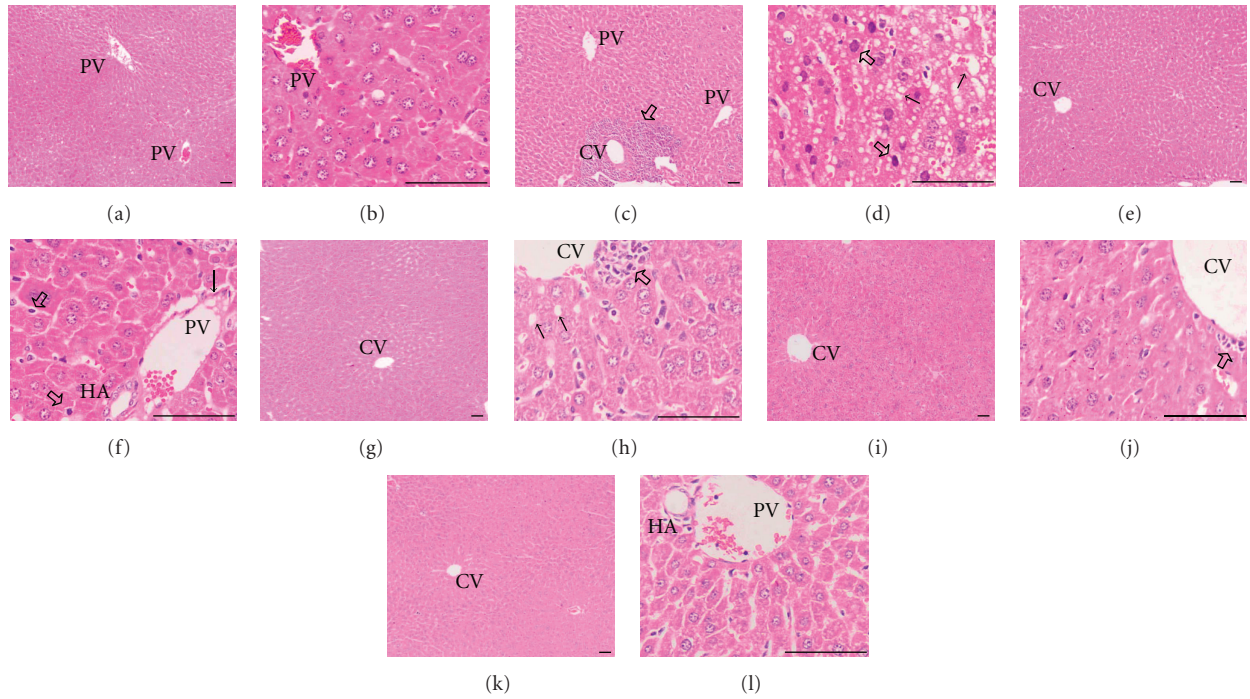

Figure 1: Histological micrograph of liver sections of mice stained with haematoxy lin and eosin. Liver sections excised from the normal control mice (a, ×40) showed normal structure of the hepatocytes with defined cell lining and round nuclei (b, ×100). Histological sections of mice fed with 50% ethanol showed damage of the liver structure as indicated by infiltration of monocytes (block arrow head) (c, ×40), steatosis (filled arrow), and the presence of necrotic cells (block arrow) (d, ×100). Treatment with 15 mg/kg BW of *P. niruri*, the positive control, indicated normalization of the damaged hepatocytes (e, ×40). A great reduction of fat droplets (filled arrow), and the presence of a minute quantity of necrotic cells (block arrow) (f, ×100) could also be observed in this group. On the other hand, section of liver treated by ESL showed recovery of hepatocytes into normal shape (g, ×40), along with lesser fat droplets (filled arrow) and mild monocytes infiltration (block arrow) (H, ×100). When the concentration was increased to 15 mg/kg BW (i, ×40), mild monocytes infiltration was observed (j, ×100). Upon treatment with ESH, structure of the hepatocytes reverted to normal (k, ×40) with defined cell lining and round nuclei (l, ×100). Centrilobular vein (CV), peripheral vein (PV), hepatic artery (HA). Bar = 50 μm.

to a spectrum of liver injuries with varying degree of severity, with some common pathologies including steatosis, foamy degeneration, steatonecrosis, venous lesion, and cirrhosis [26].

Generally, alcohol is metabolized in the liver as a process of detoxification. The metabolism of alcohol occurs mainly via alcohol dehydrogenases (ADH), which requires the cofactor NAD^+. The reduced form of NAD^+ (NADH) is attenuated when the alcohol concentration is in excess, and this could cause hepatic NADH accumulation [7]. As a result, more fatty acids and triglycerides would be synthesized whereas β-oxidation of fatty acids will be impeded [7]. Accumulation of ROS and polyunsaturated fatty acids would increase the oxidative stress and toxicity to the hepatic cells [27]. In this study, the increased level of ALT, ALP and AST, as well as elevated TG and TB content (Table 2) after 7 days of continual feeding with high concentration (50% v/v) of ethanol were indications for alcohol intoxication to the liver. Besides, results from histological images that showed accumulations of fatty droplets in the hepatocytes also provided clear evidence that the preinduction with 50% of ethanol induced liver damage, including loss of cell membrane integrity, accumulation of fatty acids, and necrotic cell death in the mice (Figures 1(c) and 1(d)). Treatment with ESL, ESM, and ESH was able to reduce the accumulation of fats in the mice (Figures 1(e), 1(f), 1(g), 1(h), 1(i), and 1(j)) and decline of TG content in the serum (Table 2). Moreover, the group treated by ESH showed the most substantial improvement of steatosis and the greatest reduction of TG concentration. The ethanolic extract of *E. scaber* was chosen for the present study as it was shown to exhibit a wide range of bioactivities and most of the compounds in *E. scaber* were isolated from this extract [28]. Therefore, it was hypothesized that the *E. scaber* ethanol extract would show potent liver protection activity against ethanol-induced liver damage.

In contrast, the recovery of liver injury by treatment with *P. niruri* was less significant. When comparing histological appearance of the hepatic cells and TG content to ESH treatment group, mild monocytes infiltration and a few fat droplets were still detectable in the former group. On the other hand, increase of ALP and TB concentrations in the serum of alcohol-induced mice might reflect blockage of the bile ducts that obstructed the secretion of bile. Hepatic ALP is present on the lining of biliary ducts and is secreted via biliary bile into blood circulation [29]. ALP synthesis

Table 3: Clinical signs and necropsy findings in mice after acute treatment with *E. scaber*.

Treatment group	No. of mice	Clinical sign	Gross necropsy finding	Mortality
Untreated control	8	No observable abnormalities	No findings	None
E. scaber	8	No observable abnormalities	No findings	None

Table 4: Effect of acute treatment with *E. scaber* ethanol extract on the weight of mice.

		Mean average values (g)	
		Untreated control	*E. scaber*
Body weight	before	20.40 ± 1.18	21.95 ± 0.53
	after	23.09 ± 1.97	23.45 ± 0.65
Body weight gain		2.69 ± 1.19	1.50 ± 0.90

Data represents mean ± SEM of sample size (n) of 8. *Statistical significance ($P < 0.05$) between the control cells and treatment groups.

is stimulated during pathological conditions like bile duct obstruction, primary biliary cirrhosis, primary sclerosing cholangitis, drug-induced cholestasis, adult bile ductopenia, metastatic liver diseases, and bone diseases [30]. Bilirubin, on the other hand, is a bile pigment produced from the enzymatic breakdown of heme within the reticuloendothelial system where its elevation in bloodstream could be related to increased bilirubin production (or increased hemolysis), decreased conjugation, or defects in bilirubin transport [31]. Therefore, both ALP and TB levels measure how well the liver functioned instead of the extent of hepatic injury. Treatment by ESH was most effective in reducing the concentrations of ALP and TB that were elevated by alcohol in comparison to treatment by ESL, ESM, and *P. niruri*. Therefore, it could be deduced that *E. scaber* provided protection to the liver against harmful effect of alcohol by preserving the functionality of this organ.

In this study, *P. niruri* was used as the positive control as this plant had been studied extensively and was shown to possess protective activity against liver injuries that were caused by a number of hepatotoxins including acetaminophen [32], nimesulide [33], carbon-tetrachloride [34–36], and paracetamol [37]. Biochemical analysis from this study was in good agreement with previous finding where *P. niruri* was capable of restoring levels of the liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST) that were elevated following liver injury induced by alcohol [32] (Table 2). ALT is found primarily in the cytoplasm of hepatic cells, while AST is present in both cytoplasm and mitochondria [29]. Both the enzymes catalyse gluconeogenesis from noncarbohydrate sources and are important markers for liver injury [30]. Elevation of the serum concentrations of these 2 markers implied disruption of plasma membrane integrity, which eventually lead to leakage of the enzymes into the blood circulation [31]. Similar to the positive control group, treatment with *E. scaber* also successfully decreased the elevated concentrations of AST and ALT in a dosage-dependent manner whereby the highest concentration of *E. scaber* (ESH) reduced both ALT and AST most. Restoration of both AST and ALT levels suggested that *E. scaber* could potentially restore hepatic cells, or more specifically the integrity of cell membrane and the normalization of hepatocytes into their normal tissue architecture.

The reduced concentrations of both these aminotransferases were consistent with previous studies on lipopolysaccharide-(LPS-) treated rats, which showed that theprotective effect of *E. scaber* over LPS-stressed acute hepatic injury was contributed in part by the antioxidant property of the herb [19]. Previously, the leaf, stem, and root of *E. scaber* had been shown to exhibit antioxidant activities [38, 39] and this property had also been suggested to be attributed by its constituting phenolic content [38]. A few studies have also shown that the hepatoprotective activity is highly correlated to the phenolic content of a given plant and the flavonoid content was suggested as the main contributor to this bioactivity [40–42]. Therefore, the hepatoprotective activity against ethanol-induced liver damage that was demonstrated by the *E. scaber* ethanolic extract could be attributed to the high phenolic and flavonoid content of the plant. However, this would require further assessment through identification and screening of the phenolic constituents of this plant extract.

From this study, ethanol extract of *E. scaber* leaves showed promising hepatoprotection activity in mice with alcohol-induced liver damage. The ability of *E. scaber*-treated mice to cope with the oxidative stress induced by alcohol could be accountable to the antioxidant capacity of the herb. These activities might be contributed, or at least in part by its high total phenolic and flavonoid contents. On the other hand, it was also noted that the hepatoprotective effect of the extract was concentration dependent. Whilst 30 mg/kg BW appeared to most effectively restore the liver damage to near normal, lower concentrations of the extract also showed moderate liver protection activities. More importantly, all the concentrations of *E. scaber* selected for this study were lower than the highest concentration used in acute oral toxicology study (5000 mg/kg BW). With validation from the safety of consuming the ethanol extract of *E. scaber,* this plant extract may be useful as a natural protecting agent against liver damage.

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Research Article

Total Saponin from Root of *Actinidia valvata* Dunn Inhibits Hepatoma 22 Growth and Metastasis *In Vivo* by Suppression Angiogenesis

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The root of *Actinidia valvata* dunn has been widely used in the treatment of hepatocellular carcinoma (HCC), proved to be beneficial for a longer and better life in China. In present work, total saponin from root of *Actinidia valvata* Dunn (TSAVD) was extracted, and its effects on hepatoma H22-based mouse *in vivo* were observed. Primarily transplanted hypodermal hepatoma H22-based mice were used to observe TSAVD effect on tumor growth. The microvessel density (MVD), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) are characterized factors of angiogenesis, which were compared between TSAVD-treated and control groups. Antimetastasis effect on experimental pulmonary metastasis hepatoma mice was also observed in the study. The results demonstrated that TSAVD can effectively inhibit HCC growth and metastasis *in vivo*, inhibit the formation of microvessel, downregulate expressions of VEGF and bFGF, and retrain angiogenesis of hepatoma 22 which could be one of the reasons.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, also the leading cause of cancer-related deaths in China [1]. The prognosis of patients with this type of cancer is primarily determined by the incidence of recurrence after surgery and the occurrence of invading metastases into the remaining liver parenchyma. New metastatic nodules of HCC were supplied by plentiful blood. Therapeutic angiogenesis describes an emerging field of antimetastasis medicine of HCC whereby new blood vessel growth is induced to supply oxygen and nutrients to tumor cells. The growth of this field has exploded in the past decade as a result of the development of recombinant growth factors, the best characterized are the soluble mediators basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Both of these factors stimulate *in vivo* angiogenesis [2, 3].

Actinidia valvata Dunn is a shrub mainly growing in eastern China, the roots of which have been widely used for hepatoma treatment for a long time. Continuous studies mainly on its antitumor effects and active ingredients have been carried out in our laboratory for many years. Previous studies have confirmed that injections of *Actinidia valvata* Dunn were proved to restrain the growth of primary hypodermal tumor with no cell-mediate immunity inhibitive effects *in vivo* [4–6]. TSAVD were extracted from the root of *Actinidia valvata* Dunn, which was proved to prevent the metastasis of human hepatocellular carcinoma cells *in vitro* in poir experiments [7], so this study expands on work by observing its effects on hypotransplanted and experimental pulmonary metastasis hepatoma H22 based mouse *in vivo*.

2. Materials and Methods

2.1. Plant Material. The roots of *Actinidia valvata* dunn were collected in Changshan county, Zhejiang province, China, in October 2006, and identified by Professor Han-Chen Zheng. Department of Pharmacognosy, School of Pharmacy, Second Military Medical University (SMMU). A voucher specimen (no. 20061005) was deposited in the same department.

2.2. Extraction and Isolation of TSAVD. The powdered plant material of roots of *A. valvata* was refluxed with 8 times of 80% alcohol solution for 3 times, 1.5 hours each time. The extracting solution was concentrated under reduced pressure twice with 1.5 hours each time to brown syrup, and 1700 mL brown residue was obtained. Centrifugal method of 5000 rpm which lasted 4 min was used to obtain 1400 mL supernatant, which was absorbed by D101 macroporous resin column subsequently, eluted with 5000 mL 80% ethanol to yield major fractions, which were later concentrated under reduced pressure. Finally, the juice was exsiccated and crushed into powder. The HPLC analysis indicated that there were triterpenoids in the TSAVD and the major peaks were identified by comparison with standard compounds and literature, and they were determined, respectively, as 2α, 3β, 20β, 23, 24, 30-hexahydroxyurs-12-en-28-oic acid O-β-D-glucopyranosyl ester (a), 2α, 3α, 6α, 20α, 24, 30-hexahydroxyurs-12-en-28-oic acid (b), 2α, 3β, 24-trihydroxyurs-12-en-28-oic acid (c), 2α, 3α, 24-trihydroxyurs-12-en-28-oic acid (d), corosolic acid (e) (Figure 1) [8].

2.3. Cell Culture. Hepatoma H22 cells were purchased from Chinese Academy of Sciences (Shanghai, China), preserved in the Tumor Research Institute of TCM Department, Changhai Hospital, SMMU (Shanghai, China), grown in RPMI-1640 medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO^2 in a humidified incubator.

2.4. Animals. Kunming mice weighing 18–22 g, purchased from Animal Center, SMMU, received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH publication 86–23 revised 1985).

2.5. Transplanted Hypodermal Hepatoma H22-Bearing Mice. We observed the cell morphology in the microscope, quantitated, and suspended cell density to about 2×10^7 cells/mL. About 0.2 mL cell suspensions were then subcutaneously inoculated into the left oxter region of each mouse [6]. Calculate mice weight and tumor size each other day. When the tumor size reached an average diameter of 5-6 mm (about 5 days later), tumor-bearing mice were divided into 3 groups randomly, each group with 10 mice. (1) High-TSAVD: mice in this group were injected with 0.2 mL TSAVD in abdominal cavity with a concentration of 1 g/kg/d for 10 days, TSAVD was dissolved completely in DMSO, and dissolved in distilled water containing 0.5% tween-80 and 0.5% carboxymethylcellulose. (2) Low-TSAVD group: mice in this group were injected with TSAVD at half concentration of first group. (3) Control group: mice in this group were treated with solventia at concentration of the first group.

2.6. Evaluation of Antitumor Effect In Vivo. Therapeutic response was evaluated by mouse activities, body weights, and tumor growth. The long (a) and short (b) diameters of the tumors were measured with a slide caliper each other day after treatment. Tumor size was calculated as follows: volume = $(a \times b^2)/2$. Mice were sacrificed and peeled next day after injection, and tumors were weighted. Inhibitive ratio (IR) was calculated as follows: IR = (1 − average tumor weight of treated group/control group) ×100%.

The fixed tumor tissues were progressively dehydrated in solutions containing an increasing percentage of ethanol embedded in paraffin, sectioned at 5 μm thickness, deparaffinized, and stained with hematoxylin and eosin. Immunohistochemical staining for CD34 was performed by the immunoperoxidase technique. Microvessel density (MVD) was determined in the five most intense vascularization areas (hotspots) of each section by observing at 200x magnification. All discrete clusters or single cells stained positive for CD34 were counted as one vessel. The average count was regarded as MVD for each case. IQAS medical image quantitative analysis system was used to know vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) expression rate. VEGF, bFGF-positive expression rates were determined by brown spot in the microscope visual field [9]. Three visions were selected randomly in each specimen at high magnification. The spleen, thymus, kidney, liver, and lung were also observed by naked eye to see whether they had adverse reactions, while pathochanges of the organs were also viewed in microscope. Evaluation criterion: the therapy was inefficient when IR was less than 30%, efficient when IR was more than 30%.

2.7. Experimental Pulmonary Metastasis Hepatoma H22 Bearing Mice. In this experiment, we quantitated the density of hepatoma H22 cells to about 2.5×10^7 cells/mL, injected each mouse by vena caudalis with 0.2 mL. Thirty mice were divided into 3 groups randomly the next day. Each mouse was treated in the same way of the former experiment for 2 weeks. The mice were weighted every other day and activities were observed in treatment. They were sacrificed the next day after the last injection. Metastatic nodes number, distribution, and size in lung were observed by naked eye and microscope, pathochanges of lung tissues were detected by hematoxylin and eosin stain [10]. The metastasis inhibitory rate was calculated as follows: IR = (1 − average number of lung metastatic nodes in treated group/average number of lung metastatic nodes in control group) × 100%. The weight of spleen was read in electronic scale, the spleen index was calculated to see whether it had vice effects on immune system. The white blood cells, red blood cells, and platelets were also observed in the three groups to test whether it had vice effects on blood system.

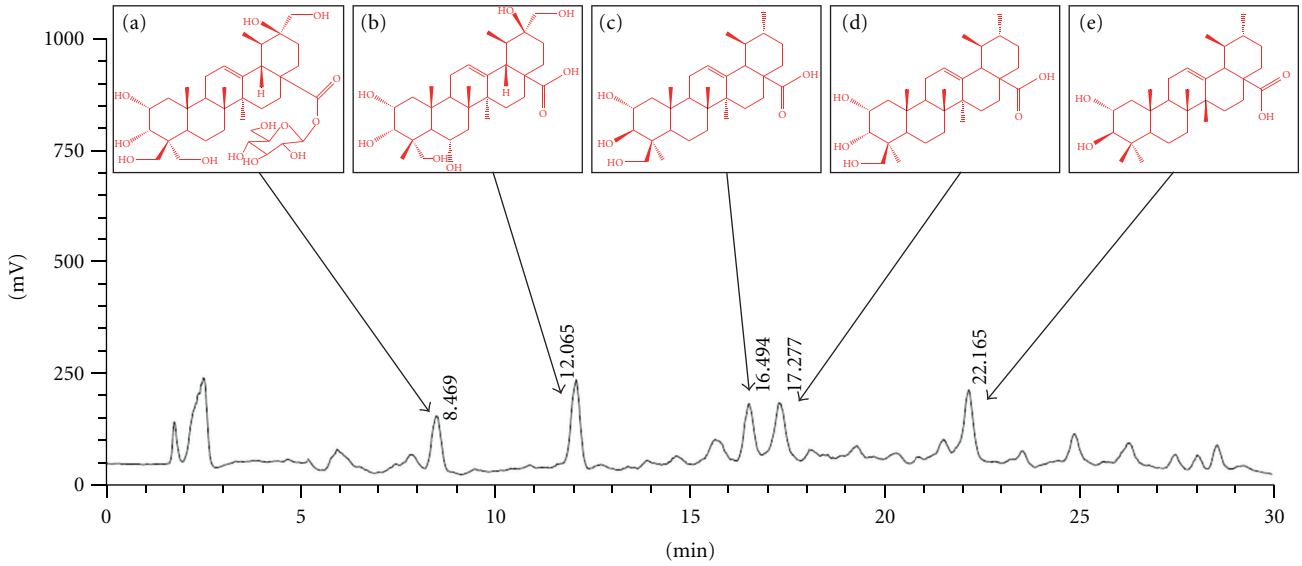

Figure 1: HPLC chromatogram of TSAVD. The major peaks were identified by comparison with standard compounds. Note: (a) 2α, 3β, 20β, 23, 24, 30-hexahydroxyurs-12-en-28-oic acid O-β-D-glucopyranosyl ester. (b) 2α, 3α, 6α, 20α, 24, 30-hexahydroxyurs-12-en-28-oic acid; (c) 2α, 3β, 24-trihydroxyurs-12-en-28-oic acid. (d) 2α, 3α, 24-trihydroxyurs-12-en-28-oic acid. (e) corosolic acid.

2.8. Statistical Analysis. All data were processed by SPSS, and results were expressed as mean ± standard deviation. The statistical significance of differences among different groups was determined with a 95% confidence interval by the ANOVA test for normally distributed data, or the Kruskal-Wallis H Test, Fridman Test for nonnormally distributed data.

3. Results

3.1. HCC Growth Inhibition In Vivo. From the experiments, we could see that tumor in TSAVD-treated group was smaller than that of control group as shown in Figures 2 and 3. The tumor weight in TSAVD-treated groups was obviously less than that of control group at the 8th and 10th day. The tumor weight in TSAVD-treated group was less than that of control group, and the difference showed a statistical significance. IRs were 53.2% of H-TSAVD group, 43.5% of L-TSAVD group. No metastasis tumors and changes were found in the other organs such as lung, liver, and kidney. The spleen, thymus indexes in three groups show no differences.

As Figure 4 shows, in our experiments, the expressions of MVD within the three groups were different ($P < 0.05$): about (1.68 ± 0.50), (10.63 ± 1.08), and (13.36 ± 3.77) in H-TSAVD, L-TASVD group and control group. Expressions of VEGF in H-TSAVD were 1.6, different from control group ($P < 0.05$), while no difference was shown in H-TSAVD and L-TSAVD groups. The expressions of bFGF within the three group were different ($P < 0.05$), about 3.4, 3.1, and 4 in H-TSAVD, L-TASVD group, and control group.

3.2. HCC Metastasis Inhibition In Vivo. No difference was shown in the weights of mice in three groups, but the numbers of pulmonary metastases tumor were different

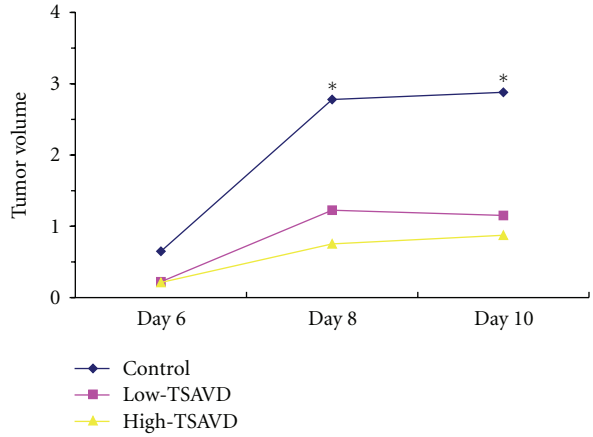

Figure 2: Tumor volume of transplanted hypodermal hepatoma H22 bearing mice in 3 groups.

in H-TSAVD, L-TSAVD, and control groups, which were (4.5 ± 2.33), (6.5 ± 1.51), and (10.1 ± 3.52), respectively, as shown in Figure 5. The number in TSAVD-treated groups was significantly less than that of control group ($P < 0.01$), but no difference was shown between H-TSAVD and L-TSAVD groups. IRs were 55.49% and 35.71% in H-TSAVD, L-TSAVD groups.

The white blood cells, red blood cells, and platelets were different between TSAVD-treated group and control group ($P < 0.01$), as shown in Figure 6, but no difference was shown in H-TSAVD, L-TSAVD groups ($P > 0.05$). The spleen index of the three groups showed statistic significance as Figure 7 show ($P < 0.01$).

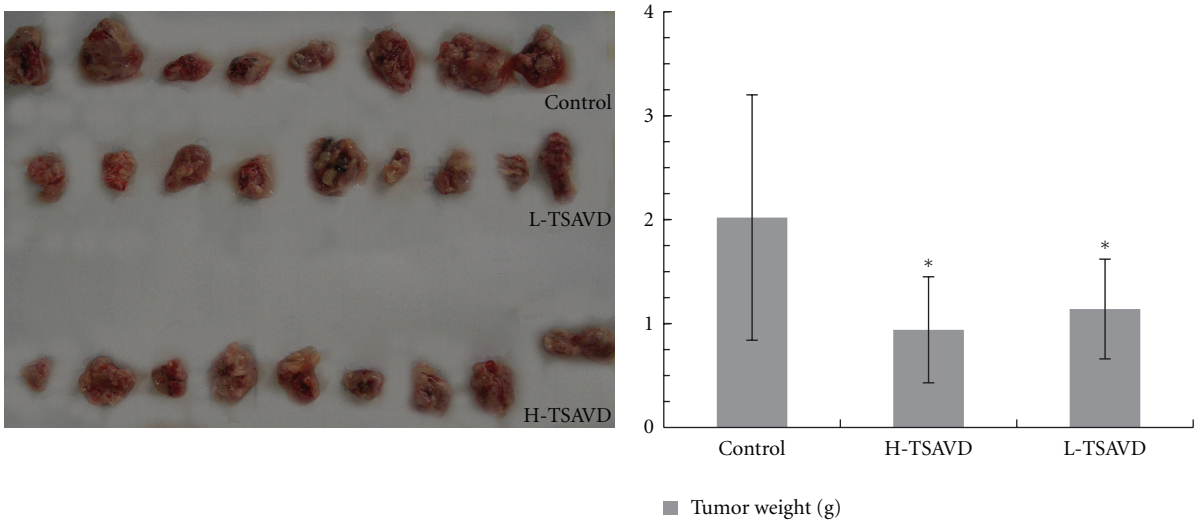

Figure 3: Anti-growth effect of TSAVD on transplanted hypodermal hepatoma H22 bearing mice (*$P < 0.05$ versus control group).

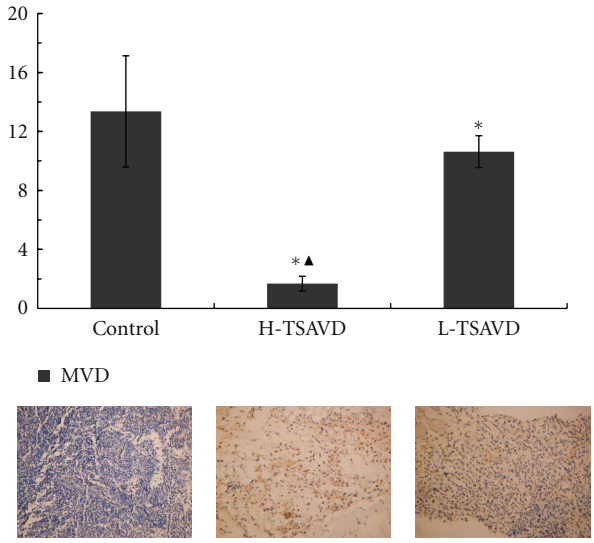

Figure 4: CD34 expressions of transplanted hypodermal hepatoma H22 bearing mice (*$P < 0.05$ versus control group, ▲$P < 0.05$ versus H-TSAVD group).

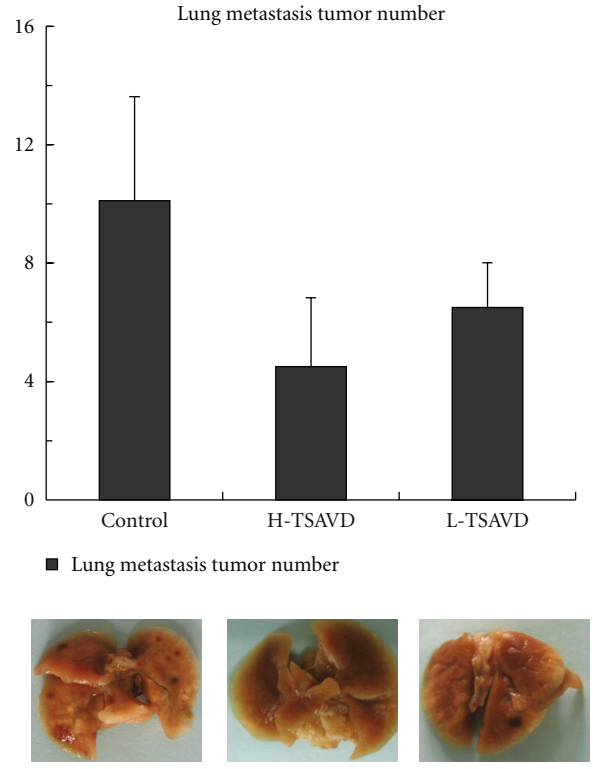

Figure 5: Antimetastasis effect of TSAVD on pulmonary metastasis hepatoma H22 bearing mice (*$P < 0.05$ versus control group).

4. Discussion

Overgrowth and metastasis are the two major characteristics of malignancy, so researches of effective Chinese herbs used for cancer therapy mainly focus on affecting each progress of carcinoma, involving precancerous lesion prevention, antitumor, antimetastasis, and so forth [11]. In solid tumors, angiogenesis is well characterized as a critical step for growth, invasion, and metastasis [12]. Angiogenesis refers to the process of new blood vessel formation from a preexisting vasculature which occurs in either physiological or pathological conditions [13]. This process is tightly regulated by a series of pro- and antiangiogenic molecules in normal physiology; however, serious consequences may arise when this equilibrium is broken [14]. Tumor angiogenesis shows a markedly increasing proliferation of endothelial cell and has significant functional and structural differences in the vascular plexus. Tumor cells promote vessel formation through the expression of angiogenic molecules or their induction in the microenvironment [15, 16].

Among the proangiogenic molecules, vascular endothelial growth factor (VEGF) and basic fibroblast growth

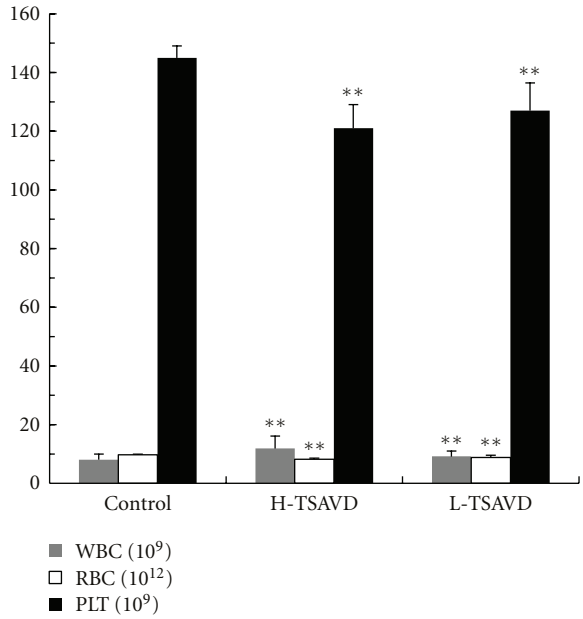

Figure 6: Blood cells of pulmonary metastasis hepatoma H22 bearing mice (**$P < 0.01$ versus control group).

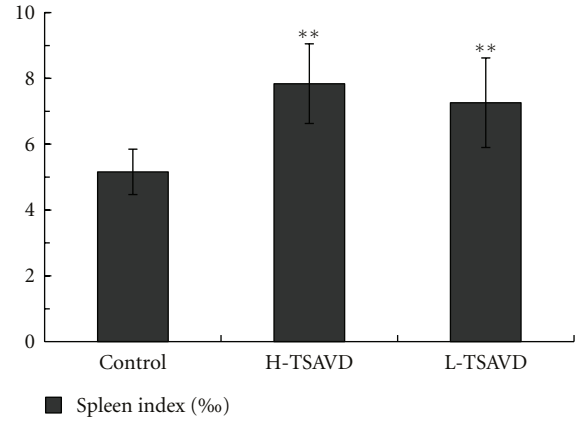

Figure 7: Spleen index of pulmonary metastasis hepatoma H22 bearing mice (**$P < 0.01$ versus control group).

factor (bFGF) have been identified to drive tumor-related angiogenesis. VEGF is the best-characterized angiogenic cytokine and the most potent angiogenesis inducer [2]. The crucial regulators of the angiogenesis process associated with tumor development and metastasis are VEGF and their receptors [17]. Hepatoma H22 tumor cells with significant invasion and metastasis can propagate easily in mice, so they are extensively applied for basal investigation and pharmic screening. Also in this study, transplanted hypodermal and experimental pulmonary metastasis hepatoma H22-bearing mice were used.

Actinidia valvata Dunn was widely used for hepatoma therapy by traditional Chinese doctor in clinical practice, exhibited antitumor and anti-inflammatory effects, and it had been used widely for hepatoma, lung carcinoma, and myeloma therapy for a long time. It is abundant in amino acids and inorganic elements that people need. Antitumor effects of *Actinidia valvata* Dunn had been investigated for the past five years, its injection had been confirmed to have a significant antitumor effect, significantly restrain the growth of primarily hypodermal tumor with no cell-mediate immunity inhibitive effects *in vivo* [4, 5]. TSAVD was isolated for the first time from the root of *Actinidia valvata* Dunn. In previous studies, we have found that TSAVD has inhibitory effects on HCC metastasis *in vitro*. The present study aimed to extend the previous study of TSAVD and to evaluate its anti-HCC mechanism *in vivo*.

Experimental data presented here showed that TSAVD played the potent antiangiogenic activity in mice *in vivo*. The antiangiogenic mechanism of TSAVD lies in the ability to inhibit hepatoma tumor growth and migration, the results showed that TSAVD can effectively restrain the growth and metastasis of hepatoma H22 *in vivo*.

According to the volume and immunohistochemistry of the tumors, the TSAVD-treated group showed a lower tumor volume and CD34 expression level (a lower blood vessel density). The result that TSAVD decreased the tumor volume and MVD implied TSAVD had the action of antitumor and antiangiogenesis activity. The expressions of bFGF in TSAVD-treated groups were less than control group, and also in H-TSAVD group, the expressions were less than that of L-TSAVD group. Expressions of VEGF in H-TSAVD were different from other two groups. In the process of tumor growth, the two-way paracrine action between tumor cells and vascular endothelial cells leads to obvious increase of tumor blood vessels and promote the tumor growth.

The pathochanges of metastasis tumor also show that TSAVD can accelerate putrescence of tumor in pulmonary metastasis hepatoma H22-bearing mouse experiments, Results also showed that TSAVD may improve levels of WBC, RBC, and PLT in mice, having no restraint effects like most chemotherapy medicine.

To sum up, the present study demonstrates that TSAVD can restrain growth and metastasis of HCC with no vice blood effects *in vivo*, restrain angiogenesis maybe one of the mechanism. Further studies will be done in our laboratory to konw the effects of TSAVD on other tumors.

Acknowledgments

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Research Article

The Chinese Herbal Decoction Danggui Buxue Tang Inhibits Angiogenesis in a Rat Model of Liver Fibrosis

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In this study, we investigated the anti-angiogenic effect of the Chinese herbal decoction Danggui Buxue Tang (DBT; *Radix Astragali* and *Radix Angelicae sinensis* in 5 : 1 ratio) in a rat model of liver fibrosis, in order to elucidate its mechanisms of action against liver fibrosis. Liver fibrosis was induced with CCl_4 and high-fat food for 6 weeks, and the rats were treated with oral doses of DBT (6 g raw herbs/kg/d) and N-Acetyl-L-cysteine (NAC; 0.1 g/kg/d). The results showed that both DBT and NAC attenuated liver fibrosis and neo-angiogenesis. Furthermore, DBT and NAC improved SOD activity but decreased MDA content and 8-OH-dG in fibrotic livers, with DBT being more effective than NAC. DBT decreased the expression of VEGF, Ang1 and TGF-β1 and their signaling mediators, whereas NAC had no effect on VEGF and VEGFR2 expression. Both DBT and NAC reduced HIF-1α gene and protein expression in fibrotic livers, with DBT being more effective. These data clearly demonstrate that the anti-fibrotic properties of DBT are related to its ability to inhibit angiogenesis and its anti-angiogenic mechanisms are associated with improving oxidative stress, regulating the expression and signaling of angiogenic factors, and especially modulating HIF-1α in fibrotic livers.

1. Introduction

Angiogenesis is a hypoxia-driven and growth factor-dependent process that leads to the formation of neovasculature from preexisting blood vessels. Experimental and clinical studies have unequivocally shown that pathological angiogenesis, irrespective of etiology, plays a key role in the fibrogenic progression of chronic liver diseases [1–3], and the inhibition of pathological angiogenesis in liver not only can stop liver cancer development, but also regress or reverse liver fibrosis [4, 5].

Danggui Buxue Tang (DBT), an ancient traditional Chinese herbal formula composed of Huangqi (*Radix Astragali*) and Danggui (*Radix Angelica sinensis*) with a weight ratio of 5 : 1, has wide pharmacological actions, including regulation of immune functions and protection against liver injuries [6]. Although there are no reports concerning the effect of DBT on liver cirrhosis, several studies have reported antifibrotic effects for its components. For example, the combination of *Astragali* and *Angelicae sinensis* significantly inhibited the progression of renal fibrosis. This treatment led to a decrease in histologic damage, type III and IV collagen expression, fibronectin, and laminin in a rat model of chronic puromycin-induced nephrosis [7]. *Astragali* significantly attenuated liver tissue collagen and hydroxyproline (Hyp) content in a rat model of liver fibrosis induced by albumin immune complex [8]. In a rat model of pulmonary fibrosis induced by intratracheal instillation of bleomycin,

Table 1: Antibodies used in the study.

Antibody	Isotype	Suppliers	Cat. no.	Dilution
Collagen type I	Mouse IgG1	Sigma	C2456	1 : 400
α-SMA	Rabbit polyclonal IgG	Abcam	ab5694	1 : 400
vWF	Rabbit polyclonal IgG	Abcam	ab6994	1 : 200
PECAM-1	Goat polyclonal IgG	Santa Cruz	sc-1506	1 : 200
HIF-1α	Rabbit monoclonal IgG	EPIT MICS	#2015-1	1 : 200
VEGF	Rabbit polyclonal IgG	Abcam	ab46154	1 : 400
VEGFR2	Rabbit polyclonal IgG	Abcam	ab39638	1 : 500
Angiopoietin 1	Rabbit polyclonal IgG	Abcam	ab95230	1 : 200
Tie-2	Rabbit polyclonal IgG	Abcam	ab71712	1 : 250
TGF-β1	Mouse IgG1	R&D systems	MAB240	1 : 500
TGFβ-R1	Rabbit polyclonal IgG	Cell signal technology	#3712	1 : 1000
TGFβ-R2	Mouse IgG_{2a}	Santa Cruz	sc-17792	1 : 200
ERK	Mouse IgG_{2b}	Santa Cruz	sc-1647	1 : 200
p-ERK	Mouse IgG_{2a}	Santa Cruz	sc-7383	1 : 200

Angelica sinensis ameliorated fibrosis by inhibiting thromboxane B_2 level and transforming growth factor-β1 (TGF-β1) expression [9].

Our previous study found that DBT was able to ameliorate liver fibrosis induced by carbon tetrachloride (CCl_4) in rats, with the best results seen with a 5 : 1 ratio of *Radix Astragali* and *Radix Angelicae sinensis* [10]. In a subsequent study, we showed that this protective effect of DBT was associated with the prevention of lipid peroxidation and the inhibition of matrix metalloproteinases 2/9 (MMP 2/9) activities in fibrotic livers [11].

In the present study, we observed the effects of DBT on angiogenesis in fibrotic livers, with the antioxidant N-Acetyl-L-cysteine (NAC) as a positive control drug. To test the hypothesis that the antifibrotic properties of DBT are related to its ability to inhibit angiogenesis, we also analyzed its effects on oxidative stress injury, expression of VEGF, TGF-β1, and Ang1, expression of VEGF-R2, TGFβ-R1/2 and Tie2 receptors, and ERK phosphorylation, in particular HIF-1α expression in the fibrotic liver. Collectively, our data demonstrate that the anti-angiogenic mechanisms of DBT in fibrotic livers are associated with improving oxidative stress, regulating angiogenic factors expression and signaling, and especially modulating the gene and protein expression of HIF-1α.

2. Materials and Methods

2.1. Reagents. CCl_4, olive oil and N-Acetyl-L-cysteine (NAC) were purchased from Shanghai National Chemicals Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Sino-American Biotechnology Co. (Henan, China). The primary antibodies used in this study are listed in Table 1. Nitrocellulose membrane (Hybond-C, optimized for protein transfer) was purchased from Amersham Biosciences UK, Ltd. (Buckinghamshire, UK). Horseradish peroxidase-labeled goat anti-mouse antibody and goat anti-rabbit antibody were obtained from Santa Cruz Biotechnology (California, USA). Cy3-labeled goat anti-rabbit IgG (H+L), Cy3-labeled donkey anti-goat IgG (H+L), and 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were provided by the Beyotime Institute of Biotechnology (Jiangsu, China). The BCA Protein Assay Kit and the SuperSignal West Pico Chemiluminescent Substrate (ECL) were obtained from Pierce Chemical Company (Rockford, USA). TRIzol was obtained from Invitrogen (California, USA). The First-Strand cDNA Synthesis Kit (K1622) was purchased from Fermentas (St. Leon-Roth, Germany). The SYBR Green Real Time PCR Kit (DRR041A) was from TakaRa Biotechnology Co., Ltd (Dalian, China). Superoxide dismutase (SOD), malondialdehyde (MDA), and 8-hydroxydeoxyguanosine (8-OH-dG) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Preparation of Danggui Buxue Decoction. DBT consists of *Radix Astragali* and *Radix Angelica sinensis* in a 5 : 1 ratio. The herbs originated from Gansu province, China. Slices of the herbs were purchased from Shanghai Huayu Chinese Herbs Co., Ltd. The medicinal herbs were extracted twice. Radix Astragali (1000 g) and *Radix Angelica sinensis* (200 g) were first boiled together in 6x volume of water for 1 h, and then the residue from first extraction was boiled in 8x volume of water for 1.5 h. Finally, the filtered solutions were combined and concentrated into the resulting aqueous extracts containing 0.9 g/mL raw herbs. The quantitative analyses of active compounds were verified by Professor Li. Yang (Table 2).

2.3. Animal Models of Liver Fibrosis and Drug Treatment. Fifty-four male Wistar rats (SCXK [Shanghai] 2007-005) were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All animal protocols were carried out in accordance with ethical guidelines, and animals had free access to chow and water throughout the

Table 2: The amounts of six compounds in DBT.

Compound	DBT (*Radix Astragali* : *Radix Angelica sinensis* = 5 : 1)
Astragaloside IV	61.63 ± 2.06
Calycosin	2.29 ± 0.08
Calycosin-7-O-β-D-glucoside	2.20 ± 0.03
Formononetin	0.68 ± 0.00
Formononetin-7-O-β-D-glucoside	1.12 ± 0.07
Ferulic acid	0.98 ± 0.02

Values are expressed in 10^{-2} mg/g of raw herbs and are in $\overline{X}$ ± sd, $n = 3$.

experiments. Liver fibrosis was induced by subcutaneous injection of CCl_4 and the administration of food with a high lipid content and lower protein content [12]. Briefly, the rats received a single injection of 100% CCl_4 at 5 mL/kg and then 3 mL/kg of 40% CCl_4 dissolved in olive oil twice every week for 6 weeks. These rats were pair-fed with a high lipid and low protein diet containing 79.5% corn flour, 20% lard, and 0.5% cholesterol for the first 2 weeks, then with pure corn flour feeds for the following 4 weeks. Rats in the normal group ($n = 8$) did not receive CCl_4 treatment and were fed a normal diet.

The model rats were randomly divided into three groups: model ($n = 12$), DBT ($n = 12$), and NAC ($n = 12$). The rats in the DBT group received intragastric administrations of DBT at 6 g (raw herbs)/kg/d. Rats in the NAC group received intragastric administrations of NAC at 0.1 g/kg/d. Both drug treatment duration is 6 weeks, which means from the beginning of intoxication to the end of experiment, and both dosages are equivalent to 10 times the clinical dosage for a 60 kg adult. Rats in the normal and model groups received the same volume (10 mL/kg) of normal saline.

2.4. Pathological Examination. Liver specimens were preserved in 4% formaldehyde and dehydrated in a graded alcohol series. The specimens were then embedded in paraffin blocks, cut into 4 μm thick sections and placed on glass slides. Sections were then stained with Sirius red.

2.5. Hepatic Hyp Content Examination. Hepatic Hyp content was measured with a modified version of the method developed by Jamall et al. [13]. Briefly, 100 mg of liver samples were homogenized and hydrolyzed in 12 M HCl at 110°C for 18 h. After filtration of the hydrolysate through filtration paper, chloramine T was added to a final concentration of 2.5 mM for 10 min at room temperature. The mixture was then treated with 25% (w/v) p-dimethylaminobenzaldehyde and 27.3% (v/v) perchloric acid in isopropanol (Ehrlich's reagent solution) and incubated at 50°C for 90 min. After cooling to room temperature, the samples were examined at 558 nm against a reagent blank that contained the complete system without tissue. The concentration of Hyp in each sample was determined from a standard curve, which was generated from a serial of known quantities of Hyp from 0.2 to 1.6 μg Hyp (Peptide Co. Japan). The Hyp content is expressed as μg/g of liver wet weight.

2.6. Immunohistochemistry. 4 μm thick sections were used for immunohistochemical examinations. After deparaffinization and dehydration, microwave antigen retrieval was performed for 5 min prior to peroxidase quenching with 3% H_2O_2 in PBS for 15 min. Consequently, the sections were preblocked with 5% bovine serum albumin for 30 min. Slides were incubated at 4°C with primary antibodies (Table 1) at 37°C for 70 min and then with biotinylated secondary antibodies for 45 min. They were then developed with DAB for 3 min and finally counterstained with hematoxylin. For the negative controls, the primary antibody was replaced with PBS.

2.7. Immunofluorescence. Frozen liver tissue slices (7 μm thick) were fixed with cold acetone for 10 min and dried in air for 30 min. The slices were rinsed with PBS for 3 × 5 min and blocked with 0.2% BSA for 1 h at 37°C before being incubated with the specific primary antibodies (Table 1). To visualize the primary antibodies, the slices were stained with cy3-labeled secondary antibodies for 1 h at 37°C. After nuclear staining with DAPI (1 : 1000; Beyotime) for 1 min, the slices were observed using immunofluorescence microscopy (OLYMPUS ZX70). Negative control staining was performed using PBS instead of the primary antibody. Microvessel density was determined in the tissue as the mean number of labeled vessel sections in 5 successive high-magnification fields (×400) with IPP software. The results are expressed as the means ± SD.

2.8. Western Blotting Analysis. Liver tissues were homogenized in RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, and 1×Roche complete mini protease inhibitor cocktail). The supernatants were collected by centrifugation at 10,000 ×g at 4°C for 15 min. Protein concentration was determined using a BCA Protein Assay Kit. Equal amounts of protein were separated by 10% SDS gel electrophoresis (SDS-PAGE) under denaturing and nonreducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST at room temperature for 1 h and then incubated with primary antibody (Table 1) at 4°C overnight. After washing in TBST, the blots were incubated with a horseradish-coupled secondary antibody. The signals were visualized using the enhancement system (ECL).

2.9. Fluorescence Western Blot. The fluorescence western blot protocol is the same as the general western blot protocol until the membrane transformation step. The membranes were blocked in blocking buffer (Odyssey, LI-COR, USA) for 1 h at room temperature followed by incubation with primary antibodies (Table 1) at 4°C overnight. After washing in PBS, the blots were incubated with the second antibody (Donkey anti-Rabbit IRDye 680 antibody; 1 : 10000; Odyssey) for 1 h at room temperature. After washing, the densities of

Table 3: Primers used for real-time PCR.

Gene	Primer sequences	Gene bank accession no.	Length (bp)
HIF1α	5′-CCAGATTCAAGATCAGCCAGCA-3′ 5′-GCTGTCCACATCAAAGCAGTACTCA-3′	NM_024359	100
β-actin	5′-TGA CGA GGC CCA GAG CAA GA-3′ 5′-ATG GGC ACA GTG TGG GTG AC-3′	DQ237887	331

immunoreactive bands were quantified and corrected for GAPDH signal with Li-Cor Odyssey 2.1 software (LI-COR, USA).

2.10. RNA Extraction and Quantitative Real-Time PCR. Total RNA was isolated from the liver tissues with TRIzol according to the manufacturer's instructions. RNA purity was determined spectrophotometrically, and its integrity was verified by agarose gel electrophoresis. First-Strand cDNA was synthesized by reverse transcribing 4 μg of total RNA in a final reaction volume of 20 μL using a First-Strand cDNA Synthesis Kit according to the manufacturer's instructions. Primer oligonucleotide sequences specific for real-time PCR are shown in Table 3 and were designed and synthesized by Sangon Biotech Inc. (Shanghai, China). The PCR mixtures contained 1 μL cDNA, 10 μL SYBR Premix Ex Tq 2X, and 0.25 μmol/L forward and reverse primers in a final volume of 20 μL. Triplicate reactions were performed with a Rcorbett 6.0 system (Rotor-Gene 3000), starting with a polymerase activation step for 10 s at 95°C, followed by 40 cycles of 5 s at 95°C and 20 s at 60°C. Fluorescence data were acquired after each cycle. The absence of primer dimers and unspecific products was verified after every run by melting curve analysis (72 to 95°C) and agarose gel electrophoresis.

2.11. Hepatic Oxidative Injury Examination. SOD, MDA and 8-OH-dG levels in the liver homogenates were quantified using commercially available kits according to the manufacturer's instructions (Nanjing Jiancheng Co. Ltd.). Results were normalized to the total amount of protein measured by bicinchoninic acid (BCA).

2.12. Statistical Analysis. Statistical tests were performed using SPSS software version 12.0. Differences between two groups were analyzed by the SNK-q' test. P values lower than 0.05 were considered statistically significant.

3. Results

3.1. DBT and NAC Ameliorated Liver Fibrosis Induced by CCl_4. The model rats had increased collagen deposition in the liver, which formed fibrous septa and cirrhotic nodules or pseudolobules. The doses of DBT and NAC used in this study were calculated according to their usage in patients with liver cirrhosis. Figures 1(a) and 1(b) show that DBT- and NAC-treated rats had much less liver collagen accumulation compared to the model control. This was confirmed with Hyp content as a specific marker for collagen synthesis. Compared to normal rats, Hyp content was increased significantly in model rats and significantly decreased by DBT and NAC treatment ($P < 0.01$; Figure 1(c)).

Table 4: Effect of DBT on hepatic HIF-1α mRNA expression in CCl_4-induced fibrosis in rats ($\overline{X} \pm$ sd).

Group	n	HIF-1α /β-actin mRNA
Normal	3	0.98 ± 0.03
Model	3	$3.32 \pm 0.46^{\#\#}$
DBT	3	$0.49 \pm 0.04^{**\wedge\wedge}$
NAC	3	$0.86 \pm 0.05^{**}$

$^{\#\#}P < 0.01$ versus normal group, $^{**}P < 0.01$ versus model group, $^{\wedge\wedge}P < 0.01$ versus NAC group.

3.2. Effect of DBT and NAC on Hepatic Stellate Cell (HSC) Activation. α-SMA is a marker of HSC activation. We measured the expression of α-SMA by immunohistochemical staining and western blot analysis. CCl_4 treatment caused a significant increase in the expression of α-SMA positive HSCs around damaged hepatocytes and fibrotic bands compared to the normal group. In DBT- and NAC-treated animals, the number of α-SMA-positive HSCs was significantly reduced. Western blot analysis revealed that the α-SMA expression in model rats was almost 5 times higher than that in normal rats. Treatment with DBT and NAC resulted in significant reductions. In addition, there was a significant difference between the DBT and NAC group ($P < 0.01$; Figures 2(a), 2(b) and 2(c)); DBT was more effective.

3.3. Effect of DBT and NAC on Tissue Angiogenesis. Immunofluorescence for von Willebrand factor (vWF) and platelet/endothelial cell adhesion molecule-1 (PECAM-1, also referred to as CD31) was performed to analyze microvessel growth. In normal livers, vWF is expressed in the great vessels of the portal tract and central veins but not along sinusoids, and CD31 is rarely expressed. In CCl_4-treated livers, we observed vWF/CD31-positive microvessels located in fibrotic areas surrounding larger vessels, as well as in emerging fibrotic septa. As illustrated in Figure 3(c), DBT and NAC reduced the number of vWF/CD31-positive microvessels, which were also concentrated in fibrotic areas. DBT was more effective than NAC in reducing vWF expression ($P < 0.01$).

3.4. Effect of DBT and NAC on Hypoxia Inducible Factor (HIF)-1α. Real-time PCR analysis showed that the expression of HIF-1α mRNA in the model livers was 3 times higher than in the normal group (Table 4). DBT and NAC

Table 5: Effect of DBT and NAC on hepatic SOD activity and MDA and 8-OH-dG contents in CCl_4-induced fibrosis ($\overline{X} \pm$ sd).

Group	n	MDA (μmol/g)	SOD (NU/g)	8-OHDG (ng/mL)
Normal	8	457.9 ± 140.6	42.9 ± 8.7	1.6 ± 0.6
Model	10	1054.5 ± 255.0$^{\#\#}$	24.7 ± 4.1$^{\#\#}$	2.8 ± 0.8$^{\#\#}$
DBT	12	642.8 ± 277.2**	37.7 ± 7.2$^{**\wedge}$	2.0 ± 0.4*
NAC	12	617.9 ± 344.5**	30.3 ± 15.8*	1.7 ± 0.9**

$^{\#\#}P < 0.01$ versus normal group, $^{**}P < 0.01$ versus model group, $^{\wedge\wedge}P < 0.01$ versus NAC group. Two rats in model group died in 6 weeks of modeling.

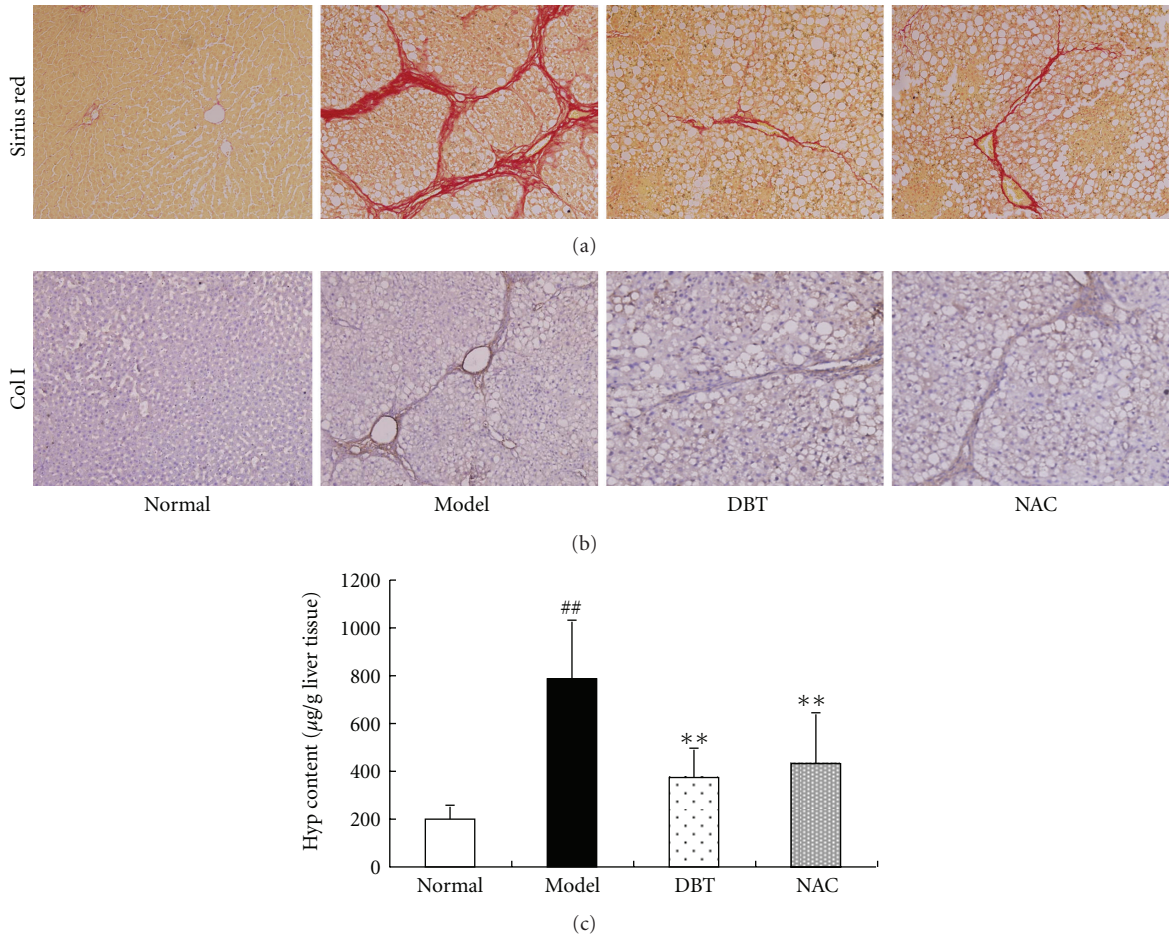

Figure 1: Effects of DBT and NAC on liver fibrosis induced by CCl_4. (a) Sirius red staining of collagen deposition in liver fibrosis. CCl_4 induced an increase in liver collagen. DBT and NAC decreased the collagen deposition in the fibrotic liver tissue. (b) Immunohistochemical staining of type I collagen protein expression in liver tissue (DAB). The model group had much stronger and more extensive positive staining among fibrotic septa compared with the normal group. DBT and NAC significantly decreased the amount of positive staining. (c) Liver Hyp content was determined using Jamall's method. The Hyp content was increased significantly in the model group compared with the normal group. DBT and NAC treatment significantly decreased liver Hyp content. $^{\#\#}P < 0.01$ versus normal group, $^{**}P < 0.01$ versus model group.

significantly decreased this expression (Table 4). This was confirmed with HIF-1α immunostaining (Figure 4). In the livers of normal animals, HIF-1α immunolabeling was rarely detected. In the fibrotic livers, HIF-1α immunolabeling was highly expressed, especially in zone III of the acinus. DBT and NAC decreased HIF-1α expression in the fibrotic livers ($P < 0.05$). Consistent with the immunohistochemistry findings, western blot analysis showed that HIF-1α expression was significantly increased in the fibrotic livers ($P < 0.01$, versusnormal group). DBT and NAC decreased HIF-1α expression ($P < 0.01$), with a significant difference between the two treatment groups ($P < 0.01$).

3.5. Effects of DBT and NAC on Hepatic Oxidative Injury. CCl_4 intoxication significantly increased hepatic contents of MDA and 8-OH-dG, and decreased hepatic SOD activity (Table 5) ($P < 0.01$, versus normal group). DBT and NAC

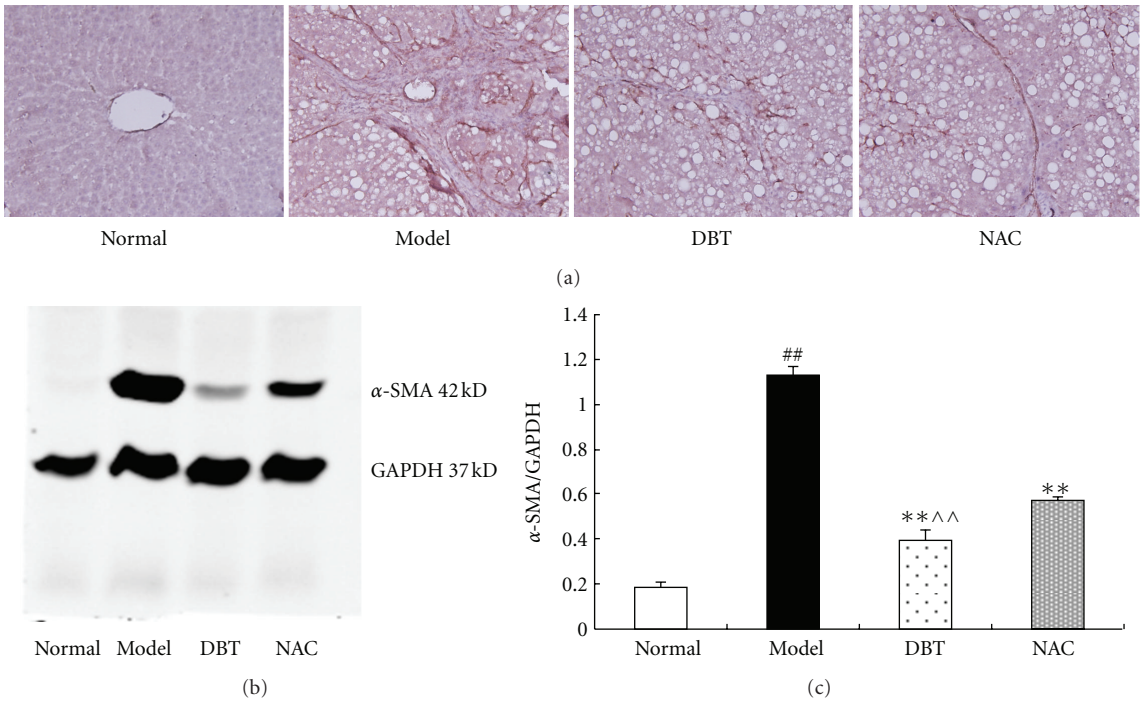

Figure 2: Effect of DBT and NAC on α-SMA protein expression in CCl_4-induced fibrotic liver tissue in rats. (a) Immunohistochemical staining, DAB × 200. Normal liver tissue had little positive staining. Tissue from the model group had much stronger and more extensive positive staining among fibrotic septa and in the sinusoidal area. DBT and NAC significantly decreased the amount of positive staining. (b) A representative western blot. Consistent with the immunohistochemistry findings, the semi-quantitative value of the α-SMA western blot was significantly increased in fibrotic livers. Compared to the model control group, DBT significantly decreased α-SMA expression, and the effect of DBT was more pronounced than that of NAC. (c) A histogram plot showing the densitometric analysis corresponding to the mean ± SD of three independent experiments. $^{\#\#}P < 0.01$ versus normal group, $^{**}P < 0.01$ versus model group, $^{\wedge\wedge}P < 0.01$ versus NAC group.

reduced hepatic MDA and 8-OH-dG contents, increased the SOD activity ($P < 0.05$). In addition, DBT has better effect than NAC on increasing SOD activity in fibrotic liver.

3.6. Effects of DBT and NAC on Expression of VEGF, Ang1 and TGF-β1 and Their Signaling Mediators. Western blot analysis (Figure 5) showed that the expressions of pro-angiogenic growth factors such as VEGF, TGF-β1, and Ang1 elevated in fibrotic livers ($P < 0.01$, versus normal group), and their respective receptors—VEGF-R2, TGFβR-1/2 and Tie2 also increased. ERK is common down-stream signaling mediator for VEGF, TGF-β1, and so forth, the result showed that phosphorylation of ERK-1/2 increased remarkably, although total ERK1 has no change. DBT, but not NAC, significantly decreased VEGF and VEGF-R2 protein expression ($P < 0.01$), and there was a significant difference in VEGF expression between DBT- and NAC-treated groups. ($P < 0.01$). Both DBT and NAC down-regulated TGFβ-R1/2 and Tie2 expression and ERK-1/2 phosphorylation in fibrotic liver ($P < 0.05$), compared to the model control.

4. Discussion

The liver fibrosis, caused by many etiologies such as viruses, toxins, alcohol and cholestasis and so forth, is an essential pathological process in chronic liver diseases, and lead into cirrhosis which is end stage of chronic liver diseases. Therefore, it is very important to find an approach for curing or regressing liver fibrosis. Angiogenesis is the main process of new vessel formation, and accompanies liver fibrosis [2]. Recently a increasing body of evidences has shown that angiogenesis play a crucial role in liver fibrogenesis [14], and the inhibition of angiogenesis with multitargeted receptor tyrosine kinase inhibitors sunitinib or sorafenib could regress or reverse liver fibrosis in animals [15, 16]. Angiogenesis, a hypoxia-driven and growth factor-dependent process, also plays a key role in the development of cirrhosis [1, 2, 5, 17–20]. Since the conventional therapies are not desirable for curing liver fibrosis, the manipulation of angiogenesis could be a promising approach for treatment of liver fibrosis.

According to traditional Chinese medicine (TCM) theory, liver fibrosis is caused by a dual deficiency of Qi-yin and blood stasis [21]. DBT has good function of nourishing Qi (energy flow) and Xue (blood), as well as resolving Yuxue (blood stasis), and has been used traditionally to treat menopausal disorders [6]. In our previous study, we found that DBT could ameliorate CCl_4-induced liver fibrosis in rats [11]. In this study, we found that in normal livers, vWF and CD31, two markers of angiogenesis, are expressed few and limited to the large vessels of the periportal region and could not be found along sinusoids. However, their

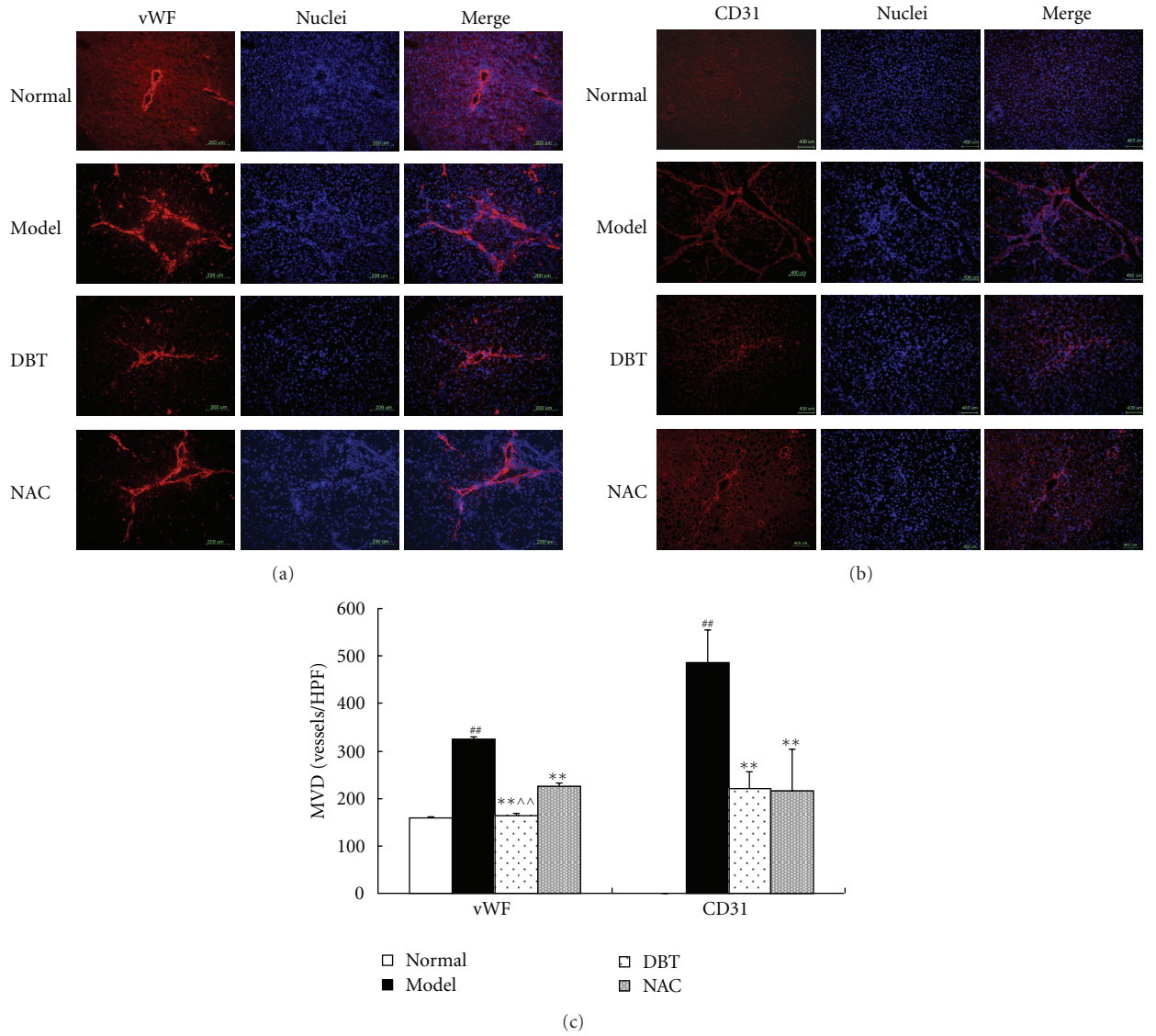

Figure 3: Effect of DBT and NAC on vWF and CD31 expression in CCl_4-induced fibrotic liver tissue in rats, (a, b) Microvessel density in different groups. Immunofluorescence for vWF and CD31 was performed to analyze microvessel growth. In normal livers, the expression of vWF was limited to large vessels located in the periportal zone with no expression along the sinusoids, and no CD31 expression was observed. In CCl_4-treated livers, increased vWF/CD31-positive microvessels were seen primarily in fibrotic areas. DBT- and NAC-treated livers showed reduced amounts of vWF/CD31-positive microvessels, which were also concentrated in fibrotic areas. A significant difference in vWF expression was seen between DBT- and NAC-treated groups. (c) A bar graph representation of microvessel density reported as the mean number of labeled vessel sections in 5 successive high-magnification fields (×400). The results are expressed as mean ± SD for three separate experiments. $^{\#\#}P < 0.01$ versus normal group, $^{**}P < 0.01$ versus model group, $^{\wedge\wedge}P < 0.01$ versus NAC group.

expression increased remarkably in fibrous septa and along sinusoids in fibrotic livers, which means that rich neovessels formed in fibrotic livers. While DBT and NAC treatment could decrease the expression of vWF and CD31 proteins, indicating that DBT and NAC can inhibit liver angiogenesis, which may be one of the action mechanisms of DBT and NAC antifibrotic property. It is reported recently that the thiol antioxidant NAC had effect against liver fibrosis [22] and decreased tumor angiogenesis and tumorigenesis [23]; our current study confirmed NAC's effect on liver fibrosis and find its new action on fibrotic liver angiogenesis.

Angiogenesis is a dynamic, hypoxia-stimulated and growth factor-dependent process. A recent document has identified that there are number of common cellular and molecular mechanisms between angiogenesis and liver fibrosis, with a specific emphasis on the crucial role of hypoxic conditions, angiogenic factors and HSC activation. The hypoxia play a central role in angiogenesis through

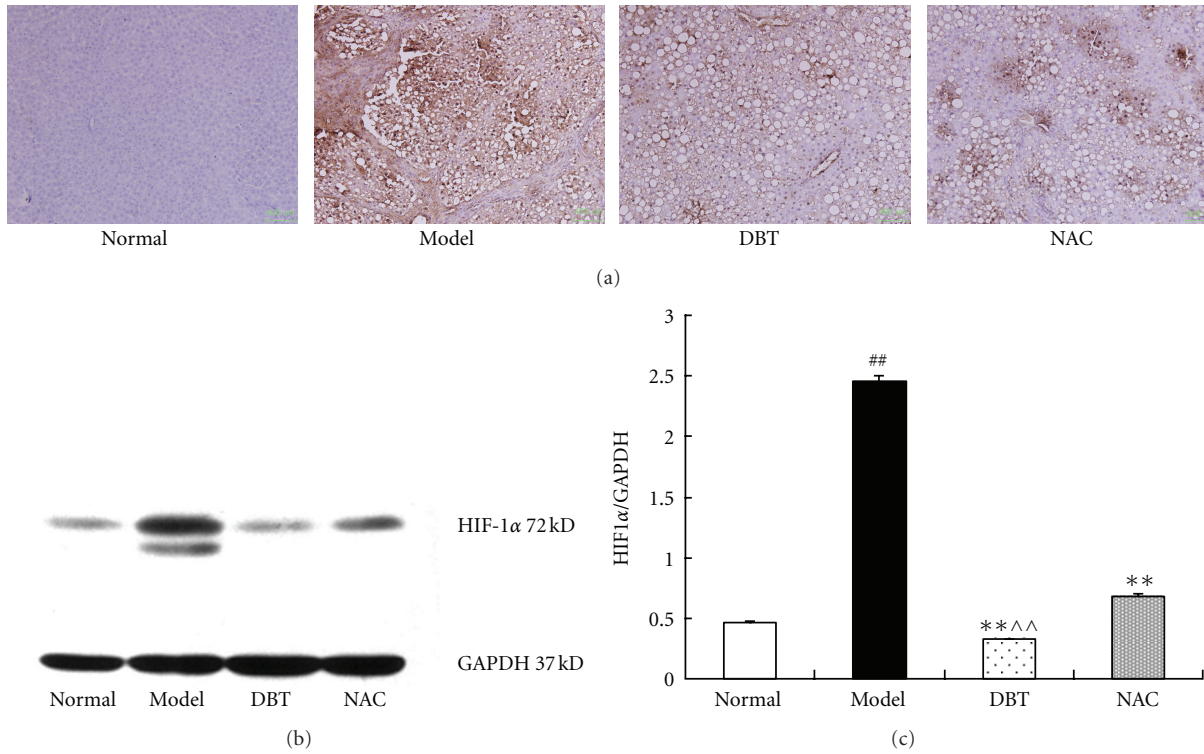

Figure 4: Effect of DBT and NAC on HIF-1α protein expression in CCl_4-induced fibrotic liver tissue in rats. (a) Immunohistochemistry (DAB × 200). In normal livers, HIF-1α immunolabeling was rarely detected. In fibrotic livers, HIF-1α immunolabeling was highly expressed, especially in zone III of the acinus. DBT and NAC decreased HIF-1α expression in the fibrotic livers. (b) A representative western blot. Consistent with the immunohistochemistry findings, the semiquantitative value of the HIF-1α western blot was significantly increased in fibrotic livers. Compared to the model rats, DBT and NAC significantly decreased the expression of HIF-1α, with DBT being more effective than NAC. (c) A histogram showing HIF-1α protein semiquantified by densitometric analysis and expressed as the mean±SD for three separate experiments. $^{\#\#}P < 0.01$ versus normal group, $^{**}P < 0.01$ versus model group, $^{\wedge\wedge}P < 0.01$ versus NAC group.

inducing HIF-1α [24]. HIF-1α is a heterodimeric DNA binding complex, it serve as a key regulator of the molecular hypoxia response and mediates a wide range of physiological and pathological processes. In the study, although we did not check hepatic hypoxia by assaying pO_2 etc., HIF-1α, a hypoxia-driven factor, was mainly expressed in perivenous region (hepatic zone III area) where O_2 concentration is lowest in liver and susceptible to be damaged. And fibrotic liver had much higher levels of gene and protein expression of HIF-1α, while DBT and NAC decrease this pathological elevated HIF-1α levels, in particular HIF-1α expression in perivenous region, these suggested that DBT and NAC action against liver angiogenesis was associated with down-regulation of HIF-1α, which at least partly own to improving hypoxia condition.

However, HIF-1α upregulation could be hypoxia-independent, as elicited by growth factors, oncogenes, and oxidative stress and injury and so forth. The oxidative stress could cause lipid peroxidative products such as MDA and 8-OH-dG, leading to liver injury and angiogenesis [25], which can be regulated by endogenous antioxidant enzymes such as SOD and thioredoxin [26]. Recent studies have shown that antioxidants such as NAC can inhibit tumor angiogenesis [27]. In the study, we found DBT and NAC both improve SOD activity but decrease contents of MDA and 8-OH-dG in fibrotic liver, indicating that DBT and NAC inhibition of HIF-1α and angiogenesis is related to their effect on oxidative stress and injury. VEGF, TGF-β1, and Ang1 are potent angiogenic and fibrogenic factors, which may induce angiogenesis directly through their signaling pathway or indirectly by inducing HIF-1α which in turn promote its target genes including VEGF expression. VEGF, TGF-β1 and Ang1 stimulate vascular development and stabilization through signaling mediators, including their receptors—VEGF-R1/2 and TGFβ-R1/2, Tie2 and mitogen-activated protein kinase (MAPK) cascade [28, 29]. In the present study, DBT not only inhibited the expression of growth factors including VEGF, TGF-β1, and Ang1, but also downregulated their receptors expression and ERK phosphorylation which is common cytoplasmic mediator, indicating that the inhibition of VEGF, TGF-β1 and Ang1 and their signaling is another important mechanism for DBT action on HIF-1α, and against angiogenesis and liver fibrosis.

In addition, MMP-2/9 play potent role in initiation of angiogenesis and activation of HSC, while activated HSCs synthesizing Ang 1 and VEGF contribute to both fibrogenesis and neovascularization [20]. In a previous study, we showed that the protective effect of DBT was associated with the

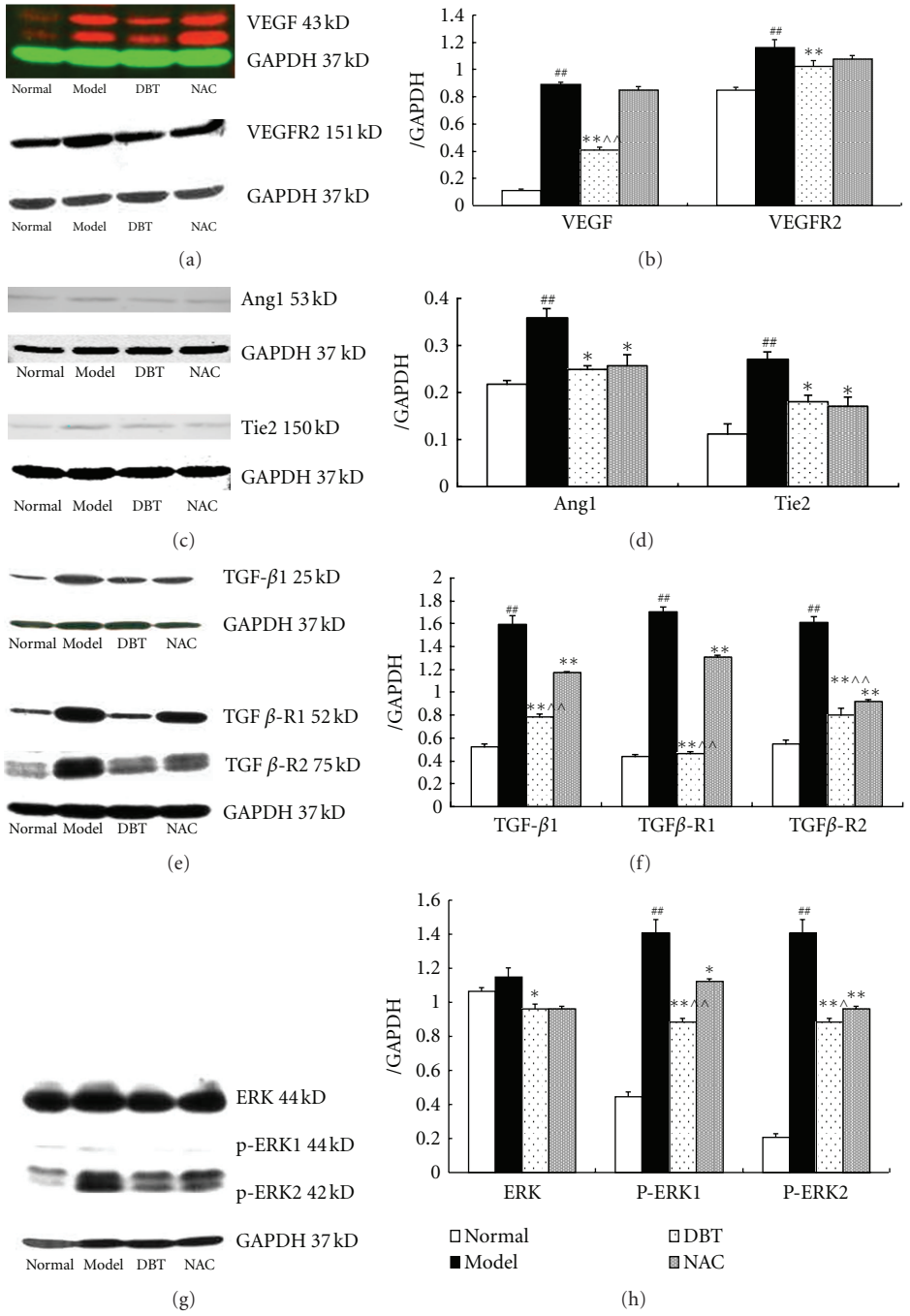

Figure 5: Effect of DBT and NAC on VEGF, Angiopoietin 1 and TGF-β1 signaling in CCl_4-induced fibrotic liver tissue in rats. (a) The expression of VEGF and VEGFR2 protein was detected by western blot. Compared to the normal group, the expression of VEGF and VEGFR2 was significantly increased in fibrotic livers. DBT, but not NAC, significantly decreased VEGF and VEGFR2 expression, and there was a significant difference in VEGF expression between DBT- and NAC-treated groups. (c) The expression of the Angiopoietin 1 and Tie 2 protein was detected by western blot. Compared to the normal group, the expression of Ang1 and Tie 2 was increased in fibrotic livers. DBT and NAC decreased Ang1 and Tie 2 expression. (e) The expression of the TGF-β1/TGFβ-R protein was detected by western blot. Compared to the normal group, TGFβ1/TGFβ-R protein expression was significantly increased in fibrotic livers. DBT and NAC significantly decreased the expression of TGFβ1/TGFβ-R. (g) The expression of ERK and p-ERK protein was detected by western blot. Although there was no significant difference in ERK protein expression among the four groups, p-ERK protein expression was significantly increased in fibrotic livers. DBT and NAC significantly decreased p-ERK protein expression, and there was a significant difference between DBT- and NAC-treated groups. (b, d, f, h) Histograms of VEGF/VEGFR2, Ang1/Tie 2, TGFβ1/TGFβ-R, and ERK/p-ERK expression. The proteins were semi-quantified by densitometric analysis and expressed as the mean $\pm$ SD for three separate experiments. $^{\#\#}P < 0.01$ versus normal group, $^{**}P < 0.01$ versus model group, $^{\wedge\wedge}P < 0.01$ versus NAC group.

inhibition of MMP 2/9 activities in fibrotic livers [11]. And the current experiment reconfirmed that DBT and NAC inhibited HSC activation *in vivo*, which suggest that inhibition of MMP-2/9 and HSC activation is the third mechanism of DBT action mechanism against angiogenesis in fibrotic liver.

It is interesting to note that DBT can stimulate the production of erythropoietin, a specific hematopoietic growth factor, mediated by increasing the mRNA and protein expression of HIF-1α as well as the activation of Raf/MEK/ERK signaling pathway in cultured cells [30]. While these data appear to contradict our findings, these data collectively illustrate the potential of DBT and other herbal formulations in modulating cellular pathophysiology to achieve the desirable clinical outcome.

In summary, we found that DBT inhibits angiogenesis in CCl_4-induced liver fibrosis in rats in the current study, which is closely related to DBT anti-fibrotic property; and that DBT reduces the expression of HIF-1α, VEGF, TGF-β1, and Ang1, decreases the receptors expression of VEGF-R2, TGFβ-R1/2 and Tie2, downregulates ERK phosphorylation, and improves hepatic oxidative injury in fibrotic liver; these effects contribute to the overall action mechanisms of DBT against liver angiogenesis and fibrosis.

Glossary

DBT: Danggui Buxue Tang
NAC: N-Acetyl-L-cysteine
CCl_4: Carbon tetrachloride
α-SMA: Alpha-smooth muscle actin
HIF-1α: Hypoxia inducible factor-1α
vWF: von Willebrand factor
PECAM-1: Platelet/endothelial cell adhesion molecule-1, also referred to as CD31
VEGF: Vascular endothelial growth factor
VEGF-R2: Vascular endothelial growth factor receptor 2, also referred to as KDR
TGF-β1: Transforming growth factor-β1
TGFβ-R: Transforming growth factor-β1 receptor
p-ERK: Phospho-extracellular signal-regulated kinase
BSA: Bovine serum albumin
ECM: Extracellular matrix
CLDs: Chronic liver diseases
DAPI: 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride
Hyp: Hydroxyproline
HSC: Hepatic stellate cell
MMPs: Metalloproteinases
SOD: Superoxide dismutase
MDA: Malondialdehyde
8-0H-dG: 8-hydroxy-deoxyguanosine
Ang1: Angiopoietin 1
Tie2: Tyrosine kinase with immunoglobulin G-like and endothelial growth factor-like domain 2.

Acknowledgments

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Research Article

Evaluation of the Chinese Medicinal Herb, *Graptopetalum paraguayense*, as a Therapeutic Treatment for Liver Damage in Rat Models

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The incidence of cirrhosis is rising due to the widespread occurrence of chronic hepatitis, as well as the evident lack of an established therapy for hepatic fibrosis. In the search for hepatoprotective therapeutic agents, *Graptopetalum paraguayense* (GP) showed greater cytotoxicity toward hepatic stellate cells than other tested herbal medicines. Histopathological and biochemical analyses suggest that GP treatment significantly prevented DMN-induced hepatic inflammation and fibrosis in rats. Microarray profiling indicated that expression of most of metabolism- and cell growth and/or maintenance-related genes recovered to near normal levels following GP treatment as classified by gene ontology and LSM analysis, was observed. ANOVA showed that expression of 64% of 256 liver damage-related genes recovered significantly after GP treatment. By examining rat liver samples with Q-RT-PCR, five liver damage-related genes were identified. Among them, *Egr1* and *Nrg1* may serve as necroinflammatory markers, and *Btg2* may serve as a fibrosis marker. *Oldr1* and *Hmgcs1* were up- and down-regulated markers, respectively. A publicly accessible website has been established to provide access to these data Identification of 44 necroinflammation-related and 62 fibrosis-related genes provides useful insight into the molecular mechanisms underlying liver damage and provides potential targets for the rational development of therapeutic drugs such as GP.

1. Introduction

Hepatic fibrosis is a wound-healing process that follows chronic liver injury and is characterized by the activation of hepatic stellate cells (HSCs) and excess production of extracellular matrix (ECM) components. HSC activation involves transdifferentiation from a quiescent state into myofibroblast-like cells and includes the appearance of alpha smooth muscle actin (α-SMA) and loss of cellular vitamin A storage [1]. Activated HSCs are distinguished by accelerated proliferation and enhanced production of ECM components. Major cascades of the injury process have been reported, including interactions between damaged hepatocytes and Kupffer cell activation, HSC proliferation and activation and the excess production of ECM components during hepatic fibrosis [2]. However, the types of genes involved and their reactions to liver injury and healing are unclear. Moreover, cirrhosis caused by these risk factors often progresses insidiously.

Patients with end-stage liver cirrhosis usually die without liver transplantation, which has a 75% five-year survival rate [3].

The identification of both effective drug treatments and molecular markers for liver fibrosis diagnosis is an important task. Numerous agents, including Silymarin and penicillamine, have been employed to treat hepatic fibrosis [4, 5]. Silymarin, a mixture of flavonoids extracted from the seeds of *Silybum marianum* containing three structural isomers (silybin, silydianin, and silychristin), has exhibited hepatoprotective effects both *in vivo* and *in vitro* [4]. Silymarin suppresses the expression of both profibrogenic procollagen alpha (I) and Timp1, most likely via down-regulation of *Tgfb1*, in rat models [6]. It is also used to protect liver cell membranes against hepatotoxic agents and has been shown to improve liver function in both experimental animals and humans [7]. Corticosteroids and methotrexate may play roles in primary sclerosing cholangitis (PSC) treatment. Penicillamine is an effective treatment for Wilson's disease, which is characterized by excessive copper accumulation in the liver and other organs. However, due to Penicillamine's serious side effects, there is not yet a generally definitive treatment [8–11]. Herbal medicines have been used in China for thousands of years. Traditional Chinese herbal medicines may contain therapeutic agents for treating hepatic fibrosis [12]. Moreover, there is a growing trend in Western countries to use Chinese medicine to treat a wide range of diseases, such as inflammatory diseases and chronic liver diseases (including hepatitis and fibrosis). For example, Radix Polygoni Multiflori, the root tubers of *Polygonum multiflorum* Thunb, is a traditional Chinese treatment for liver disease and has been shown to impede the hepatic deposit of collagen and significantly improve survival rates in mice with DMN-induced liver cirrhosis [13]. Sho-saiko-to (TJ-9), a potent antifibrosis drug that inhibits lipid peroxidation in hepatocytes and HSCs, is an effective treatment for liver inflammation and fibrosis [14, 15]. Inchin-ko-to (TJ-135) is a possible treatment for liver fibrosis and portal hypertension that acts through suppression of activated HSC function by regulating PDGF-dependent events in HSCs and attenuating the development of liver fibrosis [16, 17]. *Graptopetalum paraguayense* (GP), a traditional Chinese medicine, has been identified as a possible hepatoprotective therapeutic agent. GP, which has been used as a health food in Taiwan, exhibits potentially beneficial effects on hypertension, diabetes, hyperuricemia, inflammation, and chronic liver diseases. However, the lack of information regarding these compounds' molecular mechanisms diminishes their clinical utilities.

This study aims to characterize the therapeutic effects of GP on liver fibrosis using microarray profiling. Silymarin was used as a positive drug control. Dimethylnitrosamine (DMN), a potent nongenotoxic hepatotoxin, has been demonstrated to induce liver damage rapidly and is empirically proven to be useful in the study of early human fibrosis formation [18]. We employed DMN to induce liver fibrosis in rats and performed a six-week time course Affymetrix microarray study [19]. A quantitative depiction of transcriptional regulation over the course of liver fibrosis was achieved using statistical analysis of histopathological grading of the rats. The histopathological, clinical biochemical, and microarray data are freely available at http://ehco.iis.sinica.edu.tw:8080/LFZ/.

2. Materials and Methods

2.1. Preparation of GP. GP was purchased from a herb farm in Taiwan. GP leaves were washed with distilled water and air dried overnight, then freeze-dried at −50°C by a frozen dryer, and ground into powder (100 mesh). Lyophilized GP powder was stored in a sealed container at 4°C until use. To prepare GP extracts, GP powder was first dissolved in water, and ethanol was gradually added to a final concentration of 80%. After centrifugation at 1400 ×g for 20 min, the resulting precipitates were discarded; the supernatant was filtered through a 0.22 μm filter and evaporated to dryness on a rotary evaporator. The recovery of GP extracts after ethanol treatment was 65%. The dried extracts were dissolved in water at a concentration of 50 mg/mL as a stock solution. The stock solution was then diluted in culture medium to the appropriate working solution.

2.2. Isolation of HSCs and Cell Viability Determination. HSCs were isolated from the livers of male SD rats (500 to 600 g). Briefly, the livers were perfused *in situ* through the portal vein with a 16-guage cannula, first with Ca^{2+}/Mg^{2+}-free HBSS solution at 37°C for 10 min at a flow rate of 10 mL/min, followed by 0.1% pronase E (Merck, Darmstadt, Germany) in HBSS solution for 10 min and 0.3% collagenase (Wako, Osaka, Japan) in HBSS solution for 30 min. The digested liver was excised, minced with scissors, and incubated in HBSS solution containing 0.05% pronase E and 20 μg/mL DNase for 30 min. The resulting suspension was filtered through a nylon mesh (150 mm in diameter). An HSC-enriched fraction was obtained by centrifugation of the filtrate in an 8.2% Nycodenz (Nycomed, Oslo, Norway) solution at 1400 ×g and 4°C for 20 min. The cells in the upper layer were washed by centrifugation at 450 ×g at 4°C for 10 min and suspended in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% L-glutamine. The purity of the isolated HSCs was assessed both by direct cell counting under phase-contrast microscopy using intrinsic vitamin A autofluorescence and by immunohistochemistry using a monoclonal antibody against desmin (DAKO; diluted 1 : 40). Cell viability was examined using trypan blue dye exclusion. Both cell purity and viability were in excess of 90%. HSCs were plated at a density of 5×10^5 cells per well in 1 mL of culture medium on uncoated plastic culture dishes. The culture medium was changed 2 days after plating. GP extracts were first prepared as PBS stock solutions (0.5 g/mL) and then diluted in culture medium to the appropriate working solutions. Cells were maintained at 37°C in a 5% CO_2 incubator for indicated time periods.

2.3. Animal Treatments. Liver fibrosis in the rat models was induced using DMN as previously described [19]. DMN treatments took place over three weeks (Figure 2(a)).

GP extracts (1.4 g/kg) or Silymarin (0.4 g/kg) was orally administered daily to the rats beginning on day 7 of DMN treatment. All rats received distilled water and libitum. Food intake and body weights were recorded weekly, and rats were observed daily for clinical signs of ill health. Rats were euthanized with CO_2. All animals that were euthanized or found dead were autopsied, and their tissues were fixed in 10% neutral buffered formalin. Rats were separated into groups of 4 to 7, and each group was treated with either DMN or an equal volume of normal saline without DMN as a control. All rats were subjected to biochemical and histopathological analyses. Two rats from each group were subjected to microarray analysis every week. Rats were weighed and euthanized on days 11, 18, 25, 32, 39, and 46, designated as weeks 1 through 6, respectively (Figure 2(a)).

2.4. Histopathological Examination. Liver specimens were fixed with phosphate-buffered formaldehyde, embedded in paraffin, and stained with hematoxylin-eosin. Differential staining of collagenous and noncollagenous proteins was performed with 0.1% Sirius red and 0.1% fast green as a counterstain in saturated picric acid, resulting in red-stained collagens. The scoring system, based on the histology activity index (HAI) [20, 21], included necroinflammatory, fibrosis, and fatty change scores as previously described [19]. Three images of each histology sample section (at 100× magnification) from each rat were selected randomly, scored, and deposited on the publicly accessible website (http://ehco.iis.sinica.edu.tw:8080/LFZ/).

2.5. Serum Biochemical Data. Blood samples collected from the animals at autopsy were used to measure serum concentrations or activities of albumin, aspartate aminotransaminase (AST), alanine aminotransferase (ALT), total bilirubin, acid phosphatase (ACP), α-fetoprotein (AFP), blood urea nitrogen (BUN), lactate dehydrogenase (LDH), globulin, prothrombin time (PT), and blood platelets (PLT) using a Hitachi 747 and an ACL 3000 clinical chemistry analyzer system (MYCO, Renton, Washington) at Taichung Veterans General Hospital, Taiwan.

2.6. RNA Extraction, Reverse Transcription, and Quantitative Real-Time-Reverse Transcriptase-Polymerase Chain Reaction (Q-RT-PCR). The same total RNA samples were used for both microarray and Q-RT-PCR analyses, which were performed as previously described [19]. Applied Biosystems Assays-on-Demand was used to identify transforming growth factor beta-1 (*Tgfb1*), tissue inhibitor of metalloproteinase 1 (*Timp1*), tissue inhibitor of metalloproteinase 2 (*Timp2*), peroxisome proliferator-activated receptor gamma (*Pparg*), B-cell translocation gene 2 (*Btg2*), early growth response 1 (*Egr1*), oxidized low-density lipoprotein (lectin-like) receptor 1 (*Olr1*), neuregulin 1 (*Nrg1*), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (*Hmgcs1*), and 18s ribosomal RNA (as an internal control).

2.7. Microarray Analysis, Data Analysis, and Clustering Algorithm. Protocols and reagents for hybridization, washing, and staining followed Affymetrix instructions (http://www.affymetrix.com/support/technical/manuals.affx). The images were transformed into text files containing intensity information using Affymetrix GeneChip Operating Software. Microarray datasets were then analyzed using GeneSpring 7.3.1 software (Silicon Genetics, Redwood City, CA).

2.8. Statistical Analysis. All statistical analyses were performed using SAS/STAT 8e (SAS Institute, Cary, NC). Biochemical data are expressed as mean ± standard deviation (mean ± S.D.). Two-way analysis of variance (ANOVA) was used to build an explicit model of the sources of variances in the measurements. The least squares means (LSM) method was used to identify significant differences among groups across treatment and time course factors. Differences between control groups and treated groups were evaluated on the basis of mean biochemical data from the first and the second weeks, the third and the fourth weeks, and the fifth and the sixth weeks. Similarities between Q-RT-PCR and microarray data of *Timp1*, *Tgfb1*, and *Pparg* are presented using Pearson's correlation coefficients. Necroinflammatory and fibrosis-associated genes were identified by statistical analysis. LSM, separately estimated for variations in each three-subgroup variations by necroinflammatory score, were used for necroinflammatory-related analysis. Nonparametric test methods, estimated for only two-subgroup variations by fibrosis score, were used in fibrosis-related analyses. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. GP Has Greater HSC Cytotoxicity Than Other Tested Herbal Medicines. Herbal medicines have been used in Chinese population for thousands of years. It is generally believed that traditional Chinese herbal medicines may contain therapeutic agents for treating hepatic fibrosis [12]. Moreover, there has been a growing trend in Western countries to use Chinese herbal medicines to treat a wide range of diseases, such as inflammatory diseases and chronic liver diseases (including hepatitis and fibrosis). We tested HSC drug sensitivity using 10 traditional Chinese medicines (Figure 1), including six individual herb species (*Graptopetalum paraguayense, Phyllanthus urinaria, Salvia miltiorrhiza, Bupleurum falcatum L., Panax pseudoginseng* var. *Notoginseng*, and *Astragalus membranaceus*) and four formulas, (Tao Zen Qian Cao Tang, Er Ju Tang, Jia Wei Xiao Yao San, and Da Huang Zhe Chong Wan). Among the tested substances, HSCs were the most sensitive to GP. Next, we measured the expression of the activated HSC markers α-SMA and collagen 1; these were also downregulated after GP treatment in the culture system (see below, Figure 3(b)). We therefore focus on characterization of GP's therapeutic potential.

3.2. GP Serves as a Better Therapeutic Agent Than Silymarin for DMN-Induced Necroinflammation and Hepatic Fibrosis as Indicated by Histopathological and Biochemical Data. We compared the therapeutic effects of GP and Silymarin on rats with DMN-induced liver fibrosis. A schematic illustration of

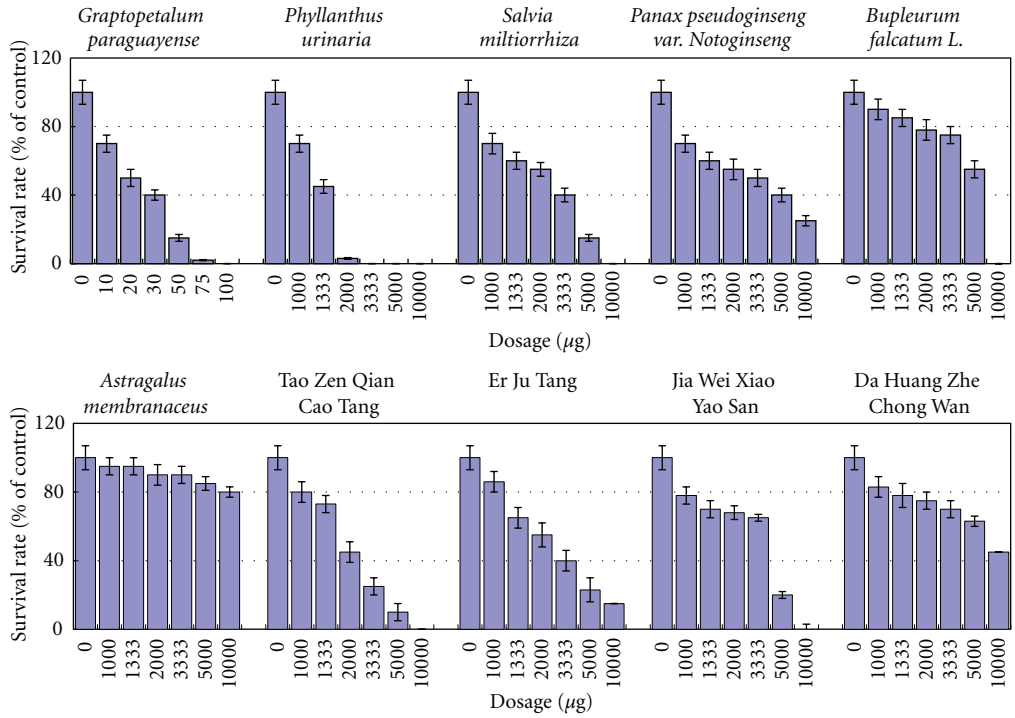
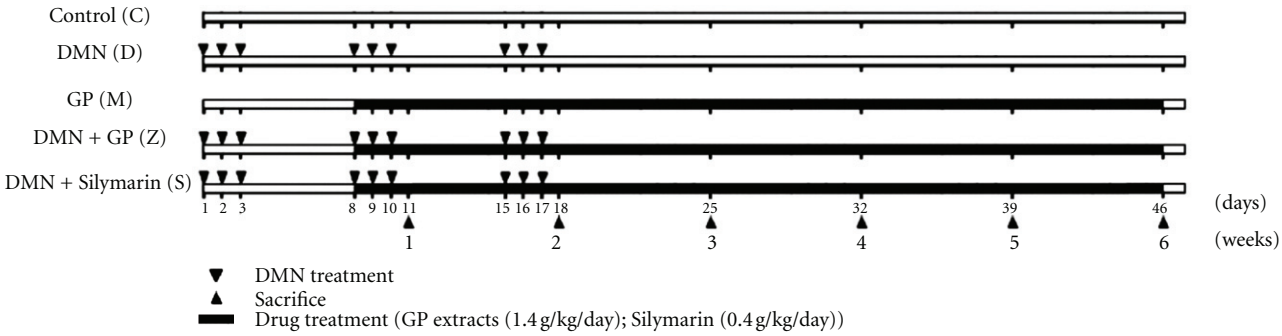


Figure 1: Screening of traditional Chinese medicines for therapeutic agents targeting liver fibrosis using HSC as a model. Characterizations of medicine-induced HSC cell death are shown. Ten traditional Chinese medicines, including six herb species and four formulas, were used: *Graptopetalum paraguayense*, *Phyllanthus urinaria*, *Salvia miltiorrhiza*, *Panax pseudoginseng var. Notoginseng*, *Bupleurum falcatum L.*, *Astragalus membranaceus*, Tao Zen Qian Cao Tang, Er Ju Tang, Jia Wei Xiao Yao San and Da Huang Zhe Chong Wan.

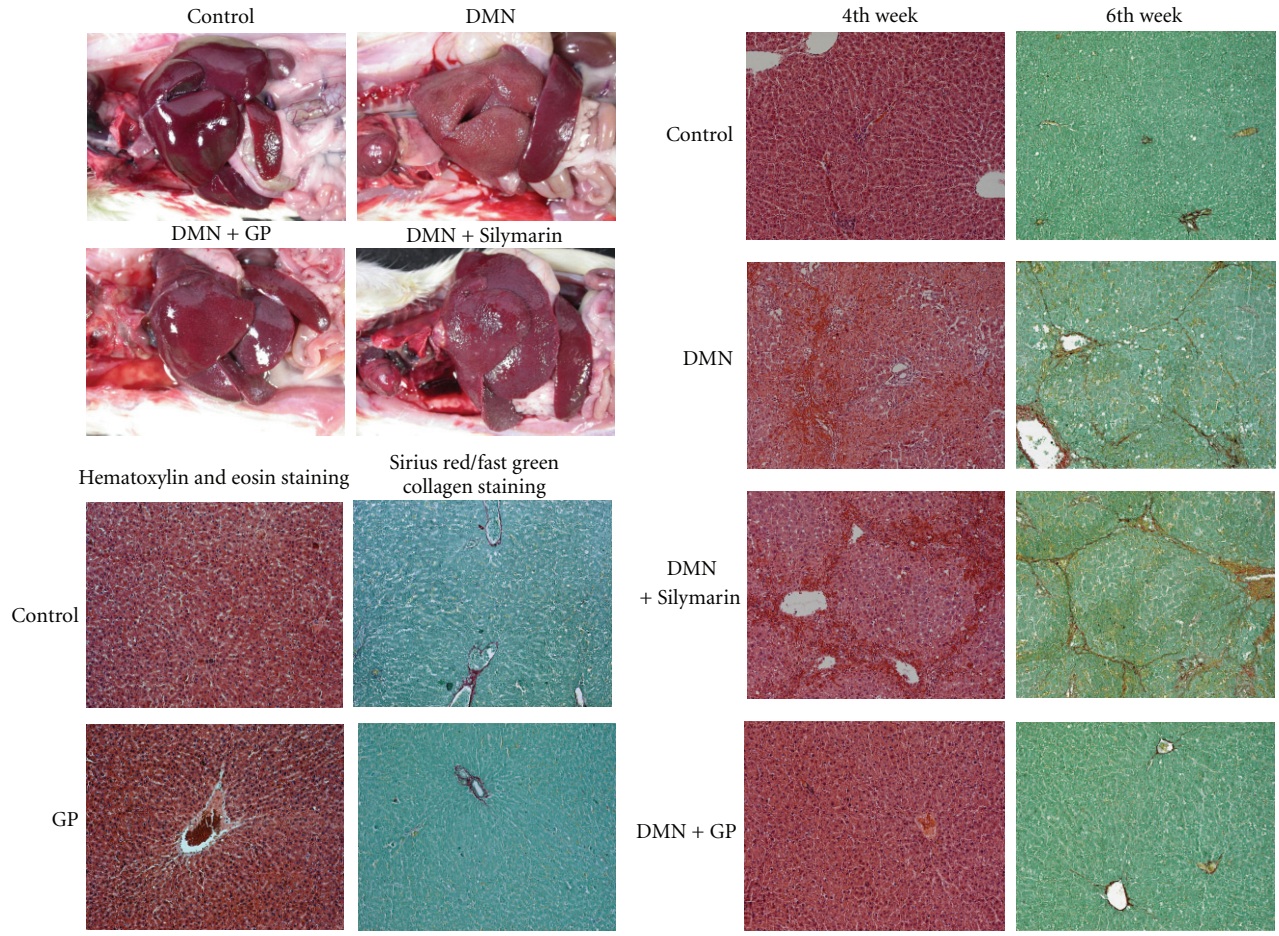
the course of administration of DMN, GP, and Silymarin is shown in Figure 2(a). GP treatment resulted in increased survival rates in pilot studies (data not shown). The livers of GP-treated rats externally appeared much healthier than those treated with Silymarin, during liver damage (Figure 2(b)). To further delineate the biological and histopathological characteristics of GP treatment, experimental rats were weighed and euthanized and their livers excised and weighed. A scoring system, as previously described [19], was used to characterize phenotypic changes due to DMN-induced liver damage. Treatment with DMN caused significant decreases in both rat body and liver weights that were alleviated by GP treatment (data not shown). We next investigated GP's effect on necrosis and inflammatory responses in the liver following DMN exposure. Massive necrosis in the pericentral and midzonal area, with infiltration of inflammatory cells, was observed 3 weeks following DMN treatment. Following GP treatment, histopathological examinations found a 31% reduction in necroinflammatory response in the third and forth weeks and a 30% reduction of bridging fibrosis in the fifth and sixth weeks. These results indicated greater therapeutic value than those of Silymarin (19% reduction in necroinflammatory response and no effect on bridging fibrosis) (Table 1) and that GP treatment may significantly reduce DMN-induced hepatic fibrosis and necroinflammatory responses.

The sera of the control and DMN-treated rats both treated and untreated GP were subjected to various biochemical examinations. The rats were further divided into three subgroups (first to second weeks, third to fourth weeks, and fifth to sixth weeks) for statistical analysis. Biochemical analyses of the GP-treated subgroups indicated significant recovery compared to the DMN-treated groups as illustrated in Tables 2 and 3. Two-way ANOVA at a 5% significance level was performed to distinguish the variations between the treatments (e.g., DMN + GP versus DMN alone or differences due to the time course) and to estimate the variance of each individual variable in the ANOVA model. In the GP-treated groups, 9 of 14 serum markers, including ALT, AST, bilirubin, AKP, AFP, BUN, PT, and PLT, showed significant differences. These differences were not due to changes over the time course (1–6 weeks). These data indicate that DMN-induced increases in serum GTP, AST, and bilirubin levels were negated through GP treatment. Treatment with DMN resulted in marked reductions of serum albumin levels, but recovery at these levels was observed in the DMN + GP-treated groups. Although there were also significant effects on LDH, globulin, and ACP after GP treatment, two-way ANOVA indicated that changes in these three serum markers could be due to the time factor during 6-week experiments. Taken together, these pathological and biochemical data suggest that GP may have therapeutic effects on liver damage in rat models.

3.3. GP Suppresses α-SMA Expression in DMN-Treated Rats. Next, the expression of α-SMA, an activated HSC marker, in the liver was measured by IHC staining 6 weeks after initial



(a)



(b)

Figure 2: Histopathological analysis of the therapeutic effects of *Graptopetalum paraguayense* (GP) and Silymarin on liver damage in rat models. (a) Schematic illustration of DMN-induced fibrosis in rats. Each rat was injected with either DMN or saline, as a control, three times per week for three consecutive weeks (triangles). Subsequently, either Silymarin or GP was dissolved in water and given orally from days 8 to 46 (black rectangles). Rats were weighed and euthanized each week (starting on day 11, which begins what are referred to as the first through sixth weeks). Blood samples were collected for biochemical analysis (summarized in Table 1), and livers were excised and weighed, followed by either fixing in formaldehyde for histopathology or isolation of RNA for microarray analysis. (b) Anatomical evaluation of damaged livers (left upper panel). Based on exterior views, the GP had greater therapeutic effect than Silymarin. Representative phenotypes of DMN-induced rat liver fibrosis and therapeutic effects of GP and Silymarin were characterized by histopathological scoring. The fixed liver samples were then processed for paraffin embedding and prepared for hematoxylin and eosin staining (to score necroinflammation in the fourth week) and for Sirius red/fast green collagen staining (to score for fibrosis in the sixth week) (right panel). Drug toxicity effects were also evaluated by scoring. No notable liver damage patterns were observed after the six-week course of treatment (left lower panel). The original magnification was 100×.

Table 1: Summary of rat model histopathological scores.

Factor (scores)		DMN			DMN + GP			DMN + Silymarin			GP	Control
		1-2 wk	3-4 wk	5-6 wk	2 wk	3-4 wk	5-6 wk	2 wk	3-4 wk	5-6 wk	2–6 wk	1–6 wk
		n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n*(%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Necroinflammatory	A0	0 (0)	1 (9)	4 (44)	3 (50)	4 (45)	9 (64)	0 (0)	2 (22)	2 (20)	10 (100)	100 (24)
	A (1–3)	5 (62.5)	3 (27)	4 (44)	3 (50)	2 (22)	1 (7)	3 (75)	3 (33)	2 (20)	0 (0)	0 (0)
	A (4–6)	3 (36.5)	7 (64)	1 (12)	0 (0)	3 (33)	4 (29)	1 (25)	4 (45)	6 (60)	0 (0)	0 (0)
Fibrosis	F (0-1)	6 (75)	1 (9)	2 (22)	6 (100)	8 (67)	7 (50)	3 (75)	3 (33)	0 (0)	10 (100)	100 (24)
	F (2-3)	2 (25)	10 (91)	7 (78)	0 (0)	4 (33)	7 (50)	1 (25)	6 (67)	10 (100)	0 (0)	0 (0)
Fatty change	−	7 (87.5)	11 (100)	9 (100)	6 (100)	12 (100)	14 (100)	4 (100)	8 (89)	10 (100)	10 (100)	100 (24)
	+	1 (12.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (11)	0 (0)	0 (0)	0 (0)

Results are ranked by time course. Necroinflammatory change was scored as A (0), no change; A (1–3), mild; A (4–6), moderate necroinflammation. Fibrosis was graded as F (0-1) for observations ranging from normal tissue to fibrous expansion of portal tracts and F (2-3) for bridge fibrosis to frequent bridging fibrosis with nodule formation. The fatty change was classified by its presence or absence (+/−). The number of rats was counted and used to calculate the percentage of each histopathological level at each time point.
n: number of rats.

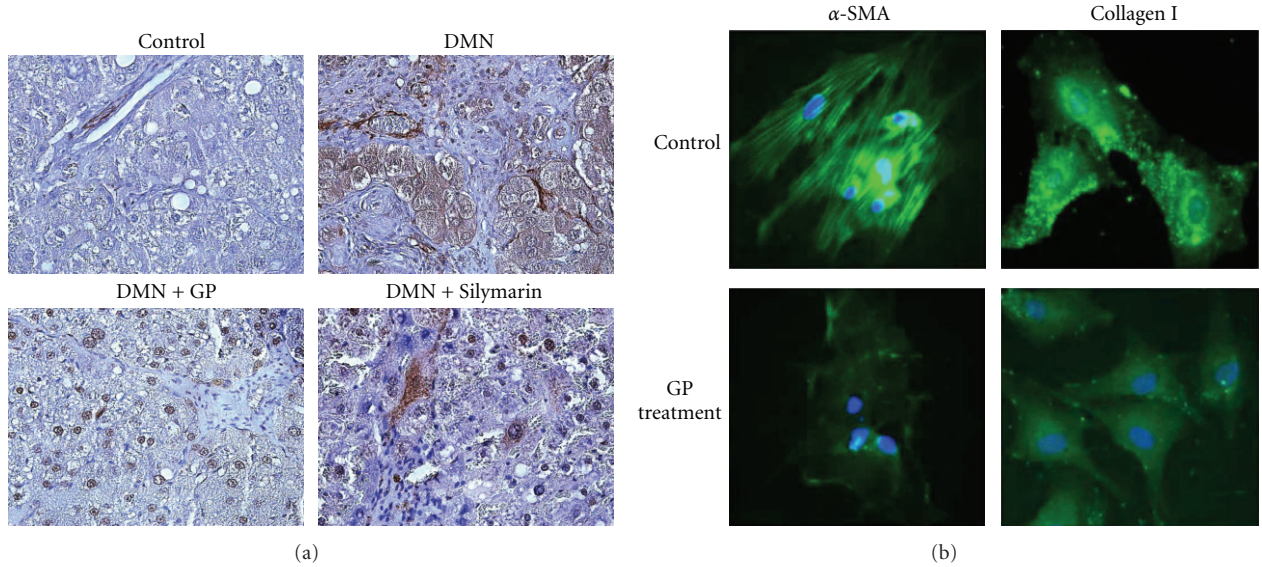


Figure 3: GP-derived reduction of DMN-induced α-SMA expression in livers and HSCs. Liver samples were excised 6 weeks after the first DMN injection. (a) The expression and localization of α-SMA in the liver were detected by immunohistochemical staining using a monoclonal antibody for α-SMA. Representative photomicrographs from controls, DMN treatment alone, DMN – Silymarin treatment, and DMN – GP treatment are shown. The original magnification was 100×. (b) Immunocytochemical staining of α-SMA and collagen I expression in cultured HSC. Cells were treated with or without 0.5 mg/mL GP extract for indicated time points and stained with FITC-conjugated α-SMA and collagen I antibodies. Representative photomicrographs from controls, DMN treatment alone, DMN – Silymarin treatment, and DMN – GP treatment are shown. The original magnification was 400×.

DMN administration. As expected, intense specific staining for α-SMA was observed in the pericentral area of the DMN-treated livers (Figure 3(a)). This expression was suppressed dramatically by oral administration of GP. Staining did not detect α-SMA in livers from vehicle control group rats or rats given GP or Silymarin alone, as expected (Data not shown). These findings indicate that GP prevented fibrogenic responses in the liver following DMN administration. In addition, TUNEL staining of histologic sections indicated an increase in the number of TUNEL-positive cells in GP-treated rat liver in regions containing α-SMA. Dual staining for TUNEL and α-SMA implied colocalization. These data suggest that the observed reduction in the number of activated HSCs in fibrotic liver in response to GP was mediated via apoptosis (data not shown).

3.4. Inhibitory Effect of GP on the Activation of Cultured Rat HSCs. Immunocytochemical analysis confirmed that GP suppressed α-SMA expression and stress fiber formation. HSCs were the major cellular source of extracellular matrix in hepatic fibrosis and were transformed into myofibroblast-like cells specifically expressing α-SMA. To examine the direct effect of GP on HSC activation, primary cultured HSCs were incubated with various doses of GP for 5 days (from days 5 to 10), and the expression of α-SMA was measured by indirect

Table 2: Therapeutic effects of *Graptopetalum paraguayense* (GP) on DMN-induced liver fibrosis in rats via biochemical analysis (1).

Numeric variable	Categorical variable						
		DMN			DMN + GP		
	Control (n)[a]	1st to 2nd wk (n)[b]	3rd to 4th wk (n)[b]	5th to 6th wk (n)[b]	2nd wk (n)[c]	3rd to 4th wk (n)[c]	5th to 6th wk (n)[c]
Albumin (g/dL)	4.6 ± 0.3 (23)	3.9 ± 0.7 (07)	3.5 ± 0.6 (11)	3.2 ± 0.1 (07)	3.7 ± 0.5 (06)	4.0 ± 0.7 (12)	4.3 ± 0.6 (14)
GPT (U/L)	58.8 ± 19.6 (23)	459.5 ± 78.5 (08)	566.6 ± 313.5 (11)	763.6 ± 405.2 (07)	235.0 ± 128.6 (06)	207.0 ± 111.7 (12)	262.5 ± 386.2 (14)
GOT (U/L)	101.9 ± 30.4 (23)	661.5 ± 134.4 (08)	1006.1 ± 749.6 (11)	1572.9 ± 965.3 (07)	271.3 ± 106.0 (06)	190.4 ± 114.5 (12)	218.0 ± 259.6 (14)
Bilirubin (mg/dL)	0.11 ± 0.04 (24)	0.72 ± 0.53 (08)	1.00 ± 0.74 (11)	1.13 ± 1.00 (07)	0.25 ± 0.14 (06)	0.25 ± 0.20 (12)	0.3 ± 0.32 (14)
AKP (KA)	45.9 ± 7.5 (12)	600.8 ± 93.0 (04)	668.3 ± 222.0 (03)	468 ± 12.7 (02)	298.2 ± 65.8 (04)	353.3 ± 62.3 (08)	374.0 ± 88.3 (11)
LDH (IU/L)	280.5 ± 49.0 (11)	414.8 ± 102.7 (04)	562.0 ± 120.8 (03)	853.5 ± 91.2 (02)	352.0 ± 21.9 (04)	378.6 ± 36.1 (08)	329.5 ± 67.6 (11)
Globulin (g/dL)	7.0 ± 0.4 (11)	6.65 ± 0.1 (02)	5.0 ± 0.84 (04)	3.6 ± 03 (02)	5.9 ± 0.6 (04)	5.7 ± 0.5 (08)	5.9 ± 1.0 (11)
Triglyceride (mg/dL)	148.0 ± 33.7 (12)	151.3 ± 107.3 (04)	180.9 ± 144.4 (07)	102.8 ± 35.1 (05)	73.0 ± 26.9 (02)	96.5 ± 29.9 (08)	98.3 ± 32.2 (11)
AFP (ng/dL)	0.26 ± 0.06 (10)	0.40 ± 0.19 (04)	0.38 ± 0.05 (04)	0.35 ± 0.07 (02)	0.20 ± 0.00 (02)	0.28 ± 0.13 (08)	0.26 ± 0.08 (10)
CHOL (mg/dL)	83.2 ± 0.14.1 (12)	76.5 ± 7.9 (04)	69.5 ± 12.8 (06)	66.6 ± 17.5 (05)	60.0 ± 5.0 (04)	65.9 ± 15.5 (08)	70.8 ± 18.6 (11)
BUN (mg/dL)	27.2 ± 6.0 (12)	32.5 ± 3.9 (04)	36.25 ± 2.2 (04)	30.5 ± 4.9 (02)	18.8 ± 3.9 (04)	20.9 ± 2.2 (08)	30.6 ± 23.0 (11)
ACP (mg/dL)	2.4 ± 0.6 (12)	1.9 ± 0.6 (04)	6.2 ± 1.1 (04)	8.2 ± 0.6 (02)	3.8 ± 0.6 (04)	5.0 ± 1.5 (08)	3.7 ± 1.1 (11)
PT (sec)	13.1 ± 1.1 (22)	18.5 ± 4.0 (08)	19.7 ± 4.3 (09)	21.5 ± 4.6 (06)	16.1 ± 2.6 (06)	17.2 ± 5.4 (10)	16.3 ± 2.9 (13)
PLT (10^3/mL)	871.5 ± 191.8 (24)	406.6 ± 71.7 (07)	300.2 ± 164.7 (11)	228.6 ± 302.4 (07)	535.3 ± 137.9 (06)	548.9 ± 259.1 (12)	704.5 ± 301.1 (14)

[a]: Mean ± SD of value from 1st to 6th week in control groups.
[b]: Mean ± SD of value from 1st to 2nd, 3rd to 4th or 5th to 6th week in DMN treatment groups.
[c]: Mean ± SD of value from 2nd, 3rd to 4th or 5th to 6th week in DMN+GP treatment groups.
n: number of rats.

Table 3: Therapeutic effects of *Graptopetalum paraguayense* (GP) on DMN-induced liver fibrosis in rats via biochemical analysis (2).

Numeric variable	p[a]			Categorical variable[b]		
	Drug[c]	Week[d]	Drug × Week	1st to 2nd wk	3rd to 4th wk	5th to 6th wk
Albumin	0.1285	0.3895	0.2342	−0.17	0.38	0.83
GPT	**0.0003**	0.6769	0.7826	−225	−360	−501
GOT	**<0.0001**	0.3298	0.2164	−390	−816	−1355
Bilirubin	**<0.0001**	0.6728	0.7539	−0.48	−0.75	−1
AKP	**<0.0001**	0.6172	0.1147	−303	−230	−107
LDH	**<0.0001**	**0.0132**	**0.0013**	−63	−141	−363
Globulin	0.1341	**0.029**	**0.0254**	−0.75	0.58	1.65
Triglyceride	0.0658	0.4116	0.355	−78	−84	−5
AFP	**0.0031**	0.8992	0.6289	−0.2	−0.1	−0.1
CHOL	0.324	0.9847	0.3248	−16.5	−3.6	4.2
BUN	**0.0444**	0.5896	0.4293	−13.8	−13.6	−1.6
ACP	**0.097**	**0.0004**	**0.0006**	1.8	−0.7	−3.6
PT	**0.0285**	0.7278	0.9627	−2.4	−2.5	−5.3
PLT	**0.0008**	0.3879	0.4526	129	249	476

[a]: Significant correction is marked in bold at the 0.05 level by two-way ANOVA.
[b]: $\text{Average}_{\text{DMN+GP}}$-$\text{Average}_{\text{DMN}}$ in 1st to 2nd, 3rd to 4th or 5th to 6th week.
[c]: DMN or DMN + GP treatment groups.
[d]: 1st to 2nd, 3rd to 4th or 5th to 6th week.

immunofluorescence analysis (Figure 3(b), left panel). At day 10 of culture, control HSC cultures demonstrated increased expression of α-SMA, which appeared to form stress fiber. However, continuous administration of GP for 5 days drastically inhibited α-SMA expression compared to these controls. Next, the effect of GP on expression of collagen I in activated HSCs *in vitro* was evaluated by immunocytochemical staining. At day 10, marked expression of collagen I in the cytoplasm of the control HSCs was observed (Figure 3(b), right panel). This expression was inhibited by the addition of GP extract. These observations confirmed that GP inhibited transactivation and production of α-SMA and collagen I in primary cultured HSCs *in vitro*.

3.5. GP Reverses Gene Expression Patterns of Liver-Damage-Related Markers. Previous biochemical studies have identified several well-known fibrosis and cirrhosis markers, both invasive and noninvasive [3, 22, 23]. For example, tissue inhibitors of metalloproteinases (TIMPs), secreted by activated HSCs, can prevent matrix degradation by inhibiting the enzymatic activities of matrix degrading metalloproteinases (MMPs) [3] and transforming growth factor beta-1 (TGFB1). TGFB1 is also the strongest known inducer of fibrogenesis in the effecter cells of hepatic fibrosis and can stimulate the adipocyte transformation [24–27]. To study the molecular characteristics of liver fibrosis in DMN-treated rats and the therapeutic efficacy of GP, we examined the gene expression profiles of rat liver samples using Affymetrix rat oligochips (RG-U34A) and Q-RT-PCR analysis. Consistent with previous observations, both microarray and Q-RT-PCR indicated higher levels of *Tgfb1* and *Timp1* mRNA expression in DMN-treated rat livers than in the controls (Figures 4(a) and 4(b)). Expression of these markers was significantly reduced when the DMN-damaged rats were treated with GP, but only *Timp1* expression was reduced when Silymarin was administered. Regulation of peroxisome proliferator-activated receptors (PPARs), a transcription factor family involved in the retinoic-acid- (RA-) mediated signal pathway of lipid metabolism [28], usually takes place when HSCs are activated in culture systems [29]. In this study, downregulated expression of *Pparg* was found in DMN-treated rat liver, consistent with the hypothesis that downregulation of *Pparg* may be connected to liver inflammation and fibrosis mechanisms (see Supplementary Figure 1 available online at doi: 10.1155/2012/256561). Microarray and Q-RT-PCR results indicated that *Pparg* expression recovered after GP treatment, suggesting potential for protecting or preventing liver damage.

3.6. Microarray Analysis. A set of 256 previously identified liver damage-related discriminator genes [19] were further analyzed using principal component analysis (PCA), a decomposition technique that produces a set of expression patterns known as principal components. Rats in the DMN-damaged group treated with GP fared much better than those treated with Silymarin, especially in the sixth week (Figure 5(a), left), in this analysis. Hierarchical clustering found similar results (Supplementary Figure 2). Additional ANOVA found that the expression of 64% of the 256 genes recovered significantly after GP treatment, but only 9% recovered after Silymarin treatment (Figure 5(a), right). Significantly, treatment with GP returned the expression of more than 60% of these discriminators to the levels of the controls. The 256 genes were further classified via gene ontology analysis (http://www.fatigo.org/) [30]. In each category, the largest group (approximately 50%) was found to be uncharacterized genes in the summary results (data not shown). Hierarchical clustering was further employed to

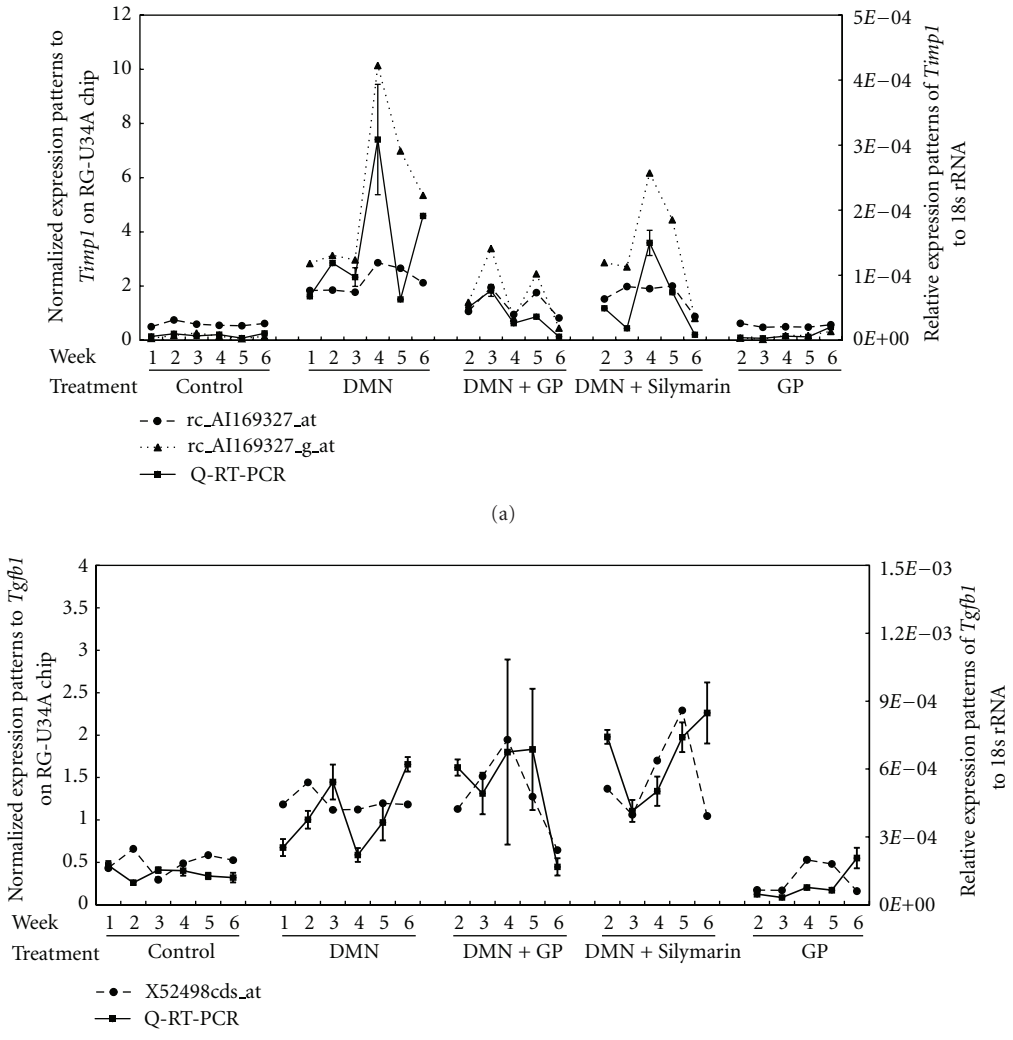
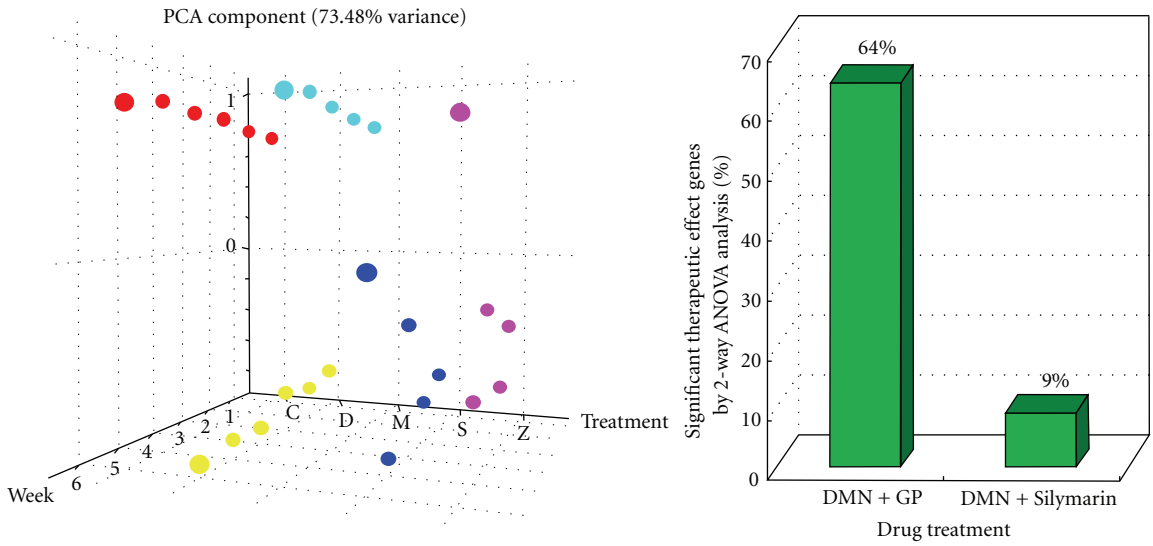


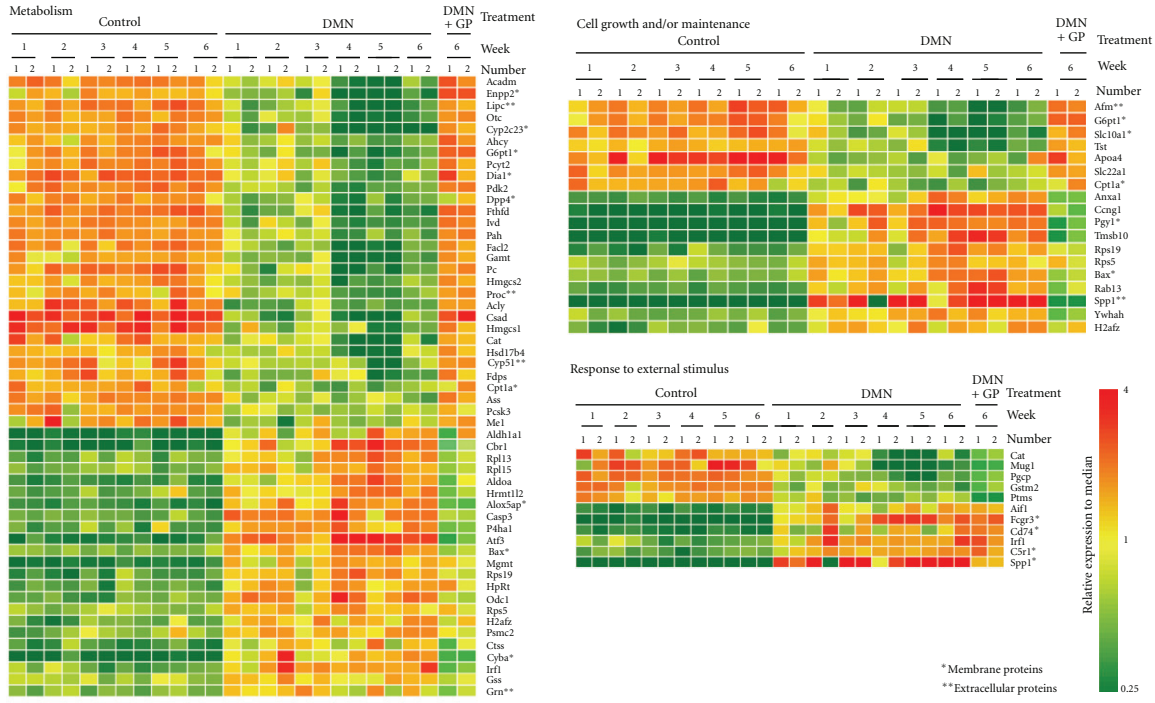
Figure 4: The therapeutic effects of GP were validated using marker expression patterns. A comparison of gene expressions measured by Q-RT-PCR and microarray is shown (a and b). TaqMan® assays were conducted in triplicate for each sample, and the mean value was used to calculate expression levels (square markers). To standardize the quantification of *Timp1* (a) and *Tgfb1* (b), 18s rRNA from each sample was quantified at the same time as the target gene and is shown on the right-hand log scale. For the *Timp1* transcripts, rc_AI169327_at and rc_AI169327_g_at (circles and triangles, resp., in a) and *Tgfb1* transcripts, X52498cds_at (triangles in b), expression levels from microarray data are relative to the average of all gene expression levels and are shown on the left-hand scale.

organize each of the top three biological process categories into a dendrogram. Interestingly, with the exception of stress stimulus-related genes, most of the metabolism- and cell growth and/or maintenance-related genes recovered to near normal liver expression levels (Figure 5(b)). Identification of these discriminators allows them to serve as molecular classifiers, providing novel biological insights into the early development of liver damage, and aids in the development of new therapeutic drugs for liver disease.

Overall expression patterns for the 2,409 microarray transcripts (8,799 probe sets analyzed) were further analyzed for evidence of GP therapeutic action. GP treatment-related genes were clustered using two-way ANOVA at a 5% significance level to distinguish the various variations (e.g., GP treatment with DMN-damage versus only DMN-damage and differences due to the time course) and to estimate the variance of each individual variable in the ANOVA model. The resulting 813 transcripts gave rise to distinct molecular signatures of liver fibrosis progression that could be partly reversed GP treatment. Out of the 813 genes, 168 were also present in the list of 256 liver damage-related genes and reverted to normal levels significantly after treatment with GP or Silymarin (Supplementary Table 3). Interestingly, expression of 145 of the 168 genes was significantly reversed by GP treatment but not by Silymarin. In contrast, the expression of only 3 genes was significantly reversed by Silymarin but not by GP. Twenty genes were reversed by both GP and Silymarin, suggesting that they share same expression mechanism. Statistical analysis indicates that these 168 genes might serve as therapeutic target genes and



(a)



(b)

Figure 5: The 256 gene expression patterns from experimental samples. (a) Principal component analysis (PCA) of liver damage-related gene expression profiles from 5 different groups, including normal (C, marked in red), DMN treatment (D, yellow), GP treatment only (M, cyan), Silymarin-therapeutic (S, blue), and GP-therapeutic (Z, pink). PCA analyses were conducted on the expression of the 256 genes on the array (left side). The 3D distribution patterns indicate that there were excellent therapeutic effects and no notable liver damage patterns at molecular levels. Based on the ANOVA results (right), expression of 64% of the genes (165 genes out of 256) was significantly reversed in the GPtherapeutic treatment, whereas only 9% (23 genes) were reversed using Silymarin. (b) Hierarchical clustering identified three biological process categories: metabolism, cell growth and/or maintenance and response stimulus. Here, rows represent individual transcripts and columns represent each time course sample. The right two columns of each classification indicate the expression patterns of the GP therapeutic groups in the 6th week. Expression of metabolism and cell growth and/or maintenance genes in the GP therapeutic groups reverted to normal levels; response stimulus gene expression did not. The color in each cell reflects the expression level of the corresponding sample relative to its mean expression level, and the scale extends from fluorescence ratios of 0.25 to 4 relative to the mean level for all samples.

GP could regulate the gene expression patterns better than Silymarin.

3.7. Necroinflammatory and Fibrosis-Related Gene Expression Profiling. It has been suggested that necroinflammation and fibrosis play important roles in liver cirrhosis progression in rat models [18–22, 31]. To clarify the factors responsible for this histopathological phenotype, all histopathological samples were scored for necroinflammation and fibrosis as previously reported [19]. The mRNA expression levels of 44 genes, assayed by microarray, were significantly correlated with unchanged to higher scores according to a statistical analysis that separately estimated variations in necroinflammatory score and the 62 differentially expressed genes in fibrosis score, as previously reported [19]. These discriminators are shown in the left panels of Figures 6(a) and 6(b) (necroinflammation and fibrosis, resp.) and plotted over the time course for each group (Figures 6(a) and 6(b), right panel). Hierarchical clustering analysis indicates that gene expression reservation patterns after GP treatment were much closer to those of normal liver than those after Silymarin.

3.8. Q-RT-PCR Validations for Novel Liver Damage Markers. From Q-RT-PCR assays of rat liver samples, 5 novel liver damage-related genes, including *Btg2*, *Egr1*, *Oldr1*, *Nrg1*, and *Hmgcs1*, were subjected to further statistical analysis (Figure 7). An internal control, 18 s ribosomal RNA, was used to obtain relative expression patterns and for comparison. A high Pearson correlation was found between the microarray and Q-RT-PCR results. Based on the expression patterns, *Egr1* and *Nrg1* may act as necroinflammatory markers, and *Btg2* may act as a fibrosis-related marker. *Oldr1* and *Hmgcs1* were up- and downregulated markers for liver damage, respectively. These genes show potential as commercial diagnostic markers.

4. Discussion

The gene expression profiling described here provides a powerful and robust tool that is able to reveal molecular markers for liver damage at an early stage. Although several liver damage-related gene expression profiling analyses have been reported in animal models [32, 33], these gene discriminators have rarely been applied for therapeutic purposes. However, the identification of a drug treatment that improves outcomes for patients with liver fibrosis and molecular markers for liver fibrosis diagnosis are important therapeutic needs. Comparison of our dataset with earlier related studies reveals multiple overlapping gene identities that may potentially serve as markers for fibrosis, cirrhosis, and/or HCC diagnosis [19]. The expression patterns enabled us to identify 256 differentially expressed genes, including 44 necroinflammatory and 62 fibrosis-related genes. To our knowledge, this is the first paper to delineate the therapeutic effects of GP, ranging from histopathologic and biochemical analyses to a molecular portrait of liver fibrosis based on the courses of necroinflammation and fibrosis over time. Our findings suggest that GP has excellent anti-inflammatory, hepatoprotective, and antifibrotic activities and has potential as a novel antifibrotic medication.

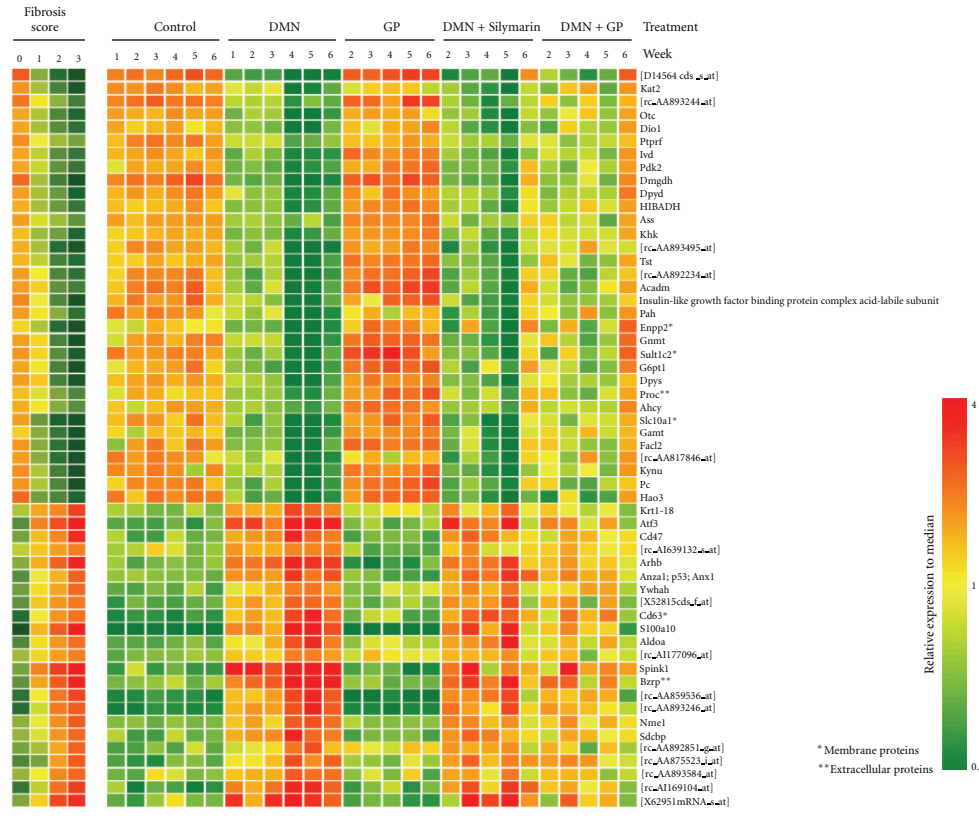
CD63, a transmembrane protein, which was upregulated after DMN treatment, was identified as a fibrosis gene signature. Inhibition of CD63 inhibits collagen secretion and HSC migration [34]. In an alcoholic liver disease (ALD) study [35], Annexin A1 (*Anxa1*) was highly expressed after liver injury. A recent study has indicated that alcohol-initiated liver injury occurs via inflammation. ALD progression involves continuing liver injury, fibrosis and impaired liver regeneration. In the current study, the expression patterns of both *Cd63* and *Anxa1*, both of which were present in the list of potential therapeutic target genes, significantly recovered following GP, but not Silymarin, treatment. Extending the previous study [19], identification of these necroinflammatory and fibrosis-related genes provides useful insight into the molecular mechanisms underlying liver damage and provides potential targets for the rational development of therapeutic drugs such as GP.

Although limited by the complex cellular components, there were still dramatic damage-related patterns present. For example, early growth response 1 (Egr1) was an immediate-early gene transcription factor and was identified as an important contributor to increased LPS-stimulated TNF-alpha secretion by Kupffer cells after chronic ethanol exposure [36]. Egr1 also controls the expression of a number of inflammatory mediators contributing to liver fibrosis. Based on our observations, Egr1 expression in DMN-damaged liver was higher than that in normal liver, but the expression levels recovered to near normal after GP treatment (Figure 7). These results indicate that Egr1 may be an effective therapeutic target in the treatment of liver damage.

Models for fibrosis were first developed in rodents. Carbon tetrachloride (CCl_4) [37] and DMN are the most commonly used hepatotoxic agents, and they ensure bridge fibrosis first developed in pericentral areas and secondarily between central and portal areas eventually leading to cirrhosis. Another secondary biliary fibrosis model is common bile duct ligation (BDL) [38]. However, it is obvious that they are not human and there are large species differences, for example, pharmacological, metabolic, or tissue responses. The whole organ is more intact than *in vitro* observation system, and the animal models still are better way and allow for comprehensive study of questions that cannot be addressed in human studies, especially in drug development. In addition, the preliminary results indicated that GP also diminished CCl_4- and BDL-induced fibrosis and effectively alleviated fibrogenic progression by image morphometric analysis (data not shown).

Currently available human hepatic fibrosis treatments remain unsatisfactory due to a lack of antifibrosis agent selectivity against fibrogenesis. GP is a potent candidate for development as an antifibrosis drug. Despite GP's attractive antifibrotic properties, its safety has not been fully evaluated. GP toxicity in animal models requires further study to move the substance toward human clinical trials. Acute toxicity effects demonstrate the relationship between dosage and the proportion of individuals responding with a quantifiable effect such as death. The half-lethal dose (LD_{50}), the slope

(a)



(b)

Figure 6: Hierarchical clustering illustrates necroinflammatory and fibrosis-related gene expression patterns. Rows represent individual transcripts and columns represent time course samples. Transcripts were ranked using hierarchical clustering of necroinflammatory (a) and fibrosis scores (b). Colors reflect the expression level of the each sample relative to its mean expression level.

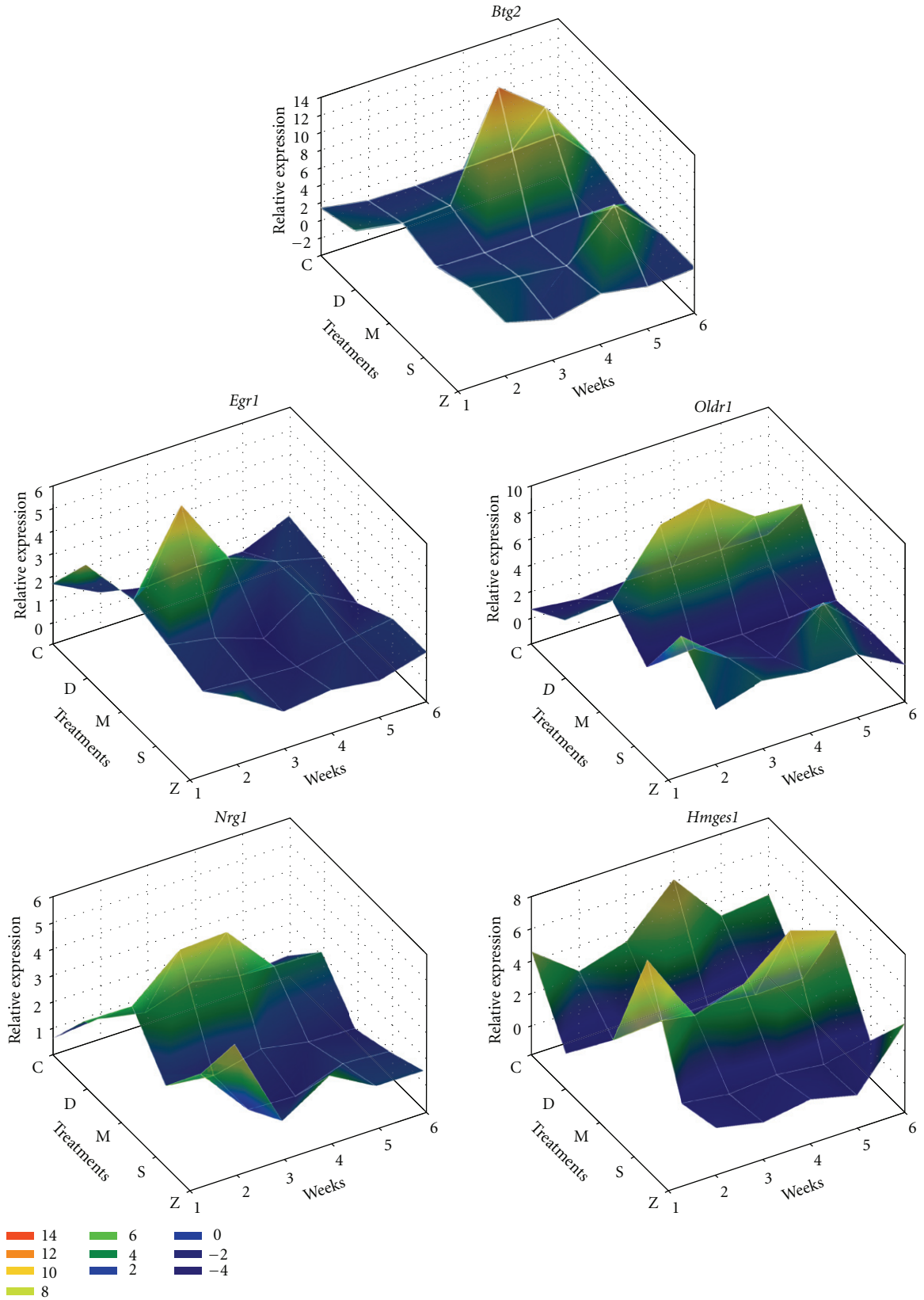


Figure 7: Q-RT-PCR validation of novel therapeutic markers. Five candidate markers, *Btg2*, *Egr1*, *Oldr1*, *Nrg1*, and *Hmgcs1*, were validated by Q-RT-PCR and are illustrated on 3D mesh plots. 18s rRNA from each sample was quantified at the same time as the target gene. *Btg2*, *Egr1*, *Oldr1*, *Nrg1* were upregulated with liver damage and recovered following both drug treatments. However, *Hmgcs1* was downregulated and recovered after GP administration only. The five different groups are shown as normal (C), DMN treatment (D), GP treatment only (M), Silymarin therapeutic (S), and GP-therapeutic (Z).

of the lethality curve, and prominent clinical effects are all required to characterize the potency of the drug. GP's acute toxicity was analyzed to determine these characteristics (data not shown). In future studies, we will evaluate the therapeutic effects of GP on liver-fibrosis in more detail and in comparison to clinical applications of GP. The mechanism and active components of GP require further study.

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Research Article

Anti-Hepatitis B Virus Effect and Possible Mechanism of Action of 3,4-*O*-Dicaffeoylquinic Acid *In Vitro* and *In Vivo*

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The anti-hepatitis B activity of 3,4-*O*-dicaffeoylquinic acid isolated from *Laggera alata* was studied using the D-galactosamine- (D-GalN-) induced hepatocyte damage model, HepG2.2.15 cells, and with HBV transgenic mice. *In vitro* results showed that 3,4-*O*-dicaffeoylquinic acid improved HL-7702 hepatocyte viability and markedly inhibited the production of HBsAg and HBeAg. At a concentration of 100 μg/mL, its inhibitory rates on the expression levels of HBsAg and HBeAg were 89.96% and 81.01%, respectively. The content of hepatitis B virus covalently closed circular DNA (HBV cccDNA) in HepG2.2.15 cells was significantly decreased after the cells were treated with the test compound. In addition, 3,4-*O*-dicaffeoylquinic acid significantly increased the expression of heme oxygenase-1 (HO-1) in HepG2.2.15 cells. *In vivo* results indicated that the test compound at concentrations of 100 μg/mL significantly inhibited HBsAg production and increased HO-1 expression in HBV transgenic mice. In conclusion, this study verifies the anti-hepatitis B activity of 3,4-*O*-dicaffeoylquinic acid. The upregulation of HO-1 may contribute to the anti-HBV effect of this compound by reducing the stability of the HBV core protein, which blocks the refill of nuclear HBV cccDNA. Furthermore, the hepatoprotective effect of this compound may be mediated through its antioxidative/anti-inflammatory properties and by the induction of HO-1 expression.

1. Introduction

Hepatitis B is an infectious illness caused by hepatitis B virus (HBV), which infects the liver of Hominoidea, including humans, and causes an inflammation reaction called hepatitis. Although there is an effective vaccine against HBV, chronic infection poses a huge health burden on the global community [1]. Its prevalence approaches 10% in hyperendemic areas such as Southeast Asia, China, and Africa [2]. Furthermore, approximately one-third of the world's population (more than 2 billion people) have been infected with the hepatitis B virus, which includes 350 million chronic carriers of the virus [3]. Some antiviral agents such as interferon-α and nucleosides (including lamivudine and adefovir dipivoxil) have been approved for the treatment of chronic HBV infection. However, a significant number of patients develop drug resistance after long-term use of these agents [4]. Therefore, there is a pressing need to continue developing safer and more effective anti-hepatitis B agents. The development of natural substances as antiviral agents is thought to be a promising approach towards solving this public health concern [5].

Laggera alata belongs to the genus *Laggera* (Asteraceae) and is distributed mainly among the tropical areas of Africa, Southeast Asia, and China. This plant has been used as a folk medicine for over 300 years, especially for the treatment of some ailments associated with hepatitis [6]. Most of the previous studies examining *L. alata* have focused on its folk use and phytochemical analyses [7–9]. In previous investigations, we examined the anti-inflammatory and hepatoprotective activities of an *L. alata* extract containing dicaffeoylquinic acids and confirmed its potent effects [10, 11]. In this study, we utilized the D-galactosamine- (D-GalN-) induced HL-7702 hepatocyte damage model, HBV-transfected HepG2.2.15 cells, and HBV transgenic mice to evaluate the anti-hepatitis B activity and possible hepatoprotective mechanisms of 3,4-*O*-dicaffeoylquinic acid isolated from *L. alata* (Figure 1). The study is the first to demonstrate

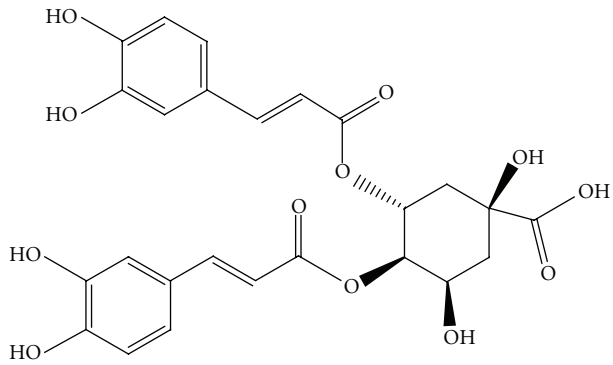

Figure 1: Structure of 3,4-*O*-dicaffeoylquinic acid isolated from *L. alata*.

that 3,4-*O*-dicaffeoylquinic acid possesses an anti-hepatitis B activity.

2. Materials and Methods

2.1. Reagents. Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM) and 1640 medium were purchased from Gibco-BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), silibinin, and D-galactosamine were purchased from Sigma Chemical Co., USA. Lamivudine was provided by GlaxoSmithKline Investment Co., Ltd. The HBV DNA PCR-fluorescence quantitation kit and the enzyme immunoassay (EIA) kits for the detection of HBsAg, and HBeAg were obtained from Shanghai Kehua Bio-Engineering Co., Ltd. The Plasmid Mini Preparation kit was obtained from Axygen Biosciences. Plasmid safe ATP-dependent DNase was purchased from EPICENTRE Biotechnologies. The TA cloning kit was obtained from Invitrogen Corporation. The heme oxygenase-1 ELISA kit was purchased from Beijing Yonghui Biological Technology Co., Ltd. Conventional PCR reagents were obtained from Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. The HBsAg immunohistochemical detection kit was purchased from Boster Biological Technology Co., Ltd., China. All other reagents were of the highest available commercial grade.

2.2. Compound. 3,4-*O*-Dicaffeoylquinic acid was isolated from *Laggera alata* and its structure was authenticated according to a previously reported method [10]. All of the *Laggera alata* (D. Don) Sch.-Bip ex Olivier was collected from Yunnan Province, China. A voucher specimen (ZY982003LA) was deposited in the herbarium of the College of Pharmaceutical Sciences, Zhejiang University, China. 3,4-*O*-Dicaffeoylquinic acid (HPLC purity ≥ 98%) was initially dissolved in dimethyl sulfoxide (DMSO) and further diluted in cell culture medium to achieve a final concentration of 0.1% DMSO, which was not toxic to either HL-7702 hepatocytes or HepG2.2.15 cells.

2.3. Cells and Transgenic Mice. HL-7702 hepatocytes were maintained in 1640 medium containing 2 mM glutamine and 10% (v/v) fetal bovine serum at 37°C (95% humidity, 5% CO_2). HepG2.2.15 cells were maintained in DMEM containing 2 mM glutamine, 10% (v/v) fetal bovine serum, and 380 μg/mL of G418 at 37°C (95% humidity, 5% CO_2). HBV transgenic mice were generated in the Shanghai Research Center for Model Organisms by routine microinjection of the linearized HBV DNA of clone no. 25-8 (GenBank ID: AF461363) into fertilized eggs of C57BL/6J mice [12]. The transgenic mice were kept in a room maintained at 22 ± 2°C and at relative humidity between 40% and 70%. The experimental protocol was approved by the Animal Ethics Committee of Zhejiang University, in accordance with "Principles of Laboratory Animal Care and Use in Research" (Ministry of Health, Beijing, China).

2.4. Hepatoprotective Assay against D-GalN-Induced Hepatocyte Damage. HL-7702 hepatocytes were transferred to 96-well plates at a density of approximately 1.0×10^5 cells/mL. Cytotoxicity induced by the test compound was measured using the MTT assay as reported previously [11]. Hepatocyte injury was induced by D-GalN in the following manner: after HL-7702 hepatocytes were incubated for 8 h with 80 mM D-GalN, the cells were then cultured for another 48 h in fresh culture medium containing 1–100 μg/mL 3,4-*O*-dicaffeoylquinic acid. Hepatocyte viability was detected using the MTT assay. The hepatoprotective effect of the test compound was assessed by the cell viability assay and expressed as percent protection. Silibinin was used as the reference drug at a concentration of 100 μg/mL.

2.5. Anti-HBV Assay in HepG2.2.15 Cells. Cytotoxicity induced by 3,4-*O*-dicaffeoylquinic acid was analyzed as follows: HepG2.2.15 cells were transferred to 96-well plates at a concentration of 1.0×10^5 cells/mL. Different concentrations of the test compound were applied to the culture wells in triplicate. After the cells were incubated for 8 days, the MTT assay was carried out as described previously [11]. To measure the effect of 3,4-*O*-dicaffeoylquinic acid on the expression of HBV antigens and HBV DNA, HepG2.2.15 cells were treated with various concentrations of the test compound in the 96-well plates. The medium with the compound was replaced every 4 days. On the fourth day, the replaced medium was assayed for HBsAg and HBeAg. On the eighth day, the replaced medium was measured for HBsAg, HBeAg and HBV DNA. Lamivudine was used as the reference drug. The levels of HBsAg and HBeAg in the replaced culture supernatants were determined by HBsAg and HBeAg enzyme-immunoassay kits, respectively. The results were read at 450 nm by a multiwell plate reader (MULTISKAN MK3, Thermo Fisher Scientific Inc., USA). The HBV viral load in the replaced culture supernatants was detected with a HBV DNA PCR-fluorescence quantitation kit as follows: HBV DNA was extracted and amplified with a Bio-Rad iQ5 real-time PCR system. The forward primer was 5-CCG TCT GTG CCT TCT CAT CTG-3, the reverse primer was 5-AGT CCA AGA GTA CTC TTA TAG AAG ACC TT-3, and the Taqman probe was FAM-CCG TGT GCA CTT CGC TTC ACC TCT GC. The thermal program comprised of an initial

Table 1: Cytotoxicity of 3,4-dicaffeoylquinic acid in HL-7702 hepatocytes.

Groups	Concentration (μg/mL)	Absorbency (570 nm)	Cell survival (%)
Vehicle	—	1.275 ± 0.042	100
Silybin	100	1.235 ± 0.069	96.86
	50	1.238 ± 0.038	97.10
	10	1.259 ± 0.048	98.74
3,4-Dicaffeoylquinic acid	100	1.230 ± 0.048	96.47
	50	1.246 ± 0.027	97.72
	10	1.255 ± 0.046	98.43

All determinations were performed in six replicates, and values were expressed as mean ± SD. No significant difference compared with the vehicle control.

denaturation at 94°C for 2 min followed by 40 amplification cycles with each of the two following steps: 95°C for 5 s and 60°C for 30 s. A plasmid containing the full-length insert of the HBV genome was used to prepare the standard curve.

2.6. Assay for Elimination HBV cccDNA in HepG2.2.15 Cells. After HepG2.2.15 cells were incubated for 48 h in 6-well plates at a density of 1.0×10^5 cells/mL, new DMEM medium containing different concentrations of the test compound (50, 25, and 10 μg/mL) was added. Three parallel controls were performed, including positive controls with oxymatrine (50 μg/mL), a vehicle control of 0.1% DMSO, and a normal control with no antiviral drug. The medium with the compound was replaced every 3 days. On the sixth day, the cells of each well were harvested. Based on the similarity of cccDNA and plasmid structures, the cell pellet containing 1.0×10^6 cells was extracted with the Mini Plasmid Extraction Kit. The extracted plasmid was further purified by plasmid safe ATP-dependent DNase to remove the residual HBV relaxed circular DNA. Hepatitis B virus covalently closed circular DNA (HBV cccDNA) was detected by selective real-time fluorescent quantitative PCR with specific primers and a Taqman MGB probe [13]. The forward primer was 5-TGA ATC CTG CGG ACG ACC-3, the reverse primer was 5-ACA GCT TGG AGG CTT GAA CAG-3 and the Taqman probe was 5-FAM-CCT AAT CAT CTC TTG TTC ATG TC-MGB-3. According to the structural differences between cccDNA and rcDNA, only the cccDNA should have been amplified with the designed primers and probe.

2.7. Assay for Induction of HO-1 of HepG2.2.15 Cell. After HepG2.2.15 cells were incubated for 48 h in 6-well plates at a density of 1.0×10^5 cells/mL, the cells were treated with various concentrations of test compound, and the medium with the compound was replaced every 3 days. Oxymatrine was used as the reference drug. On the sixth day, the cells were collected, and their heme oxygenase-1 (HO-1) levels were determined by an HO-1 ELISA kit according to the protocol provided with the kit. The absorbency was measured at 450 nm by a multiwell plate reader. The content of HO-1 in these cells was then determined by comparing the absorbency of the samples to the standard curve.

2.8. Anti-HBV Assay in HBV Transgenic Mice. HBV transgenic mice were divided into four groups. The vehicle control group received a normal saline solution at a dose of 10 mL/kg. The drug control group received lamivudine at a dose of 100 mg/kg. Experimental drug groups received 3,4-*O*-dicaffeoylquinic acid at doses of 50 and 100 mg/kg. The vehicle and drugs were administered orally to the different groups of mice once per day for 30 days. Five hours after the last administration, the mice were briefly anesthetized with ether, and blood samples were taken from the orbital sinus. The serum was separated for the measurements of HBsAg, and HO-1. The serum HBsAg and HO-1 levels were determined using the HBsAg and HO-1 detection kits according to the respective protocols provided with the ELISA kits. For histopathological analysis, formalin-fixed, paraffin-embedded liver specimens were routinely stained with hematoxylin and eosin (HE). The liver HBsAg expression level was determined using the HBsAg immunohistochemical detection kit according to the manufacturer's instructions. The pathological and immunohistochemical changes were evaluated and photographed under the microscope.

2.9. Statistical Analysis. Data were expressed as the mean ± standard deviation. Statistical analyses were carried out by the application of one-way analysis of variance (ANOVA) and student's t-test. $P < 0.05$ was chosen as the criterion for statistical significance.

3. Results

3.1. Effect of 3,4-O-Dicaffeoylquinic Acid on D-GalN-Induced Hepatocyte Damage. The cytotoxicity test indicated that 3,4-*O*-dicaffeoylquinic acid was not toxic to HL-7702 hepatocytes at concentrations of 10–100 μg/mL (Table 1). Hepatocyte injury was induced by exposure to 80 mM D-GalN, and the cells were subsequently treated with 3,4-*O*-dicaffeoylquinic acid. The results show that test compound improved cell viability at concentrations of 10–100 μg/mL (Table 2).

3.2. Anti-HBV Activity of 3,4-O-Dicaffeoylquinic Acid in HepG2.2.15 Cells. After hepG2.2.15 cells were treated with 3,4-*O*-dicaffeoylquinic acid for 8 days, the cell viability was determined using the MTT assay. The results indicated that 3,4-*O*-dicaffeoylquinic acid was not cytotoxic at concentrations of 10–100 μg/mL (Table 3). The HBsAg and HBeAg levels in culture supernatants were assayed after the cells were

Table 2: Effect of 3,4-dicaffeoylquinic acid on the survival of D-GalN injured HL-7702 hepatocytes.

Groups	Concentration (μg/mL)	Absorbency (570 nm)	Protection rate (%)
Vehicle	—	$1.289 \pm 0.055^{**}$	—
D-GalN-control	—	0.769 ± 0.053	—
Silybin-D-GalN	100	$0.868 \pm 0.036^{*}$	18.90
	50	0.832 ± 0.055	12.07
	10	0.810 ± 0.031	7.79
3,4-Dicaffeoylquinic acid- D-GalN	100	0.846 ± 0.044	14.77
	50	0.812 ± 0.031	8.18
	10	0.801 ± 0.019	6.16

Silybin was used as the positive control. 0.1% DMSO was used as the vehicle control. Values are expressed as the means ± SD of four replicates. Protection rate (%) = (the mean absorbency value in experimental group − the mean absorbency value in model control group)/(the mean absorbency value in negative control group − the mean absorbency value in model control group) × 100%. $^{*}P < 0.05$ and $^{**}P < 0.01$ represent the significance of the difference from the D-GalN control.

Table 3: Cytotoxicity of 3,4-dicaffeoylquinic acid in hepG2.2.15 cells.

Groups	Concentration (μg/mL)	Absorbency (570 nm)	Cell survival (%)
Vehicle	—	0.922 ± 0.031	100
Lamivudine	100	0.906 ± 0.101	98.26
	50	0.912 ± 0.092	98.92
	10	0.918 ± 0.141	99.57
3,4-Dicaffeoylquinic acid	100	0.893 ± 0.061	96.85
	50	0.895 ± 0.105	97.07
	10	0.909 ± 0.078	98.59

All determinations were performed in six replicates, and values were expressed as mean ± SD. No significant difference compared with the vehicle control.

incubated with the test compound for 4 days (Table 4). The results showed that the test compound significantly inhibited HBsAg expression at concentrations of 50–100 μg/mL and markedly repressed HBeAg expression at a concentration of 100 μg/mL.

The HBsAg, HBeAg and HBV DNA levels in culture supernatants were measured after the cells were treated with the test compound for 8 days (Table 5). At concentrations of 50–100 μg/mL, the test compound significantly inhibited the expression of HBsAg and HBeAg. At a concentration of 100 μg/mL, the test compound inhibited the expression rates of HBsAg and HBeAg by 89.96% and 81.01%, respectively.

3.3. Effect of 3,4-O-Dicaffeoylquinic Acid on HBV cccDNA Content of HepG2.2.15 Cells. The effect of 3,4-*O*-dicaffeoylquinic acid on the level of HBV cccDNA is shown in Table 6. The results indicated that 3,4-*O*-dicaffeoylquinic acid significantly reduced the HBV cccDNA content of HepG2.2.15 cells at a concentration of 50 μg/mL. Furthermore, the test compound exhibited a larger effect than the reference drug oxymatrine.

3.4. Effect of 3,4-O-Dicaffeoylquinic Acid on HO-1 Expression in HepG2.2.15 Cell. The expression level of HO-1 in HepG2.2.15 cells was determined after the cells were treated with various concentrations of test compound for 6 days (Table 7). At concentrations of 10–50 μg/mL, 3,4-*O*-dicaffeoylquinic acid significantly increased HO-1 expression. Oxymatrine, which was the reference drug, showed a similar effect.

3.5. Anti-HBV Activity of 3,4-O-Dicaffeoylquinic Acid in HBV Transgenic Mice. The anti-HBV activity of 3,4-*O*-dicaffeoylquinic acid was determined in HBV transgenic mice (Table 8). These results show that the test compound significantly reduced the serum HBsAg level at concentrations of 50–100 μg/mL. Meanwhile, the test compound markedly induced HO-1 expression at a concentration of 100 μg/mL. Histological analysis revealed almost normal lobule architecture and slight swelling of liver cells, but no obvious pathological changes were observed in the control and drug-treated mice (Figure 2). Immunohistochemical detection indicated that the strongest HBsAg-positive signals were detected in the control group, and the different concentrations of test compound clearly repressed the expression of liver HBsAg (Figure 3).

4. Discussion

Patients with hepatitis B are often treated with antiviral agents, hepatoprotective drugs, and immunomodulatory drugs. Typically, the beneficial role of hepatoprotectors in viral hepatitis is achieved through their inhibitory action on the inflammatory and cytotoxic cascades induced by viral infection. In addition, these agents can also improve the regeneration process and normalize liver enzymes through their effects on protein synthesis [14]. Among the numerous models of experimental hepatitis, D-GalN-induced liver damage is very similar to human viral hepatitis in its morphological and functional features [15]. D-GalN reduces the intracellular pool of uracil nucleotides in hepatocytes,

Table 4: Anti-HBV activity of 3,4-*O*-dicaffeoylquinic acid in HepG2.2.15 cells. (After the cells were treated with the test compound for 4 days.)

Groups	Concentration (μg/mL)	HBsAg		HBeAg	
		Absorbency	Inhibition (%)	Absorbency	Inhibition (%)
Vehicle	—	1.175 ± 0.085	—	2.947 ± 0.273	—
Lamivudine	100	1.058 ± 0.101	10.01	3.132 ± 0.034	—
	50	1.097 ± 0.083	6.67	3.140 ± 0.039	—
	10	1.197 ± 0.116	—	3.274 ± 0.100	—
3,4-Dicaffeoylquinic acid	100	0.585 ± 0.024**	50.23	2.070 ± 0.198*	29.77
	50	0.840 ± 0.020**	28.50	2.505 ± 0.077	15.02
	10	1.111 ± 0.096	5.48	2.750 ± 0.250	6.70

Lamivudine was used as the positive control in the anti-HBV assay. 0.1% DMSO was used as the vehicle control. Inhibition (%) = (the mean absorbency value in negative control group − the mean absorbency value in experimental group)/(the mean absorbency value in negative control group) × 100%. Data are expressed as the means ± SD of three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the vehicle group.

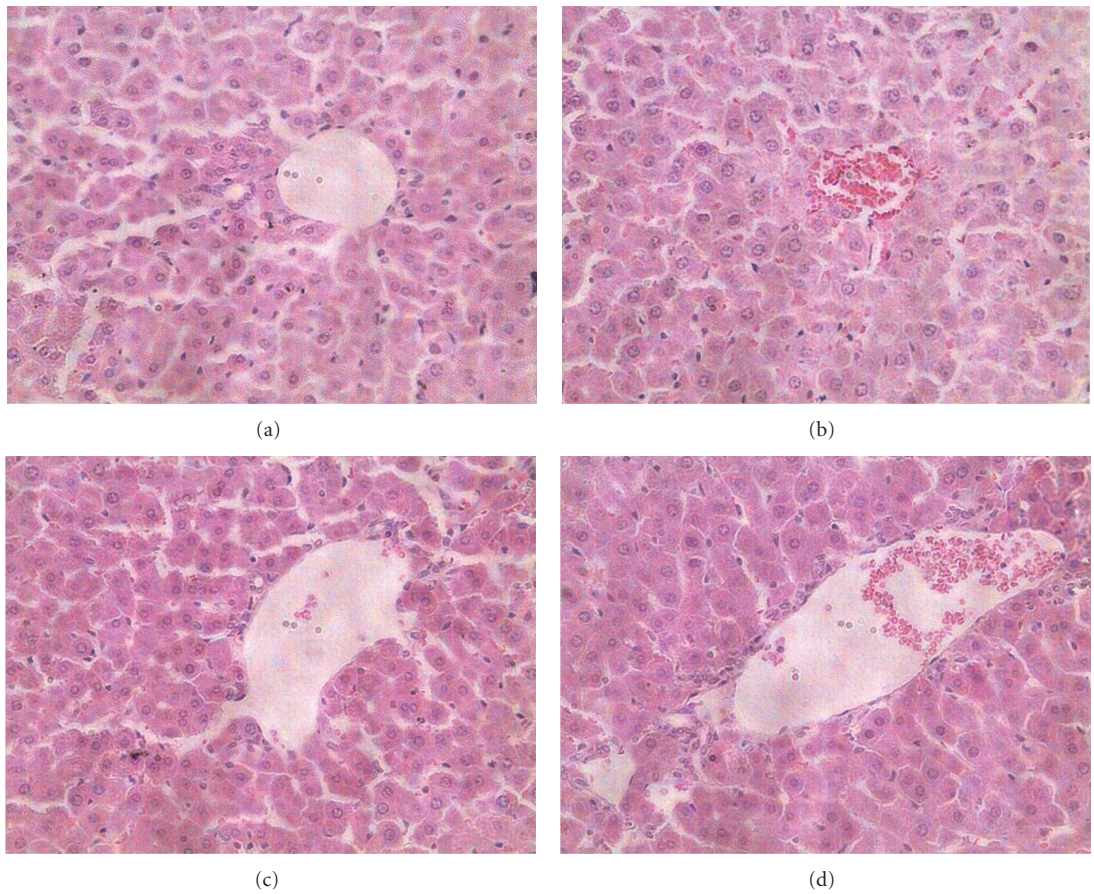

Figure 2: Histopathological changes of liver tissue from HBV transgenic mice (HE × 40). (a) A control untreated HBV transgenic mouse; (b) a lamivudine (100 mg/kg) treated HBV transgenic mouse; (c) a 3,4-dicaffeoylquinic acid (100 mg/kg) treated HBV transgenic mouse; (d) a 3,4-dicaffeoylquinic acid (50 mg/kg) treated HBV transgenic mouse. (a), (b), (c), and (d) do not show obvious pathological changes, which is probably related to the immunotolerance of HBV transgenic mice to HBV.

thereby inhibiting the synthesis of RNA and proteins [16]. Oxygen-derived free radicals released from activated hepatic macrophages are the primary cause of D-GalN-induced liver damage [17]. In previous studies, the potent anti-inflammatory and hepatoprotective activities of an *L. alata* extract containing dicaffeoylquinic acids were confirmed [10, 11]. Furthermore, dicaffeoylquinic acids exhibit a variety of pharmacological activities, such as antioxidative, anti-inflammatory, and antiviral effects [18–20]. In the current study, 3,4-*O*-dicaffeoylquinic acid protected D-GalN-injured

Table 5: Anti-HBV activity of 3,4-*O*-dicaffeoylquinic acid in HepG2.2.15 cells. (After the cells were treated with the test compound for 8 days.)

Groups	Concentration (μg/mL)	CC_{50}	HBsAg Absorbency	HBsAg Inhibition (%)	IC_{50}	SI	HBeAg Absorbency	HBeAg Inhibition (%)	IC_{50}	SI	cccDNA (Log) (copy/μL)
Vehicle	—	—	1.023 ± 0.062	—	—	—	3.213 ± 0.109	—	—	—	5.10 ± 0.04
Lamivudine	100	>400	0.867 ± 0.008*	15.19	—	—	3.211 ± 0.195	—	—	—	4.57 ± 0.02**
	50		1.045 ± 0.060	—			3.388 ± 0.082	—			4.65 ± 0.04**
	10		1.078 ± 0.157	—			3.510 ± 0.054	—			4.77 ± 0.11*
3,4-Dicaffeoylquinic acid	100	>400	0.103±0.010**	89.96	31.90	>12.54	0.610±0.060**	81.01	50.06	>7.99	5.04 ± 0.05
	50		0.342±0.026**	66.56			2.081±0.095**	35.21			5.11 ± 0.04
	10		0.910 ± 0.078	11.02			2.894 ± 0.177	9.92			5.14 ± 0.14

Lamivudine was used as the positive control in anti-HBV assay. 0.1% DMSO was used as the vehicle control. CC_{50}(μg/mL): value of the 50% cytotoxic concentration. Inhibition (%) = (the mean absorbency value in negative control group − the mean absorbency value in experimental group)/(the mean absorbency value in negative control group) × 100%. IC_{50} (μg/mL): value of the 50% inhibition concentration. SI: selectivity index (CC_{50}/IC_{50}). Data are expressed as means ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$ compared with the vehicle group.

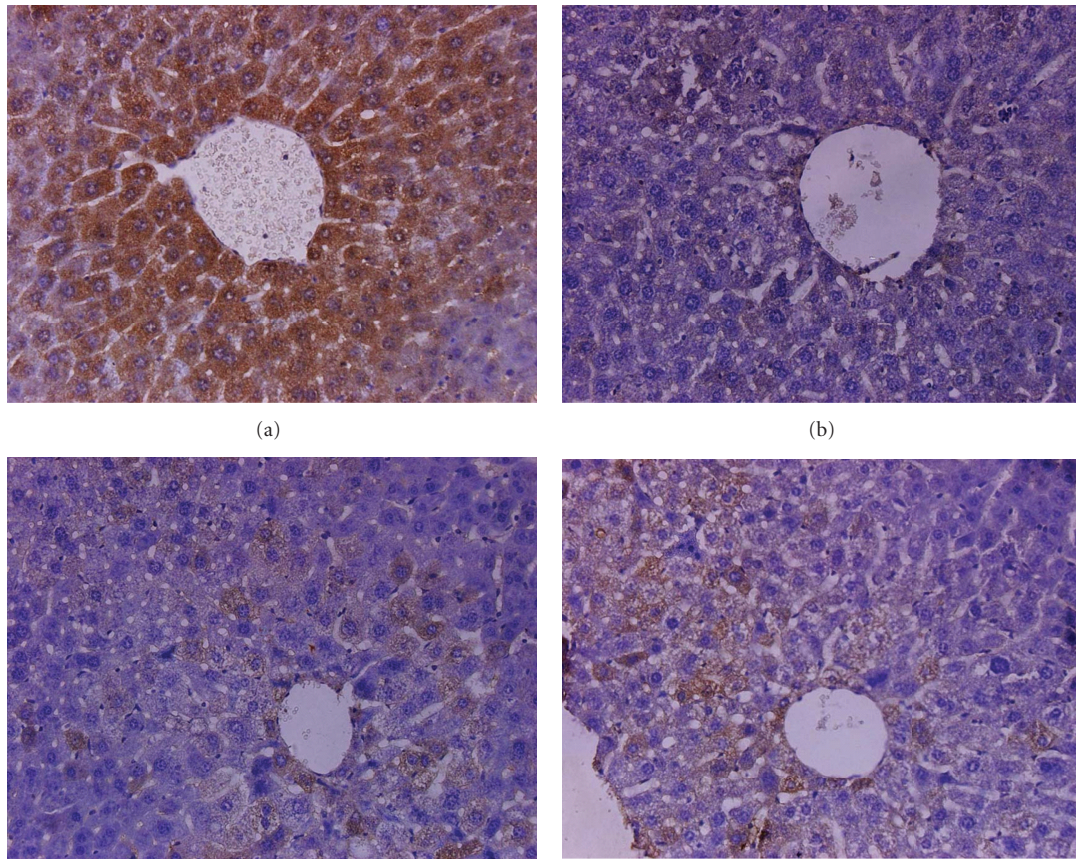

Figure 3: Immunohistochemical staining of HBsAg in the liver of HBV transgenic mice (×40). (a) A control untreated HBV transgenic mouse showing the positive expression of HBsAg (brown stain); (b) a lamivudine (100 mg/kg) treated HBV transgenic mouse; (c) a 3,4-dicaffeoylquinic acid (100 mg/kg) treated HBV transgenic mouse; (d) a 3,4-dicaffeoylquinic acid (50 mg/kg) treated HBV transgenic mouse. (b), (c), and (d) show clear inhibition of HBsAg expression.

Table 6: Effect of 3,4-dicaffeoylquinic acid on the HBV cccDNA content of HepG2.2.15 cells.

Groups	Concentration (μg/mL)	HBV cccDNA (Log) (copy/μL)
Normal	—	2.79 ± 0.03
Vehicle	—	2.73 ± 0.02
Oxymatrine	50	2.59 ± 0.12*
3,4-Dicaffeoylquinic acid	50	2.54 ± 0.05**
	25	2.71 ± 0.08
	10	2.83 ± 0.03

Oxymatrine was used as the positive control. 0.1% DMSO was used as the vehicle control. Data are expressed as the means ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$ compared with the vehicle group.

Table 7: Effect of 3,4-dicaffeoylquinic acid on HO-1 expression in HepG2.2.15 cells. (After the cells were treated with the test compound for 6 days.)

Groups	Concentration (μg/mL)	HO-1 content (ng/g protein)
Normal	—	36.00 ± 0.45
Vehicle	—	33.61 ± 1.51
Oxymatrine	50	57.70 ± 3.21**
	25	51.09 ± 4.80**
	10	49.49 ± 1.99**
3,4-Dicaffeoylquinic acid	50	57.18 ± 3.37**
	25	48.31 ± 2.52**
	10	45.12 ± 0.87**

Oxymatrine was used as the positive control. 0.1% DMSO was used as the vehicle control. Data are expressed as the means ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$ compared with the vehicle group.

hepatocytes, thereby implying that its antioxidative and anti-inflammatory properties may have contributed to the amelioration of hepatocyte damage.

HepG2.2.15 cells are derived from human hepatoblastoma HepG2 cells that were transfected with a plasmid containing HBV DNA. These cells can stably secrete viral particles in culture medium [21]. The presence of HBsAg is the most common marker of HBV infection, whereas HBeAg is used as an ancillary marker primarily to indicate active HBV replication and associated progressive liver disease [22].

Table 8: Anti-HBV activity of 3,4-*O*-dicaffeoylquinic acid in HBV transgenic mice.

Groups	Concentration (mg/kg)	HBsAg P/N	HO-1 (ng/L)
Vehicle	—	55.33 ± 1.10	609.62 ± 39.54
Lamivudine	100	56.82 ± 1.67	646.53 ± 29.19
3,4-Dicaffeoylquinic acid	100	53.16 ± 1.15**	676.31 ± 31.81*
	50	53.62 ± 1.49*	630.51 ± 56.48

Lamivudine was used as the positive control in the anti-HBV assay. Normal saline solution was used as the vehicle control. P/N (positive-to-negative) ratios were determined as the mean absorbency value of the test compounds divided by that of the negative control. Data are expressed as the means ± SD of four samples. *$P < 0.05$ and **$P < 0.01$ compared with the vehicle group.

Hepadnaviruses have a relaxed circular DNA genome. Following infection of hepatocytes, this DNA is transported to the nucleus and converted to a covalently closed form (cccDNA) that serves as a transcriptional template. Viral DNA is synthesized within nucleocapsids via reverse transcription of a viral RNA known as the pregenome [23]. Nucleocapsids containing mature forms of viral DNA are packaged into viral envelopes and secreted from the cell. cccDNA does not replicate; however, additional copies (up to 50 per cell) may be formed from viral DNA synthesized in the cytoplasm [24]. The cccDNA plays a key role in the life cycle of the virus and permits the persistence of infection. The formation of cccDNA is inhibited by viral envelope proteins [25]. In this study, 3,4-*O*-dicaffeoylquinic acid significantly inhibited expression of HBsAg and HBeAg in HepG2.2.15 cells. Although the response of lamivudine on HBV DNA replication is on the low side, its suppression on HBV DNA replication is statistically significant when compared to the vehicle group. Under the different experimental conditions, the test results of the same compound may have certain difference because of the different cell states. The low response of HBV DNA to lamivudine does not affect the judgement of the results, only if the anti-HBV activities of lamivudine and test compound are measured and compared under the same test condition. Furthermore, the test compound also significantly reduced the HBV cccDNA content of HepG2.2.15 cells and its effect was stronger than the reference drug oxymatrine, thereby indicating the anti-HBV effect of this compound was probably related to inhibiting the formation of cccDNA.

HBV transgenic mice with a known genetic background and a well-characterized HBV isolate have been employed as an animal model of the HBV-carrier state and are thought to be a good model to evaluate the anti-HBV efficacy of candidate compounds *in vivo* [12, 26]. Based on *in vitro* results, we further studied the anti-hepatitis B activity of 3,4-*O*-dicaffeoylquinic acid in HBV transgenic mice. The results indicated that 3,4-*O*-dicaffeoylquinic acid significantly inhibited the serum and liver HBsAg levels and significantly increased HO-1 expression in these transgenic mice, which was in good agreement with the results of *in vitro* research. Upon histopathological analysis, no obvious pathological changes were found in both the control and experimental groups of mice, which is probably related to the immunotolerance of HBV transgenic mice to HBV. Because these transgenic mice are immunotolerant to HBV, they did not present with any of the disease signs that would normally be associated with immunopathological responses [27].

Heme oxygenases catalyze the initial and rate-limiting step in the oxidative degradation of heme. Among the three known heme oxygenases, HO-1 is the only inducible form of these enzymes [28]. Overexpression of HO-1 protects organs and/or tissues from immune-mediated organ injury, which can occur either through the prevention of oxidative damage or *via* local immunomodulatory influence on inflammatory cells [29]. Induction of HO-1 has been shown to be beneficial in immune-mediated liver damage. In addition, liver injury was significantly reduced after HO-1 induction in an acute hepatitis B model [30]. In addition to its hepatoprotective effect, HO-1 exhibited a pronounced antiviral effect, which was confirmed in stably HBV-transfected hepatoma cells and in persistently HBV replicating transgenic mice. HO-1 induction repressed HBV replication directly in hepatocytes at a posttranscriptional step by reducing the stability of the HBV core protein, which resulted in blocking the refill of nuclear HBV cccDNA. Small interfering RNAs directed against HO-1 have demonstrated that this effect is dependent on the expression level of HO-1 [30]. Therefore, the induction of HO-1 might be a novel therapeutic option for inflammatory flares of hepatitis B. In this study, 3,4-*O*-dicaffeoylquinic acid significantly increased the expression of HO-1 *in vitro* and *in vivo*, thereby suggesting that the hepatoprotective and anti-HBV effects of 3,4-*O*-dicaffeoylquinic acid were achieved by HO-1 induction.

In conclusion, this study verifies the *in vitro* and *in vivo* anti-hepatitis B effects of 3,4-*O*-dicaffeoylquinic acid isolated from *L. alata*. The upregulation of HO-1 may contribute to the anti-HBV effect of this compound by reducing the stability of the HBV core protein and by blocking the refill of nuclear HBV cccDNA. Additionally, the hepatoprotective effect of this compound was mediated by its antioxidative/anti-inflammatory properties and the induction of HO-1. Therefore, 3,4-*O*-dicaffeoylquinic acid should be considered a potential candidate or lead compound for the development of novel antiviral agents.

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Research Article

Evaluation of Hepatoprotective Effect of Leaves of *Cassia sophera* Linn.

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In the present study, the hepatoprotective activity of ethanolic extracts of *Cassia sophera* Linn. leaves was evaluated against carbon-tetrachloride- (CCl_4-) induced hepatic damage in rats. The extracts at doses of 200 and 400 mg/kg were administered orally once daily. The hepatoprotection was assessed in terms of reduction in histological damage, changes in serum enzymes, serum glutamate oxaloacetate transaminase (AST), serum glutamate pyruvate transaminase (ALT), serum alkaline phosphatase (ALP), total bilirubin, and total protein levels. The substantially elevated serum enzymatic levels of AST, ALT, ALP, and total bilirubin were restored towards the normalization significantly by the extracts. The decreased serum total protein level was significantly normalized. Silymarin was used as standard reference and exhibited significant hepatoprotective activity against carbon tetrachloride-induced hepatotoxicity in rats. The biochemical observations were supplemented with histopathological examination of rat liver sections. The results of this study strongly indicate that *Cassia sophera* leaves have potent hepatoprotective action against carbon tetrachloride-induced hepatic damage in rats. This study suggests that possible activity may be due to the presence of flavonoids in the extracts.

1. Introduction

Cassia sophera Linn (Family Caesalpiniaceae), popularly known as kasundi, is a shrubby herb found throughout India and in most tropical countries. In the ethnobotanical claims, the leaves are considered to be used for their anti-inflammatory, antirheumatic, and purgative property, as an expectorant for cough, cold, bronchitis, and asthma, and in the treatment of liver disorders. Previous studies have investigated on its pharmacological activities of the seeds of *C. sophera* including analgesic and anticonvulsant [1], antidiabetic [2], inhibition of lipid peroxidation [3], herbicidal [4], and fungicidal [5] effects.

The chemical constituents of *C. sophera* include the flavonoids [6, 7] and anthraquinone [8, 9]. To the best of our knowledge, there is no scientific report of hepatoprotective effect of *C. sophera*. Thus, the present study was to investigate the hepatoprotective activity of ethanol extract of leaves of *C. sophera* against CCl_4-induced hepatic damage in rats.

2. Materials and Methods

2.1. Plant Material. The fresh leaves of *Cassia sophera* Linn was collected from Tiruvannamalai district of Tamilnadu, India, in October and November. The plant was identified by B. Velmurugan, Taxonomist, Sri Ramana Maharishi Natural Society, Tiruvannamalai, India. A voucher specimen (Reg. no. GPT/8/2003) was deposited in our laboratory for future references. The leaves of the plant were dried under the shade and then milled into coarse powder, stored in an air tight closed container.

2.2. Extraction and Isolation. The dried coarse powdered *Cassia sophera* leaves (1.5 kg) were first defatted with petroleum ether (60–80°C) and then extracted with 5 L of ethanol (90%) in a soxhlet apparatus. The solvent was then removed under reduced pressure, to obtain petroleum ether (PECS, yield 8.5%) and ethanol extract (EECS, yield 22.5%), respectively. The ethanol extract was partitioned

successively between chloroform and ethyl acetate (3 × 1 L). The respective solvents were removed similarly under reduced pressure, which produced ethyl acetate fraction (EAF) (150 g) and chloroform fraction (CF) (50 g). Both fractions were evaluated for hepatoprotective activity against CCl_4-induced hepatic damage in rats. EAF was found to be more potent than CF. Hence, EAF was further exploited for isolation, which led to the isolation of rhamnetin, O-methylated flavonol. The isolated bioactive metabolite was characterized as rhamnetin based on melting point and spectroscopic (IR, ^{1}H NMR and MS) data [10, 11].

7 g of the ethyl acetate fraction was adsorbed on silica gel (silica gel 60 G, Merck, 600 g) and applied to a column of silica gel. A gradient of chloroform : ethyl acetate : methanol was used to elute the column, collecting 100 fractions of 50 mL each. Fractions, 35–42, were combined and, on TLC, it shows a single spot having an R_f value of 0.58. These combined fractions are evaporated to dryness and were further rechromatographed on a silica gel column using a gradient elution with chloroform : ethyl acetate (8 : 2) to give one compound, which was recrystallized with methanol to give pure rhamnetin.

2.3. Animals. Adult male Wistar albino rats weighing 150–180 g were used for the present investigation. All animal experiments were duly approved by Institutional Ethical Committee (CPCSEA/ORG/CH/2006/Reg. no.95), Jadavpur University, Kolkata, India.

2.4. Chemicals and Drugs. Silymarin was purchased from Microlabs (Hosur, Tamilnadu, India), carbon tetrachloride purchased from SICCO Research Laboratory, Mumbai, India. All other chemicals and solvent were of analytical grade and commercially available.

2.5. Acute Toxicity Test. The animals were divided into five groups ($n = 6$). The EECS suspension was administrated orally in increasing dose up to 2000 mg/kg, b.w [12]. The rats were observed continuously for 2 h for behavioural, neurological, and autonomic profiles and after 24 and 72 h for any lethality [13].

2.6. Experimental Design. The animals were divided into five groups ($n = 6$). Group I served as a vehicle control, which received liquid paraffin, intraperitoneally. Groups II–V were treated with CCl_4 in liquid paraffin (1 : 2) at the dose of 1 mL/kg body weight (b.w) intraperitoneally once in every 72 h for 16 days [14]. Aqueous suspension of EECS at the doses of 200 mg/kg and 400 mg/kg, b.w, were administered orally to the animals in groups III to IV in alternate days for 16 days. Group V received silymarin as a standard drug at the dose of 25 mg/kg, b.w., p.o. in alternate days for 16 days. At the 17th day, all the rats were sacrificed by cervical dislocation after collecting the blood from retroorbital plexus under ether anesthesia for biochemical estimations. The blood samples were allowed to clot and the serum was separated by centrifugation at 5000 rpm for 5 min and used for the assay of biochemical marker enzymes.

2.7. Biochemical Estimations. Different biochemical parameters like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin and total protein were determined by using commercially available kits (Span Diagnostic Limited, Surat, India).

2.8. Histological Observation. The washed liver tissues were fixed by using fixative (picric acid, formaldehyde, and 40% glacial acetic acid) for 24 h and dehydrated with alcohol. Liver tissues were cleaned and embedded in paraffin (melting point 58–60°C), cut in 3–5 μm sections, stained with the haematoxylin-eosin dye and finally, observed under a photomicroscope and morphological changes such as cell necrosis, ballooning degeneration, fatty changes or inflammation of lymphocytes were observed [15].

2.9. Statistical Analysis. The results were analyzed from statistical significance by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test using Statistical Package of the Social Science (SPSS) software. Results are expressed as mean ± SD for six rats in each group. Differences among groups were considered significant at $P < 0.05$ level.

3. Results

3.1. Phytochemical Screening and Isolation of Rhamnetin. Preliminary phytochemical screening of the ethanol extract of *C. sophera* revealed the presence of steroids, alkaloids, tannins, saponins, and flavonoids. Different compositions of the mobile phase were tested and the desired resolution of rhamnetin with symmetrical and reproducible peak was achieved by using the mobile phase chloroform and ethyl acetate. The structure of the compound was characterized by UV, IR, MS, and 13C-NMR methods as rhamnetin (Figure 1). Structures and the IR, 13C-NMR, and MS data obtained independently in these studies are in close conformity with reported literature [10, 16].

yellow colour crystals, TLC: (chloroform : methanol, 9 : 1 v/v) R_f 0.59: UV λ_{max} (C_2H_5OH): 360.1 nm; m.p. 282–285°C. MS m/z 316 (calculated value $C_{16}H_{12}O_7$, 316.26). ^{1}H NMR (CD_3OD): δ 13.04 (s, 1H, OH-5), δ 12.96 (s, 2H, OH-3′, 4′), δ 7.32–7.41 (d, 1H, H-6′), δ 6.88 (d, 1H, $J = 1.2$, H-5′), δ 6.50 (d, 1H, $J = 2$, H-8), δ 6.43 (d, 1H, $J = 2$, H-6), δ 3.78 (s, 3H, OCH_3). The proton signal at δ 3.78 (s, 3H, OCH_3) suggests the location of –OCH_3 at C-7, IR (KBr) v cm^{-1}, 3388 (O–H), 1654 (>C=O), 1610 (C=O), 1029 (C–O–C).

3.2. Acute Toxicity Studies. Acute toxicity studies revealed the nontoxic nature of the ethanol extracts of *Cassia sophera*. There was no lethality or toxic reaction found at any doses selected until the end of the study period.

3.3. Hepatoprotective Activity. Rats treated with CCl_4 developed a significant hepatic damage and oxidative stress. This is evident to the significant ($P < 0.05$) increase in serum ALT, AST, ALP, and bilirubin levels in CCl_4-treated rats compared

Table 1: Effect of EECS and silymarin on serum biochemical parameters.

Biochemical parameters	Control	CCl_4 1 mL/kg	MECS (200 mg/kg) + CCl_4	MECS (400 mg/kg) + CCl_4	Silymarin (50 mg/kg) + CCl_4
ALT (IU/L)	84 ± 5.4	178.3 ± 10.1[a]	151.6 ± 11.1*	135.3 ± 3.4*	120.0 ± 3.9*
AST (IU/L)	143.3 ± 14.8	251.6 ± 11.9[a]	220.0 ± 11.5*	180.8 ± 13.1*	158.3 ± 10.6*
ALP (IU/L)	82.2 ± 7.8	190.0 ± 13.9[a]	173.3 ± 13.1*	130.5 ± 9.5*	108 ± 5.2*
Total protein (mg/dL)	6.9 ± 0.32	3.5 ± 0.46[a]	4.6 ± 0.28	5.2 ± 0.21**	6.0 ± 0.31
Total bilirubin (mg/dL)	0.29 ± 0.04	0.98 ± 0.07[a]	0.79 ± 0.07	0.63 ± 0.05**	0.48 ± 0.07*

Values are mean ± SEM ($n = 6$). *$P < 0.01$ (moderately significant), **$P < 0.05$ (significant) as compared with CCl_4, [a]: significant as compared with control ($P < 0.01$).

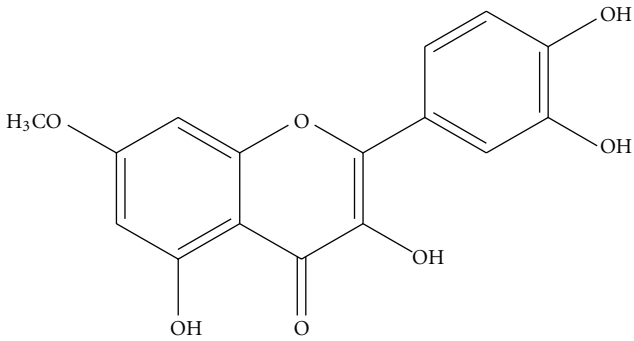

Figure 1: Structure of Rhamnetin.

to normal rats. However, the serum total protein level was significantly ($P < 0.05$) decreased in CCl_4-intoxicated rats. The toxic effects of CCl_4 were controlled in the animals treated with methanol extract of *Cassia sophera* at the doses of 200 and 400 mg/kg, p.o. significantly ($P < 0.05$) decreased the elevated serum marker enzymes. Total bilirubin and total proteins were found to be restored to almost normal level. The effects of EECS on serum ALT, AST, ALP, bilirubin and total protein levels in CCl_4 intoxicated rats are summarized in Table 1.

Histological observations of the liver tissue of the normal animals showed hepatic cells with well-preserved cytoplasm, nucleus, nucleolus, and central vein (Figure 2(a); 10x). Treatment with CCl_4 caused fatty degeneration with severe necrosis of the parenchyma cells in the central lobular region of the liver. Furthermore, hepatocytic necrosis was predominant surrounding the central vein, which formed a streak-like appearance (Figure 2(b); 10x). Figures 2(c), 2(d) and 2(e) (10x) show animals treated with EECS (200 and 400 mg/kg, p.o.) and Silymarin (25 mg/kg) and restored the altered histopathological changes, respectively.

4. Discussion

Preventive action in liver damage induced by carbon tetrachloride has widely been used as an indicator of the liver protective activity of drugs in general [17]. It was found that chronic administration of CCl_4 produces liver cirrhosis in rats. It is well documented that carbon tetrachloride is biotransformed under the action of cytochrome P-450 system in the microsomal compartment of liver to trichloromethyl or peroxytrichloromethyl free radical. These free radicals bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides followed by pathological changes such as triacylglycerol accumulation, polyribosomal disaggregation, depression of protein synthesis, cell membrane breakdown, and even death [18, 19].

Estimating the activities of serum marker enzymes like AST, ALT, ALP, and bilirubin can make assessment of liver function. When liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released into the blood stream. Their estimation in the serum is a useful quantitative marker of the extent and type of hepatocellular damage [20].

The increased level of AST, ALT, ALP, and bilirubin is conventional indicator of the liver injury. In the present study, it is observed that administration of CCl_4 elevates the levels of serum marker enzymes AST, ALT, ALP, and bilirubin. Levels of total proteins are lowered. Ethanol extracts of *Cassia sophera* and reference drug silymarin-treated groups exhibited lower levels of AST, ALT, ALP, and bilirubin as compared to CCl_4 treated groups. The treatment with MECS also significantly elevated total protein levels. The stabilization of serum AST, ALT, ALP and bilirubin by EECS is clear indication of the improvement of the functional status of the liver cells. The characteristic feature of experimental hepatic damage observed is significant decrease in protein level. The rats in group IV, which receive EECS, showed restoration of protein levels.

These findings can be further corroborated with histopathological studies. The histopathological examination clearly reveals that the hepatic cells, central vein, and portail triad are almost normal in EECS (400 mg/kg. p.o.) group in contrast to group IV, which receive CCl_4 only. Thus EECS can be considered to be an effective hepatoprotective as it ameliorates almost to normalcy the damage caused by CCl_4 to hepatic function.

As the flavonoid compound isolated from *Artemisia scorparia* [21] is reported to possess hepatoprotective activity,

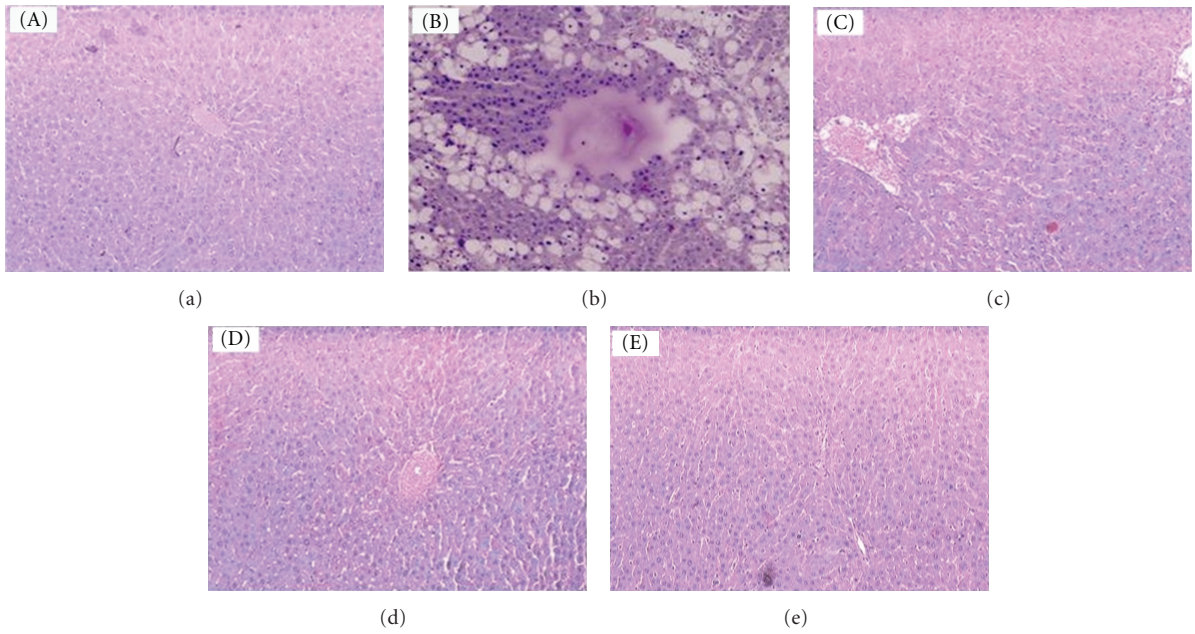

Figure 2: Photomicroscopy of liver sections from CCl_4-intoxicated rats (10x). Histological observations of the liver tissue of the normal animals showed hepatic cells with well-preserved cytoplasm, nucleus, nucleolus, and central vein (a). Treatment with CCl_4 caused fatty degeneration with severe necrosis of the parenchyma cells in the central lobular region of the liver. Furthermore, hepatocytic necrosis was predominant surrounding the central vein, which formed a streak-like appearance (b), (c), (d), and (e) showed animals treated with EECS (200 and 400 mg/kg, p.o.) and silymarin (25 mg/kg) and restored the altered histopathological changes, respectively.

it is very likely that the flavonoid glycoside in *C. sophera* [22] may be responsible for hepatoprotective activity, but further exploration is needed.

5. Conclusion

The ethanolic extract of *Cassia sophera* could effectively control the AST, ALT, ALP, and total bilirubin levels and increase the protein levels in the protective studies. The histopathological studies also substantiate the activity of the drug. Therefore, the study scientifically supports the usage of this plant in traditional medicine for treatment of liver disorders and as a tonic.

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