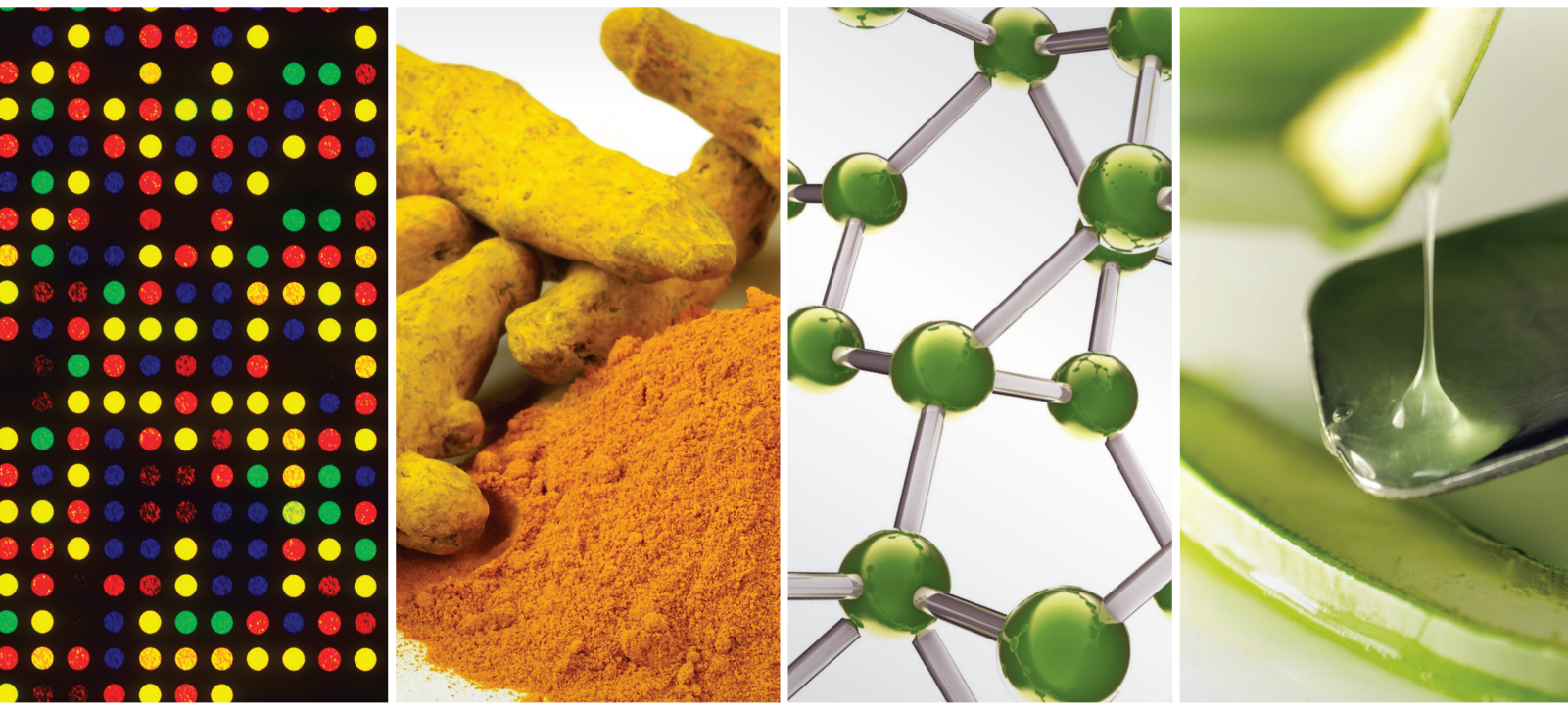
eCAM Evidence-Based Complementary and Alternative Medicine

Role of Complementary and Alternative Medicine in Controlling Dyslipidemia

Guest Editors: Waris Qidwai, Firdous Jahan, and Kashmira Nanji



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Editorial Board

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Editorial

Role of Complementary and Alternative Medicine in Controlling Dyslipidemia

Waris Qidwai,[1] Firdous Jahan,[2] and Kashmira Nanji[1]

[1] *Family Medicine Department, Aga Khan University, Karachi 74800, Pakistan*
[2] *Family Medicine Department, Oman Medical College, P.O. Box 391, Sohar, Oman*

Correspondence should be addressed to Waris Qidwai; waris.qidwai@aku.edu

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1. Introduction

This special issue focuses on the role of complementary and alternative medicine (CAM) in controlling dyslipidemia. Complementary and alternative medicine (CAM), also known as nonconventional medicine, includes a wide and heterogeneous array of health care practices (such as herbal medicine, acupuncture, yoga, meditation, and homeopathic medicine) that are not part of a health care system.

The popularity of CAM has dramatically increased in many developed countries since the 1990s. This could be attributed to the aging of population, prevalence of chronic diseases, and concern about the adverse reaction of chemical drugs. All these aspects have contributed greatly to the worldwide popularity of CAM. In the United States, consumers spend over $34 billion per year on CAM therapies spent outside the conventional health care financing system. This out-of-pocket expenditure is evidence of the belief that CAM therapies have benefits that outweigh their costs. CAM is generally more popular in most developed countries, especially in North America, Europe, and Australia. According to the 2007 National Health Interview Survey on more than 32,800 Americans, 38.2% of adults and 12% of children used some form of CAM within the previous 12 months.

Dyslipidemia is an independent preventable risk factor of coronary heart disease and has been shown to increase the risk of cardiovascular mortality manyfold. Therefore, the study on various indicators and risk factors of dyslipidemia appears to be significant for future health outcomes. It is critically important to recognize the need for treatment of dyslipidemia and to institute necessary therapies to reduce the long-term risks of disease recurrence or modify the metabolic derangements that promote atherosclerosis. Drugs used in dyslipidemia may cause adverse effects if used for longer duration. Therefore patients use CAM to reduce lipids without any major side effect.

The exact reasons for the popularity of CAM are complex; as they change with time and space, they vary from individual to individual and from therapy to therapy. In general, there are a broad range of positive motivations to the present popularity of CAM.

Although the use and expenditure of CAM have been increased radically, the potential role of CAM in modern clinical practice and health care system seems to be limited. As the efficacy and safety have been the major concerns in the recognition of CAM and integrating it into the conventional medicine, hence, there is an urgent need to provide evidence regarding the merits of the numerous techniques of CAM. The best way to achieve this is through rigorous research. Since then, in CAM, simple answers or broad generalizations are not possible. Each of the numerous techniques has to be evaluated separately. It is necessary to bring the practice of CAM in line with evidence. The wide dissemination of its findings is also important to include CAM into the modern clinical practice.

Studies have reported that CAM can be ineffective for a specific condition, but still they could be used as they do not harm the patients. Some argue that CAM should be used regardless of the results of placebo-controlled clinical trials, particularly when its use is not associated with serious risks.

The main purpose of this issue is to illustrate the inevitability of researches in the field of CAM to integrate it into the modern clinical practice. Altogether, 14 manuscripts were submitted for publication, out of which 11 manuscripts got accepted. The articles in this issue represent a wide range of therapeutic approaches of CAM in preventing dyslipidemia.

This special issue includes a review that discusses the progress and perspective of studies on dyslipidemia with single Chinese herb and its monomers or effective extracts during the past 10 years. The review concludes that traditional Chinese medicine (TCM) has some beneficial effects on the treatment of patients with dyslipidemia and has less adverse effects as compared to chemical agents. However, future clinical trials are needed to be confirmed about the effects of TCM.

This edition also includes a randomized, double-blind, placebo-controlled clinical trial on 50 hyperlipidemic patients. The results of this trial show that daily consumption of the fruit extract of *Vaccinium arctostaphylos* significantly reduces the serum levels of total cholesterol, LDL-C, and triglyceride (TG) and oxidative stress in hyperlipidemic patients. Therefore, this extract could be considered as a potential agent for treatment of dyslipidemia.

Another study in this issue is on Chaihu-Shugan-San (CSS), an ancient classical formula composed of seven Chinese herbs, which found that integrated recipe might work as a significant anti-inflammatory effect in Kupffer cells. In addition, another study conducted by Han et al. on the molecular docking of echinocystic acid (EA) (isolated from *G. sinensis* fruits) and the effects of it on the possible targets in *in vitro* rat liver microsomes has been added.

This review also includes a research paper which concluded that preventive acupuncture and moxibustion can significantly decrease the plasma TG and LDL, increase the plasma HDL, and prevent fat accumulation during climacteric period in rats. Moreover, a paper included is on intestinal transportations of main chemical compositions of Polygoni Multiflori Radix (PMR, originated from the root of *Polygonum multiflorum* Thunb.). This has been used in the treatment of hyperlipidemia in some countries for centuries. Another paper using the same herb PMR has been added to this special issue which discusses the mechanisms of the water extracts of Polygoni Multiflori Radix (PMR) and its processed products (PMRP) on liver lipid metabolism. Moreover, another paper was included in this review on propolis, which is a brownish resinous material collected by worker bees from the leaf buds of numerous plants that have strong pharmacological and biological properties.

Another study analyzed the effects of acupuncture on interleukin, IL-17expression in fat excess liver on adult mice, and provided some basic evidences that the inflammatory damage of hyperlipidemic fatty liver could be restricted through acupuncture. A study in this review investigates the beneficial effects of ethanol extract of *Gastrodia elata* Blume (EGB) on lipid metabolism and endothelial dysfunction in a high-fructose (HF) diet animal model.

2. Conclusion

The articles included in this issue highlight the need of sufficient scientific evidence from CAM research to clarify their mechanism of action and demonstrate their efficacy and safety. Patients and health care providers need to know which forms are safe and effective through unbiased scientific evaluation. Through the rigorous researches, the benefits of CAM therapies will be highlighted, and this will help in integration of CAM into the mainstream medicine. This integrative approach will ultimately lead to a safer and more effective patient-centered health care system.

Acknowledgments

We hope that this special issue informs us about the rationale use of CAM in dyslipidemia patients. We also hope that the papers included in this issue play a role in reflecting the latest trends in the field of CAM. We would like to thank the contributors to this special issue for their insightful papers. We would also like to acknowledge the many reviewers for their detailed comments and constructive suggestions.

Waris Qidwai
Firdous Jahan
Kashmira Nanji

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Research Article

In Vivo Lipid Regulation Mechanism of Polygoni Multiflori Radix in High-Fat Diet Fed Rats

Pei Lin, Yan Ran He, Jian Mei Lu, Na Li, Wan Gen Wang, Wen Gu, Jie Yu, and Rong Hua Zhao

Yunnan University of Traditional Chinese Medicine, Kunming, Yunnan 650500, China

Correspondence should be addressed to Jie Yu; yujie.ynzyxy@gmail.com and Rong Hua Zhao; kmzhaoronghua@hotmail.com

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Mechanisms of the water extracts of Polygoni Multiflori Radix (PMR) and its processed products (PMRP) on liver lipid metabolism were observed in this paper. Aqueous extract of PMR and PMRP was given to nonalcoholic fatty liver model rats, respectively. PMR was better in reducing the contents of very low density lipoprotein (VLDL) than PMRP and the positive control groups. In the aspect of regulating TG, medium dose PMR reduced the activity of diacylglycerol acyltransferase (DGAT) to 1536 ± 47.69 pg/mL ($P < 0.001$) and promoted the expression of hepatic lipase (HL) to 23.59 ± 0.2758 U/mL ($P < 0.05$). HL promotion ability of medium dose PMR was similar with the simvastatin positive control. Both medium and high dose of PMR showed significant alterations in TC, which were related to the downregulation effects on hydroxyl methyl-glutaryl coenzyme A reductase (HMGCR) and upregulation effects on cholesterol 7-alpha-hydroxylase or cytochrome P450 7A (CYP7A). Quantitative relationships research indicated that the prominent effect on inhibiting the content of HMGCR ($r = 0.756$, $P < 0.05$) was strongly positive correlated with to the TC regulation effects. Effects of PMR on enhancing decomposition rate or reducing *de novo* synthesis rate of TG and TC were better than PMRP.

1. Introduction

Fatty liver disease (FLD), a kind of lipid metabolic disorder of 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). According to the different inducements of fatty liver, FLD is divided into alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD). NAFLD is increasingly recognized as the hepatic manifestation of insulin resistance and the systemic complex known as metabolic syndrome [1, 2]. NAFLD is the most common form of chronic liver disease in adults in the United States, Australia, Asia, and Europe [3–5]. NAFLD is also gaining recognition as a significant early sign of liver cirrhosis and liver cancer [6]; prevalence estimates of NAFLD have used a variety of laboratory and imaging assessments.

In NAFLD pathogenic process, the accumulation of lipid within the liver, especially total cholesterol (TC) and triglycerides (TG) accumulation, has been confirmed by dynamics research. These researches point out that TC, TG, and esterification of free fatty acid (FFA) accumulation in the liver for the treatment of NAFLD might have direct influence [7, 8].

Polygoni Multiflori Radix (PMR, Heshouwu in Chinese) and Polygoni Multiflori Radix Praeparata (PMRP, Zhiheshouwu in Chinese) are originated from the root of *Polygonum multiflorum* Thunb. (Polygonaceae) (Figure 1). They mainly contain anthraquinone, stilbene glycosides, phospholipids, and other ingredients and are used in the prevention and treatment of NAFLD, hyperlipidemia, or related diseases in oriental counties for centuries [9].

Previously, a sensitive, accurate, and rapid *in vitro* model (the steatosis L02 cells, obtained after being cultured with 1% fat emulsion, 10% fetal bovine serum (FBS), and RPMI 1640 medium for 48 h) was used in our research group to investigate the lipid regulation effects of 2, 3, 5, 4′-tetrahydroxystilbene-2-O-β-D-glucoside (TSG), emdin, and physcion [10]. Hereafter, *in vivo* model (high-fat diet fed rats) was applied to explore the lipid regulation effects of extracts

Hand drawing (a) PMR (b) PMRP (c)

Figure 1: Photographs of Polygoni Multiflori Radix and its processed products.

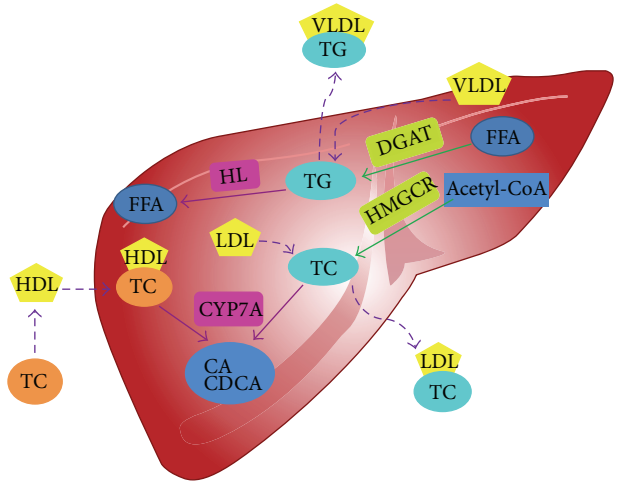

Figure 2: Lipid synthesis and lipolysis procedure of TG and TC.

of PMR and PMRP [11]. The results show that both the raw crude drug and its processed products have significant lipid-lowering activities; however, obvious target organ selectivity was found. PMR was considered to possess better effects in lipid regulation in liver sample and was recommended for the treatment of early stage NAFLD. On the other hand, PMRP displayed better effects in lipid regulation in blood circulation system for the treatment of hyperlipidemia. However, the lipid regulation mechanism of *P. multiflorum* is still not clearly elucidated. So we chose the following four key enzymes as the investigation objectives, in this paper (Figure 2).

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR), most abundantly expressed in the liver, plays a central role in the regulation of TC concentration. HMGCR is a key enzyme catalyzing cholesterol in *de novo* synthesis pathway *in vivo*. HMGCR activity directly affects the speed of cholesterol synthesis and the level of cholesterol [12]. Clinical results confirm that HMGCR inhibitor reduced plasma concentrations of TC and TG, low density lipoprotein (LDL), and very low density lipoprotein (VLDL) and increased plasma concentrations of high density lipoprotein (HDL). Therefore, the inhibition of this enzyme could contribute to reduction of synthesis of cholesterol.

Cholesterol 7a-hydroxylase (CYP7A) is the first and rate-limiting enzyme in bile acid synthesis pathway and is expressed only in the liver. Lack of CYP7A results in high levels of plasma cholesterol, whereas induction of CYP7A prevents elevation of blood cholesterol in rodents fed by a cholesterol-rich diet indicating its importance in maintaining plasma cholesterol homeostasis. CYP7A is tightly regulated by feedforward of cholesterol and negative feedback of bile acids [13].

The diacylglycerol acyltransferase (DGAT) is rate-limiting enzyme for triglyceride synthesis. DGAT catalyzes the final step in TG biosynthesis by converting diacylglycerol (DAG) and fatty acyl-coenzyme A into TG [14].

Hepatic lipase (HL) is a lipolytic enzyme that contributes to the regulation of TG levels. Hepatic lipase facilitates the clearance of TG from VLDL pool, and this function is governed by the composition and quality of HDL particles. HDL regulates the release of HL from the liver and HDL structure

Table 1: Animal grouping and treatments in this research.

Groups	Diets	Treatment (from the nineteenth 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 PMR	0.4050
D	High-fat diets	Water extraction of PMR	0.8100
E	High-fat diets	Water extraction of PMR	1.620
F	High-fat diets	Water extraction of PMRP	0.8100
G	High-fat diets	Water extraction of PMRP	1.620
H	High-fat diets	Water extraction of PMRP	3.240
I	High-fat diets	Simvastatin	0.001200
J	High-fat diets	Fenofibrate	0.03300

controls HL transport and activation in the circulation [15]. HL could catalyze the chylomicrons (CM) and promote the hydrolysis of triglycerides in VLDL.

Based on the key role of above enzyme in the lipid metabolism, we used the *in vivo* model (high-fat diet fed rats) to investigate lipid regulation mechanisms and possible regulatory targets of TC and TG by PMR and PMRP further and systematically.

2. Materials and Methods

2.1. Samples [11]. PMR was collected in Luquan, Yunnan, by the authors. The plants were identified as the root of *Polygonum multiflorum* Thunb. by Prof. Rong-hua Zhao, Yunnan University of Traditional Chinese Medicine. PMRP was made by PMR with black bean decoction according to the method recorded in the Pharmacopoeia of People's Republic of China [8]. Processed products were detected by high liquid chromatography (HPLC); the content of stilbene glycoside was greater than 0.70%, in accordance with the Pharmacopoeia standards, while the content of stilbene glycoside of raw products was greater than 1.0%.

2.2. Preparation of Extraction of PMR and PMRP [11]. 300 g powder of PMR and 472 g powder of PMRP were extracted for 1 hr with 10 times boiling water. Then the residue was extracted for 40 min with 10, 8, and 6 times volume boiling water, respectively. Extracts were combined, condensed, and lyophilized. The concentrations of PMR and PMRP extracts were 0.6980 g/mL and 0.8580 g/mL, respectively.

2.3. Animals Groups [11]. SD rats were provided by the Experimental Animal Center of Yunnan University of Traditional Chinese Medicine. They were aged 8 weeks and weighed 245 ± 20 g and 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.

120 SD male rats were randomly divided into 10 groups (Table 1): normal control group (A), model group (B), water extraction group of PMR (low, medium, and high dose groups: C, D, and E), water extraction group of PMRP (low, medium, and high dose groups: F, G, and H), and positive control groups (fenofibrate and simvastatin control: I and J). In addition to the normal control group, other groups were fed with a high-fat diet (containing 1% cholesterol, 10% lard, 0.2% methyl thiouracil, and 88.8% usual feed) to the end of the experiment (42 days).

2.4. Drug Delivery Process after the Success of Modeling [11]. After giving high-fat diet for 18 days, group C to H received the PMR and PMRP treatments till 42 days, the end of the research. In the meantime, normal control group and hyperlipidemia model group were given 0.9% saline 1 mL. Positive groups I and J received 0.033 $g \cdot kg^{-1}$ fenofibrate and 0.0012 $g \cdot kg^{-1}$ simvastatin daily, respectively. The low dose group of PMR and PMRP was given 0.405 $g \cdot kg^{-1}$ and 0.810 $g \cdot kg^{-1}$ daily [15]; the middle and high dosages of PMR and PMRP were 2 and 4 times of the low dosages, respectively. All rats were fasted for 2 h every day before administration of therapeutic agents (Table 1).

2.5. The Preparation and Detection of Animal Liver Homogenate. Rats were sacrificed by cervical dislocation. Liver samples were collected (Figure 3) and weighed after washing with 0.9% saline. 100 mg tissues were rinsed with PBS and homogenized in 1 mL of PBS and then stored overnight at −20°C. After two freeze-thaw cycles were performed to break all cell membranes, the homogenates were centrifuged for 5 minutes at 5000 g, 2–8°C. The supernatants were collected and analysed immediately.

Contents of TC, TG, LDL, and HDL in the supernatant were measured by AB-1020 automatic biochemical analyzer (Sunostik Medical Technology Co., Ltd.) and assay kits purchased from Shanghai Rongsheng Biological Pharmaceutical Co., Ltd., and Sichuan Maker Biotechnology Co., Ltd., China.

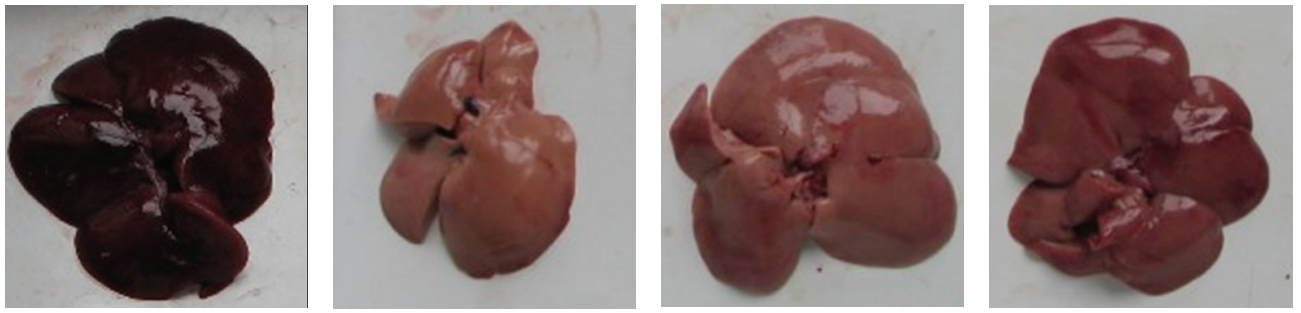

Figure 3: Liver tissue samples. Group A normal control group. Group B model group. Group D water extraction group of PMR (medium dose). Group I positive control group (simvastatin).

The VLDL, DGAT, HMGCR, HL, and CYP7A contents were tested by assay kits purchased from Cusabio Biotech Co., Ltd., China.

2.6. Statistical Analysis. All data in this research were expressed in the form of mean ± SD. All data were analyzed by single factor analysis of variance (ANOVA) statistics and the test results of $P < 0.05$, $P < 0.01$, and $P < 0.001$ as a statistically significant difference criterion.

Relationships between these enzymes and proteins and TG and TC were assessed with Pearson's correlation coefficient. Results were classified into two significance levels using the P value of 0.05.

3. Results

3.1. Effects of Lipid Regulation Using Raw and Processed Radix Polygoni Multiflori [11]. Morphologic observations were carried in every group. Livers in high-diet fed group were obviously smaller and paler than normal livers. Treatment of simvastatin, PMR, and PMRP relieved the steatosis procedure; however, none of these treatments could reverse it (Figure 3).

As listed in Table 2, TC, TG, and LDL-C in liver tissue were all significantly higher than in model rats. Both PMR and PMRP revealed TC-lowering effects; however, dose-dependent TC- and TG-lowering effects were observed only in PMR groups.

3.2. Effects of PMR and PMRP on VLDL in Liver Samples. 42 days of high-fat diet intake significantly increased the liver VLDL level from 33.22 ± 6.445 ug/mL to 64.36 ± 6.455 ug/mL ($P < 0.001$) in rats. Fortunately, the liver VLDL levels were reduced in PMR, PMRP, and positive control drugs. From comparison of PMR groups and PMRP groups, PMR was better in reducing VLDL than PMRP. Low dosage of PMR could reduce the content of VLDL to 35.20 ± 15.03 ug/mL, which was similar to the normal group. VLDL-lowering effects were even better than the positive control groups (Figure 4).

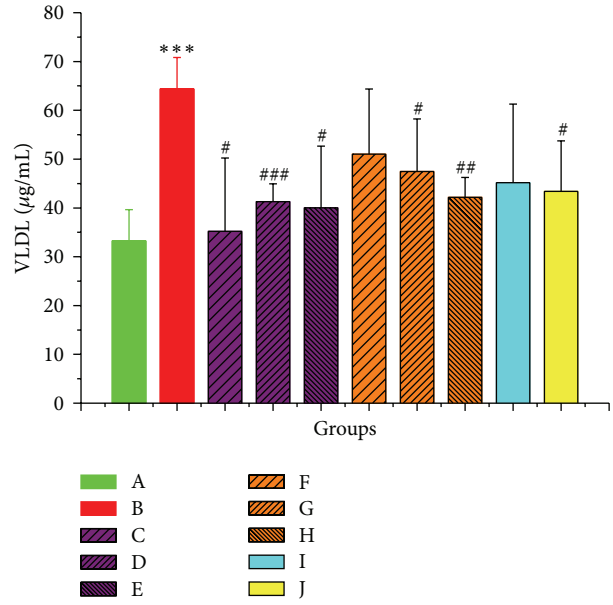

Figure 4: The content of VLDL of liver homogenate in all groups. The ∗ indicates a significant difference compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The # indicates a significant difference compared with model group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

3.3. Expression of Key Enzyme of Triglyceride (TG) Metabolism. The activities of DGAT and HL were investigated as the key enzymes of TG metabolism.

Considering the two indexes in Figure 5, the effects of aqueous extract of PMR on enzyme activity were better than those of PMRP. Medium dose group of PMR showed more prominent effect; they reduced the activity of DGAT from 1776 ± 50.44 pg/mL to 1536 ± 47.69 pg/mL and promoted the expression of HL to 23.59 ± 0.2758 U/mL. HL expression regulation ability of PMR was even similar to the positive control group. The way of PMR to regulate the TG content in the liver was to reduce the activity of DGAT and promote the expression of HL. The regulations of DGAT were not very

Table 2: Lipid indexes in the liver samples.

Groups	TC (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)
A	66.63 ± 4.093	147.22 ± 6.180	10.74 ± 2.186
B	100.2 ± 19.22***	200.0 ± 32.56***	28.36 ± 12.57**
C	105.8 ± 15.01***	179.8 ± 18.56***	28.79 ± 7.821***
D	87.71 ± 17.19**	180.8 ± 15.94***	24.49 ± 6.547***
E	57.18 ± 6.754$^{**,\#\#\#}$	153.6 ± 27.34$^{\#}$	24.86 ± 4.385***
F	66.29 ± 28.08$^{\#}$	162.1 ± 39.88	38.39 ± 18.53***
G	89.48 ± 18.75**	205.8 ± 29.90***	48.98 ± 12.02$^{***,\#}$
H	69.82 ± 24.30$^{\#}$	165.0 ± 32.11	41.89 ± 16.49***
I	43.67 ± 2.936$^{***,\#\#\#}$	150.8 ± 18.82$^{\#\#}$	9.990 ± 3.548$^{\#\#}$
J	44.66 ± 5.379$^{***,\#\#\#}$	195.8 ± 25.96***	15.79 ± 9.505

The (∗) indicates a significant difference compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.
The (#) indicates a significant difference compared with model group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

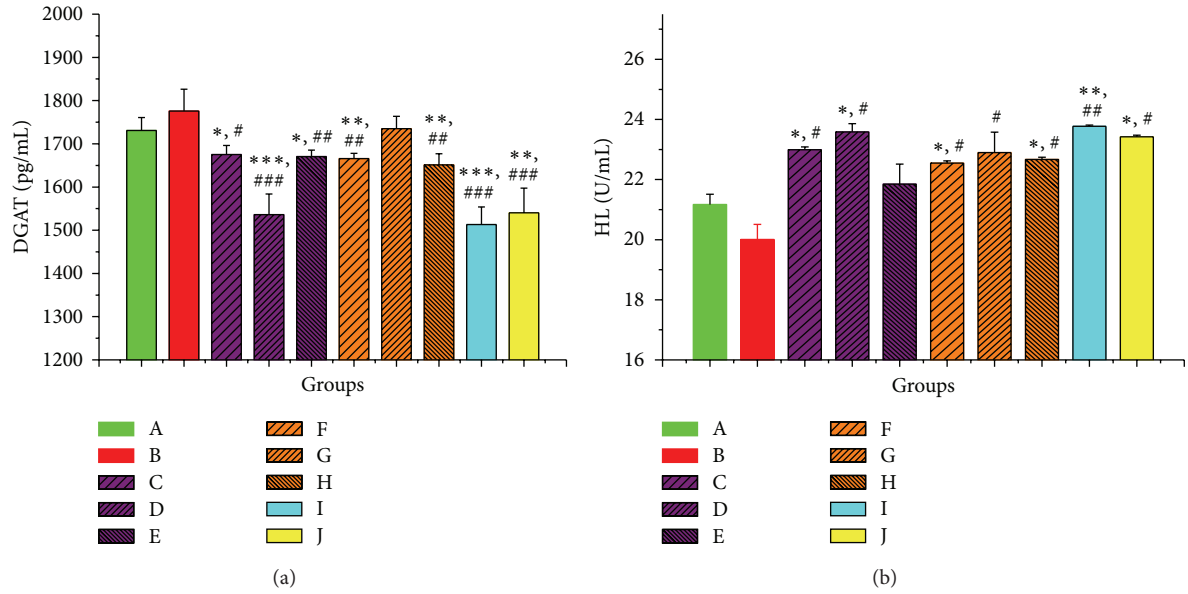

Figure 5: The activity of key enzymes of TG metabolism. The ∗ indicates a significant difference compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The # indicates a significant difference compared with model group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

obvious in all PMRP groups, while the promotion effects of HL were much better.

3.4. Expression of Key Enzyme of Total Cholesterol (TC) Metabolism. TC level in high-fat diet group was significantly higher than that in the control group (Table 3); no TC melioration effect was observed in the low dosage PMR group; however, both the middle (group D) and high dosage (group E) PMR groups showed significant changes in TC regulation. These TC regulation effects were related to the downregulation on HMGCR and upregulation on CYP7A. Comparing the expression of these two key enzymes (Figure 6), HMGCR played a leading role in TC metabolism; the ability of middle and high dosage PMR was similar to the positive control group. In the meantime, all dosage groups of PMRP showed the TC melioration effect; however, these are not better than PMR.

3.5. Relationships between Enzymes and Proteins and TC and TG. Pearson's correlation coefficients between these enzymes and proteins and TG and TC were displayed in Table 4. HMGCR activities ($r = 0.756$, $P < 0.05$) were strongly positive correlated with TC. Moreover, LDL was positive correlated with TC, CYP7A was negative correlated with TC, and VLDL was positive correlated with TG. However, we did not find any significant relationship between DGAT and HL and TG regulation effects.

4. Discussion and Conclusion

Liver was the main organ responsible for lipid metabolism. Liver cells played important roles in lipid uptake, transport, metabolism, and excretion. Generally, the fat metabolism disorder leaded to liver steatosis which was the initial step of fatty liver disease. Fat metabolism related to fatty liver induced

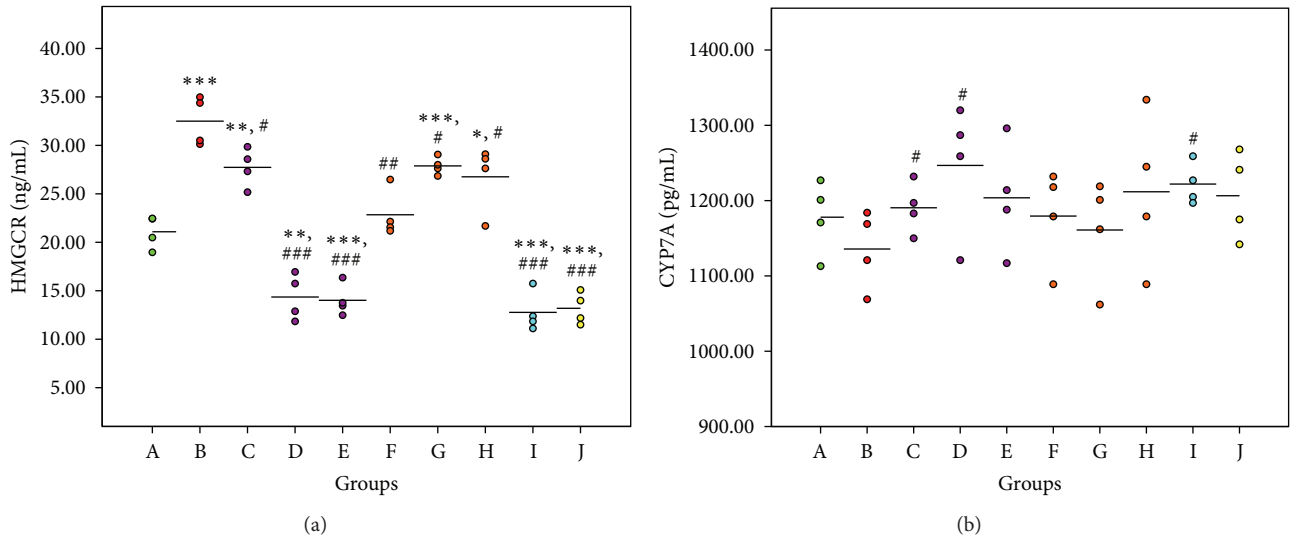

Figure 6: The activity of key enzymes of TC metabolism. The ∗ indicates a significant difference compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The # indicates a significant difference compared with model group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

Table 3: Contents of TC, HMGCR, and CYP7A in every group.

	TC (mg/dL)	HMGCR (ng/mL)	CYP7A (pg/mL)
A	66.63 ± 4.093	21.09 ± 1.687	1178 ± 49.00
B	$100.2 \pm 19.22^{***}$	$32.50 \pm 2.533^{***}$	1110 ± 54.64
C	$105.8 \pm 15.01^{***}$	$27.73 \pm 1.994^{**,\#}$	$1191 \pm 33.96^{\#}$
D	$87.71 \pm 17.19^{**}$	$14.35 \pm 2.389^{**,\#\#\#}$	$1247 \pm 87.46^{\#}$
E	$57.18 \pm 6.754^{**,\#\#\#}$	$14.01 \pm 1.655^{***,\#\#\#}$	1203 ± 73.91
F	$66.29 \pm 28.08^{\#}$	$22.84 \pm 2.464^{\#\#}$	1180 ± 64.37
G	$89.48 \pm 18.75^{**}$	$27.88 \pm 0.920^{***,\#}$	1161 ± 70.16
H	$69.82 \pm 24.30^{\#}$	$26.76 \pm 3.432^{*,\#}$	1212 ± 103.59
I	$43.67 \pm 2.936^{***,\#\#\#}$	$12.77 \pm 2.050^{***,\#\#\#}$	$1222 \pm 27.74^{\#}$
J	$44.66 \pm 5.379^{***,\#\#\#}$	$13.19 \pm 1.640^{***,\#\#\#}$	1207 ± 58.09

The (∗) indicates a significant difference compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.
The (#) indicates a significant difference compared with model group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

Table 4: Relationships between enzymes and proteins and TG and TC.

	Correlation coefficient and significance	Pearson's correlation coefficient	Significance (P)
TC	LDL	0.487	0.153
	HMGCR	0.756	0.011
	CYP7A	−0.453	0.189
TG	VLDL	0.490	0.150
	DGAT	0.192	0.596
	HL	−0.019	0.958

triglyceride metabolism, cholesterol metabolism, phospholipid metabolism, and priority to triglycerides metabolic disorder.

However, there was no specific drug for NAFLD treatment. Considering the effectiveness and acceptable prices of traditional Chinese medicine (TCM), the prevention and treatment of NAFLD and hyperlipidemia by TCM were a research hotspot. PMR and PMRP, which displayed great clinical effect in treating NAFLD, seemed to be potential choices.

Our research group had focused on the lipid regulation effect of PMR and PMRP for decades; we had confirmed the great lipid reducing effect of PMR and PMRP by both *in vitro* [10] and *in vivo* [11] assays. However, the lipid regulation mechanism of *P. multiflorum* was still not clearly elucidated. So in this research we chose four key enzymes, DGAT, HL,

HMGCR, and CYP7A, as the major objectives, to seek the possible regulatory targets in the metabolisms of TC and TG by PMR and PMRP.

After giving high-fat diet for 42 days, we found that TC, TG, LDL, and VLDL in liver tissue were all significantly higher than in model rats. DGAT and HMGCR in model group were upregulated, and CYP7A and HL were all down-regulated. These showed that the method of the rats induced into NAFLD was successful.

Compared with model group, PRM and PRMP had good lipid-lowering effects; they could not only enhance decomposition activity but also reduce synthase activity. TC and TG melioration effects of PRM were better. In particular, middle dose group of PMR showed more prominent effect on inhibiting the content of HMGCR and DGAT, which could block the synthesis of TC and TG, respectively. Key enzyme regulation ability of PMR was similar to the positive control group. At the same time, low dose group of PMR showed the best VLDL-reducing effect, and the VLDL-reducing effect of PMRP was in a dose-dependent manner.

According to the analysis of Pearson's correlation coefficients, HMGCR activity ($r = 0.756$, $P < 0.05$) was strongly positive correlated with TC regulation effects. This was consistent with the above results. In a conclusion, TC regulation of PMR was mediated by HMGCR. In some extension, PMR showed similar activities and mechanisms with statins. We also found the better TG regulation effects in some dosage of PMR and PMRP; however, there was no significantly relationship between DGAT, HL, VLDL, and TG. Therefore, we assumed that the regulation on TG might be related to some other mechanisms or targets.

Previous researches [16, 17] pointed out that water extract and total glycosides of PMR had shown a good lipid-lowering activity in animal experiments; they could reduce the contents of the TG and TC in rats caused by high-fat diet. Moreover, the effect of total glycosides on reducing the TG in hyperlipidemia rat lack of Apo E gene was even better than the positive drugs. These were in line with our results that high dosage of PMR had the similar TG-lowering effect with statins.

Other researches [18, 19] consider that the lipid-lowering activity of PMR was associated with the inhibition of HMGCR, the reduction of VLDL and LDL contents, and the decreasing of TG and TC absorption. In this paper, we reported the effects on the activity of DGAT, HL, and CYP7A for the first time. This research could contribute to our knowledge on the lipid regulation mechanism of PMR in high-fat diet fed rats.

TG content in the liver was affected not only by the amount directly absorbed from the food but also by the *de novo* synthesis by FFA [8, 20]. Therefore, whether FFA supply chain was affected by PMR and PMRP will be in great worthy of study in the future. On the other hand, previous literatures [21, 22] also showed that insulin resistance was often observed in the NAFLD patients. The increasing of insulin concentration could reduce the oxidation and decomposition rate of TG, so that TG content in the cells would increase [23]. Therefore, whether PMR and PMRP could control the insulin resistance status in NAFLD patients also will be a subject in our future research plan.

Abbreviations

CM:	Chylomicrons
CYP7A:	Cholesterol 7-alpha-hydroxylase or cytochrome P450 7A
DAG:	Diacylglycerol
DGAT:	Diacylglycerol acyltransferase
FBS:	Fetal bovine serum
FFA:	Free fatty acid
FLD:	Fatty liver disease
HMGCR:	3-Hydroxy-3-methylglutaryl-CoA reductase
HDL:	High density lipoprotein
HL:	Hepatic lipase
HPLC:	High liquid chromatography
LDL:	Low density lipoprotein
NAFLD:	Nonalcoholic fatty liver disease
PMR:	Polygoni Multiflori Radix
PMRP:	Polygoni Multiflori Radix Praeparata
RPMI 1640:	Roswell Park Memorial Institute medium 1640
TC:	Total cholesterol
TCM:	Traditional Chinese medicine
TG:	Triglyceride
TSG:	2, 3, 5, 4′-Tetrahydroxystilbene-2-O-β-D-glucoside
VLDL:	Very low density lipoprotein.

Conflict of Interests

The authors declare that there is no conflict of interests. They declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work; there are no professional or other personal interests of any nature or kind in any product, service, or company that could be construed as influencing the position presented in this paper.

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Research Article

Propolis Reduces Phosphatidylcholine-Specific Phospholipase C Activity and Increases Annexin a7 Level in Oxidized-LDL-Stimulated Human Umbilical Vein Endothelial Cells

Hongzhuan Xuan,[1,2] Zhen Li,[1] Jiying Wang,[3] Kai Wang,[2] Chongluo Fu,[1] Jianlong Yuan,[1] and Fuliang Hu[2]

[1] *School of Life Science, Liaocheng University, Liaocheng 252059, China*
[2] *College of Animal Sciences, Zhejiang University, Hangzhou 310029, China*
[3] *Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences, Jinan 250100, China*

Correspondence should be addressed to Fuliang Hu; flhu@zju.edu.cn

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To understand the mechanisms underlying the regulating dyslipidemia action of Chinese propolis and Brazilian green propolis, we investigated their effects on phosphatidylcholine-specific phospholipase C (PC-PLC) activity and annexin a7 (ANXA7) level which play crucial roles in the control of the progress of atherosclerosis. Furthermore, active oxygen species (ROS) levels, nuclear factor-KappaB p65 (NF-κB p65), and mitochondrial membrane potential (MMP) were also investigated in oxidized-LDL- (ox-LDL-) stimulated human umbilical vein endothelial cells (HUVECs). Our data indicated that the treatment of both types of propolis 12.5 μg/mL significantly increased cell viability and attenuated apoptosis rate, increased ANXA7 level, and decreased PC-PLC activity. Both types of propolis also inhibited ROS generation as well as the subsequent MMP collapse, and NF-κB p65 activation induced by ox-LDL in HUVECs. Our results also indicated that Chinese propolis and Brazilian green propolis had similar biological activities and prevented ox-LDL induced cellular dysfunction in HUVECs.

1. Introduction

Propolis is a brownish resinous material collected by worker bees from the leaf buds of numerous plants like birch, poplar, *Baccharis dracunculifolia*, and *Dalbergia ecastaphyllum* [1–4]. It has been extensively used as a folk medicine since ancient time because of its special chemical components, strong pharmacological and biological properties, and low toxicity [5].

In recent years, the regulation of dyslipidemia actions of propolis has been widely documented, resulting in the genesis and progression of atherosclerosis. A recent report indicated ethanolic extract of propolis inhibited atherosclerosis in ApoE-knockout mice [6]. Furthermore, we also reported that Chinese propolis regulated lipid metabolism of diabetes *in vivo* by regulating triglycerides, total cholesterol, high-density lipoprotein, and low-density lipoprotein cholesterol [7, 8]. However, the molecular mechanisms underlying such protect effects of propolis have not been fully elucidated.

Phosphatidylcholine-specific phospholipase C (PC-PLC), an important member of phospholipase C family, has been implicated in several cellular signaling pathways such as cell growth, differentiation, senescence, apoptosis, and autophagy of mammalian cells [9–13]. Accumulating evidence demonstrated that PC-PLC was a key inducing element of atherosclerosis and contributed to the progression of atherosclerosis [14]. Pharmacological blockade of PC-PLC inhibited the progression of atherosclerosis [15]. And a recent

study indicated that annexin a7 (ANXA7), a member of the annexin family of calcium-dependent phospholipid binding proteins, was an endogenous regulator of PC-PLC. ANXA7 also participated in the progression of atherosclerosis and targeting ANXA7 inhibited atherosclerosis in apoE$^{-/-}$ mice. ANXA7/PC-PLC signaling pathway may represent a novel target for the treatment of atherosclerosis [16].

Chinese propolis affected PC-PLC activity. Our previous study indicated that Chinese propolis played an anti-inflammatory role partly through its inhibitory effect on the activity of PC-PLC [17]. Considering the important roles of ANXA7 and PC-PLC in atherosclerosis and propolis modulated atherosclerosis and affected PC-PLC activity, we hypothesized that propolis may also affect ANXA7, the endogenous regulator of PC-PLC. In present study we tested the hypothesis by investigating the effects of Chinese propolis and Brazilian green propolis on PC-PLC activity and ANXA7 level in ox-LDL-stimulated HUVECs; ox-LDL plays crucial role in triggering the development of atherosclerosis. Furthermore, we investigated the effects of both types of propolis on reactive oxygen species (ROS) levels, nuclear factor-KappaB p65 (NF-κB p65), and mitochondrial membrane potential (MMP) which were regulated by PC-PLC in HUVECs.

2. Materials and Methods

2.1. Chemicals and Reagents. DMEM was from Gibco (USA). Fetal bovine serum (FBS) was from Hyclone Lab Inc. (USA). L-α-phosphatidylcholine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH), and JC-1 were from Sigma Co. (USA). Acridine orange was from Amresco (USA). Ox-LDL was from Beijing Union-Biology Co. (China). Primary antibodies against ANXA7, NF-κB p65, GAPDH, and secondary antibody (horseradish peroxidase) were from Santa Cruz Biotechnology (USA). Secondary antibody for immunofluorescence, donkey anti-rabbit IgG Alexa Fluor-488, was purchased from Life Technologies (USA). All other reagents were of ultrapure grade.

2.2. Preparation of Propolis Extracts. Propolis used in present study was Chinese propolis and Brazilian green propolis. Both types of propolis had been used in previous studies [17, 18]. The extraction method was as before. Briefly, extracted Chinese propolis was obtained from colonies of honeybees, *A. mellifera* L., in Shandong Province of North China and the main plant origin was poplar (*Populus* sp.). Extracted Brazilian green propolis was collected in Minas Gerais State of Brazil, where *Baccharis dracunculifolia* DC. is the main botanical source. Both types of propolis were stored at −20°C until used. Chinese propolis and Brazilian green propolis samples were extracted with ethanol and then filtered under reduced pressure, and the filter liquid was concentrated under reduced pressure at 40°C until reaching a constant weight and then redissolved in ethanol. The ethanol-extracted Chinese propolis (EECP) and ethanol-extracted Brazilian green propolis (EEBP) had a brown color. The prepared EECP and EEBP were stored under a dry condition at 4°C.

2.3. Cell Culture. HUVECs were gifted by Atherosclerosis Research Institute of Taishan Medical University of China purchased from ATCC. HUVECs were cultured in DMEM (high glucose) supplemented with 100 U/mL of penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated FBS at 37°C under humidified 95%–5% (v/v) air and CO_2.

2.4. Exposure of HUVECs to EECP and EEBP. When the HUVEC cultures reached 80% confluence, then the cells were divided for treatment: (a) culture in 3.5% DMEM medium with ethanol at <0.1% (v/v) (control); (b) culture in basal DMEM medium with 45 μg/mL ox-LDL with ethanol at <0.1% (v/v); and (c) culture in basal DMEM medium with 45 μg/mL ox-LDL with EECP and EEBP (12.5 μg/mL), respectively. EECP and EEBP were dissolved in ethanol, with final concentration of ethanol in the culture medium <0.1% (v/v). Ethanol at 0.1% (v/v) did not affect cell viability.

2.5. Cell Viability Assay. The viability of HUVECs was determined by MTT assay. HUVECs were seeded in 96-well cell culture plates and grown to 80% confluence and then treated with ox-LDL or EECP and EEBP for 12 and 24 h, respectively. MTT solution was added to each well and incubated for 4 h. The MTT-formazan product dissolved in DMSO to estimate by measuring absorbance at 570 nm in an ELISA plate reader. The viability (%) was expressed as (OD of treated group/OD of ox-LDL group) × 100%. The viability of the ox-LDL group was set at 100%.

2.6. Nuclear Fragmentation Assay. The morphological changes of nuclei were detected by acridine orange staining. At 24 h, cells were stained with 5 μg/mL acridine orange at room temperature for 5 min and observed under a laser scanning confocal microscopy (Olympus FV1200, Japan).

2.7. PC-PLC Activity Assay. PC-PLC activity assay was performed as the described methods in [19, 20]. Briefly, we used L-α-phosphatidylcholine as the substrate of PC-PLC. The optical density was measured at 660 nm. Enzyme activity was expressed as nanomoles per minute per milligram protein.

2.8. Immunofluorescence Microscopy. After treatment, cells were fixed with 4% paraformaldehyde and blocked with 5% normal donkey serum for 20 min at room temperature. Cells were incubated with ANXA7 and NF-κB p65 primary antibodies (1/100) at 4°C overnight. After three rinses in 0.1 M phosphate-buffered saline, cells were treated with a corresponding FITC-conjugated secondary antibody (1/200) in a humid chamber at 37°C for 1 h. Cells were rinsed three times with 0.1 M phosphate-buffered saline to eliminate the uncombined secondary antibody. A laser scanning confocal microscope (Olympus FV1200, Japan) was used for fluorescence detection. Analysis used the Image-Pro Plus software (USA). Images are representative of three independent experiments.

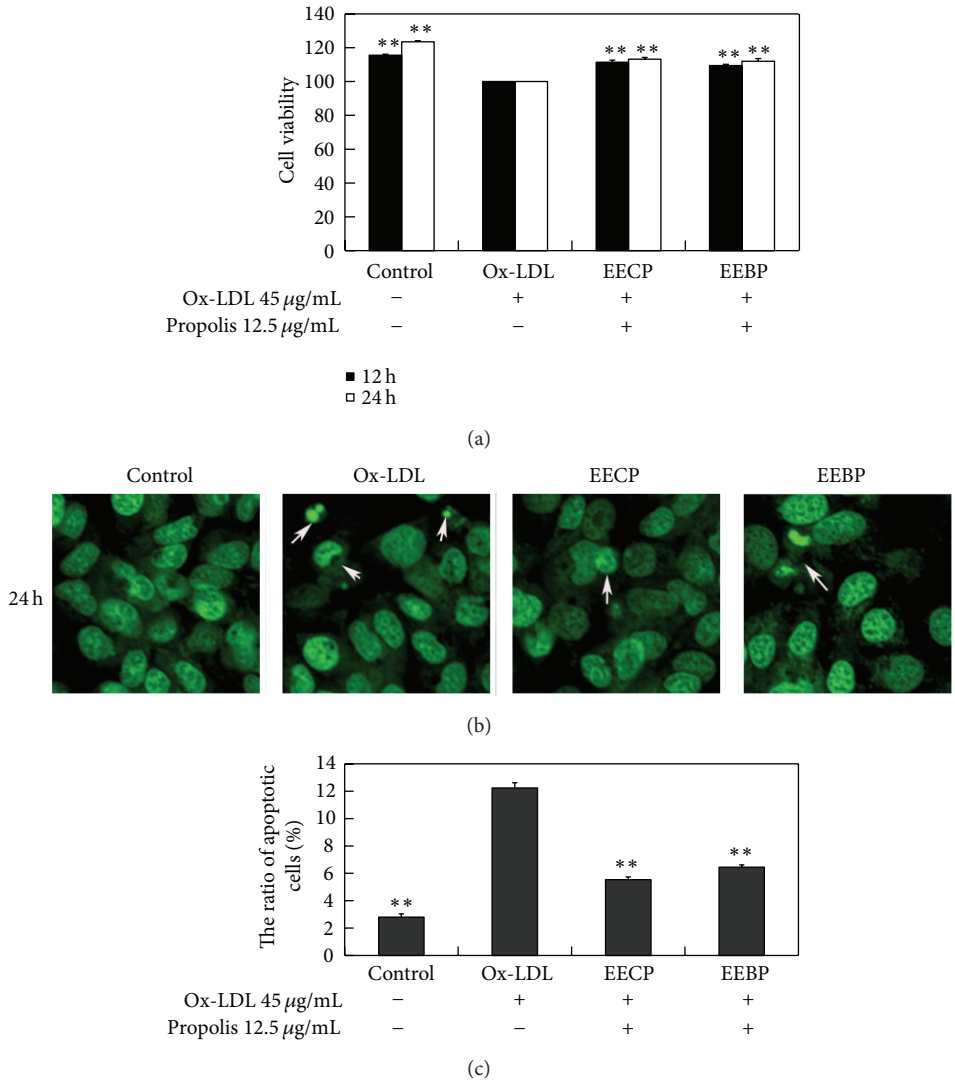

Figure 1: EECP and EEBP increased cell viability and inhibited apoptosis rate in ox-LDL-stimulated HUVECs. (a) Effect of EECP and EEBP on cell viability induced by ox-LDL. EECP and EEBP (12.5 μg/mL) and ox-LDL (45 μg/mL for HUVECs) were used. (b) Effects of EECP and EEBP on nuclear fragment were detected by acridine orange staining. (c) The ratio of apoptotic cells induced by ox-LDL ($^{**}P < 0.01$ versus ox-LDL group, $n = 3$). Data are means ± SEM.

2.9. Western Blot Analysis. Western blot assay of ANXA7 level was performed as previously described [21]. Thirty micrograms of protein was separated by 12% SDS-PAGE and transferred onto PVDF membrane. The relative quantities of the proteins were evaluated by the use of Quantity One software.

2.10. Measurement of ROS Production. ROS production in HUVECs was determined by the use of a fluorescent probe, DCFH, which can be oxidized into fluorescent dichlorofluorescin (DCF) by intracellular ROS [22]. After treating cells for 24 h, cells were incubated with DCFH for 30 min at 37°C. Then cells were washed with basal DMEM medium 3 times and observed on laser scanning confocal microscopy (Olympus FV1200, Japan). The level of ROS was quantified by Image-Pro Plus software (USA). Results were shown as relative fluorescence intensity of three independent experiments.

2.11. Measurement of Mitochondrial Membrane Potential. The fluorescent dye JC-1 was used to measure mitochondrial membrane potential. JC-1 exists as a monomer at low mitochondrial membrane potential and emits green fluorescence but forms aggregates and emits red fluorescence at high mitochondrial membrane potential [23]. After treating cells for 24 h, cells were incubated with JC-1 for 15 min at 37°C. Then cells were washed with basal DMEM medium 3 times and observed on laser scanning confocal microscopy (Olympus FV1200, Japan). The mitochondrial membrane potential was quantified by the use of the Image-Pro Plus software (USA). Results were shown as ratio of red to green fluorescence of three independent experiments.

2.12. Statistical Analysis. All experiments were performed in duplicate and repeated at least 3 times. Data are expressed as means ± SEM. Statistical analyses were performed using

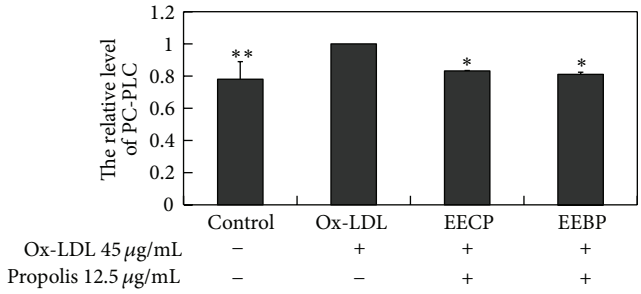

Figure 2: EECP and EEBP decreased PC-PLC activity in ox-LDL-stimulated HUVECs. Cells were treated with EECP and EEBP 12.5 μg/mL for 24 h, respectively ($^{*}P < 0.05$, $^{**}P < 0.01$ versus ox-LDL group, $n = 3$).

independent t-tests and analysis of variance (ANOVA), followed by the Tukey *post hoc test*. A $P < 0.05$ was considered significant.

3. Results

3.1. Effect of EECP and EEBP on HUVEC Viability. Ox-LDL is a major cause of endothelial dysfunction. MTT assay revealed that ox-LDL significantly inhibited cell viability, and after treatment with EECP and EEBP 12.5 μg/mL for 12 and 24 h, the cell viability obviously increased compared with ox-LDL group, respectively ($^{**}P < 0.01$; Figure 1(a)).

3.2. Effect of EECP and EEBP on HUVEC Apoptosis. We further examined the effects of EECP and EEBP on HUVEC apoptosis induced by ox-LDL. The results of AO staining showed that there were evidently condensation and fragmentation of chromosomes in ox-LDL group (Figure 1(b)), and cell apoptosis was significantly decreased by EECP and EEBP at 24 h ($^{**}P < 0.01$; Figure 1(c)).

3.3. Effect of EECP and EEBP on PC-PLC Activity. The activity of PC-PLC in ox-LDL treated HUVECs was significantly increased, whereas EECP and EEBP evidently depressed PC-PLC activity at 24 h ($^{*}P < 0.05$, $^{**}P < 0.01$; Figure 2).

3.4. Effect of EECP and EEBP on ANXA7 Level. ANXA7 was the endogenous regulator of PC-PLC. To further investigate the relationship between PC-PLC and ANXA7, we investigated the effect of EECP and EEBP on ANXA7 expression and distribution in cells treated with ox-LDL. Western blotting results showed that EEBP obviously increased ANXA7 level at 12 h, and both EECP and EEBP significantly increased ANXA7 level at 24 h ($^{**}P < 0.01$; Figure 3). And immunofluorescence assay results showed that cells treated with EECP and EEBP exhibited higher fluorescence intensity of ANXA7 per cell in a noticeable punctate pattern compared with ox-LDL group (Figure 3(a)).

3.5. Effect of EECP and EEBP on NF-κB p65 Level. Ox-LDL induced NF-κB activation in HUVECs. Both EECP and EEBP 12.5 μg/mL significantly decreased NF-κB p65 level by immunofluorescence assay, and both types of propolis inhibited translocation of NF-κB p65 from cytoplasm to nucleus ($^{**}P < 0.01$; Figure 4).

3.6. Effect of EECP and EEBP on ROS Level. Both EECP and EEBP 12.5 μg/mL significantly decreased ROS generation in HUVECs at 24 h as compared with the ox-LDL group ($^{*}P < 0.05$; Figure 5).

3.7. Effect of EECP and EEBP on Mitochondrial Membrane Potential. Ox-LDL damages mitochondria membrane potential. Both EECP and EEBP 12.5 μg/mL significantly increased mitochondrial membrane potential compared with ox-LDL group. ($^{**}P < 0.01$; Figure 6).

4. Discussion

Atherosclerosis is considered to be a chronic inflammatory disease. Ox-LDL is believed to be a key step in endothelial cell injury and in the process of initiation and progression of atherosclerotic disease [24, 25]. According to the documents on ox-LDL roles in HUVEC apoptosis [26], in current study, 45 μg/mL of ox-LDL was used. There are more than 300 active components in propolis. Because of different plant source, the chemical constituents of Chinese propolis and Brazilian green propolis are different. Our previous researches suggested that both Chinese propolis and Brazilian green propolis 12.5 μg/mL averted apoptosis and protected HUVECs with nutrient deprivation [17, 18]. Munari et al. (2010) also suggested that 12.5 μg/mL *Baccharis dracunculifolia* extract, the major plant resource of Brazilian green propolis, was the most effective in antigenotoxic chemoprevention [27]. Therefore, we have chosen 12.5 μg/mL Chinese propolis and Brazilian green propolis used in current study and compared their biological activity in ox-LDL-stimulated HUVECs.

Accumulating evidence indicates that PC-PLC plays an important role in progression of atherosclerosis and PC-PLC is an attractive target for antiatherosclerosis therapy [14]. A recent study showed that ANXA7, having different roles in autophagy, tumor suppression, and exocytosis [28–30], was negative regulation of PC-PLC in HUVECs and suggested that ANXA7/PC-PLC signaling pathway may present a novel target to treat atherosclerosis [17]. In present study, the data indicated that both Chinese propolis and Brazilian green propolis reduced PC-PLC activity and increased ANXA7 level in ox-LDL-stimulated endothelial cells, which suggested that ANXA7/PC-PLC might be the targets of both types of propolis in modulating dyslipidemia.

ROS play a critical role in vascular pathology as well as in the maintenance of normal physiological vascular function. Overproduction of ROS will lead to oxidative stress, which cause the endothelial dysfunction and promote the development of many cardiovascular diseases such as atherosclerosis by activating downstream signal molecules such as NF-κB [31]. Our previous study also showed that elevating ROS levels triggered apoptosis in HUVECs with nutrition deprivation [18]. Ox-LDL is a potent inducer of ROS, and this was con-

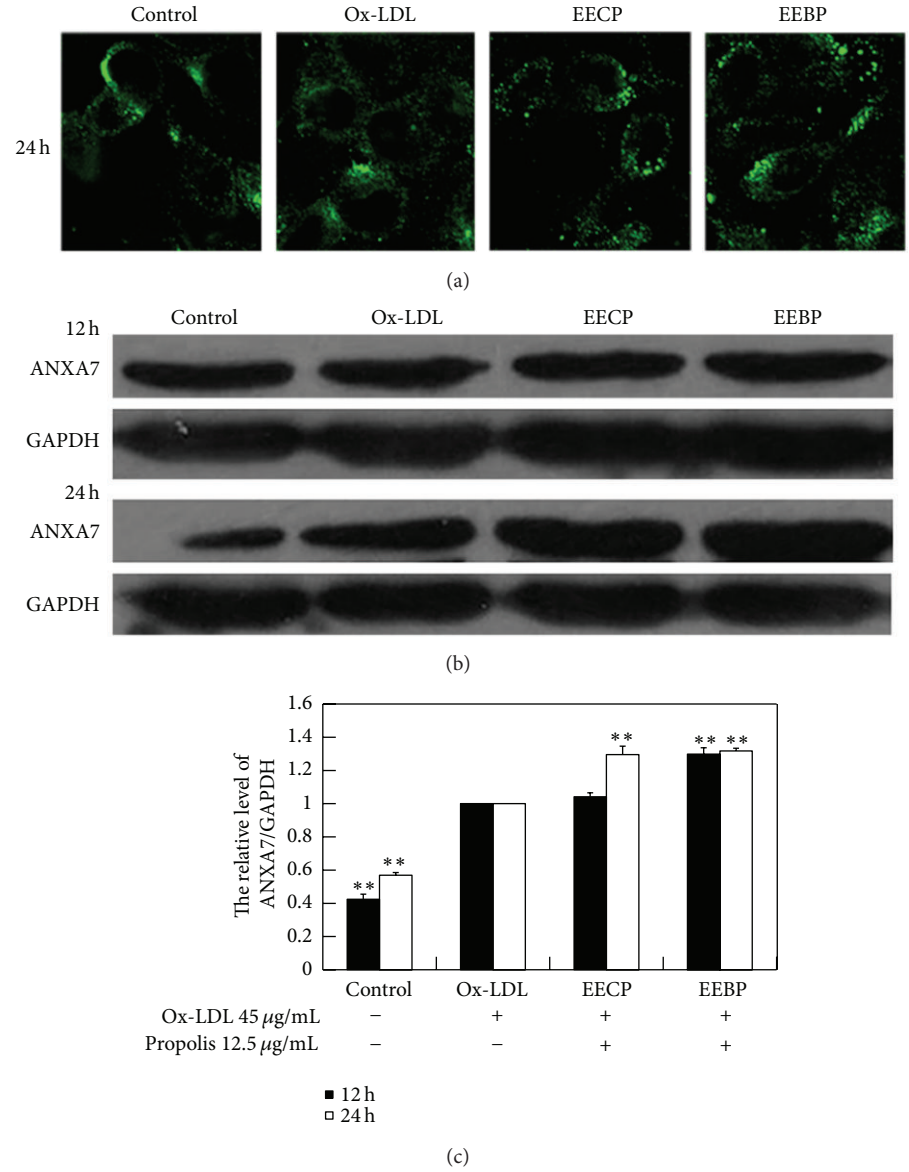

Figure 3: EECP and EEBP increased ANXA7 level in ox-LDL-stimulated HUVECs. (a) Fluorescent micrographs obtained at 24 h. (b) ANXA7 levels were detected by western blot analysis at 12 and 24 h. (c) The hemiquantification of ANXA7 level in HUVECs ($^{**}P < 0.01$ versus ox-LDL group, $n = 3$).

firmed in the present study; ROS level was increased in HUVECs treated with ox-LDL, whereas both types of propolis could depress ROS level, which lend support to the theory that ROS scavenging could reduce the risk of cardiovascular diseases [32]. Furthermore, transcription factor NF-κB is activated by high level of ROS [33]. In present study, ox-LDL induced ROS increase and subsequent activation of NF-κB p65 were all attenuated by EECP and EEBP. NF-κB signaling pathway is involved in multiple cell processes including apoptosis, proliferation, and gene expression. Moreover, recent studies have suggested that several natural products including propolis suppress inflammatory responses by regulating the NF-κB pathway [34, 35]. Atherosclerosis is a kind of chronic inflammatory disease. These findings concur with our finding that the transcriptional inhibition of proinflammatory mediators by propolis is associated with the blockade of NF-κB signaling pathway.

Mitochondria are the most important intracellular source of ROS, and elevated ROS levels can also decrease mitochondrial membrane potential [36]. Ox-LDL damages mitochondrial membrane potential, leading the cytochrome c release to induce apoptosis in HUVECs [37]. We previously reported that high concentration of propolis damaged mitochondrial membrane potential in endothelial cells with nutrition deprivation. Here we found that both types of propolis 12.5 μg/mL significantly protected mitochondrial membrane potential. Together with these results, we confirmed the protective effect of propolis on HUVECs induced by ox-LDL, and it may be

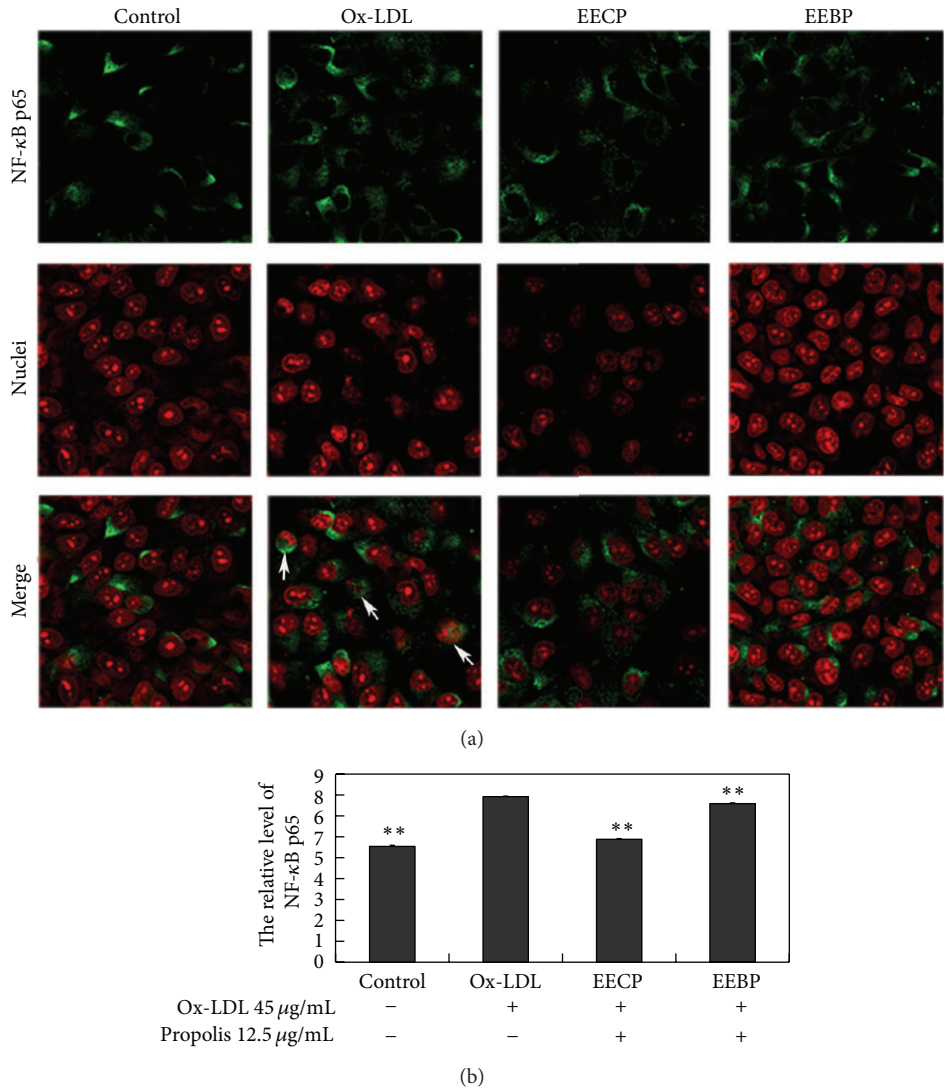

Figure 4: EECP and EEBP decreased NF-κB p65 level and inhibited translocation of NF-κB p65 from cytoplasm to nucleus in ox-LDL-stimulated HUVECs. (a) Fluorescent micrographs obtained at 24 h. Nuclei were counterstained with PI. (b) The relative level of NF-κB p65 in HUVECs ($^{**}P < 0.01$ versus ox-LDL group, $n = 3$).

the major mechanisms of propolis modulating atherosclerosis.

Many studies indicate that propolis from different areas has similar biological activity although the chemical constituents are different. Hu et al. (2011) reported that Chinese propolis and Brazilian green propolis had similar biological activities on streptozotocin-induced type 1 diabetes mellitus in rats [38]. And we also previously found that Chinese propolis and Brazilian green propolis had similar protective effects on hepatocytes injury induced by hydrogen peroxide [39]. In current study, we confirmed that both Chinese propolis and Brazilian green propolis had similar activity on ANXA7 expression and PC-PLC activity and other signal molecules in HUVECs induced by ox-LDL. We proposed that it was not a simple chemical constituent in propolis playing crucial role modulating dyslipidemia diseases; it might be a synergy effect of various chemical constituents of propolis. However, in other cells, such as breast cancer MCF-7 and MDA-MB-231 cells, we found that the cytotoxicity of Chinese propolis and Brazilian green propolis was different. So the activities of propolis on different diseases and cells should be further studied.

Altogether, the present findings indicated that both types of propolis 12.5 μg/mL significantly increased cell viability and attenuated apoptosis rate, increased the expression of ANXA7, and decreased PC-PLC activity. Both kinds of propolis also inhibited ROS generation as well as the subsequent MMP collapse and NF-κB p65 activation induced by ox-LDL in HUVECs, which may be the major mechanisms of propolis protecting endothelial injury and preventing atherosclerosis. Our results also indicated that Chinese propolis and Brazilian green propolis had similar biological activities and prevented ox-LDL induced cellular dysfunction in HUVECs. Both types of propolis may be potent alternative

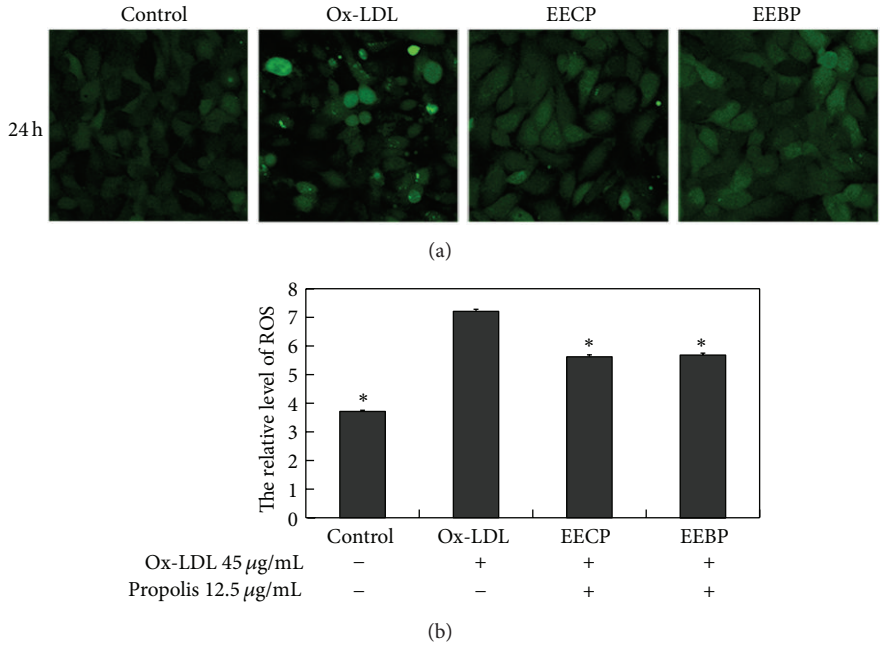

Figure 5: EECP and EEBP decreased ROS level in ox-LDL-stimulated HUVECs. (a) Fluorescent micrographs obtained at 24 h. (b) The relative quantity of ROS level in HUVECs ($^{*}P < 0.05$ versus ox-LDL group, $n = 3$).

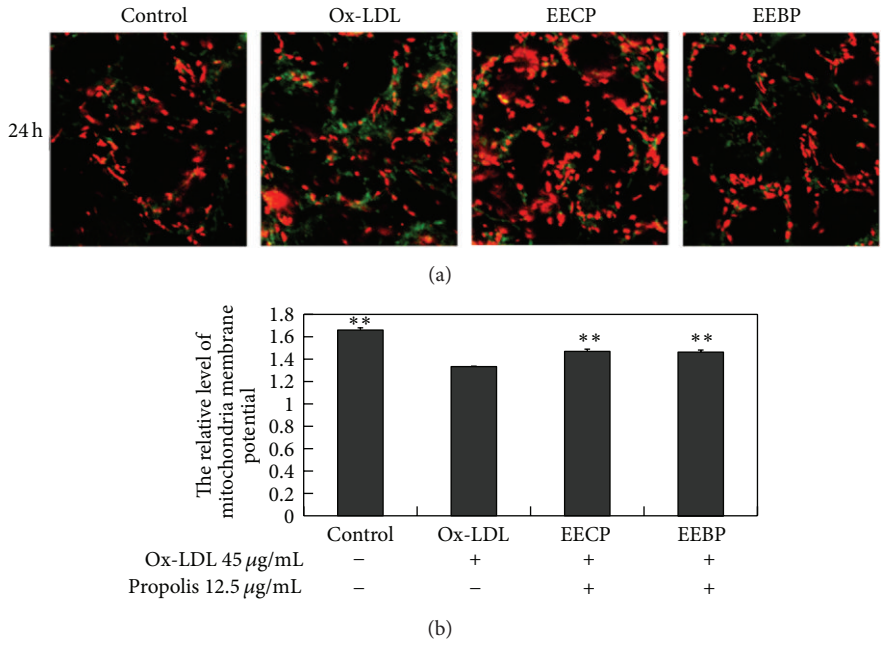

Figure 6: EECP and EEBP increased mitochondria membrane potential in ox-LDL-stimulated HUVECs. (a) Fluorescent micrographs obtained at 24 h. (b) The relative quantity of mitochondrial membrane potential in HUVECs ($^{**}P < 0.01$ versus ox-LDL group, $n = 3$).

drugs for the prevention of atherosclerosis. However, the mechanism of propolis regulating dyslipidemia should be further studied.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Effects of Chaihu-Shugan-San and Shen-Ling-Bai-Zhu-San on p38 MAPK Pathway in Kupffer Cells of Nonalcoholic Steatohepatitis

Qin-He Yang,[1] Yong-Jian Xu,[1] Yi-Zhen Liu,[1] Yin-Ji Liang,[1] Gao-Fei Feng,[1] Yu-Pei Zhang,[1] Hui-Jie Xing,[2] Hai-Zhen Yan,[1] and Yuan-Yuan Li[1]

[1] *Medical School of Jinan University, 601 Huangpu Road West, Guangzhou, Guangdong 510632, China*
[2] *Experimental Animal Management Center of Jinan University, 601 Huangpu Road West, Guangzhou, Guangdong 510632, China*

Correspondence should be addressed to Qin-He Yang; tyangqh@jnu.edu.cn

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This study aimed to investigate the effects of Chaihu-Shugan-San (CSS), Shen-Ling-Bai-Zhu-San (SLBZS), and integrated recipe of the above two recipes on inflammatory markers and proteins involved in p38 MAPK pathway in Kupffer cells of NASH rats induced by high fat diet (HFD). Rats were administered at low or high dose of CSS, SLBZS, and integrated recipe except normal group and model group for 16 weeks. The levels of hepatic lipid, TNF-α, IL-1, and IL-6 in liver tissues were measured. Kupffer cells were isolated from livers to evaluate expressions of TLR4, p-p38 MAPK, and p38 MAPK by Western blotting. The results showed that the NASH model rats successfully reproduced typical pathogenetic and histopathological features. Levels of hepatic lipid and liver tissues inflammatory factors in high-dose SLBZS group and integrated recipe group were all lower than that of model group decreased observably. Expressions of TLR4, p-p38 MAPK, and p38 MAPK in Kupffer cells were decreased in all treatment groups, but there was no significant difference between treatment groups. The high-dose SLBZS group had the lowest expression levels of TLR4, and the most visible downtrend in the expression levels of p-p38 MAPK and p38 MAPK was found in the high-dose integrated recipe group. The ratio of p-p38 MAPK to total p38 MAPK protein was obviously increased in all treatment groups. Therefore, our study showed that the activation of p38 MAPK pathway in Kupffer cells might be related to the release of inflammatory factors such as TNF-α, IL-1, and IL-6 in NASH rats. High dose of SLBZS and integrated recipe might work as a significant anti-inflammatory effect in Kupffer cells of NASH rats induced by HFD through suppression of p38 MAPK pathway. It indicated that p38 MAPK pathway may be the possible effective target for the recipes.

1. Introduction

Traditional Chinese medicine (TCM) has been clinically used in China for thousands of years for the treatment of many diseases. Chaihu-Shugan-San (CSS), an ancient classical formula from "Jingyue Quanshu", is composed of seven Chinese herbs: *Bupleurum Chinese DC*, *Pericarpium Citri Reticulatae*, *Ligusticum chuanxiong Hort*, *Rhizoma Cyperi*, *Fructus Aurantii*, *Radix Paeonia Alba*, and *Glycyrrhiza uralensis Fisch* with a traditional dose ratio of 6 : 6 : 5 : 5 : 5 : 5 : 3. Shen-Ling-Bai-Zhu-San (SLBZS) is also a famous classical formula recorded in "Taiping Huimin Heji Ju Fang" which consists of ten Chinese herbs: *Panax Ginseng, Atractylodes Ovata, Poria Cocos, Dioscorea Batatas*, *Dolichos lablab*, *Coix lacryma-jobi*, *Nelumbo nucifera*, *Glycyrrhiza uralensis Fisch*, *Platycodon grandiflorum*, and *Amomum xanthioides* in a ratio of 5 : 5 : 5 : 5 : 4 : 3 : 3 : 3 : 2 : 2.

CSS and SLBZS are traditionally used to treat some chronic diseases such as fatty liver disease (FLD) or gastroenteropathy. Many studies have demonstrated that CSS protects against lipid peroxidation [1, 2], liver fibrosis [3, 4], and insulin resistance [5]. And SLBZS has inhibitory activities on oxidative stress [6], lipid peroxidation [7], and inflammatory reaction [6, 8].

Some of the major compounds from CSS and SLBZS, like saikosaponins [9–11], total glucosides of peony [12, 13],

ginsenoside [14, 15], atractylenolide [16], atractylodes macrocephalaon polysaccharide [17], and Carboxymethylpachymaran [18], which also have been identified their potential protection on liver. Based on the theory of TCM, CSS dredges liver qi and dispels the stagnation and is prescribed mainly for the liver qi stasis. SLBZS has the functions of tonifying spleen and stomach qi and is mainly used for deficiency of spleen and stomach.

Nonalcoholic steatohepatitis (NASH) is an important stage from simple steatosis development to fibrosis, and cirrhosis in nonalcoholic fatty liver disease (NAFLD), characterized by hepatocellular ballooning degeneration and necroinflammation based on hepatic steatosis [19–21]. Kupffer cells (KCs), which are resident macrophages of the liver, account for 80%–90% of the total innate macrophages [22]. KCs are an important source of both inflammatory and anti-inflammatory mediators [23]. Researches have showed that amounts of inflammatory cytokines and biologically toxic mediators from activated KCs have been strongly implicated in the pathogenesis of hepatic injury, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), interleukin-13 (IL-13), and tumor necrosis factor-alpha (TNF-α) [23–25]. Modern researches have also indicated that p38 mitogen-activated protein kinase (p38 MAPK) is closely related to inflammatory cellular signal transduction and gene regulation during the course of NASH [26].

In accordance with our previous study supported by Natural Science Foundation of China (number 30371726), we observed that CSS and SLBZS were significantly effective for the treatment of FLD, respectively [27]. And we found that the high expression levels of phosphor-p38 MAPK (p-p38 MAPK) and p38 MAPK in KCs isolated from 12 weeks high fat diet (HFD)-induced NAFLD rats, which preliminarily revealed the relationship between NAFLD and p38 MAPK pathway [28]. So, how is the NASH rats HFD induced for 16 weeks? In this paper, we studied the effects of soothing liver and invigorating spleen recipe on inflammatory markers and proteins involved in p38 MAPK pathway in KCs of NASH rats induced by HFD in order to explore part of the underlying mechanisms.

2. Materials and Methods

2.1. Preparation of CSS and SLBZS. CSS is composed of seven Chinese herbs: *Bupleurum Chinese DC*, *Pericarpium Citri Reticulatae*, *Ligusticum chuanxiong Hort*, *Rhizoma Cyperi*, *Fructus Aurantii*, *Radix Paeonia Alba*, and *Glycyrrhiza uralensis Fisch* with a traditional dose ratio of 6 : 6 : 5 : 5 : 5 : 5 : 3. Invigorating spleen recipe includes *Panax Ginseng*, *Atractylodes Ovata*, *Poria Cocos*, *Dioscorea Batatas*, *Dolichos lablab*, *Coix lacryma-jobi*, *Nelumbo nucifera*, *Glycyrrhiza uralensis Fisch*, *Platycodon grandiflorum*, and *Amomum xanthioides* in a ratio of 5 : 5 : 5 : 5 : 4 : 3 : 3 : 3 : 2 : 2. Integrated recipe contains is the mixture of CSGS and SLBZS at a ratio of 1 : 1. All Chinese medicines were formula granules purchased from Shenzhen Sanjiu Medical Co., Ltd. (1005001S). The formula granules were put in the solvent of distilled water and preserved at −4°C refrigerator.

2.2. Animals, Grouping, and Modeling. 120 Specific Pathogen-Free Male Sprague-Dawley rats (6 weeks old, 200 g ± 20 g) were obtained from the Laboratory Animal Research Center of Guangzhou University of Traditional Chinese Medicine (Approval number SCXK (Yue) 2008-0020), Guangdong province, China. The rats were housed under conditions of controlled temperature (24°C ± 2°C) and humidity (70% ± 10%) in 12 h of light and 12 h of dark cycle (lights on from 8:00 am to 8:00 pm), with free access to diet and water. After one week of adaptive breeding, the rats were randomly divided into 8 groups, 15 rats in each group: normal group, model group, low-dose CSS group (L-CG), high-dose CSS group (H-CG), low-dose SLBZS group (L-SG), high-dose SLBZS group (H-SG), low-dose integrated recipe group (L-IG), and high-dose integrated recipe group (H-IG). Rat models of NASH were duplicated according to method as we previously reported [29] with some minor modifications. Normal group of rats got free access to a normal chow diet, model group of rats were fed with HFD (composed of regular chow 88%, axungia porci 10%, cholesterol 1.5%, and bile salt 0.5%). All rats in treatment groups were fed with decoction (1 mL/100 g body weight by gastrogavage [30]), while the rats in the normal group and model group were fed with the same dose of distilled water once at 8:00 am every day. Low-dose equaled human clinical equivalent dosage, and high-dose was 3-fold volume of low-dose. The treatment lasted for 16 weeks.

At the end of treatment, rats in each group were divided into two groups by table of random number: 9 rats for liver samples collection, 6 rats for isolation of KCs. All rats were treated in compliance with the Guiding Principles for Animal Experiments and the protocols were approved by the Animal Experimental Ethnics Committee of Jinan University, China.

2.3. Biochemical Test in Liver. After rats were anesthetized by intraperitoneal injection of 3% pentobarbital (0.2 mL/100 g body weight), livers were taken out quickly. Liver tissues were put into isopropanol. Homogenates were manufactured using a TissueLyser-II homogenizer (QIAGEN, Germany), centrifuged at 3000 ×g, 4°C for 10 min, and then clear supernatants were collected. Total cholesterol (TC) and triglyceride (TG) in the liver tissue were determined with automatic biochemical analyzer (Olympus, Japan).

2.4. Histopathological Examination of Liver. The paraffin-embedded liver tissue (about 1 cm × 0.5 cm × 0.5 cm) which selected the same part of the liver, about 0.5 cm from the edge of the right hepatic lobule, was sliced at a thickness of 4 μm and examined by hematoxylin-eosin (HE) staining. The steatosis grade, fibrosis stage, and inflammation of NASH were evaluated according to the NASH histological scoring system [31].

2.5. Determination of Inflammatory Cytokines in Liver Tissue. Liver homogenates were centrifuged at 3000 ×g, 4°C for 10 min. Clear supernatants were used to determine the cytokines. The contents of TNF-α, IL-1, and IL-6 were tested following the recommended procedures provided by the enzyme-linked immunosorbent assay (ELISA) kits.

2.6. Separation and Identification of KCs. KCs were isolated and identified from 6 rats in each group as we previously described [32], and some modifications were made. After rats were anesthetized, the liver was perfused in situ with 200 mL 0.5 mmol/L Ethylene Glycol Tetraacetic Acid (EGTA) in D-Hanks at 20 mL/min, 37°C until the colour of liver changed into amber. Then the liver was transferred to a culture dish and was perfused ex situ with 0.03% collagenase IV in Hanks, which contains 5 mmol/L calcium ion and should be preheated to 37°C, at 20 mL/min in a recirculating fashion for 15 min. The liver was then placed into 10 mL RPMI-1640 culture medium containing 10% fetal calf serum (FBS), capsule and fibrous tissue were removed, and the remaining tissue was cut into small pieces. After the obtained liver homogenate was filtered through 200 μm and 300 μm nylon mesh, the cell suspension was centrifuged at 50 ×g, 4°C for 3 min and clear supernatant was collected in another tube and centrifuged at 400 ×g, 4°C for 10 min. The cell pellet was subsequently resuspended in RPMI-1640 containing 10% FBS.

Then some 15 mL centrifuge tubes were carefully laid into 2.5 mL 24% Nycodez working solution in the bottom, 2.5 mL 11% Nycodez working solution in the middle layer, and 2.5 mL the cell suspension in the top. Then it was centrifuged at 800 ×g, 4°C for 15 min. KCs which have a clouding appearance between 11% Nycodez layer and 24% Nycodez layer were collected to another 15 mL tube and resuspended in GBSS, and then centrifuged at 400 ×g, 4°C for 15 min twice. The cell pellet was then resuspended and seeded on culture dish at a density of $2–5 \times 10^6$ cells/mL with RPMI-1640 containing 10% FBS and incubated in a 5% CO_2 atmosphere for 30 min at 37°C. By further using adhesion purification, KCs purity was improved, and cell viability was tested by trypan blue dye exclusion.

2.7. Protein Extraction and Western Blot. Western blotting was used to determine proteins of KCs toll like receptor 4 (TLR4), p-p38 MAPK, p38 MAPK, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was used as an internal control. KCs were split in RIPA lysis buffer and centrifuged at 8000 ×g for 5 min at 4°C and the supernatants were collected. The supernatant protein concentration was determined by BCA protein assay. Sixty micrograms of protein was resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proceeded with transmembrane. The polyvinylidene difluoride (PVDF) membrane was blocked with 5% skim milk in Tris-Buffered Saline Tween-20 (TBST), shaken for 1 h at room temperature, and then incubated overnight at 4°C with specific primary antibodies. Then horseradish peroxidase (HRP) conjugated goat-anti-rabbit antibody were added and incubated at room temperature for 1 h. After being washed three times in TBST, the PVDF membrane was put into developer and exposed to X-ray film. The films were scanned and analyzed by gel image processing system.

2.8. Statistical Analysis. The results were expressed as the mean ± S.E.M. unless otherwise indicated. Analysis of variance (ANOVA) was used to determine the statistical significance of the differences followed by Tukey's test. Ranked data were analyzed by Rank-Sum test. Probability value (P) less than 0.05 was considered statistically significant. All data were analyzed with the Statistical Package for the Social Sciences (SPSS, USA) 13.0 Software.

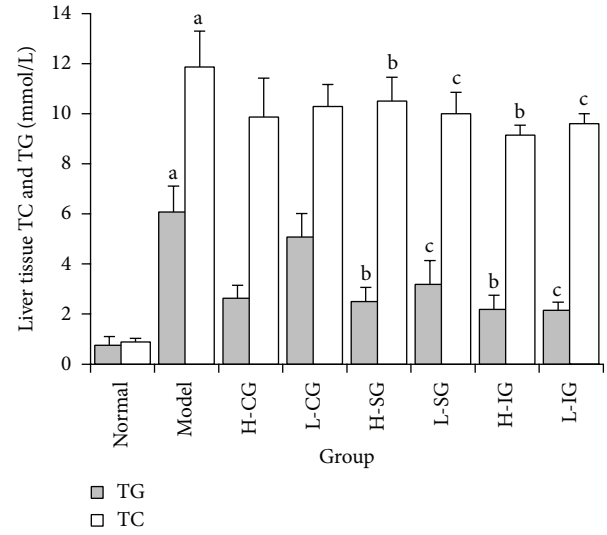

Figure 1: Levels of TC and TG in liver were determined. Rats were fed with normal chow diet or HFD with or without CSS and SLBZS for 16 weeks. The values were expressed as mean ± S.E.M. of 9 rats per group. $^aP < 0.01$ versus normal group; $^bP < 0.01$, $^cP < 0.05$ versus model group.

3. Results

3.1. Levels of TC and TG in Liver. Elevated levels of TC and TG indicated hepatic lipid accumulation and lipid metabolic disturbance in liver tissue. As shown in Figure 1, the levels of TC and TG were significantly increased in the model group compared to the normal group ($P < 0.01$). Compared with the model group, lower levels of TG and TC were shown in the H-SG, L-SG, H-IG, and L-IG ($P < 0.01$, $P < 0.05$). Results indicated the increased TG and TC induced by HFD were attenuated by high and low dose of SLBZS and integrated recipe.

3.2. Effects of CSS and SLBZS on Liver Histopathological Changes. Liver specimens with HE staining were shown in Figure 2. Sections of liver from model group showed typical NASH features, including microvesicular and macrovesicular steatosis, lobular and portal inflammation, fibrosis, and hepatocyte ballooning (Figure 2(b)). Compared with the normal group, the model group scored 12 points and had a significant difference ($P < 0.01$). The pathological changes in the treatment groups lightened to different degree as compared with the model group, particularly in H-SG, L-SG, H-IG, and L-IG ($P < 0.01$) (Figure 3). This indicated that the liver steatosis, fibrosis, and inflammation were inhibited to some extent by both high and low dose of SLBZS and integrated recipe in NASH rats.

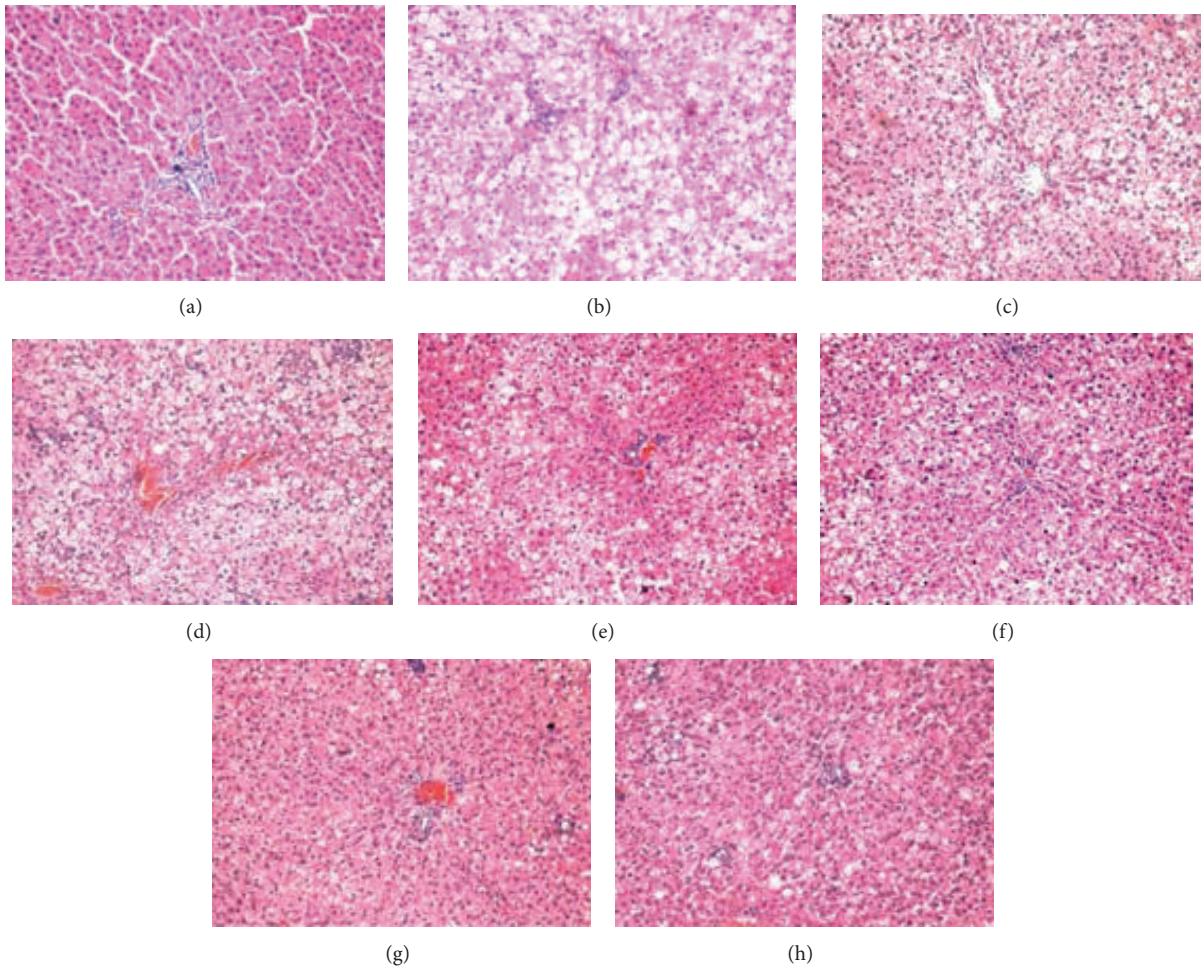

Figure 2: Histological changes of liver sections in different groups (HE stain × 100). (a): normal group; (b): model group; (c): high-dose CSS group (H-CG); (d): low-dose CSS group (L-CG); (e): high-dose SLBZS group (H-SG); (f): low-dose SLBZS group (L-SG); (g): high-dose integrated recipe group (H-IG); (h): low-dose integrated recipe group (L-IG).

3.3. Effects of CSS and SLBZS on Liver Inflammatory Cytokine Levels. Rising inflammatory cytokine levels of TNF-α, IL-1, and IL-6 are regarded as biomarkers of inflammation. As shown in Figure 4, higher levels of TNF-α, IL-1, and IL-6 were observed in the model group compared with that of the normal group ($P < 0.01$). Compared with the model group, significant decreases of TNF-α and IL-6 in the H-SG, H-IG, and L-IG ($P < 0.01$ or $P < 0.05$), and the levels of IL-1in H-SG and H-IG were clearly lower ($P < 0.01$ or $P < 0.05$). The results showed that both the high dose of SLBZS and integrated recipe reduced the TNF-α, IL-1 and IL-6 levels of liver inflammatory cytokine in NASH rats induced by HFD.

3.4. The Population, Purity, and Viability of KCs. The yields of purified cell of KCs in each rat were $1.5–2.0 \times 10^7$. The viability of KCs isolated was higher than 95%, with purity over 90.18%. The number and purity degrees of KCs complied with the requirement of the follow-up testing.

3.5. Effects of CSS and SLBZS on p38 MAPK Signal Pathway Related Proteins in KCs. To explore the mechanism of the anti-inflammatory effect of soothing liver and SLBZS in KCs of NASH rats, we assayed three important proteins of TLR4, p-p38 MAPK, and p38 MAPK involved in p38 MAPK signal pathway which is one important mediator in inflammatory response. Figures 5(a) and 5(b) showed that protein expression levels of TLR4, p-p38 MAPK, and p38 MAPK in the model group were significantly higher than those in the normal control group ($P < 0.01$). Compared with the model group, the expression levels of TLR4, p-p38 MAPK, and p38 MAPK ($P < 0.01$, $P < 0.05$) were inhibited in all treatment groups, but there was no significant difference between treatment groups. The group of H-ISG had the lowest expression levels of TLR4, and the most visible downtrend in the expression levels of p-p38 MAPK and p38 MAPK was found in the group of H-IG. Compared with the model group, Figure 6 showed that the ratio of p-p38 MAPK to total p38 MAPK protein increased obviously ($P < 0.01$), but H-CG and L-CG did not ($P > 0.05$). The result indicated that p38 MAPK signal pathway may be activated in KCs of NASH rats. The high dose of SLBZS and integrated recipe inhibited activation of p38 MAPK signal pathway in different degrees.

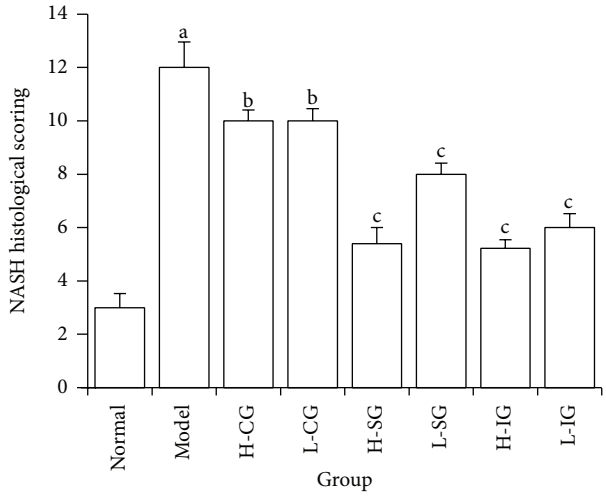

Figure 3: NASH histological scoring in different groups. Rats were fed with normal chow diet or HFD with or without CSS and SLBZSs for 16 weeks. The values were expressed as mean ± S.E.M. of 9 rats per group. $^{a}P < 0.01$ versus normal group; $^{b}P > 0.05$, $^{c}P < 0.01$ versus model group.

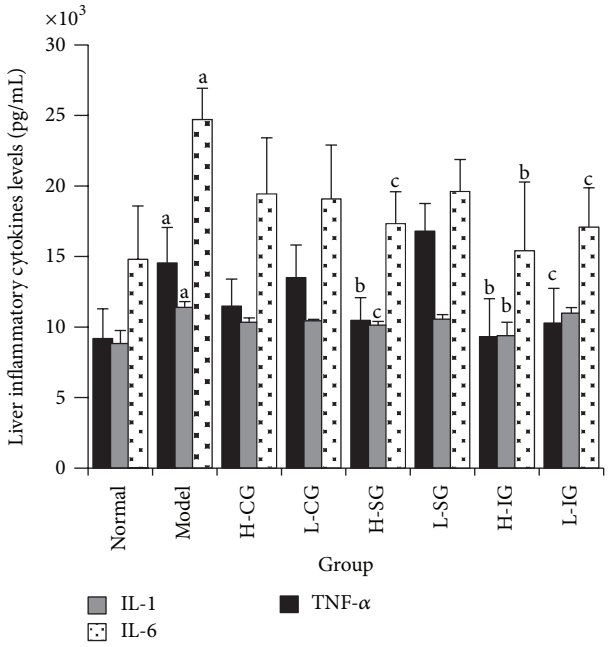

Figure 4: Related inflammatory cytokines of TNF-α, IL-1, and IL-6 in liver tissues were determined by ELISA. Rats were fed with normal chow diet or HFD with or without CSS and SLBZSs for 16 weeks. The values were expressed as mean ± S.E.M. of 9 rats per group. $^{a}P < 0.01$ versus normal group; $^{b}P < 0.01$, $^{c}P < 0.05$ versus model group.

4. Discussion

NASH is a common chronic liver disease, and it has been one of the important factors in leading to hepatocirrhosis and liver cancer [33, 34]. It turns out that the excessive inflammatory cytokines such as TNF-α [35–38], IL-1 [36, 39], and/or IL-6 [36, 40–42] exacerbated cell lipid peroxidation and liver injury and promoted NASH progression in different ways.

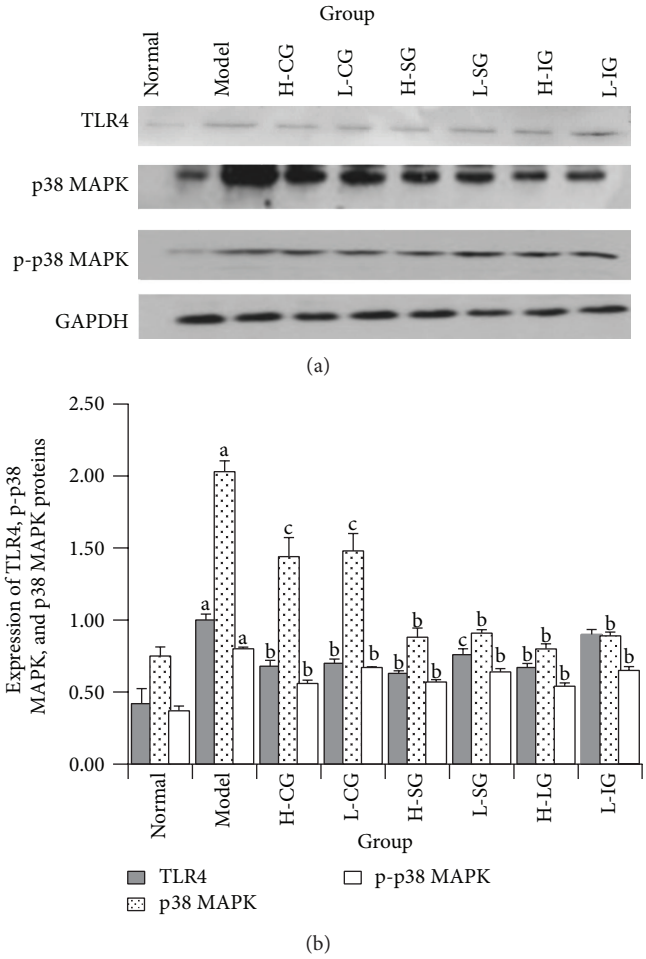

Figure 5: Western blot analysis of proteins involved TLR4, p-p38 MAPK, and p38 MAPK in Kupffer cells (a). Expression of TLR4, p-p38 MAPK, and p38 MAPK proteins in Kupffer cells (b). Rats were fed with normal chow diet or HFD with or without CSS and SLBZSs for 16 weeks. KCs were isolated and identified from 6 rats in each group. Values represent the mean ± S.E.M. $^{a}P < 0.01$ versus normal group; $^{b}P < 0.01$, $^{c}P < 0.05$ versus model group.

On present understanding, MAPKs are a highly conserved family of serine/threonine kinases including known ERK 1/2, JNK/SAPK, p38 MAPK, and ERK5/BMK1, which are all important signaling molecules in the control of cellular biological effects to extracellular stimuli. Following stimulation, the proteins of p38 MAPK signal pathway are phosphorylated and then activate several downstream factors to regulate the corresponding gene expression [43]. And the study of Wagner EF showed that p38 MAPK signal pathway played an important role in the stress responses of inflammatory reaction [44]. TLR4 is the main receptor in the lipopolysaccharide- (LPS-)mediate immune responses [45]. After TLR4 is integrated with LPS, MAPKs cascade reactions are activated by the pathway of myeloid differentiation factor

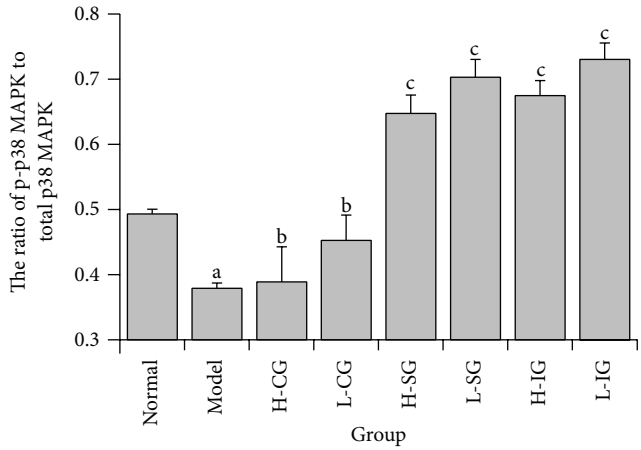

Figure 6: The ratio of p-p38 MAPK to total p38 MAPK protein in Kupffer cells. Rats were fed with normal chow diet or HFD with or without CSS and SLBZSs for 16 weeks. KCs were isolated and identified from 6 rats in each group. Values represent the mean ± S.E.M. $^{a}P < 0.05$ versus normal group; $^{b}P > 0.05$, $^{c}P < 0.01$ versus model group.

(MyD88), interleukin-1 receptor related kinase-1 (IRAK-1), tumour necrosis factor receptor correlation factor (TRAF6), and transforming factor activating kinase (TAK1). Then the p38 MAPK protein is phosphorylated, leading to release of inflammatory factor and starting cell damage mechanism [46, 47]. Moreover, the activated TLR4 pathway turned out to be playing a critical role in the inflammatory immune response of NASH [48] and it was demonstrated that nosogenesis of many inflammatory diseases was mediated with TLR4-p38 MAPK signal pathway [49, 50].

In the present research, rat model of NASH induced by HFD successfully replicated several typical histopathological characteristics of NASH in human, such as hepatocyte steatosis and ballooning and lobular and portal inflammation. And levels of TC, TG, and inflammatory factors in liver were increased in different degrees. It was consistent with the previous reporter [29]. Our preliminary studies have suggested that CSS, SLBZS, and integrated recipe have certain therapeutic effect on FLD [27, 28, 51] and NFLD [52]. In this research, the results showed that the high and low dose of SLBZS and integrated recipe protected against liver injury, moderated NASH progression, and decreased liver lipid and inflammatory factors levels.

To elucidate how CSS and SLBZS affect p38 MAPK signal pathway and the anti-inflammatory, we detected several proteins which were closely related to the signal transduction of p38 MAPK pathway in KCs of NASH rats. The results demonstrated that the activation of TLR4-p38 MAPK signal pathway in KCs was involved in the development of NASH induced by HFD. The increases of TNF-α, IL-1, and IL-6 might be due to the activation of TLR4-p38 MAPK signal pathway in KCs. Both the high dose of SLBZS and integrated recipe may inhibit the related proteins expression in TLR4-p38 MAPK signal pathway to decrease inflammatory factors such as TNF-α, IL-1, and IL-6. Moreover, it was interesting to note that the high and low dose of CSS inhibited activation of TLR4, p-p38 MAPK, and p38 MAPK in different degrees, but there was no significant difference compared with the model group on the contents of TNF-α, IL-1, and IL-6 and the ratio of p-p38 MAPK to total p38 MAPK protein. So we did not observe that the phosphorylation of p38 MAPK was suppressed by the high and low dose of CSS.

Based on the theory of TCM, CSS dredges liver qi and dispel the stagnation and is prescribed mainly for the liver qi stasis. SLBZS has the functions of tonifying spleen qi and is mainly used for deficiency of spleen and stomach. In accordance with the previous study, we suggested that the basic pathogenesis of FLD was closely correlated to liver stagnation and spleen deficiency from the point of TCM theory [27, 28, 51, 53, 54]. In this study, the effects of high-dose SLBZS and high-dose integrated recipe are better than that of high- or low-dose CSS. Thus we suggested that the pathogenesis of NASH might be closely related to Pixu in NASH rats induced by HFD for 16 weeks.

5. Conclusion

In conclusion, this study revealed that the activation of p38 MAPK pathway in Kupffer cells might be related to the release of inflammatory factors such as TNF-α, IL-1, and IL-6 in NASH rats. High dose of SLBZS and integrated recipe might work as a significant anti-inflammatory effect in Kupffer cells on NASH induced by HFD through suppression of p38 MAPK pathway. At the same time, p38 MAPK pathway may be the effective targets for the recipes. Thus, SLBZS and integrated recipe might be a potentially complementary medicine used in the treatment of NASH.

The Chinese medicinal herbs exert their pharmacological effects usually through a multicomponent and multitarget way. Further study is needed to find out whether there is some other signal transduction pathways involved in the course and to elucidate the other beneficial effect of CSS and SLBZS on NASH. Moreover, it is essential to do some evidence-based medical research on CSS and SLBZS in clinical applications.

Conflict of Interests

All of the authors of this paper declare that they have no direct financial relation with the commercial identities mentioned in this paper. And all of the authors declare that they have no competing interests.

Acknowledgment

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Research Article

The Effects of Needling Fenglong (ST40) and Neiguan (PC6) on IL-17 of ApoE-Gene-Knockout Mice's Liver

Fu Yun Lee,[1] Ze Jun Huo,[2] Li Zhang,[1] Jia Guo,[2] Huan Chen,[1] Tong Liu,[1] Bo Peng,[1] Pei Xin Hong,[1] Yuan Yuan Peng,[1] Yi Fan Fan,[1] and Yu Pei Chen[1]

[1] *School of Acupuncture and Moxibustion, Beijing University of Chinese Medicine, No. 11 North 3rd Ring Road, Chaoyang District, Beijing 100029, China*
[2] *Acupuncture and Moxibustion Department, The Third Hospital of Peking University, No. 49 North Garden Road, Haidian District, Beijing 100191, China*

Correspondence should be addressed to Li Zhang; zhangli1572@sina.com

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The aim of the present paper was to observe the effects of needling ST40 and PC6 on IL-17 of ApoE$^{-/-}$ mice with fatty liver. Forty male ApoE$^{-/-}$ mice were randomized into Needling-Acupoint Group, Simvastatin Intragastric Administration Group, Needling Nonacupoint Group, and Model Group. Each was fed with high fat diet for 8 weeks since 16 weeks of age; after 8 weeks of intervention, mice were sacrificed and tested for various examinations. Result showed that the body weight, TC, and serum IL-17 in Needling-Acupoint Group decreased. Compared with Model Group, the immunohistochemical expressions of IL-17 in liver tissue were significantly decreased among the other three groups. In conclusion, acupuncture was able to lower the expression of IL-17 level both in serum and liver tissue in ApoE$^{-/-}$ mice, which is helpful to reduce the inflammation and defers the progress from fatty liver to cirrhosis.

1. Introduction

As people's living standards improved, the intake of protein, fat, and alcohol has largely increased, which might lead to metabolic disorders and hyperlipidemia and trigger fatty liver disease. It is estimated that there might be over 160 million adults suffering from dyslipidemia in China. However, long-term use of lipid lowering drugs may result in side effects such as hepatic and renal dysfunction, which endanger human health [1]. The research [2–4] indicated that acupuncture was able to reduce blood lipids which, with fewer side effects, were safer.

The inflammatory cytokines inside and outside the liver play a critical role in chronic liver diseases, including fatty liver. The significant increase of IL-17 (interleukin, IL-17) can be detected in hyperlipidemic fatty liver and also cirrhosis patients [5]. IL-17 is able to promote the release of kinds of cytokines which are involved in inflammatory diseases such as IL-6, which forms a positive feedback with IL-17 [6, 7]. If the hyperlipidemic fatty liver is not well controlled, the inflammation exacerbates, then it might develop into hepatitis, cirrhosis, cardiovascular, and cerebrovascular diseases or other liver and kidney diseases [8, 9].

In this study, from inhibiting the activity of inflammatory cytokines, we analyzed the effects of acupuncture on IL-17 expression in fat excess liver and provided some basic evidences that the inflammatory damage of hyperlipidemic fatty liver could be restricted through acupuncture.

2. Materials and Methods

2.1. Materials

Experimental Animal. Adult male ApoE-gene-knockout mice (16 weeks of age, 23.6 g–30.5 g) were purchased from Vital

River Laboratory Animal Technology Co. Ltd., batch number: SCXK (Beijing) 2011-0012.

Needling Instrument and Reagent. HuanQiu acupuncture needle, 0.20 × 20 mm, batch number: LOT/BATCH, (Suzhou Acupuncture Goods Co., Ltd.). Simvastatin, (Hangzhou MSD Pharmaceutical Co., Ltd.). Anti-IL-17 antibody (Abcam, UK).

2.2. Methods

2.2.1. Grouping Experimental Animals. After normal diet feeding for one week, the ApoE$^{-/-}$ mice were randomly and equally divided into four groups: Needling-Acupoint Group; Simvastatin Intragastric Administration Group; Needling Nonacupoint Group, and Model Group. They were kept in SPF class experimental animal room, with temperature 23 ± 2°C, relative humidity 60–65%, a 12-hour light-dark cycle (7:00 am–7:00 pm), and free access to water and food.

2.2.2. Model Preparation. After grouping, mice were fed with a high-fat diet, containing 21% fat, and 0.15% cholesterol supplied by Department of Laboratory Animal Science at Peking University, China.

2.2.3. Processing Methods. Mice in Needling-Acupoint Group were received acupuncture at both sides of ST40 and PC6 with 20 mm needles in diameter. ST40 was performed by straightly inserting a stainless steel needle to a depth of 3 mm and PC6 was obliquely toward the elbow to a depth of 2 mm. The needles were rotated slowly at the speed of 60 rounds per minute to moderate reinforcing and reducing. The entire procedure was completed in 2 minutes without retaining needle, three times a week for 8 weeks. The Simvastatin Intragastric Administration Group received Simvastatin intragastric administration (25 mg/kg/d) for 8 weeks. Needling Nonacupoint Group received nonacupoint needlings (two points in 0.5 cm and 1 cm to the end of tail), each inserted obliquely 1 mm in depth. Mice in Model Group were tied up without acupuncture and bred normally as the other groups.

2.2.4. The Measurements

(1) Body weight: measuring body weight before and after the experiment.

(2) Serum indicators: TC was detected at the beginning and the end of the experiment and IL-17 was also tested by ELISA.

(3) Histopathological examination: some fresh liver tissue was made into frozen section and stained with Oil-Red-O staining as well as Haematoxylin and eosin staining to observe the degree of hepatic steatosis.

(4) Immunohistochemical method for the expression of IL-17 in liver tissue. Three portal areas were selected randomly in each staining section and their positive expressions in cytoplasm were assessed by IOD [10].

2.3. Statistical Processing. SPSS17.0 software was employed. Comparisons between groups were analyzed by One-Way ANOVA and LSD test. The Data of each group were expressed as mean ± SD, $P < 0.05$ for statistical significance, and $P < 0.01$ for a significant difference.

3. Result

3.1. Body Weight Decreased Significantly in Needling-Acupoint Group. In first week of the experiment, the body weight among 4 groups was not significantly different ($P > 0.05$). After 8 weeks of intervention, compared with Model Group, the body weights of Needling-Acupoint Group and Simvastatin Intragastric Administration Group decreased ($P < 0.05$); The body weight in Needling Nonacupoint Group rose slightly ($P > 0.05$).

3.2. Serum TC and IL-17 Decreased in Needling-Acupoint Group. There was no distinguished difference of TC among 4 groups before the experiment ($P > 0.05$). After 8 weeks intervention, compared with Model Group, TC of the Needling-acupoint Group and Simvastatin Intragastric Administration Group were lower, but only the Needling-acupoint Group was statistically significant ($P < 0.05$), and the Needling Nonacupoint Group decreased little. ($P > 0.05$, Table 2). In comparison with model group, serum IL-17 of Needling-acupoint Group and Simvastatin Intragastric Administration Group were significantly lower ($P < 0.01$), while Needling Nonacupoint Group went down lightly, with no statistical significance ($P > 0.05$, Table 2).

3.3. The Pathological Changes of Hepatic Tissues. Frozen sections of liver tissue were prepared for Haematoxylin and eosin staining and Oil-Red-O staining. Under the microscope, frozen sections showed that the liver tissue in Model Group grew varied hepatic steatosis, such as enlarged hepatic cells, structural disorder, and many lipid droplet vacuoles within the cytoplasm. By Oil-Red-O staining, numerous deep dyeing and large lipid droplets within cytoplasm can be seen.

In Needling Nonacupoint Group, the steatosis appeared and the enlarged hepatic cells are similar to that of Model Group. There were many deep dyeing lipid droplets in portal areas.

In Needling-acupoint Group, after acupuncture treatment, steatosis of the liver tissue has significantly alleviated and its structure tended to be normal, though, only a few scattered small dyeing lipid droplets in liver cells can be seen.

Mice in Simvastatin Intragastric Administration Group also had more regular liver cell structure than Model Group. There were Oil-Red-O stained lipid droplets varied in number and size around the portal areas, which were smaller, lighter, and less compared with those of Model Group (Figure 1).

3.4. Immunohistochemistry of IL-17 Expression in Liver. Immunohistochemical results showed that the Model Group

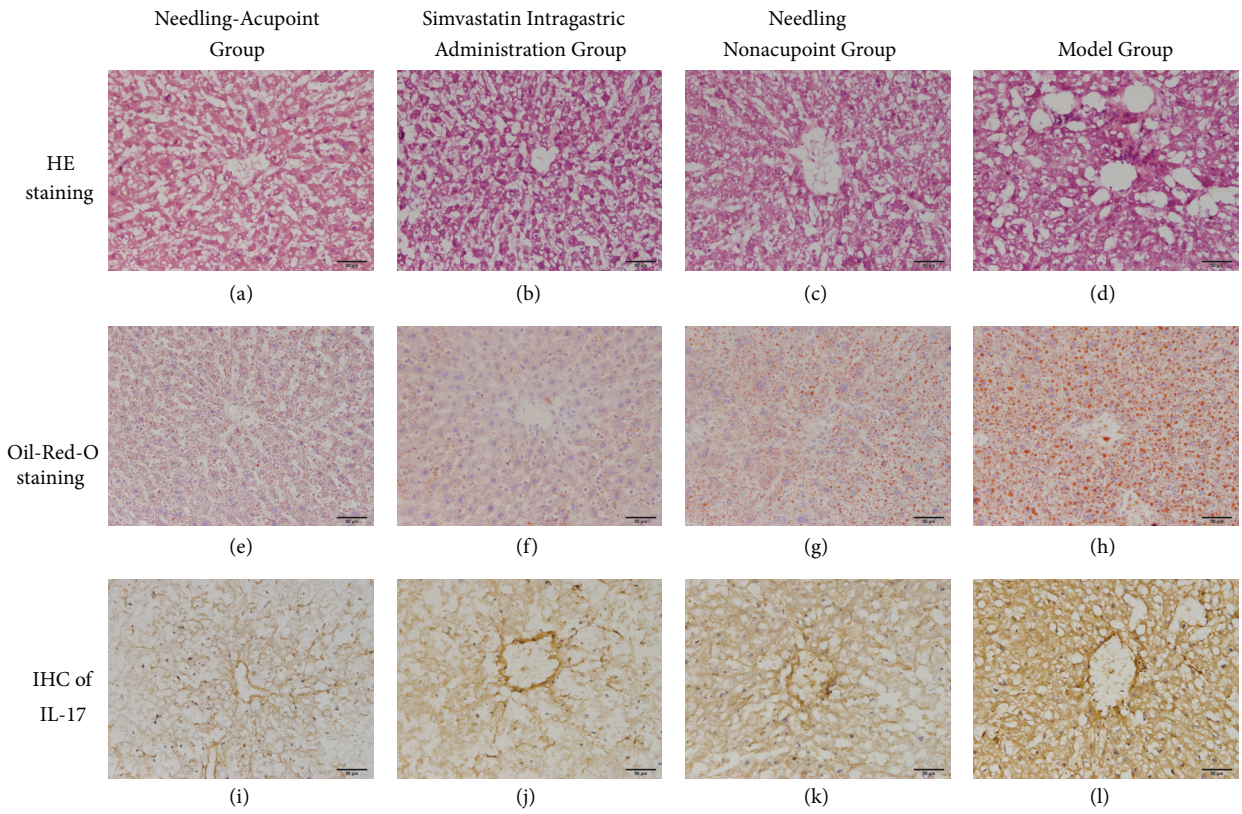

Figure 1: Histological observation of liver tissue in each group.

had strong IL-17 positive cells and brown pigmentation particles, deep in color and large in size. The pigmentation particles in Needling-acupoint Group and Simvastatin Intragastric Administration Group were lighter and smaller compared with Model Group. Unlike Model Group, the particles in Needling Nonacupoint Group were light colored, yet darker than those in Needling-acupoint Group and Simvastatin Intragastric Administration Group (Figure 1). The positive regions were measured to assess IOD with Image Pro Plus 6 software (Table 1), and the IOD of the other three groups lowered significantly than Model Group ($P < 0.01$), with no significant difference among the three.

4. Discussion

Four of the findings of acupuncture on ApoE$^{-/-}$ mice' ST40 and PC6 are worth summarizing. (1) The body weight decreased, (2) TC and serum IL-17 lowered, (3) pathological changes in hepatic tissues improved, and (4) immunohistochemical expression of IL-17 in liver significantly reduced.

The term "Fatty Liver" does not exist in Traditional Chinese Medicine, but its syndrome relates to accumulation, distention of abdomen, jaundice, hypochondriac pain, turbid phlegm, and so on, involving phlegm, dampness, blood stasis, and mass. Although the disease locates in the liver, the spleen and kidney are also related, and its first pathogenesis is the deficiency of spleen and kidney. The disease is caused by overeating greasy and sweet food and drinking excessively or by invasion of damp-heat epidemically exogenous pathogen, mental disturbance, and long illness, which could lead to the liver failing to maintain the normal flow of Qi, the spleen failing to transport and convert, and phlegm stasis. Furthermore, the kidney deficiency develops, so do the phlegm and blood stasis, afterwards the disease is formed. The treatment mainly focuses on smoothing the liver to strengthen the spleen, reducing phlegm to eliminate dampness, and on eliminating blood stasis to activate blood circulation, concurrently reinforcing the liver and kidney [2, 11–13].

Treating hyperlipidemic fatty liver with acupuncture, its mechanism operates in inhibiting activity of inflammatory factors besides improving insulin resistance, antioxidative stress [14, 15]. In this experiment, we chose Fenglong (ST40) and Neiguan (PC6) to treat fatty liver. As a key acupoint to deal with phlegm, ST40 is able to communicate Stomach Meridian Foot-Yangming (ST) and Spleen Meridian of Foot Taiyin (SP). The point functions in regulating spleen and stomach, clearing down phlegm, activating channels, and reducing tangible or intangible phlegm. As for PC6, it belongs to Pericardium Meridian of Hand-Jueyi, when compatible with ST40, it would tranquilize the mind, relieve the pain, regulate Qi flow and stomach, nourish the blood, promote blood circulation, and clear down phlegm [16–18].

Liver, as an important organ for lipid metabolism, centers on fat intake, oxidation of fatty acid, and the synthesis and secretion of cholesterol, phospholipid, and lipoprotein.

Table 1: Body weight and IOD ($n = 8$).

Group	Weight (g)		IOD
	Week 1	Week 8	
Needling-Acupoint Group	27.78 ± 0.85	26.37 ± 1.50[a]	6.75 ± 2.34[b]
Simvastatin Intragastric Administration Group	27.90 ± 1.47	26.87 ± 1.35[a]	6.16 ± 1.61[b]
Needling Nonacupoint Group	27.62 ± 1.28	29.12 ± 1.95	6.54 ± 0.91[b]
Model Group	26.68 ± 1.19	28.87 ± 2.35	9.54 ± 2.70

IOD: Integrated optical density; Note: compared with model group, [a]$P < 0.05$, [b]$P < 0.01$.

Table 2: Serum TC and IL-17 ($n = 8$).

Groups	TC (mmol/L)		Serum IL-17 (umol/L)
	Week 1	Week 8	
Needling-Acupoint Group	10.80 ± 2.64	19.84 ± 4.23[a]	25.49 ± 4.35[b]
Simvastatin Intragastric Administration Group	9.62 ± 1.41	20.89 ± 2.84	24.14 ± 6.81[b]
Needling Nonacupoint Group	11.39 ± 1.17	22.68 ± 4.53	40.56 ± 5.91
Model Group	10.34 ± 2.91	24.15 ± 4.00	43.49 ± 5.46

TC: Total cholesterol; Note: compared with model group, [a]$P < 0.05$, [b]$P < 0.01$.

When the lipid metabolism disorders, a large amount of fat enters into hepatocytes, which increases fat synthesis. If this grows beyond the hepatocytes' capability in Oxidation and Synthesis of lipoprotein, the lipids have to accumulate in the liver cells, leading to denaturing and swelling of the liver cell, consequently the inflammation, necrosis, and fibrosis of fatty liver. Hyperlipidemia shoulders large part in the formation of fatty liver, which means that it presents the positive correlation to the morbidity of fatty liver. Inflammatory factors inside or outside the liver play crucial role in the incidence of fatty liver and they are part of the early manifestations of the metabolism disorder.

IL-17 could be interpreted as the inflammatory factors mainly produced by $CD4^+$ T lymphocyte subsets (Th17). This kind of proinflammatory factors has strong induction on neutrophils and simultaneously promotes the expressions of various cytokines, such as the expression and release of IL-6, IL-18, and TNF-α, various inflammatory diseases in human body are also related to them [19]. Th17 and IL-17 can also accelerate the progress from simple fatty liver to nonalcoholic steatohepatitis (NASH) [20, 21].

In this study, $ApoE^{-/-}$ mice were used as the model of hyperlipidemic fatty liver, and ST40 and PC6 were acupunctured. It showed that acupunctural intervention on $ApoE^{-/-}$ mice would decrease IL-17 expression in serum and liver tissue. Likewise, serum total cholesterol was decreased. This result echoes Li Li Zhu et al.'s [3] and Li Zhou et al.'s [4] finding that acupuncture is capable of reducing TC in mice and rats. Although there have been many studies on observation of the impact of IL-17 on various diseases, few have been made on IL-17 control of fatty liver disease. On this basis, it can be believed that other inflammatory factors might also be reduced, for instance, inhibiting the positive-feedback loop produced by IL-6 [22]. With needling the nonacupoints, expression of IL-17 in liver tissue also could be reduced. Therefore, it could be concluded that acupuncture is helpful to reduce the hepatic inflammation and to slow down the speed of fatty liver developing into hepatitis or cirrhosis.

5. Conclusion

Needling on ST40 and PC6 of $ApoE^{-/-}$ mice is capable of lowering TC and might also be able to control the expression of IL-17. In spite of all the limitations of our conclusions, in order to obtain more reliable and objective data, further research is required in a number of directions. For instance, on the topic about the effect and mechanism of regulating lipid metabolism by acupuncture at single acupoint, how IL-17 varies in tissue or serum at different time or the IL-17 involved signaling pathways. Hopefully, future study can not only provide a better understanding of acupoint specificity, but also reflect the development of disease, through immune regulation to guide the treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Gastrodia elata Ameliorates High-Fructose Diet-Induced Lipid Metabolism and Endothelial Dysfunction

Min Chul Kho,[1,2] Yun Jung Lee,[1,2] Jeong Dan Cha,[3] Kyung Min Choi,[3] Dae Gill Kang,[1,2] and Ho Sub Lee[1,2]

[1] *College of Oriental Medicine and Professional Graduate School of Oriental Medicine, Wonkwang University, Shinyong-dong, Iksan, Jeonbuk 570-749, Republic of Korea*
[2] *Hanbang Body-Fluid Research Center, Wonkwang University, Shinyong-dong, Iksan, Jeonbuk 570-749, Republic of Korea*
[3] *Department of Research Development, Institute of Jinan Red Ginseng, Jinan, Jeonbuk 567-801, Republic of Korea*

Correspondence should be addressed to Ho Sub Lee; host@wku.ac.kr

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Overconsumption of fructose results in dyslipidemia, hypertension, and impaired glucose tolerance, which have documented correlation with metabolic syndrome. *Gastrodia elata*, a widely used traditional herbal medicine, was reported with anti-inflammatory and antidiabetes activities. Thus, this study examined whether ethanol extract of *Gastrodia elata* Blume (EGB) attenuate lipid metabolism and endothelial dysfunction in a high-fructose (HF) diet animal model. Rats were fed the 65% HF diet with/without EGB 100 mg/kg/day for 8 weeks. Treatment with EGB significantly suppressed the increments of epididymal fat weight, blood pressure, plasma triglyceride, total cholesterol levels, and oral glucose tolerance, respectively. In addition, EGB markedly prevented increase of adipocyte size and hepatic accumulation of triglycerides. EGB ameliorated endothelial dysfunction by downregulation of endothelin-1 (ET-1) and adhesion molecules in the aorta. Moreover, EGB significantly recovered the impairment of vasorelaxation to acetylcholine and levels of endothelial nitric oxide synthase (eNOS) expression and induced markedly upregulation of phosphorylation AMP-activated protein kinase (AMPK)α in the liver, muscle, and fat. These results indicate that EGB ameliorates dyslipidemia, hypertension, and insulin resistance as well as impaired vascular endothelial function in HF diet rats. Taken together, EGB may be a beneficial therapeutic approach for metabolic syndrome.

1. Introduction

Metabolic syndrome, a worldwide issue, is characterized by insulin resistance, impaired glucose tolerance and/or hyperglycemia, high blood serum triglycerides, low concentration of high-density lipoprotein (HDL) cholesterol, high blood pressure, and central obesity. The association of 3 (or more) of these factors leads to an increased morbidity and mortality from several predominant diseases such as type 2 diabetes, cancer, and cardiovascular diseases including atherosclerosis, myocardial infarction, and stroke [1, 2].

Fructose is an isomer of glucose with a hydroxyl group on carbon-4 reversed in position. It is promptly absorbed and rapidly metabolized by liver. Recent decades westernization of diets has resulted in significant increases in added fructose, enormous rised in fructose consumption typical daily [3]. The exposure of the liver to such enormous rising fructose consumption leads to rapid stimulation of lipogenesis and triglyceride accumulation, which in turn leads to reduced insulin sensitivity and hepatic insulin resistance/glucose intolerance [4]. Thus, high-fructose diet induces a well-characterised metabolic syndrome, generally resulting in hypertension, dyslipidaemia, and low level of HDL-cholesterol [5]. Recent studies suggest that high fructose intake may be an important risk factor for the development of fatty liver [6]. Rats are commonly used as a model to mimic human disease, including metabolic syndrome [7]. Similarly, emerging data suggest that experiment on fructose-diet rats tends to produce some of the changes associated with metabolic syndrome, such as altered lipid metabolism, fatty liver, hypertension, obesity, and dyslipidemia [8].

Gastrodia elata Blume is a traditional herbal medicine in Korea, China, and Japan, which has been used for the treatment of headaches, hypertension, rheumatism, and cardiovascular diseases [9]. Several major physiological substances have been identified from *Gastrodia elata* Blume such as gastrodin, vanillyl alcohol, vanillin, glycoprotein, p-endoxybenzyl alcohol, and polysaccharides including alpha-D-glucan [10–12]. Our previous studies showed that *Gastrodia elata* Blume exhibits anti-inflammatory and anti-atherosclerotic properties by inhibiting the expression of proinflammatory cytokines in vascular endothelial cells [13, 14]. However, the effect of ethanol extract of *Gastrodia elata* Blume on high-fructose (HF) diet animal model has not been yet reported. Thus, the present study was designed to determine whether an ethanol extract of *Gastrodia elata* Blume (EGB) improves high-fructose diet-induced lipid metabolism and endothelial dysfunction.

2. Materials and Methods

2.1. Preparation of Gastrodia elata Blume. The *Gastrodia elata* Blume was purchased from the Herbal Medicine Cooperative Association, Iksan, Jeonbuk Province, Korea, in May 2012. A voucher specimen (no. HBJ1041) was deposited in the herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk, South Korea. The dried *Gastrodia elata* Blume (400 g) was extracted with 4 L of 95% ethanol at room temperature for 1 week. The extract was filtered through Whatman no. 3 filter paper (Whatman International Ltd., England) and concentrated using rotary evaporator. The resulting extract (12.741 g) was lyophilized by using a freeze drier and retained until required.

2.2. Animal Experiments and Diet. All experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Utilization Committee for Medical Science of Wonkwang University. Seven week-old male Sprague-Dawley (SD) rats were obtained from Samtako (Osan, Korea). Rats were kept in a room automatically maintained at a temperature ($23 \pm 2^{\circ}$C), humidity (50~60%), and 12-h light/dark cycle throughout the experiments. After 1 week of acclimatization, animals were randomly divided into three groups ($n = 10$ per group). Control group (Cont.) was fed regular diet, high-fructose group (HF) was fed 65% fructose diet (Research Diet, USA), and the third group (HF + EGB) was fed with 65% fructose along with a single dose of 100 mg/kg/day of EGB orally for a period of 8 weeks. The regular diet was composed of 50% starch, 21% protein, 4% fat, and standard vitamins and mineral mix. The high-fructose diet was composed of 65% fructose, 20% protein, 10% fat, and standard vitamins and mineral mix.

2.3. Blood and Tissue Sampling. At the end of the experiments, the aorta, liver, adipose tissue (epididymal fat pads), and muscle were separated and frozen until analysis after being rinsed with cold saline. The plasma was obtained from the coagulated blood by centrifugation at 3,000 rpm 15 min at 4°C. The separation of plasma was frozen at −80°C until analysis.

2.4. Measurements of Blood Pressure. Systolic blood pressure (SBP) was determined by using noninvasive tail-cuff plethysmography method and recorded with an automatic sphygmotonography (MK2000; Muromachi Kikai, Tokyo, Japan). The systolic blood pressure (SBP) was measured at week 1, week 3, and week 7, respectively. At least seven determinations were made in every session. Values were presented as the mean ± SEM of five measurements.

2.5. Analysis of Plasma Lipids. The levels of triglyceride in plasma were measured by using commercial kits (ARKRAY, Inc., MINAMI-KU, KYOTO, Japan). The levels of high-density lipoprotein (HDL)-cholesterol, total cholesterol, and LDL-cholesterol in plasma were measured by using HDL and LDL assay kit (E2HL-100, BioAssay Systems).

2.6. Estimation of Blood Glucose and Oral Glucose Tolerance Test. The concentration of glucose in blood was measured which was obtained from tail vein using glucometer (One-touch Ultra) and Test Strip (Life Scan Inc., CA, USA), respectively.

The oral glucose tolerance test (OGTT) was performed 2 days apart at 7 weeks. For the OGTT, briefly, basal blood glucose concentrations were measured after 10~12 h of overnight food privation; then the glucose solution (2 g/kg body weight) was immediately administered via oral gavage, and fourth more tail vein blood samples were taken at 30, 60, 90, and 120 min after glucose administration.

2.7. Preparation of Carotid Artery and Measurement of Vascular Reactivity. The carotid arteries of the rats were rapidly and carefully isolated and placed into cold Kreb's solution of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.2, CaCl 1.5, $NaHCO_3$ 25, glucose 10, and pH 7.4. The carotid arteries were removed to connective tissue and fat and cut into rings of approximately 3 mm in length. All dissecting procedures were carried out for caring to protect the endothelium from accidental damage. The carotid artery rings were suspended by means of two L-shaped stainless-steel wires inserted into the lumen in a tissue bath containing Kreb's solution at 37°C and aerated with 95% O_2 and 5% CO_2. The isometric forces of the rings were measured by using a Grass FT 03 force displacement transducer connected to a Model 7E polygraph recording system (Grass Technologies, Quincy, MA, USA). In the carotid artery rings of rats, a passive stretch of 1 g was determined to be optimal tension for maximal responsiveness to phenylephrine (10^{-6} M). The preparations were allowed to equilibrate for approximately 1 h with an exchange of Kreb's solution every 10 min. The relaxant effects of acetylcholine (ACh, $10^{-9} \sim 10^{-6}$ M) and sodium nitroprusside (SNP, $10^{-10} \sim 10^{-5}$ M) were studied in carotid artery rings constricted submaximally with phenylephrine (10^{-6} M).

Table 1: Effect of EGB on body weight, epididymal fat pads, and blood glucose.

Groups	Control	HF	HF + EGB
Initial BW (g)	245.8 ± 7.6	244.4 ± 7.4	244.4 ± 9.0
Terminal BW (g)	449.4 ± 28.9	439.8 ± 26.5	$402.5 \pm 22.1^{\#}$
Epididymal fat pads weight (g)	2.5 ± 0.7	$3.9 \pm 1.2^{**}$	$2.5 \pm 0.5^{\#\#}$
Blood glucose (mg/dL)	94.63 ± 6.48	99.50 ± 7.30	96.70 ± 8.54

Values were expressed as mean ± SD ($n = 10$). $^{**}P < 0.01$ versus Cont.; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HF. HF: high fructose; HF + EGB: high fructose diet with EGB; BW: body weight.

2.8. Western Blot Analysis in the Rat Aorta, Liver, Muscle, and Fat. The aorta, liver muscle, and fat tissues homogenate were prepared in ice-cold buffer containing 250 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsufonyl fluoride, and 20 mM potassium phosphate buffer (pH 7.6). The homogenates were then centrifuged at 8,000 rpm for 10 min at 4°C, and the supernatant was centrifuged at 13,000 rpm for 5 min at 4°C, and as a cytosolic fraction for the analysis of protein. The recovered proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and electrophoresis transferred to nitrocellulose membranes. Membranes were blocked by 5% BSA powder in 0.05% Tween 20-Tris-bufferd saline (TBS-T) for 1 h. The antibodies against ICAM-1, VCAM-1, E-selectin, eNOS, ET-1 (in aorta), AMPK, and p-AMPK (in liver, muscle, and fat) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The nitrocellulose membranes were incubated overnight at 4°C with protein antibodies. The blots were washed several times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and then the immunoreactive bands were visualized by using enhanced chemiluminescence (Amersham, Buchinghamshire, UK). The bands were analyzed densitometrically by using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA, USA).

2.9. Histopathological Staining of Aorta, Epididymal Fat, and Liver. Aortic tissues were fixed in 10% (v/v) formalin in 0.01 M phosphate buffered saline (PBS) for 2 days with change of formalin solution every day to remove traces of blood from tissue. The tissue samples were dehydrated and embedded in paraffin, and then thin sections (6 μm) of the aortic arch in each group were cut and stained with hematoxylin and eosin (H&E). Epididymal fat and liver tissues were fixed by immersion in 4% paraformaldehyde for 48 h at 4°C and incubated with 30% sucrose for 2 days. Each fat and liver was embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen, and stored at −80°C. Frozen sections were cut with a Shandon Cryotome SME (Thermo Electron Corporation, Pittsburg, PA, USA) and placed on poly-L-lysine-coated slide. Epididymal fat sections were stained with H&E. Liver sections were assessed by using Oil Red O staining. For quantitative histopathological comparisons, each section was determined by Axiovision 4 Imaging/Archiving software.

2.10. Immunihistochemical Staining of Aortic Tissues. Paraffin sections for immunohistochemical staining were placed on poly-L-lysine-coated slide (Fisher scientific, Pittsburgh, PA, USA). Slides were immunostained by Invitrogen's HISOTO-STAIN-SP kits using the Labeled-(strept) Avidin-Biotin (LAB-SA) method. After antigen retrieval, slides were immersed in 3% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase activity and rinsed with PBS. After being rinsed, slides were incubated with 10% nonimmune goat serum for 10 min at room temperature and incubated with primary antibodies of ICAM-1, VCAM-1, and E-selectin (1:200; Santa Cruz, CA, USA) in humidified chambers overnight at 4°C. All slides were then incubated with biotinylated secondary antibody for 20 min at room temperature and then incubated with horseradish peroxidase-conjugated streptavidin for 20 min at room temperature. Peroxidase activity was visualized by 3,3′-Diaminobenzidine (DAB; Novex, CA) substrate-chromogen system, counterstaining with hematoxylin (Zymed, CA, USA). For quantitative analysis, the average score of 10~20 randomly selected area was calculated by using NIH Image analysis software, Image J (NIH, Bethesda, MD, USA).

2.11. Statistical Analysis. All the experiments were repeated at least three times. The results were expressed as a mean ±SD or mean ±SE. The data was analyzed using SIGMAPLOT 10.0 program. The Student's t-test was used to determine any significant differences. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Characteristics of Experimental Animals. During the entire experimental period, all groups showed significant increase in body weight. There was no significant change in body weight after 8 weeks of fructose feeding in HF group. However, treatment of EGB group showed significant decrease in body weight (439.8 ± 26.5 versus 402.5 ± 22.1, $P < 0.05$) (Table 1). Moreover, HF diet results in a significant increase in epididymal fat pads weight. The weight of epididymal fat pads was 60.8 ± 17.4% higher than that of the HF diet group compared with control group. However, treatment of EGB group significantly reduced the epididymal fat pads weight (57.5 ± 7.3%) compared with HF diet group (Table 1).

3.2. Effect of EGB on Blood Pressure. At the beginning of the experimental feeding period, the levels of systolic blood pressure in all groups were approximately 95~100 mmHg as investigated by the tail-cuff technique. After 4 weeks, systolic blood pressure of HF group was significantly increased than

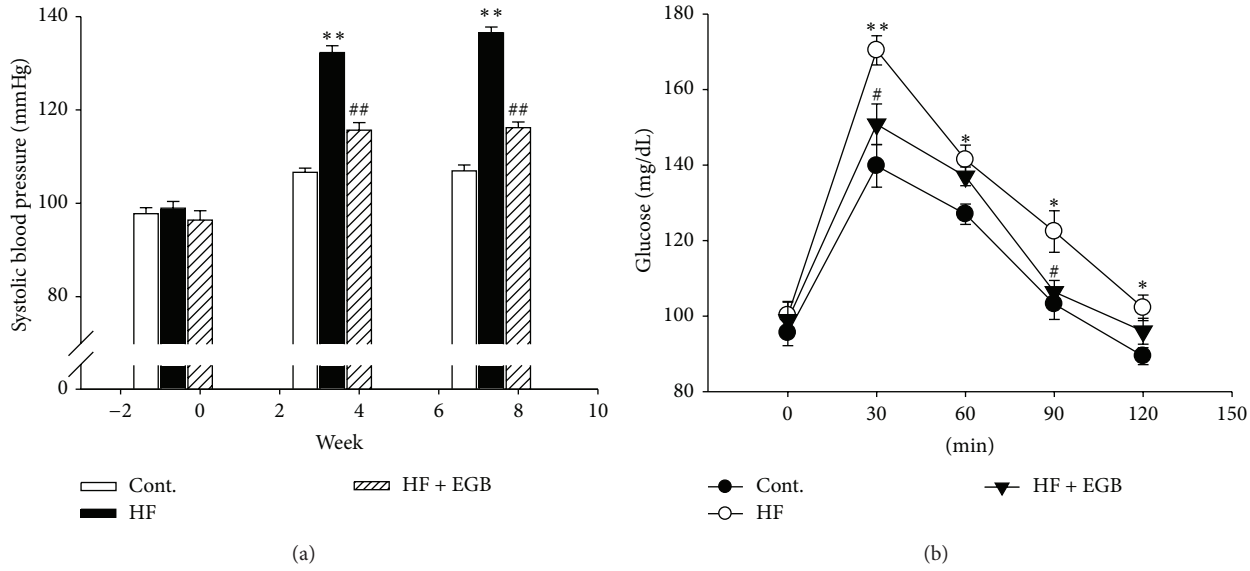

Figure 1: Effects of EGB on systolic blood pressure (a) and oral glucose tolerance test (b). Values were expressed as mean ± SE ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus Cont.; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HF.

that of control group ($P < 0.01$). However, EGB group was significantly decreased than that of HF group during all the experimental period (136.71 ± 1.24 versus 116.4 ± 1.21, $P < 0.01$) (Figure 1(a)).

3.3. Effect of EGB on Blood Glucose Level and Oral Glucose Tolerance Test. Plasma blood glucose levels were not statistically different in HF diet rats with chronic treatment of EGB (Table 1). Oral glucose tolerance test was carried out to check insulin resistance in high-fructose diet rats after 8 weeks. The results showed that HF diet group maintained the significant increase in blood glucose levels at 30, 60, 90 ($P < 0.01$), and 120 min ($P < 0.05$), respectively. However, the plasma glucose levels in treatment of EGB were significantly decreased at 30 and 90 min as compared with HF diet group ($P < 0.05$) (Figure 1(b)).

3.4. Effect of EGB on Plasma Lipids. Group fed a HF diet displayed was increased plasma triglyceride levels, total cholesterol levels, and LDL-c levels; however, treatment of EGB group significantly decreased plasma triglyceride levels (272.67 ± 107.0 versus 177.33 ± 59.6, $P < 0.05$), total cholesterol levels (102.94 ± 19.7 versus 67.79 ± 5.8, $P < 0.01$), and LDL-c levels (44.56 ± 8.1 versus 24.28 ± 3.1, $P < 0.01$), respectively. Beside the plasma levels of HDL-c levels in EGB group increased compared with HF diet group (16.02 ± 2.9 versus 20.2 ± 2.2, $P < 0.05$) (Table 2).

3.5. Effect of EGB on Vascular Tension. Vascular responses to ACh, endothelium-dependent vasodilator (1×10^{-9} to 1×10^{-6} M), SNP, and endothelium-independent vasodilator (1×10^{-10} to 1×10^{-7} M) were measured in carotid artery. Responses to ACh-induced relaxation of carotid artery rings were significantly decreased in the HF diet group compared with control group ($1 \times 10^{-7.5}$ to 1×10^{-6} M. $P < 0.05$). However, the impairment of vasorelaxation was remarkably attenuated by treatment with EGB ($1 \times 10^{-8.5}$ to $1 \times 10^{-6.5}$ M. $P < 0.01$; 1×10^{-6} M. $P < 0.05$) (Figure 2(a)). On the other hand, response to SNP-induced relaxation of carotid artery rings had no significant difference in all the groups (Figure 2(b)).

3.6. Effect of EGB on the Morphology of Aorta and Epididymal Fat Pads. EGB effectively decreased blood pressure and attenuated impairment of vasorelaxation. Thus, we examined histological changes by staining with H&E in thoracic aorta. Figure 3 showed that thoracic aorta of HF diet group revealed roughened endothelial layers and increased tunica intima-media of layers compared with control group (+24.13%, $P < 0.01$). However, treatment of EGB group significantly maintained the smooth character of the intima endothelial layers and decreased tunica intima-media thickness in aortic section (−16.10%, $P < 0.01$) (Figures 3(a) and 3(c)).

Because EGB effectively reduced the epididymal fat pads weight, we prepared frozen section of epididymal fat pads and stained with H&E. The adipocytes were hypertrophy induced by HF diet compared with control group (+40.97%, $P < 0.01$). However, treatment of EGB significantly decreased the hypertrophy of adipocytes (−13.04%, $P < 0.05$) (Figures 3(b), and 3(d)).

3.7. Effect of EGB on the Hepatic Lipids. To investigate the existence of fat accumulation of liver in all experimental groups, we prepared frozen section of liver and stained with Oil Red O. Lipid droplets were detected in HF diet groups. However, treatment of EGB showed that the number of lipid

Table 2: Effect of EGB on plasma lipid levels.

Groups	Control	HF	HF + EGB
T-Cho (mg/dL)	67.86 ± 7.6	102.94 ± 19.7**	67.79 ± 5.8##
TG (mg/dL)	83.83 ± 16.4	272.67 ± 107.0**	177.33 ± 59.6#
HDL-c (mg/dL)	13.75 ± 1.3	16.02 ± 2.9	20.2 ± 2.2#
LDL-c (mg/dL)	28.37 ± 3.9	44.56 ± 8.1**	24.28 ± 3.1##

Values were expressed as mean ± SD ($n = 10$). $^{**}P < 0.01$ versus Cont.; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HF. HF: high fructose; HF + EGB: high-fructose diet with EGB; T-Cho: total cholesterol; TG: triglyceride; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol.

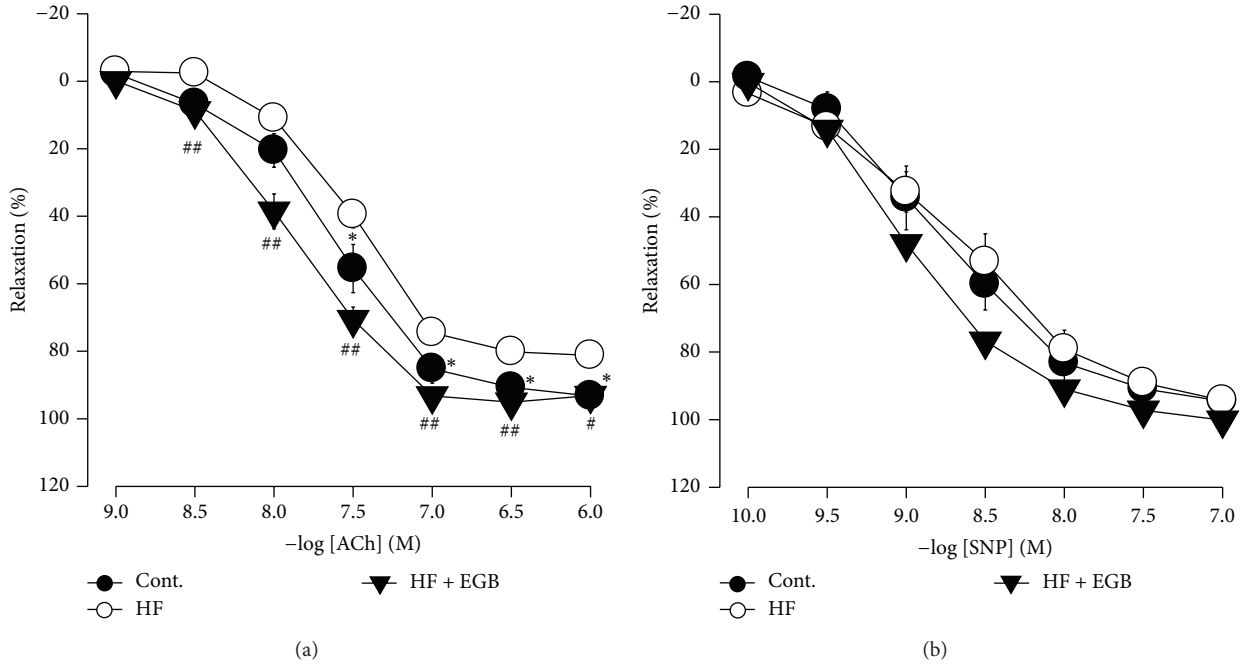

Figure 2: Effect of EGB on relaxation of carotid arteries. Cumulative concentration-response curves to acetylcholine (ACh), endothelium-dependent vasodilator (a) and sodium nitroprusside (SNP), endothelium-independent vasodilator (b) in phenylephrine precontracted carotid arteries from experiment rats. Values were expressed as mean ± SE ($n = 5$). $^{*}P < 0.05$ versus Cont.; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HF.

droplets significantly decreased compared with HF diet group (Figure 4).

3.8. Effect of EGB on the Expressions Levels of Adhesion Molecules, eNOS, and ET-1 in Aorta. Protein expression levels of VCAM-1, ICAM-1, E-selectin, eNOS, and ET-1 in aorta were determined by western blotting, respectively. Adhesion molecules (VCAM-1, ICAM-1, and E-selectin) and ET-1 protein levels were increased in the HF diet group compared with control group. However, treatment of EGB group significantly decreased expression levels of protein compared with HF diet group. Moreover, we examined the expression of eNOS levels to evaluate vascular endothelial function. The eNOS protein levels decreased in the HF diet group compared with control group. However, treatment of EGB group increased expression levels of protein compared with HF diet group (Figure 5).

Immunohistochemistry was performed to determine the direct expression of adhesion molecules in the aortic wall. Adhesion molecules expressions such as VCAM-1, ICAM-1, and E-selectin were increased in the HF diet group ($P < 0.01$); however, treatment of EGB group significantly decreased expression levels of protein (VCAM-1, ICAM-1. $P < 0.01$; E-selectin. $P < 0.05$) (Figure 6).

3.9. Effect of EGB on the Expressions Levels of AMPK in Liver, Muscle, and Fat Tissues. Because EGB effectively suppressed the development of impaired glucose tolerance, dyslipidemia, fatty liver, and endothelial dysfunction, the expression of AMPK was examined in liver, muscle, and fat tissues. The expression of AMPK was significantly decreased in HF diet group. However, treatment of EGB group increased expression levels of protein in liver, muscle, and fat tissues (Figure 7).

4. Discussion

Herb, Acupuncture, and Natural Medicine (HAN), one of the most ancient and revered forms of healing, has been used to diagnose, treat, and prevent disease for over 3,000 years. HAN

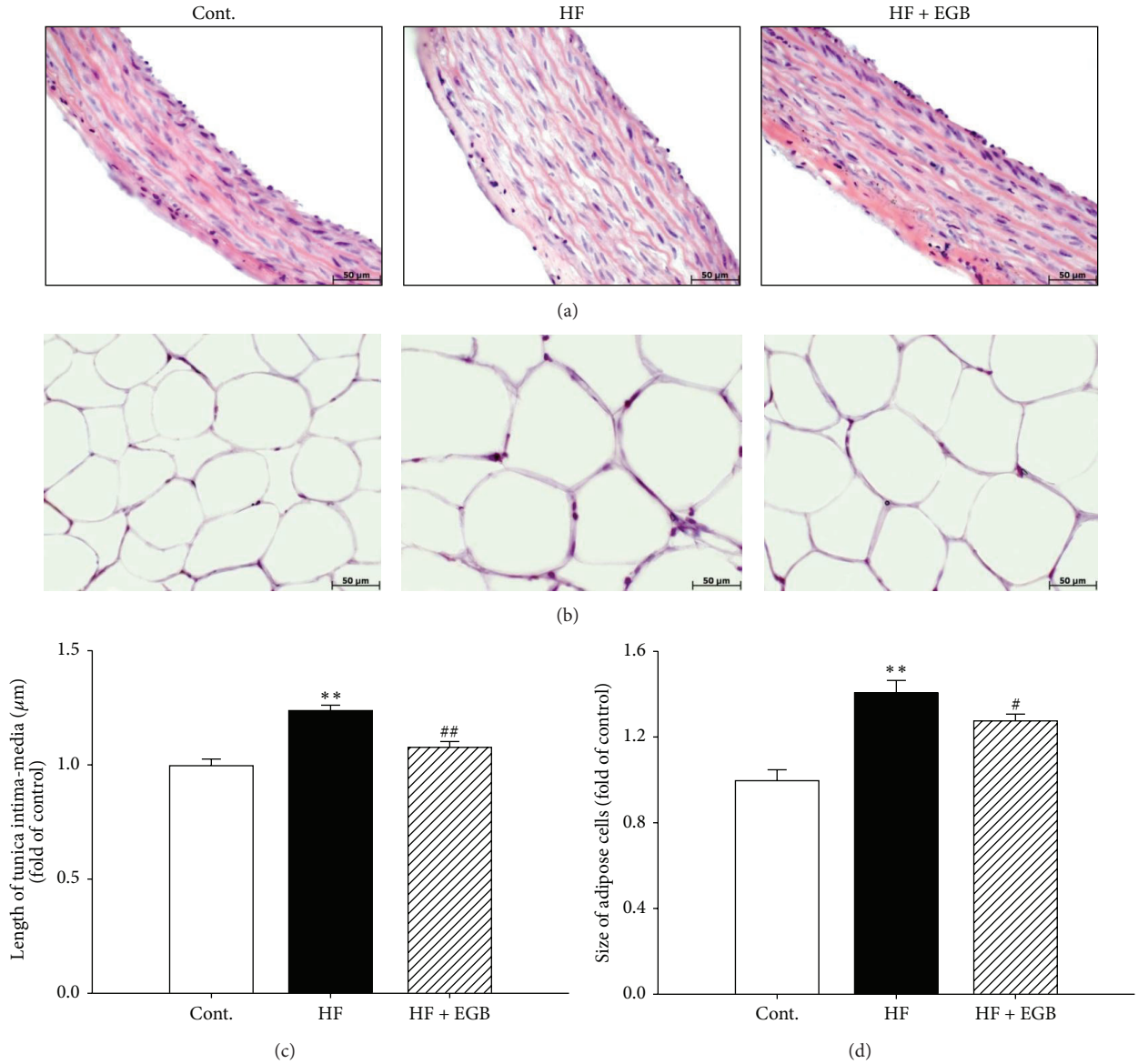

Figure 3: Effects of EGB on aortic wall and adipocytes in HF diet rats. Representative microscopic photographs of H&E stained section of the thoracic aorta (a) and epididymal fat pads (b) in HF diet rats. Lower panel indicated the length of intima-media (c) and size of adipose cells (magnification ×400). Values were expressed as mean ± SE ($n = 5$). $^{**}P < 0.01$ versus Cont.; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HF.

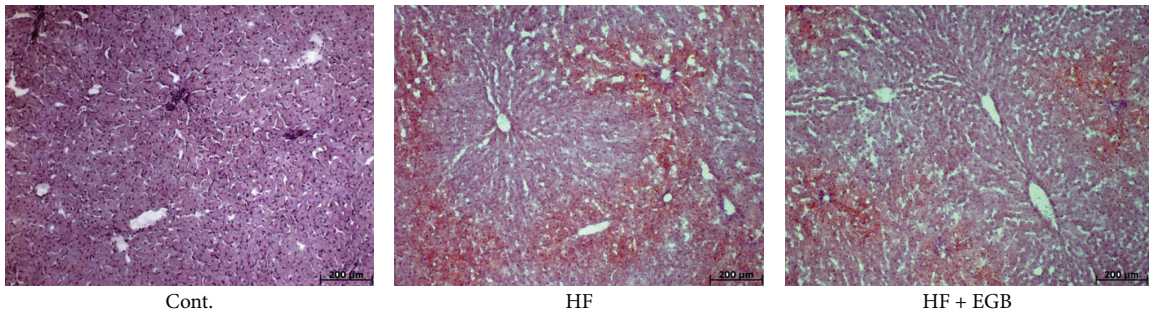

Figure 4: Effect of EGB on fatty liver in HF diet rats. Representative microscopic photographs of Oil Red O stained section of the liver.

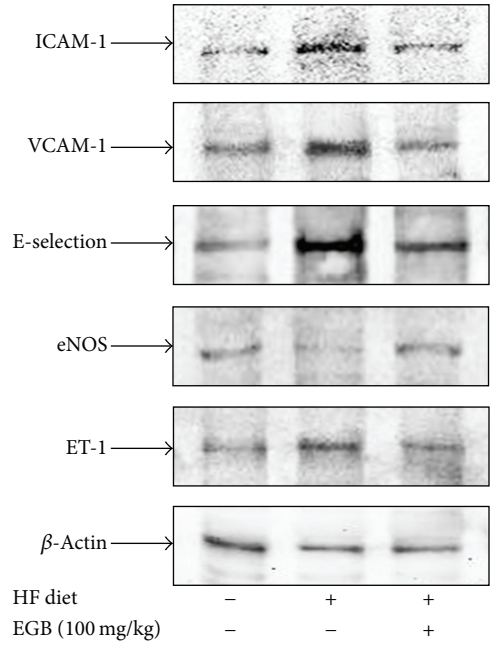

Figure 5: Effects of EGB on the expression of adhesion molecules, eNOS and ET-1 in the aorta of HF diet rats. Each electrophoretogram is representative of the results from three individual experiments.

is now used worldwide as an effective means of overcoming disease. *Gastrodia elata* is a well-known traditional Korean medicinal herb specifically for promoting blood circulation to remove blood stasis. In the present study, we provided the evidence for the beneficial effect of *Gastrodia elata* on lipid metabolism and endothelial dysfunction in high fructose-induced metabolic syndrome rat model.

Fructose is a lipogenic component, its consumption promotes the development of atherogenic lipid profile and elevation of postprandial hypertriglycemia [15, 16]. In addition, HF diet animals develop hypertriglyceridemia, obesity, impaired glucose tolerance, fatty liver, increased SBP, and vascular remodeling [17, 18]. In the present study, HF diet clearly increased visceral epididymal fat pads weight resulting from the increases in triglyceride and LDL cholesterol. Treatment with EGB lowered epididymal fat pads weight, triglyceride, and LDL cholesterol levels, whereas it elevated HDL cholesterol levels which assist lipid metabolism. Thus, EGB improves lipid metabolism by the decrease of triglyceride and LDL cholesterol. Although increased epididymal fat pads, body weight was not different from control diet and HF diet group. We suppose that proper experimental periods should be longer than the present periods for 8 weeks to increase body weight. It is sure that EGB is effective in obesity in HF diet rats, since EGB significantly decreased HF diet-induced increase in body weight.

In addition, disorder of lipid levels induced by HF diet was associated with aortic lesion. Histological analysis demonstrated that the endothelial layers were rougher in aortic sections of HF diet rats associated with a trend towards an increased development of atherosclerosis. Intima-media thickness of the thoracic aorta has been shown to correlate with prognosis and extend of coronary artery disease [19]. Treatment of EGB maintained smooth and soft intima endothelial layers and decreased intima-media thickness in aortic sections of HF diet rats.

Dyslipidemia, impaired glucose tolerance, and fatty liver are major features associated with metabolic syndrome in HF diet rats [19, 20]. Fructose induces impaired glucose tolerance via the elevation of plasma triglyceride levels. In addition, previous study demonstrated that an elevated fructose diet associated with impaired glucose tolerance and endothelial dysfunction precedes the development of hypertension [21]. Impaired glucose tolerance plays an important role in the development of such abnormalities as insulin resistance, type 2 diabetes, and dyslipidemia [22]. Similarly, HF diet induced impaired glucose tolerance and dyslipidemia, whereas treatment of EGB improved impaired glucose tolerance with the amelioration of dyslipidemia. In addition, EGB significantly suppressed the increasing adipocyte size and fatty liver. Thus, these results suggest that EGB may be useful to suppress the development of atherosclerotic lesions, obesity, and ameliorated lipid metabolism in metabolic syndrome model.

Endothelial dysfunction plays an important role in hypertension and vascular inflammation, other cardiovascular diseases, and metabolic syndrome [23, 24]. In this experimental model, the expression of ET-1 and inducible adhesion molecules such as ICAM-1, VCAM-1, and E-selectin in the arterial wall represent a key event in the development of atherosclerosis. EGB ameliorated vascular inflammation by downregulation of ET-1 as well as ICAM-1, VCAM-1, and E-selectin expressions in thoracic arota. Several studies have shown that lowering blood pressure and endothelial functions are related to an increase of eNOS reactivity, thereby increasing NO production roles as a strong vasodilator [25, 26]. In the present study, EGB upregulated eNOS levels in the aorta and recovered the HF diet-induced impairment of endothelium-dependent vasorelaxation. However, endothelium-independent vasodilator-induced vasorelaxation was not affected by EGB. These results suggest that hypotensive effect of EGB is mediated by endothelium-dependent NO/cGMP pathway. Histological study revealed that EGB suppressed vascular inflammation, compatible with the processes of atherosclerosis. In fact, endothelial dysfunction was initially identified as impaired vasodilation to specific stimuli such as ACh or bradykinin; therefore, improvement of endothelial function is predicted to regulate lipid homeostasis [27]. Thus, antihypertension and antivascular inflammatory effects of EGB contribute to the beneficial effects on endothelial function and lipid metabolism in metabolic syndrome.

To clarify the mechanism for EGB suppressing the development of visceral obesity, impaired glucose tolerance, dyslipidemia, and fatty liver, the study was focused on the expression of AMP-activated protein kinase (AMPK). There is a strong correlation between low activation state of AMPK with metabolic disorder associated with insulin resistance, fat deposition, and dyslipidemia [28–30]. AMPK is a key regulator of glucose and lipid metabolism. In the liver and muscle, activation of AMPK results in enhanced fatty acid

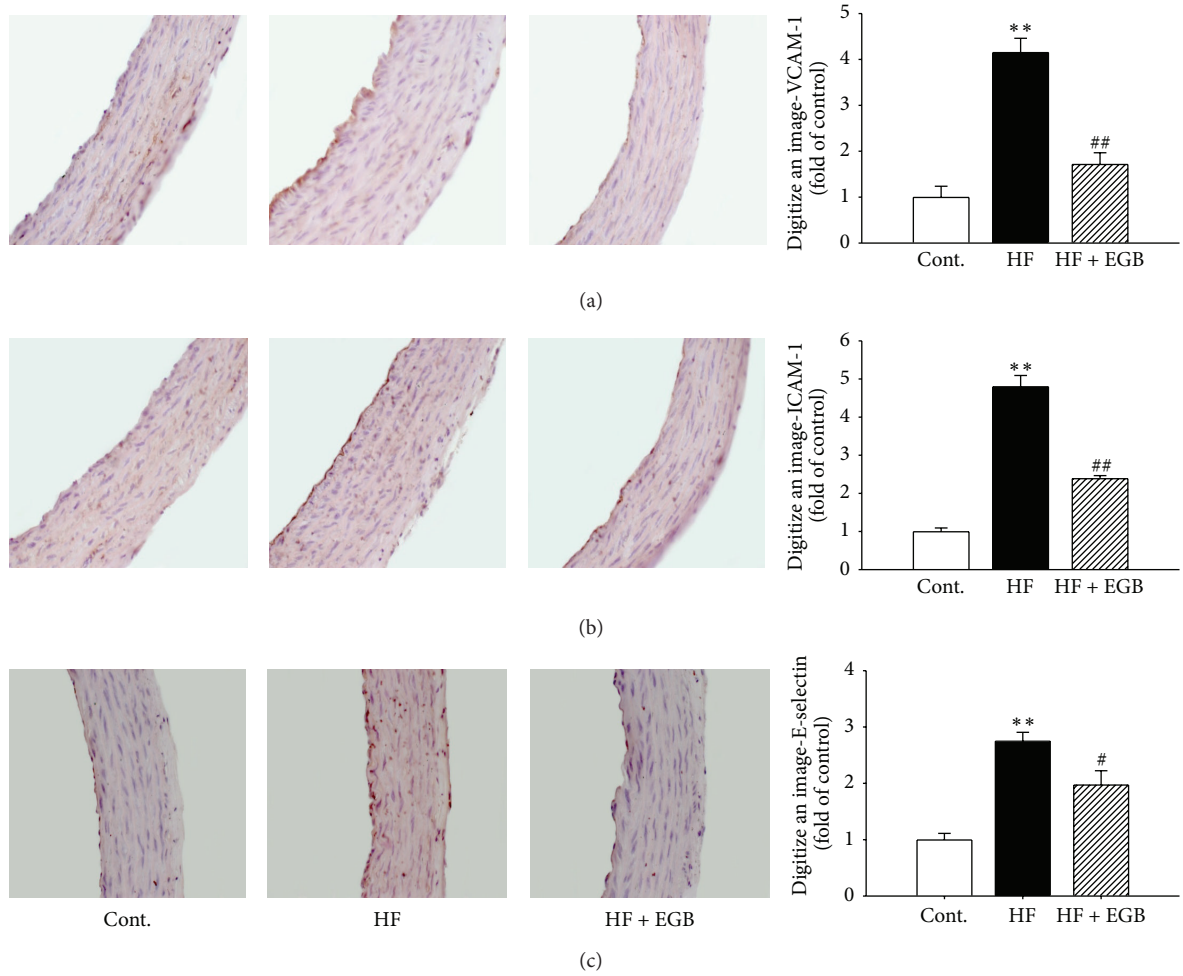

Figure 6: Effects of EGB on VCAM-1 (a), ICAM-1 (b), and E-selectin (c) immunoreactivity in aortic tissues of HF diet rats. Representative immunohistochemistry (left) and quantifications (right) are shown. Values were expressed as mean ± SE $^{**}P < 0.01$ versus Cont.; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HF.

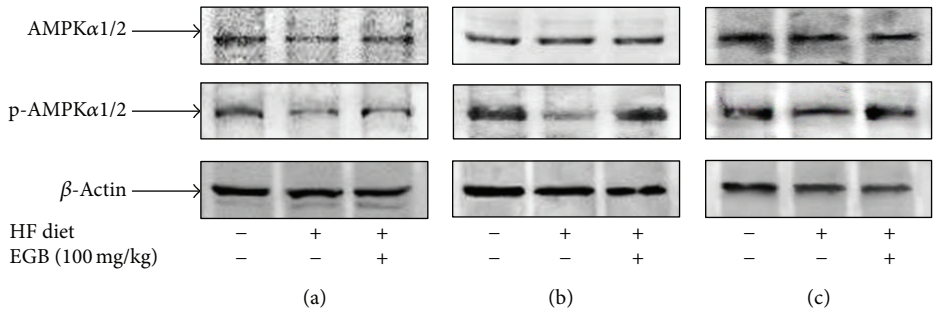

Figure 7: Effects of EGB on the expression of AMPK and p-AMPK in the liver (a), muscle (b), and fat (c) of HF diet rats. Each electrophoretogram is representative of the results from three individual experiments.

oxidation and decreased production of glucose, cholesterol, and triglycerides [31]. Recently Misra reported that the suspected role of AMPK appeared as a promising tool to prevent and/or to treat metabolic disorders [32]. Also, the activation of AMPK signaling pathway is associated with eNOS regulation and alteration of systemic endothelin pathway in fructose diet animal models [25]. AMPK is required for adiponectin-, thrombin-, and histamine-induced eNOS phosphorylation and subsequent NO production in endothelium [33]. However, our study showed that EGB induced

markedly not only activation of phosphorylation AMPKα in the liver, muscle, and fat, but also activation of eNOS levels in aorta. It could be hypothesized that EGB could lead to novel AMPK-mediated eNOS pathways which could in turn recover HF diet-induced metabolic disorders.

5. Conclusion

These results suppose that EGB ameliorates lipid metabolism, impaired glucose tolerance, hypertension, and endothelial dysfunction in HF diet-induced metabolic syndrome, at least in part, via activation of AMPK and eNOS/NO pathway. Therefore, *Gastrodia elata* Blume might be a beneficial therapeutic approach for metabolic syndrome.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Chinese Herbal Medicine on Dyslipidemia: Progress and Perspective

Ming Guo,[1,2] Yue Liu,[1,2] Zhu-Ye Gao,[1,2] and Da-zhuo Shi[1,2]

[1] *Cardiovascular Diseases Center, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China*
[2] *China Heart Institute of Chinese Medicine, China Academy of Chinese Medical Sciences, Beijing 100091, China*

Correspondence should be addressed to Da-zhuo Shi; shidazhuo@126.com

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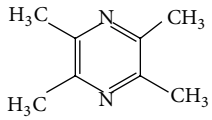
Dyslipidemia is an independent risk factor of cardiovascular diseases. The statins are a milestone in the primary and second prevention of cardiovascular diseases and significantly improved its prognosis. Along with the long-term treatment with statins in combination with other hypolipidemic drugs or alone, its safety has attracted a particular attention in clinic, such as the elevation of transaminase and rhabdomyolysis, which have raised an idea of developing the other types of lipid-lowering agents from botanic materials. Traditional Chinese medicine (TCM) has been used in clinical practice for more than 2000 years in China and showed some beneficial effects for human health and many diseases. Recently, many studies demonstrated a favorable effect of TCM for treating dyslipidemia; however, its mechanism remains unclear or totally unknown. The progress and perspective of studies on dyslipidemia with single Chinese herb and its monomers or effective extracts during the past 10 years are discussed in the present review.

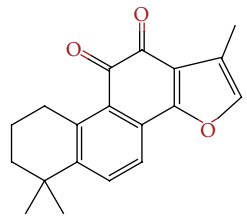
1. Introduction

Dyslipidemia is characterized by elevated level of total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) and by lowered level of high-density lipoprotein cholesterol (HDL-C) in serum. Dyslipidemia is one of the major independent risk factors for coronary heart disease (CHD) and stroke [1]. The "2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults" emphasized that the progressively regulating dyslipidemia is the pivotal controlling method for risk factors of ischemic cardiovascular events [2]. A large number of evidence indicated that the statins (3-hydroxy-3-methyl-glutaryl-coenzyme reductase inhibitor) significantly reduce the morbidity and mortality of cardiovascular and cerebrovascular events, such as MI and stroke [3]. Along with a long-term use of statins in combination with other hypolipidemic drugs or alone, however, its safety has attached a great concern from scientists and researchers, such as transaminase and creatinine elevation, skeletal muscle pain, and creatine kinase elevation. Therefore, developing novel classes of hypolipidemic agents which possess high efficiency and fewer adverse effects has still been a focus on the treatment of dyslipidemia.

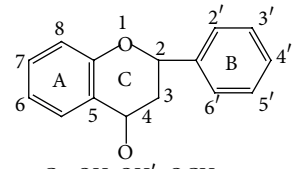
Although the hyperlipidemia has not been used in traditional Chinese medicine (TCM) term, patients with hyperlipidemia exhibited the similar etiology and pathological changes which charactered as phlegm, dampness, and blood stasis in TCM theory. Moreover, accumulating evidence has indicated that the TCM could improve phlegm, dampness, and blood stasis syndromes manifested in patients with hyperlipidemia even exhibit a beneficial effect for lowering hyperlipidemia [4, 5]. Due to the complicated mechanism of TCM on lipid lowering, most researches currently focus their attention on the effects of Chinese herb monomer or effective extracts in hyperlipidemia (see Figure 1). Studies show that the following Chinese herbs possess a favorable effect on hyperlipidemia to extent degree, which might be classified into four categories: (1) clearing heat and removing



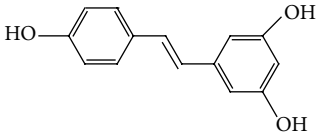
Ligustrazine



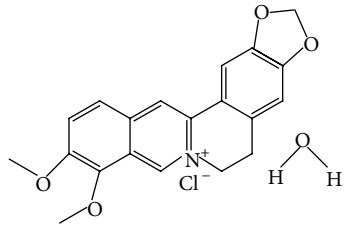
Tanshinone IIA



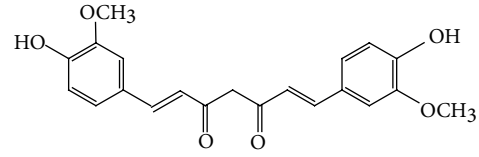
Hawthorn leaves flavonoids



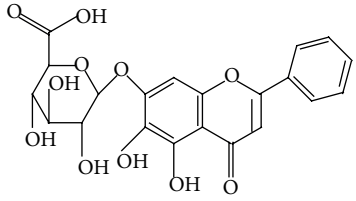
Resveratrol



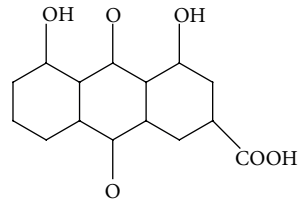
Berberine



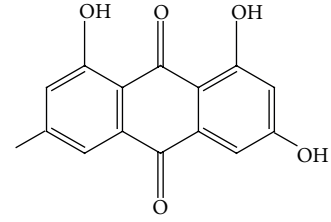
Curcumin



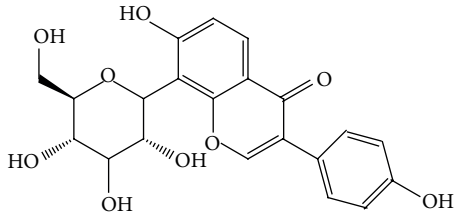
Scutellaria Baicalensis stem-leaf total flavonoid



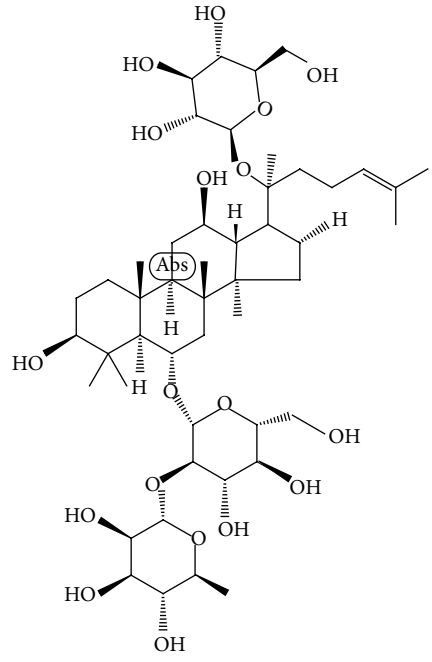
Rhein



Frangula emodin



Puerarin



Ginsenosides

Figure 1: Chemical structures of effective components of Chinese herbs for dyslipidemia.

toxicity, for example, Radix et Rhizoma Rhei, Rhizoma polygoni cuspidati, Semen Cassia, *Coptis chinensis*, *Scutellaria baicalensis*, *Gynostemma pentaphyllum*, and Radix Puerariae; (2) promoting blood circulation and removing blood stasis, for example, Fructus crataegi, Red yeast rice, Rhizoma, Radix salvia miltiorrhizae, and Turmerone; (3) eliminating dampness and phlegm, for example, Rhizoma alismatis, Plantain seed, and folium nelumbinis; (4) tonifying energy of body (including "*Qi*," *kidney*), for example, Radix Astragali, Radix Ginseng, and Radix polygoni multiflori (see Table 1). In present review, we summarized the clinical and experimental studies of single Chinese medicine and its monomers or effective extracts on dyslipidemia published during the recent 10 years.

2. Single Chinese Herb and Its Monomers or Extracts

2.1. Rheum officinale (Da Huang). Radix et. Rhizoma Rhei is derived from the root and rhizome of Rheum palmatum Linn. (Polygonaceae), Rheum tanguticum Maxim. Ex Balf. (Polygonaceae), or Rheum officinale Baill (Polygonaceae). It is used to purge fire, to remove stagnation by purgation, to cool the blood, to remove toxins, and to remove blood stasis. Anthraquinones, a main active component of Rhubarb, including rhein, aloeemodin, emodin, chrysophanol, and physcion, exhibited lipid-lowering roles by promoting intestinal peristalsis and inhibiting the intestinal absorption of cholesterol [6]. Gao et al. found that rhein, at an oral dosage of 150 mg/kg/day for 12 weeks, was proved to be lowering serum TG, TC, and LDL-c levels in db/db mice with diabetic nephropathy [7]. The powders of rhubarb administrated, at 5 g/day orally for 24 weeks, decreased serum TG and TC levels in patients with diabetic nephropathy [8]. Danthron is another extract of rhubarb, study showing that, at 0.1 μmol/L, 1 μmol/L, and 10 μmol/L of culture medium, dose-dependently promoted the phosphorylation of Adenosine monophosphate activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in both HepG2 and C2C12 cells. Likewise, danthron significantly reduced sterol regulatory element-binding protein 1c (SREBP1c) synthesis and fatty acid synthetase (FAS) gene expressions; both were closed to the lipid metabolism [9]. In addition, Li and Liu found that the powders of rhubarb administrated at 9 g/day for 5 days in 30 health volunteers caused some side-effects, such as vomiting, headache, diarrhea, and abdominal pain [10]. You reported that the decoction boiled from rhubarb, for example, at 8 g, 16 g and 32 g/kg/day for 5 days in mice, caused fatty degeneration of hepatic cell [11].

2.2. Polygonum cuspidatum (Hu Zhang). Polygonum cuspidatum (PC) is derived from the dried root and rhizome of *Polygonum cuspidatum* Sieb. et Zucc. It dispels wing dampness, removes stagnation, relieves pain, and reduces phlegm. Polydatin and resveratrol, the primary active components of PC, inhibited the cholesterol absorption from intestinal tract [12]. Du et al. found that polydatin administrated at oral dosages of 25 mg, 50 mg, and 150 mg/kg/day for 15–21 days significantly decreased TC, TG, and LDL-c levels and increased TC/HDL-c ratio in hyperlipidemic hamsters and rabbits [12, 13]. Park et al. reported that the *Polygonum cuspidatum* water extract (PCWE) at 5 ug/mL and 40 ug/mL of culture medium reduced the cholesteryl ester formation in human hepatocytes by inhibiting A-cholesterol acyltransferase activity (ACAT) in HepG2 cell *in vitro*, and PCWE at the 40 ug/mL inhibited ACAT activity by 50% [14]. Resveratrol administrated at oral dosages of 30 mg and 70 mg/kg/day for 4 weeks significantly lowered serum lipid, hepatic cholesterol (TC), and TG levels and accelerated the excretion of bile acids in hyperlipidemic rats [15]. In addition, Tong had a case report [16] showing that the oral decoction boiled containing 30 g PC caused gastrointestinal adverse reaction.

2.3. Semen Cassia (Jue Ming Zi). Semen Cassia is the ripe seed of *Senna obtusifolia* Linn. (Fabaceae) or *Cassia tora* Linn. (Leguminosae). It clears away the liver fire to improve eyesight and moistens the intestines to relax the bowels. Proteins and anthraquinone glycosides, the active components of Semen Cassiae, displayed a hypolipidemic effect, mainly due to inhibiting cholesterol absorption, synthesis, and HMG-CoA reductase expression [17]. Lin and Xiong found that the extracts from Semen Cassia administrated at oral dosages of 8 mg, 15 mg, and 25 mg/kg/day for 35 days significantly decreased TC, TG, and LDL-c and increased HDL-c in hyperlipidemic rats [18]. Li et al. showed that the administration with extracts from Semen Cassia for 1-week, at 180 mg/kg/day, significantly decreased the levels of TC, TG, and LDL-c in the mice injected intraperitoneally with 75% fresh yelkfluid [19]. Luo et al. documented that the administration with the total anthraquinone from Semen Cassia, at oral dosages of 0.1 g, 0.2 g, and 0.4 g/kg for 2 months, remarkably reduced the serum concentration of TC, TG in Sprague Dawley (SD) rats administrated with alcohol, at 12.5 mL/kg for the 1st month and 11.25 mL/kg for the 2nd and 3rd month for 2 times a day, for 3 months [20]. Zou and Li suggested that the anthraquinone, Semen Cassiae should be used cautiously because of its potential toxicity [21].

2.4. Rhizoma Coptidis (Huang Lian). Rhizoma Coptidis (RC) is derived from the dried root and rhizome of *Coptis chinensis* Franch., Coptis deltoidea C. Y. Cheng et Hsiao, and *Coptis teeta* Wall. It has the role of clearing away heat, eliminating dampness, purging fire, and removing toxin. Its main components include alkaloid and lignans. Among the alkaloids, the alkaloid berberine is an active component for lipid lowering. Zhou et al. found that berberine administrated, at oral dosages of 75 mg, 150 mg, and 300 mg/kg/day for 16 weeks, had a favorable effect in lowering serum TG, TC, and LDL-c and increasing HDL-c [22]. Chang et al. demonstrated that berberine injected intraperitoneally at 200 mg/kg/day for 16 weeks significantly decreased the serum TC, LDL-c levels and hepatic cholesterol in male SD rats treated with high-fat diet (HFD) for 8 weeks and also upregulated LDLR mRNA expression and suppressed HMGR

Table 1: The most frequently used single Chinese herbs for dyslipidemia.

Number	Herbs	Dosage/administration/time	Effects	Components	References
1	Rheum officinale (Da Huang)	Human: powder, 5 g/day, Po, 24 weeks; Db/db mice: rhein, 150 m/kg/day, Po, 12 weeks	TG↓ TC↓ LDL-C↓	Anthraquinones	[7, 8]
2	Rhizoma ploygoni cuspidate (Hu Zhang)	Rabbits: polydatin, 25 mg, 50 mg, 100 mg/kg/day Po, 3 weeks; SD rats: resveratrol 30, 70 mg/kg Po, 4 weeks	TG↓ TC↓ LDL-C↓	Polydatin, resveratrol, and emodin	[12, 13, 15]
3	Semen Cassia (Jumingzi)	SD rats: extracts, 8, 15, 25 mg/kg Po, 35 days; SD rats: anthraquinones, 0.1, 0.2, 0.4 g/kg Po, 2 months	TG↓ TC↓ LDL-C↓	Anthraquinones, protein	[18, 20]
4	*Coptis chinensis* (Huanglian)	Human: berberine, 500 mg tid Po, 12 weeks; SD rats: berberine, 200 mg/kg/day ip, 16 weeks; SD rats: berberine 75 mg, 150 mg, 300 mg/kg/day po 16 weeks	TC↓ LDL-C↓ TG↓ HDL-c↑	Alkaloid berberine	[22–24]
5	*Scutellaria baicalensis* (Huangqin)	SD rats: SSTF, 75 mg, 150 mg/kg Po, 25 days;	TC↓ LDL-C↓ TG↓ HDL-c↑	Flavonoid	[25]
6	*Gynostemma pentaphylla* (Jiaogulan)	Mice: powder, 250 mg/kg, Po, 4-day; SD rats: extract, 50 mg, 200 mg/kg/day, Po, 4 weeks	TG↓ TC↓ LDL-C↓	Gypenoside	[26, 27]
7	Radix Puerariae (Gegen)	Wistar rats: puerarin, 50 mg/kg/day ip, 30 days; ovariectomized rats: flavones, 100 mg/kg/day, po, 5 weeks	TC↓ LDL-C↓ TG↓ HDL-c↑	Puerarin	[28, 29]
8	Fructus crataegi (Shan zha)	Human: aqueous extracts, 3.6 g/kg, Po, 3 months; rats: ethanol extracts, 30 mg, 100 mg/kg/day. Po., 4 weeks	TG↓ TC↓ LDL-C↓	Flavonoids, triterpenic acids	[30, 31]
9	Red yeast rice (Hongqu)	Human: rice, 600 mg/day, Po, 8 weeks; human: rice, 1.2 mg, po, 6 months–1 year	TC↓ LDL-C↓ TG↓	Lovastatin, sterols, Isoflavones and isoflavone glycosides, and MUFA	[32, 33]
10	Rhizoma chuanxiong	Rats: ligustrazine, 20 mg, 80 mg/kg, Po, 6 weeks; rabbits: ligustrazine, 75 mg, 150 mg/kg/day, Po, 12 weeks	TG↓ TC↓ LDL-C↓	Lactones, total alkaloids	[34, 35]
11	Radix salvia miltiorrhizae (Danshen)	Rats: extracts, 50, 100, 150 mg/kg/day, Po, 4 weeks; human: tanshinone IIA, 80 mg/day, ivgtt. 14 days	TC↓ LDL-C↓ TG↓ HDL-c↑	Tanshinone IIA	[36, 37]
12	Turmerone (Jianghuang)	Hamsters: curcumin, 0.05 g/100 g, Po, 10 weeks; SD rats: curcumin, 40, 80, 160 mg/kg, Po, 4 weeks	TC↓ LDL-C↓ TG↓ FFA↓ HDL-c↑	Curcumin	[38, 39]
13	Rhizoma alismatis (Zexie)	Human: powders, 10 g/day, Po, 2 weeks; SD rats: extracts, 0.3 mL/day, Po, 21 days	TC↓ LDL-C↓ TG↓	Triterpenes	[40, 41]
14	Plantain seed (Cheqianzi)	Human: polysaccharides, 14 g/day, Po, 8 weeks; rats: powder, 15 g/kg, Po, 12 weeks; pig: plantain seed, 7.5, 10 g/100 mg, po, 4 weeks	TC↓ LDL-C↓ TG↓	Polysaccharides	[42–44]
15	Folium nelumbinis (Heye)	SD rats: aqueous extracts, 400 mg/kg/day, Po, 6 weeks; mice: flavonoids, 50, 200 mg/kg/day, Po, 28 days	TC↓ LDL-C↓ TG↓	Total flavonoids, alkaloid	[45, 46]
16	Radix Astragali (Huangqi)	Rat: extracts, 0.4%, 0.8%, Po, 5 weeks; rat: polysaccharides, 40, 100 mg/kg/day, Po, 40 days	TC↓ LDL-C↓ TG↓ HDL-c↑	Polysaccharides, flavonoid, and saponin	[47, 48]
17	Radix Ginseng (Renshen)	Mice: ginsenoside, 2 mg/kg/days, Po, 90 days; rats: ginsenoside Rb, 50 mg, 100 mg, 200 mg/kg/day, Po, 12 days	TC↓ LDL-C↓ TG↓ HDL-c↑	Ginsenoside, ginseng, and polysaccharides	[49, 50]
18	Radix Polygoni Multiflori (Heshouwu)	Rats: extracts, 12, 24 mg/kg/day, Po, 4 weeks; rats: EAEF, 30, 60 mg/kg/day, Po, 28 days	TC↓ LDL-C↓ TG↓ HDL-c↑	Anthraquinones, polysaccharides	[51, 52]

gene expression [23]. Hu et al. found that Caucasian obese human subjects were given 500 mg berberine orally 3 times a day for 12 weeks showing that the blood lipid was significantly reduced and triglyceride and cholesterol were decreased by 23% and 12.2%, respectively [24]. A meta-analysis concerning 11 randomized controlled trials and 874 participants showed that the berberine produced a significant reduction in TC, TG, and LDL-c [53]. In addition, Zhang et al. reported that berberine at more than 4 g (overdose) resulted in some adverse reactions, such as drug eruption, allergic reactions, dizziness, and shock [54].

2.5. Scutellaria baicalensis (Huang Qin). *Scutellaria baicalensis* is derived from the dried root of *Scutellaria baicalensis* Georgi. It clears away heat, eliminates dampness, purges fire, removes toxin, and stops bleeding. Flavonoid compound is an effective lipid-lowering component in *Scutellaria baicalensis* [55]. Liu et al. found that *Scutellaria baicalensis* stem-leaf total flavonoids (SSTF) administrated at oral dosages of 75 mg, 150 mg/kg/day for 25 days in type 2 diabetic rats with hyperlipidemia significantly reduced the serum TG, TC, and LDL-c levels and increased HDL-c [25]. 0.05% *Scutellaria baicalensis* radix extract was added to the diet in hyperlipidemia rats for 4 weeks, showing decrease of TG and TC of the bioflavonoids group [56]. SSTF was administrated at oral dosages of 25 mg, 50 mg, and 100 mg/kg/day in hyperlipidemia rats for 20 days, indicating that SSTF significantly reduced the serum TC, TG, and LDL-c levels and increased HDL-c and the activity of lecithin cholesterol acyltransferase (LCAT) [57].

2.6. Gynostemma Pentaphylla (Jiao Gu Lan). Gynostemma Pentaphylla (GP) is derived from the dried root and rhizome of *Gynostemma pentaphyllum* (Thunb.) Makino. It clears away heat, removes toxin, relieves cough, and eliminates phlegm. Gypenoside is an active component of GP [58]. Studies have found that the lipid-lowering effect of GP was related to inhibiting fat cells producing free fatty acid and synthesizing neutral fat [26, 59, 60]. GP administrated at oral dosages of 250 mg/kg for 4 days significantly reduced TC (by 33%), TG (by 13%), and LDL-c (by 33%) in the obese Zucker fatty diabetic rat model [26]. Zhou et al. established the hyperglycemia rat model with high-fat diet for 6 weeks and then treated them with high dose (200 mg/kg/day) or low dose (50 mg/kg/day) of GP for 4 weeks and founded that GP could decrease the concentration of serum LDL-c, TC, and TG levels remarkably and raise the concentration of HDL-c [27].

2.7. Radix Puerariae (Ge Gen). Radix Puerariae is the dried root of *Pueraria lobata* (Willd.) Ohwi. (Fabaceae). It clears away heat, purges fire, and removes toxin in the theory of Compendium of Materia Medica. Isoflavone is the active compound of Kudzuvine root, such as puerarin, isoflavoues aglycone, daidzin [61]. Yan et al. found that puerarin administrated at oral dosages of 300 mg/kg/day for 4 weeks significantly reduced the serum and hepatic cholesterol levels of hyperlipidemia rats [62]. Experimental hyperlipidemia rats were injected intraperitoneally puerarin (50 mg/kg/day) for 30 days, showing that the plasma TG, TC, and LDL-c significantly reduced and HDL-c increased [28]. Furthermore, oral administration of Kudzuvine root flavones at 100 mg/kg/day for 5 days was reported to enhance hepatic lipid metabolism in ovariectomized rats [29]. Patients with puerarin injections may cause certain adverse effects, such as allergic responses, bloody stool, and backache [63].

2.8. Fructus Crataegi (Shan Zha). Fructus crataegi (FC) is derived from the dried mature fruit of *Crataegus pinnatifida* Bunge. var. major N.E.Br. (Rosaceae) or *Crataegus pinnatifida* Bunge. (Rosaceae). FC is used to dissipate food accumulation, to improve blood circulation, and to disperse blood stasis. Flavonoids and triterpenic acids are the main active hypolipidemic components of FC [64]. FC aqueous extracts given at an oral dosage of 3.6 g/day for 3 months were demonstrated to lower blood TC, TG, and LDL-c in 45 hyperlipidemic volunteers [30]. 80% ethanolic extract administrated at oral dosages of 30, 100 mg/kg/day for 4 weeks in hyperlipidemic rats markedly reversed the increased plasma TC and HDL-c levels [31]. A study on mice that were fed with high-fat diets following the oral administration of FC extracts at a dosage of 250 mg/kg/day for 7 days *in vivo* indicated that FC's lipid-lowering action may be related to increased levels of liver PPARα [65].

2.9. Fermentum Rubrum (Red Yeast Rice). Fermentum Rubrum, popularly known as red yeast rice (RYR) which is the fermented product of Monascus purpureus on rice. It is composed of 13 kinds of natural statins, unsaturated fatty acids, ergosterol, amino acids, flavonoids, alkaloid, trace element, and so forth. 79 patients with baseline LDL-c level of 5.28 mmol/L received a twice daily dose of red yeast rice (600 mg) for 8 weeks in a randomized, double-blind, placebo-controlled study, which found that this therapy could reduce LDL-c by 27.7%, TG by 21.5%, and TC by 15.8% [32]. 72 patients with idiopathic persistent nephritic syndrome with secondary dyslipidemia were randomly given Monascus purpureus Went rice at 600 mg twice one day orally, which significantly reduced serum cholesterol after 6 months and 1 year [33]. XueZhiKang capsule is the extract of red yeast rice. In china, scholars made a systematic review on the clinical randomized controlled trials for treating hyperlipidemia with Xuezhikang, which included 22 randomized trials and a total of 6520 participants, and showed that xuezhikang remarkably lowered TC, TG, and LDL-C compared with theinositol nicotinate [66]. Animal safety evaluations indicate that RYR does not cause any toxic effects in albino rats [67]. However, dyslipidemia patients treated with RYR (1200 mg/day) experienced a few nonserious side effects, such as heartburn, flatulence, dizziness, and gastrointestinal discomfort [63].

2.10. Rhizoma Chuanxiong (Chuang Xiong). Rhizoma chuanxiong (RC) is the dried rhizome of *Ligusticum chuanxiong* Hort. (Umbelliferae). It promotes blood and qi circulation, expels wind, and alleviates pain.

RC contains a variety of esters and alkaloids. Ligustrazine in RC plays an important role in contributing to

hypolipidemic effects of RC [68]. Ligustrazine given at an oral dosage of 20 mg, 80 mg/kg/day in atherosclerosis rats decreased TG levels (by 65.2% and 76.7%), TC (by 53.2% and 77.9%), and LDL-c (by 71.2% and 79%) levels [34]. Tetramethylpyrazine administered at 75 mg, 150 mg/kg/day for 12 weeks in atherosclerosis rabbits, significantly reduced the serum TC, TG, and LDL-c levels [35]. The oral administration of RC causes headaches and injection of ligustrazine can also cause bleeding and allergic responses in certain cases [69].

2.11. Radix Salviae Miltiorrhizae (Dan Shen). Radix Salviae Miltiorrhizae (RSM) is derived from the root and rhizome of *Salvia miltiorrhiza* Bge. (Lamiaceae). It removes blood stasis and promotes blood circulation, relieves pain, regulates menstruation, removes heat from the heart, and relieve restlessness. Dan Shen is widely used to treat patients with coronary artery disease in China. Tanshinone is the main effective component in RSM [70]. Aqueous extracts of RSM given at oral dosages of 50 mg, 100 mg, and 150 mg/kg/day for 4 weeks significantly decreased TC and TG levels and increased HDL-C serum levels in hyperlipidemic rats [36]. Tanshinone IIA (T-IIA) sulfonate intravenous injected (80 mg dissolved in 250 mL 0.9% salt water) at 80 mg/day for 14 days in patients with diabetes mellitus decreased the serum TG, TC, and LDL-c obviously [37]. In addition, human HepG2 cells treated with T-IIA for 24 h exerted a dose-dependent inhibitory effect on ApoB secretion together with triglyceride [71]. RSM may cause abdominal discomfort following long-term administration and also results in internal tissue bleeding when used in combination with aspirin or warfarin [72].

2.12. Rhizoma Curcumae Longae (Jiang Huang). Rhizoma curcumae longae (RCL) is derived from the root and rhizome of *Curcuma longa* L. It removes blood stasis, promotes the circulation of Qi, regulates menstruation, and relieves pain. Curcumin is the main component in RCL [73]. Curcumin (0.05 g/100 g diet) supplementation on a high-fat diet (10% coconut oil, 0.2% cholesterol, wt/wt) fed to hamsters for 10 weeks significantly lowered the levels of free fatty acid (FFA), TG, TC, and LDL-c and elevated the levels of HDL-c and apolipoprotein (apo) A-I and paraoxonase activity in plasma [38]. Curcumin administrated at dosages of 40 mg, 80 mg, and 160 mg/kg/day for 4 weeks in hyperlipidemia rats significantly reduced the serum and hepatic TC, TG, and FFA and increased the HDL-c [39]. *In vitro*, curcumin at 5 uM concentration completely prevented LDL oxidation by $CuS0_4$ [74]. The curcumin acting on the low density lipoprotein receptor (LDLR) expression which is measured by Fluo-Microscopy and Fluorescence Flow Cytometric Methods in HepG2 cell obviously upregulated the expression of LDLR [75].

2.13. Rhizoma Alismatis (Ze Xie). Rhizoma alismatis (RA) is derived from the dried stem tuber of *Alisma orientale* (Sam.) Juzep. (Alismataceae). RA promotes diuresis to resolve dampness and expel heat. Triterpenes are the main active components from RA, which exerts its hypolipidemic effects by inhibiting the absorption and synthesis of cholesterol and improving lipid metabolism [76]. The powders of RA administered at oral dosages of 10 g/day for 2 weeks in healthy volunteers reduced the TC, LDL-C, and TG [40]. The oral administration of aqueous and alcoholic RA extracts at 0.3 mL/day for 21 days resulted in significant decreasing in serum TG, and TC, while increased the HDL-c and improved the artheriosclerosis index (AI) in hyperlipidemia SD rats [41]. The adverse effects of RA are correlated with hepatotoxicity following over dosage [42].

2.14. Semen Plantaginis (Che Qian Zi). Semen plantaginis is the ripe seed of *Plantago asiatica* L. or *Plantago depressa* Willd. Semen plantaginis clears heat, causes dieresis, excretes dampness, improves eyesight, and eliminates phlegm. The polysaccharides of Semen plantaginis (PSP) not only have the aperients effect but also the lipid-lowering role [77]. In a multicenter, double-blind, placebo-controlled, parallel, and randomized trial conducted in primary care-clinics in Spain, France, and Holland, mild-moderate hypercholesterolaemic patients (age range: 43–68 years) received 14 g/d of the soluble fibre Plantago ovate (PO)-husk ($n = 126$) for 8 weeks. Po-husk reduced plasma LDL-C by −6%, total cholesterol (TC) by −6%, triglycerides (TG) by −21.6%, and apolipoprotein (Apo) B-100 by −6.7% [78]. Wang et al. found that Plantain seed at dosage of 15 g/kg for 12 weeks can decrease content of lipid and strengthen superoxide dismutase (SOD) activity [43]. Plantain seed administered at oral dosages of 7.5 g, 10 g/100 g for 4-weeks in male Hartley guinea pig significantly reduced the level of TC and LDL-c [44].

2.15. Folium Nelumbinis (He Ye). Folium Nelumbinis is the dried leaf of *Nelumbo nucifera* Gaertn. It is used to clear away summerheat, to lift the lucid yang, to cool the blood, and to stop bleeding. The total alkaloids and flavonoids in Lotus leaves are the main active components of He Ye [79]. Aqueous extracts of Lotus leaves administered at an oral dosage of 400 mg/kg/day for 6 weeks were demonstrated to lower serum TC, TG, and LDL-C levels in rats fed a high-fat diet [45]. The flavonoids extracts of Lotus (50 mg and 200 mg/kg) were orally administered once a day for 28 days in rats, showing that the serum TC, TG, and LDL-c levels were significantly decreased, whereas serum HDL-c level was increased [46]. As demonstrated in the livers of mice that were fed high-fat diets, the mechanisms of action of Lotus leaves may be associated with suppressed expression of FAS, acetyl-CoA carboxylase, and HMG-CoA reductase and the increased phosphorylation of AMP-activated protein kinase [47].

2.16. Radix Astragali (Huang Qi). Radix Astragali is the dried root of *Astragalus propinquus* (Fisch.) Bge. var. mongholicus (Bge.) Hsiao (Fabaceae) or Astragalus membranaceus (Fish.) Bge. (Fabaceae). It replenishes the qi to consolidate superficies and promotes diuresis to relieve edema. MMR polysaccharides, flavonoids, and sponins are the main active components of membranous milkvetch root (MMR) [48]. Astragalus Mongholicus extracts at 0.4% and 0.8% for 5 weeks in rats maintained on a high-cholesterol diet significantly

reduced the serum of TG, TC, and LDL-c levels and increased the HDL-c levels and reduced levels of lipid peroxidation [80]. Polysaccharides from Astragalus administered at an oral dosage of 40 mg, 100 mg/kg/day in hyperlipidemia rats for 40 days obviously reduced the serum TC, TG, LDL-c, and MDA levels and increased HDL-c levels [81]. The hypolipidemic mechanisms of MMR polysaccharides *in vivo* may be associated with the increased expression of LDLR and 7-hydroxylase mRNAs and the decreased expression of HMG-CoA reductase mRNA in the liver [82]. The injection of Radix Astragali may cause nausea and allergic response [83].

2.17. Radix Ginseng (Ren Shen). Radix Ginseng is derived from the dried root and rhizome of *Panax ginseng* C.A. Mey. (Araliaceae). It reinforces vital energy, restores the pulse, treats exhaustion, reinforces the spleen to benefit the lungs, promotes the production of body fluids, and calms the mind. Ginseng saponins and polysaccharides are the main active components of Radix Ginseng [84]. Ginseng saponins intragastric administered at an oral dosage of 2 mg/kg/day for 90 days in C57/BL-ApoE gene knockout hyperlipidemia rats can reduce the levels of plasma TC, TG, and LDL-c [49]. Ginseng saponin is divided into Rb1, Rb2, RC, Rd, Re, and Rl [85]. Ginseng saponin Rb administered at an oral dosage of 50 mg, 100 mg, and 200 mg/kg/day in hyperlipidemia rat for 12 days significantly reduced the TG, TC, and LDL-c levels in serum and liver [50]. In addition, Compound k (CK) is a major intestinal metabolite of ginsenosides derived from ginseng radix. *In vitro*, CK significantly activated the AMP-activated protein kinase (AMPK) to affect the lipid metabolism in insulin-esistant HepG2 human hepatoma cells [86]. Ginseng saponins have poor bioavailability following oral administration. Although Ginseng is very safe for oral administration, an overdose or long-term administration of Ginseng may cause the neurotoxicity, cardiotoxicity or allergic reaction [69].

2.18. Radix Polygoni Multiflori (He Shou Wu). Radix Polygoni Multiflori (RPM) is derived from the dried root tuber of *Fallopia multiflora* Thunb. (Polygonaceae). RPM has been used in both raw and prepared pharmaceutical forms. Raw RPM prevents the recurrence of malaria, eliminates toxic materials, moistens the intestine, and relaxes the bowels. Prepared RPM blackens the hair and beard, strengthens the muscles and bones, improves the essence of the blood, and nourishes the liver and kidneys. RPM exerts its hypolipidemic effects primarily by targeting the gastrointestinal tract and inhibiting the absorption of cholesterol [87]. RPM extract administered at an oral dosage of 12 mg and 24 mg/kg/day for 4 weeks in hyperlipidemic rats reduced the serum levels of TC, TG, and LDL-c [51]. Wang et al. found the ethl acetate extracting fraction (EAEF) and stilbene glycoside from the tube of Polygonum multiflorum administered orally at dose of 30 and 60 mg/kg/day for 28 days could reduce the serum TC, TG, and LDL-c levels in hyperlipidemia rats [52]. As demonstrated in experiments with Bel-7402 cells, stilbene glucoside may be a key active component of RPM and involved in both inhibiting cholesterol synthesis and increasing the expression of low-density lipoprotein receptor (LDLR) mRNA [88]. Li et al. found that RPM extracts could regulate the lipid content within liver cell better than RPMP (Radix Polygoni Multiflori Praeparata), but RPMP displayed better effects than RPM in lipid regulation in the circulatory system [89]. Clinical reports have revealed that RPM exhibits hepatotoxicity, allergic responses, and gastrointestinal hemorrhage following chronic treatment [90, 91].

3. Perspective

During the past 10 years, the studies on lipid-lowering therapy with Chinese herbs have achieved many progresses to some extent, but some limits are also existed: (1) although the effects of Xuezhikang (extract of red yeast rice) on lowering cholesterol and LDL-c were evidenced in multicenter, large sample, and randomized clinical trials [66, 92], most clinical trials on dyslipidemia with TCM did not show enough power to identify the definite effects due to small samples or unemployment of multicenter, large samples, and randomized design; (2) because of very complicated compounds contained in one herb, even in an extract of one herb, it is a very tough work to clarify the mechanism of TCM for treating dyslipidemia and interaction with western medicines, which lead to some obstacles in clinical application in combination with statins or other chemical agents; and (3) due to different herb has different active compound and different property, which has been taken as *Han* (Cold), *Re* (Heat), *Wen* (Warm) and *Liang* (Cool) according to TCM theory, it is hard in clinical practice to optimize its benefit effects or reduce adverse effects for patients with hyperlipidemia.

Along with a long-term use of statins in combination with other hypolipidemic drugs or alone, the adverse reactions frequently occurred about statins at domestic or abroad. TCM has been widely used in China for more than 2000 years. Screening highly efficient hypolipidemic agents from TCM with fewer adverse effects has attracted more attention, and the mechanisms of TCM for hyperlipidemia become a hot topic in cardiovascular diseases research field recently.

As mentioned above, the TCM has some beneficial effects on the treatment of patients with dyslipidemia and has less adverse effects compared with chemical agents. The advantages and disadvantages of TCM, however, needed to be confirmed in the future clinical trials according to the concept of evidence based medicine. Along with the development of modern scientific techniques, which can be applied in the TCM studies, it is becoming easier to identify how many component one herb contained and which component is a main component for treating dyslipidemia. As we all know, the TCM was used in clinical practice in the formula manner and demonstrated that many formulas and herbs have some favorable effects for dyslipidemia. Therefore, to develop new agents with effectiveness and safety from TCM is a promising way for prevention and treatment of patients with dyslipidemia and even then with cardiovascular diseases.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Ming Guo and Yue Liu contributed equally to this paper and are cofirst authors.

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Research Article

Intestinal Transportations of Main Chemical Compositions of Polygoni Multiflori Radix in Caco-2 Cell Model

Jie Yu, Na Li, Pei Lin, Yunfei Li, Xiaojian Mao, Getuzhaori Bao, Wen Gu, and Ronghua Zhao

Yunnan University of Traditional Chinese Medicine, Kunming, Yunnan 650500, China

Correspondence should be addressed to Ronghua Zhao; kmzhaoronghua@hotmail.com

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Context. Polygoni Multiflori Radix (PMR) is originated from the root of *Polygonum multiflorum* Thunb. and used in oriental countries for centuries. However, little researches pay close attention to the absorption of its major constituents. *Objective*. Transepithelial transport of TSG, RL, PL, and four anthraquinones is carried out. *Materials and Methods*. Caco-2 cell monolayer, which represented a well-established model for the study of intestinal transport of nutrients and xenobiotics, was used in this paper. *Results*. The apparent permeability coefficients (P_{app}) in the Caco-2 cell monolayers were TSG (2.372×10^{-9}) < EG (2.391×10^{-9}) < EN (2.483×10^{-9}) < PL (4.917×10^{-9}) < RN (1.707×10^{-8}) < RL (1.778×10^{-8}) < AE (1.952×10^{-8}). Thus, RN, RL, and AE were considered partly absorbed, while other constituents were hardly absorbed. *Discussion and Conclusion*. Glycosides showed poor permeabilities than aglycones. In the meantime, TSG and EN gave out poor recovery rates in this assay, which indicated that TSG and EN may accumulate or metabolise in the Caco-2 cells. *In silico* prediction indicated that Gibbs energy ($r = 0.751$, $p < 0.05$) and heat of form ($r = 0.701$, $p < 0.05$) were strongly positively correlated with P_{app}.

1. Introduction

Polygoni Multiflori Radix ((PMR), heshouwu in Chinese) and Polygoni Multiflori Radix Praeparata ((PMRP), zhi-heshouwu in Chinese) are originated from the root of *Polygonum multiflorum* Thunb. (Polygonaceae) and used in the treatment of nonalcoholic fatty liver disease (NAFLD) and hyperlipidemia in oriental countries for centuries (Figure 1).

Preliminary researches [1–4] indicate that Polygoni Multiflori Radix mainly contains stilbene glycosides (2,3,5,4′-tetrahydroxystilbene-2-*O*-*β*-D-glucoside (TSG), resveratrol (RL), polydatin (PL), and others) and anthraquinones (emodin (EN), rhein (RN), aloe-emodin (AE), emodin-8-*O*-*β*-D-glucopyranoside (EG), and others). In our previous research, TSG displayed the most important role in the total cholesterol (TC) lowering effect among all the chemical constituents of *Polygonum multiflorum*. The quality of PMR was evaluated by the contents of TSG and anthraquinones as regulated by the Pharmacopoeia of the People's Republic of China, 2010 edition [5].

However, little researches pay close attention to the absorption of these major constituents of PMR. In this research, transepithelial transport of TSG, RL, PL, and the four anthraquinones is carried out using human Caco-2 cell monolayer as a model system. Caco-2 cells, derived from a human colon adenocarcinoma, spontaneously differentiate after reaching confluence in culture, exhibiting several morphological and functional characteristics of mature enterocytes. Caco-2 cell monolayers represent a well-established model for the study of intestinal transport of nutrients and xenobiotics and are widely used in pharmacology and toxicology researches [6–10]. This research provided important predictive information regarding the oral bioavailability of TSG, RL, PL, EN, RN, AE, and EG.

2. Materials and Methods

2.1. Caco-2 Cell. Caco-2 cell (the human colon adenocarcinoma cell) was purchased from Kunming Institute of Zoology, Chinese Academy of Sciences, in June 2009.

PMR (a)

PMRP (b)

Figure 1: Photographs of Polygoni Multiflori Radix and its processed products. (a) PMR: *Polygonum Multiflorum* Radix. (b) PMRP: Polygoni Multiflori Radix Praeparata.

2.2. Chemicals. TSG, EN, RN, AE, RL, and PL were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. EG was purchased from the Sichuan Xianxin Biotech Co., Ltd., China. The purities of all the standards were not less than 98%. Propranolol (PR) was used as positive control substance of high permeability. Atenolol (AT) was used as positive control substance of poor permeability. Both PR and AT were purchased from Sigma. Structures of them were listed in Figure 2.

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Invitrogen Corporation. Phosphate buffer solution (PBS), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), L-glutamine, and pyruvic acid sodium salt were of chemical or analytical grade obtained from domestic company. Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. (Deisenhofen, Germany).

2.3. Culture Conditions. Caco-2 cells were cultured in DMEM containing 1 g/L D-glucose, 2 g/L $NaHCO_3$, 165 mg/L pyruvic acid sodium salt, and 150 mg/L L-Glutamine, supplemented with 10% FBS. Cultures were maintained in a humidified incubator with 5% carbon dioxide 95% air at 37°C. 0.25% of trypsin (Sigma, USA) (0.25%) was used to passage cells at 80–90% confluence and seeded at a density of about 1×10^5 cells/mL on a 12-well Transwell (Corning Costar, Cambridge, MA, USA) insert filter (surface area = 1.13 cm^2, pore size = 3 μm). The cells were left to grow for 21 days to reach confluence and differentiation, so that Caco-2 cells were fully differentiated in this assay and used for further experiments. All cells used in this study were between passages 20 and 35.

2.4. Standardized Conditions. The Caco-2 cell system was validated by the transepithelial electrical resistance (TEER) assay (by EVOM2 from the World Precision Instrument Trading Co., Ltd.) and the alkaline phosphatase (ALP) activity difference between the two sides of the membrane. The TEER values >500 Ω/cm were required. The activities of ALP, as a specific brush border formation enzyme marker, in both AP and BL sides were estimated. The ALP_{AP}/ALP_{BL} ratio was calculated.

2.5. Permeability Studies. The stock solutions of all tested compounds were achieved by dissolving them in dimethylsulfoxide (DMSO) at a concentration of 5 mmol/mL. Then they were further diluted with PBS to graded concentrations of 100 μM and 200 μM.

The following experiment was undertaken to measure the flux of the standards. Flux describes the movement of a substance across polarized Caco-2 monolayers either in absorptive (apical → basolateral, AP → BL) or secretary direction (basolateral → apical, BL → AP). Absorption assay was carried out in this research. The monolayers were washed twice with warm transport medium PBS within 30 min and then sequentially incubated once for 30 min at 37°C with PBS. Transport medium (0.5 mL) containing TSG, EN, RN, AE, RL, PL or EG was added to the AP side, while the receiving chamber contained the corresponding volume (1.5 mL) of transport medium. After shaking at 55 r/min for 1.5 h at 37°C in a shaking water bath, samples were collected (0.4 mL and 1.2 mL in 1 der constituents the AP and BL sides, resp.) from both sides of the Caco-2 cell monolayers and immediately frozen, lyophilized, and preserved below −20°C [11].

2.6. HPLC-DAD Analysis of Samples. To determine the concentration of corresponding constituents in both AP and BL sides, the lyophilized samples of AP and BL sides were dissolved in quantitative methanol by ultrasound for 20 min and then filtered by 0.45 μm filtration membranes.

All experiments were performed with Dionex Ultimate 3000 HPLC system (Dionex Technologies, USA), which included a binary pump, an autosampler, a column oven, and a diode array detector plus on-line degasser. Data were analyzed with Chromeleon 6.8.

The separation of samples was achieved on Zorbax SB-C_{18} analytical column (4.6 × 250 mm, 5 μm particle diameter, supplied by Agilent Technologies, USA).

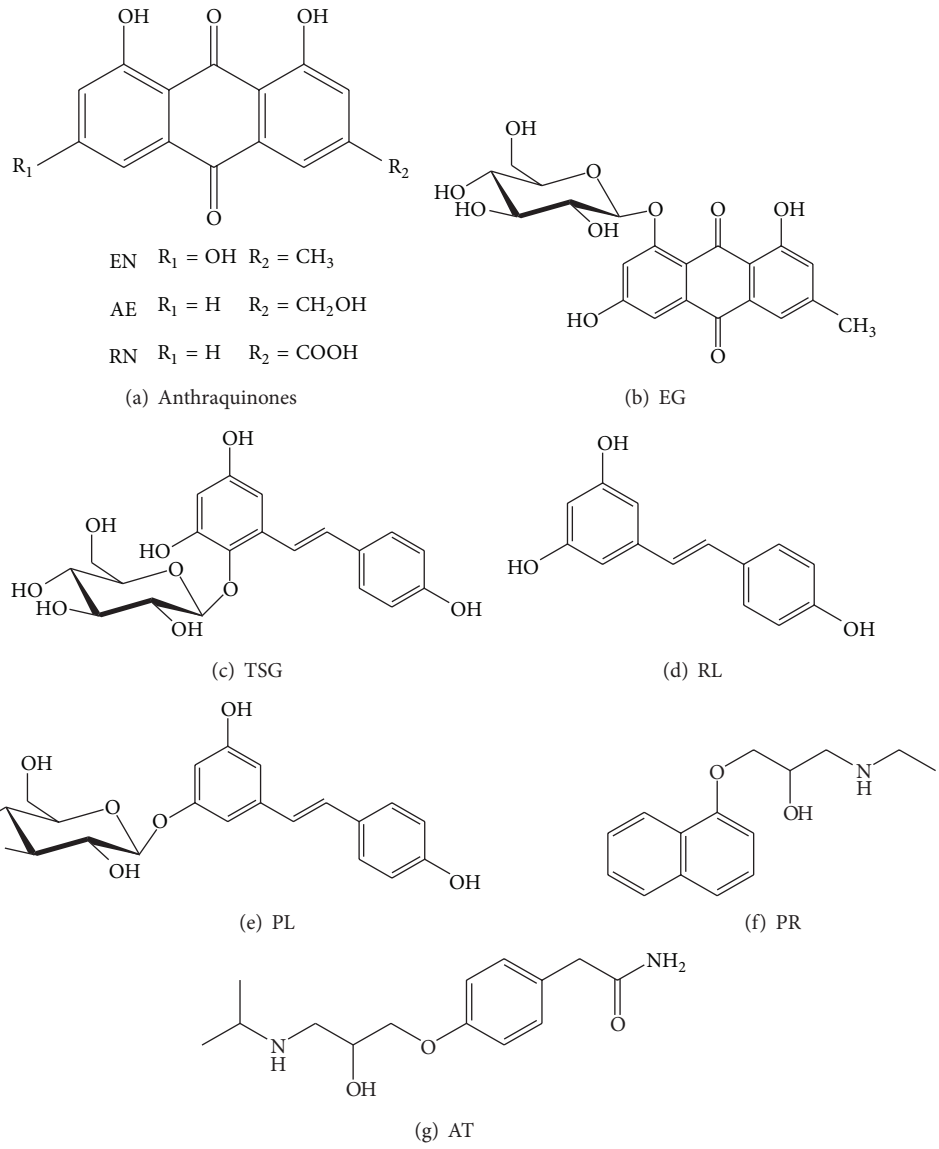

Figure 2: Structures of analytes in this research.

Isocratic and gradient elution with mobile phase consisting of (A) 0.1% H_3PO_4 and (B) methanol was used. Methanol percentage and detection wavelength were listed in Table 1. The oven temperature was set at 30°C and the flow rate was set at 1.0 mL/min.

2.7. Standard Curve. The calibration curves for TSG, EN, RN, AE, RL, and PL were constructed by plotting concentration (Y, μmol) versus peak area (X). The linear equation, linear range, and correlation coefficient were listed in Table 2.

2.8. Calculation of the Apparent Permeability Coefficient (P_{app}) and Recovery Rate. The apparent permeability coefficient (P_{app}) was calculated as follows:

$$P_{app}\,(\mathrm{cm/s}) = \frac{dQ}{dt} \times \frac{1}{60} \times \frac{1}{A} \times \frac{1}{C_0}, \quad (1)$$

where P_{app} was the apparent permeability coefficient in cm/s; dQ/dt was the rate of appearance of the test compound on the receiver side in μmol/min; C_0 was the initial concentration of test compound on the donor side in μmoL/L; and A was the surface area of the Transwell in cm^2.

Meanwhile, the recovery rate of each compound was calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{Total compound in donor and receiver at the end of the experiment } (\mu\text{M})}{\text{Initial compound present } (\mu\text{M})} \times 100\%. \quad (2)$$

2.9. Quantitative Relationships between Physical-Chemical Parameter, Transepithelial Flux, and Recovery Rate. In silico

Table 1: Elution procedure and detection wavelength of high performance liquid chromatography assays.

(a) Isocratic elution procedure, detection wavelength, and R_t of EN, AE, RL, PL, and EG

Sample	Detection wavelength (nm)	B%	R_t (min)
EN	287	75	11.24
AE	254	80	5.042
RL	306	45	8.423
PL	306	40	6.568
EG	280	55	12.11

(b) Gradient elution procedure, detection wavelength, and R_t of TSG and RN

Sample	Detection wavelength (nm)	B%			R_t (min)
		0 min	5 min	10 min	
TSG	320	40	55	70	6.513
RN	254	85	/	90	5.307

Mobile phase A: 0.1% H_3PO_4.
Mobile phase B: methanol.

prediction of physical, thermodynamic, and chemical parameters properties of analytes was made in this research. Basic physical and thermodynamic properties of these analytes, such as molecular weight, boiling point, Gibbs energy, and Henry's Law, were all predicted by ChemDraw Pro. Then we calculated the Log P (by ChemDraw Ultra 8.0), Clog P (by ChemDraw Ultra 8.0, The OSIRIS Property Explorer, and The Bio-Lim., resp.), and solubility (by The OSIRIS Property Explorer) of these constituents.

Relationships between these physical, thermodynamic, and chemical parameters and P_{app} and recovery rate were assessed with Pearson's correlation coefficient. Results were classified into two significance levels using the p values of 0.05 and 0.01.

3. Results

3.1. The Integrity and Polarity of Caco-2 Cell Monolayer Membrane. The integrity of Caco-2 cell monolayer was evaluated by TEER value between AP side and BL side on alternate days through the 21-day culture period (Figure 3). The TEER value was rising continuously from the beginning to the 18th day of this research. The TEER value tended to be stable from the 18th day (551.7 ± 76.87) to the 20th day (578.4 ± 80.40), which indicated that a tight Caco-2 monolayer had been formed.

The ALP_{AP}/ALP_{BL} ratio was increasing from the 2nd day to the 14th day and maintained in the maximum level from the 14th day to the 21st day (Figure 4). The activity of alkaline phosphatase in the AP side was almost ten times than that in BL side, which could further confirm the Caco-2 cells differentiation.

3.2. The P_{app} Values and Recovery Rate of TSG, EN, RN, AE, RL, PL, and EG through the Caco-2 Monolayer. The concentrations of analytes in AP and BL sides were analyzed after 90 mins incubation by HPLC-DAD equipment. The recovery rates and P_{app} values of seven compounds were listed in Table 3 and Figure 5.

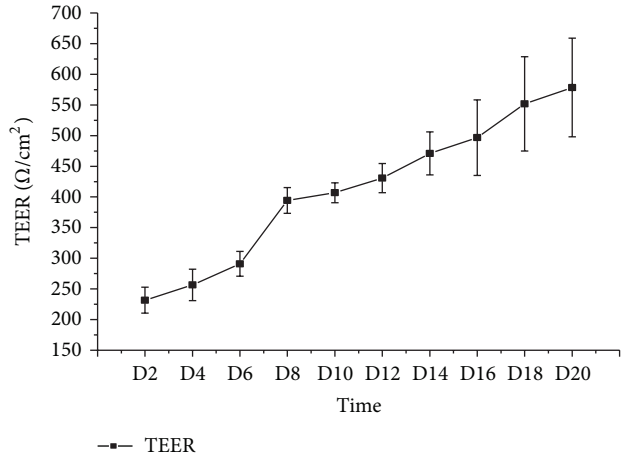

Figure 3: The value of transepithelial electrical resistance (TEER) (Mean ± SD, $n \geq 6$, Ω/cm^2).

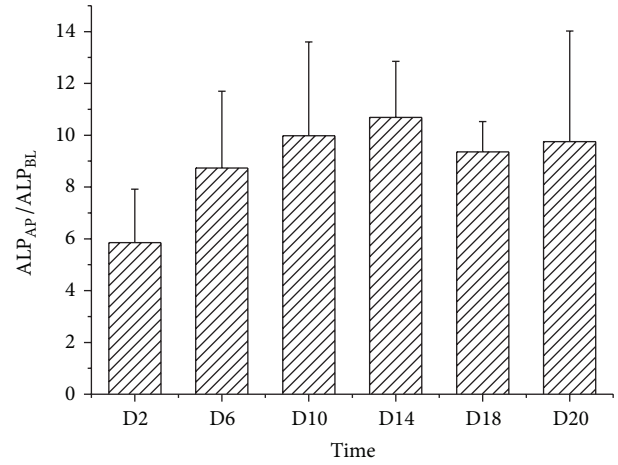

Figure 4: The alkaline phosphatase ratio (apical side to basolateral side, ALP_{AP}/ALP_{BL}) in this assay (Mean ± SD, $n \geq 6$).

RN, PR, and AT showed favorable recovery rates around 100%. AE, RL, PL, and EG exhibited middle extent (30~80%) recovery. EN and TSG gave out very poor recovery rate of only 14.48 ± 2.504% and 17.39 ± 1.600%. Usually, the poor recovery may indicate problems with poor solubility, binding of the compound to the plate, metabolism by the Caco-2 cells, or accumulation of the compound in the cell monolayer. We predict that large partitions of EN and TSG may accumulate or metabolise in the Caco-2 cells. However, further validation must be carried out to confirm this hypothesis.

Small P_{app} of TSG, EG, and PL (smaller than that of AT) reflected that they were hardly absorbed by human intestinal epithelial cells. This conformed with our consensus that glycosides were poorly absorbed than their aglycone due to the low fat solubility and low hydrophobicity. EN also showed small P_{app} probably due to its low recovery rate mentioned above. RN, RL, and AE showed middle P_{app} between atenolol

Table 2: The linear equation, linear range, and correlation coefficient of nine analytes ($n = 6$).

Sample	Linear equation	Related coefficient	Linear range (μmol)
TSG	$Y = 29836X + 0.1657$	0.9987	4.305×10^{-6}–3.444×10^{-4}
EN	$Y = 18775X + 0.0051$	1	2.664×10^{-5}–1.066×10^{-3}
RN	$Y = 22458X + 0.2069$	0.9992	2.322×10^{-5}–9.288×10^{-4}
AE	$Y = 23601X - 0.1580$	0.9996	2.664×10^{-5}–1.066×10^{-3}
RL	$Y = 50841X + 0.2434$	0.9993	1.733×10^{-5}–3.466×10^{-4}
PL	$Y = 27231X + 0.1174$	0.9999	6.892×10^{-6}–6.892×10^{-4}
EG	$Y = 25907X - 0.0244$	0.9999	7.040×10^{-6}–2.534×10^{-3}

Table 3: The apparent permeability coefficient (P_{app}) values (Mean, $n \geq 3$, cm/s) and recovery rates (Mean ± SD, $n \geq 3$) of the seven analytes.

Compound	TSG	EN	RN	AE	RL	PL	EG	PR	AT
P_{app} value (cm/s)	2.372×10^{-9}	2.483×10^{-9}	1.707×10^{-8}	1.952×10^{-8}	1.778×10^{-8}	4.917×10^{-9}	2.391×10^{-9}	6.075×10^{-8}	1.668×10^{-8}
Recovery rate (%)	17.39 ± 1.600	14.48 ± 2.504	96.96 ± 7.377	42.36 ± 6.323	62.39 ± 6.210	59.94 ± 9.90	33.95 ± 7.06	117.1 ± 8.85	125.2 ± 33.60

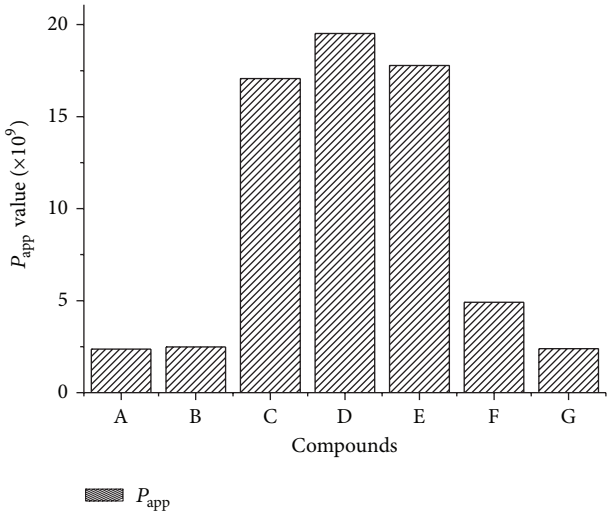

Figure 5: The apparent permeability coefficient (P_{app}, cm/s) values of TSG, EN, RN, AE, RL, PL, and EG through the Caco-2 monolayer. A: 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside (TSG), B: emodin (EN), C: rhein (RN), D: aloe-emodin (AE), E: resveratrol (RL), F: polydatin (PL), and G: emodin-8-*O*-β-D-glucopyranoside (EG).

(known human absorption of 50%) and propranolol (known human absorption of 90%) [12, 13]. Thus, RN, RL, and AE were considered partly absorbed. On the other hand, all the compounds were catalyzed to be passive-transported because the concentrations of each compound in the AP side were all higher than in the BL side.

3.3. Relationships between Physical-Chemical Properties Values and P_{app} and Recovery. Basic physical and thermodynamic properties of these analytes, such as molecular weight, boiling point, Gibbs energy, Henry's Law, Log *P*, Clog *P*, and Log *S*, were listed in Table 4.

Boiling point and melting point predicted the normal boiling and melting point for the structure, respectively. Critical temperature predicted the temperature (reported in K) above which the gas form of the structure cannot be liquefied, no matter the applied pressure. Critical pressure predicted the minimum pressure (reported as bars) that must be applied to liquefy the structure at the critical temperature. Critical volume predicted the volume occupied (reported in cm^3/mol) at the compound's critical temperature and pressure. Heat of formation was the prediction of the heat of formation for the structure (reported in kJ/mol at 1 atm and 298.15 K). Gibbs energy was the prediction of the Gibbs free energy for the structure (reported in kJ/mol at 1 atm and 298.15 K).

The log *P* value of a compound, which is the logarithm of its partition coefficient between *n*-octanol and water (coctanol/water), was a well-established measure of the compound's hydrophilicity. Low hydrophilicities and therefore high log *P* values cause poor absorption or permeation. It has been shown for compounds to have a reasonable probability of being well absorbed their log *P* value must not be greater than 5.0 [14].

The aqueous solubility of a compound significantly affects its absorption and distribution characteristics. Typically, a low solubility goes along with a bad absorption and therefore the general aim is to avoid poorly soluble compounds. The Solubility Prediction (log *S*) was calculated by The OSIRIS Property Explorer in our research, in a unit stripped logarithm (base 10) of the solubility measured in mol/liter (shown in Table 3).

Pearson's correlation coefficients between these physical, thermodynamic, and chemical parameters and P_{app} and recovery were displayed in Table 5. Gibbs energy ($r = 0.751$, $p < 0.05$) and heat of form ($r = 0.701$, $p < 0.05$) were strongly positively correlated with P_{app}. This was the first report to show that the permeability was strongly affected by the Gibbs energy and heat of form of a compound.

Table 4: The apparent permeability coefficient (P_{app}) values (Mean, $n \geq 3$, cm/s) and recovery rates (Mean ± SD, $n \geq 3$) of the seven analytes.

Compound	TSG	EN	RN	AE	RL	PL	EG	PR	AT
Molecular weight	406.38	270.24	284.22	270.24	228.24	390.38	432.38	245.32	266.34
Boiling point (K)	925.79	752.86	788.86	761.48	675.11	890.96	968.71	655.04	711.80
Melting point (K)	992.60	846.01	894.89	795.11	629.96	880.88	1096.93	450.66	524.88
Critical temperature (K)	1166.96	965.47	996.03	956.41	898.50	1111.40	1238.72	833.04	887.27
Critical pressure (Bar)	40.21	52.06	47.83	48.02	50.95	33.57	34.16	24.98	24.46
Critical volume (cm^3/mol)	1011.50	687.50	695.50	690.50	631.50	995.50	1051.5	755.50	806.50
Gibbs energy (kJ/mol)	−795.72	−357.13	−546.42	−339.33	−91.82	−641.10	−906.41	129.98	−50.00
Molar refraction index (cm^3/mol)	100.72	71.27	71.30	71.36	66.60	99.03	103.71	72.41	73.50
Henry's law constant	26.02	18.79	19.55	19.25	14.25	22.04	26.58	10.61	16.25
Heat of form (kJ/mol)	−1309.43	−622.31	−795.27	−597.23	−273.94	−1132.12	−1480.49	−173.06	−427.56
Log *P* (by ChemDraw)	0.83	1.74	1.2	1.07	3.06	1.22	−0.1	2.33	0.50
Clog *P* (by ChemDraw)	0.6538	3.617	3.529	2.700	2.833	1.517	1.650	2.444	−0.1086
Clog *P* (by OSIRIS)	0.71	2.93	2.44	2.29	3.12	1.64	0.82	2.41	0.41
Log *S* (by OSIRIS)	−2.45	−4.19	−4.15	−4.02	−2.86	−3.21	−4.07	−3.2	−2.02

Table 5: Relationships between physical-chemical properties values and apparent permeability coefficient (P_{app}).

Correlation coefficient and significance	Pearson's correlation coefficient	Significance (p)
Molecular weight	—	—
Boiling point	−0.687	0.041
Melting point	−0.768	0.016
Critical temperature	−0.703	0.035
Critical pressure	—	—
Critical volume	—	—
Gibbs energy	0.751	0.020
Molar refraction index	—	—
Heat of form	0.701	0.035
Log *P* (by ChemDraw)	—	—
Clog *P* (by ChemDraw)	—	—
Clog *P* (by OSIRIS)	—	—
Log *S* (by OSIRIS)	—	—

—: data were not listed when significance (p) was higher than 0.05.

Compounds with higher Gibbs energy and heat of form were considered to remain in higher energy state; therefore, these compounds had stronger tendency to complete the transmembrane movement. Moreover, boiling point, melting point, and critical temperature were all strongly positively correlated with P_{app} ($p < 0.05$).

4. Discussion and Conclusion

Previous studies [15, 16] reported that TSG has lipid regulation effect. Our previous researches [17, 18] also validated that the TSG possessed great TG reduction activity. Therefore, we could affirm that TSG is the main lipid regulation ingredient of PMR. However, in our previous studies, TSG content was significantly reduced after processed with black bean decoction (PMRP) according to the method recorded in the Pharmacopoeia of the People's Republic of China (2010 edition) [5].

In this research, TSG was estimated to be a poorly absorbed compound. The lower concentration of TSG in the processed PMRP together with its bad absorption all suggested that the dosage of PMRP should be increased in the treatment of hyperlipidaemia and NAFLD than the dosage of PMR. This coincided with the prescribed dosage listed in the Pharmacopoeia of the People's Republic of China (2010 edition), 3–6 g for PMR and 6–12 g for PMRP.

The human intestinal permeability of TSG, EN, RN, AE, RL, PL, and EG was evaluated using the Caco-2 cell monolayer model. In this research the permeabilities of seven compounds were passive diffusion because the drug concentrations in AP side were far above BL side.

Some researchers [19, 20] found that the absorption of passive-transport-based compounds has to do with its polarity. The greater the polarity of the compound is, the smaller the P_{app} value is. Coincidently, just from the point of view of each values, smaller P_{app} of TSG, EG, and PL in this research also proved this point.

TSG and EN both showed low recovery rates and low P_{app} in this research. We predict that large partitions of EN and TSG may accumulate or metabolise in the Caco-2 cells. TSG, EN, or their metabolites may be released from the Caco-2 cells and display their corresponding effects subsequently. However, further validation must be carried out to confirm this hypothesis.

The quantitative relationships in researches between physical-chemical properties values and P_{app} displayed higher

correlation Gibbs energy, heat of form, and apparent permeability coefficient. The higher the Gibbs energy was, the higher the apparent permeability coefficient was. The same thing applied to the heat of form. This was in accordance with a previous research [21] showing the Gibbs (free) energy contributions to the membrane partitioning of lipidated proteins. Although the mechanism why predicted boiling point, melting point, critical temperature might affect the apparent permeability coefficient, this research provided basic clues for the further research.

Abbreviations

AE: Aloe-emodin
ALP: Alkaline phosphatase
AP: Apical side
AT: Atenolol
BL: Basolateral side
Clog *P*: Calculated octanol-water partition coefficient
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: Dimethyl sulfoxide
EN: Emodin
EG: Emodin-8-*O*-β-D-glucopyranoside
FBS: Fetal bovine serum
HEPES: N-2-Hydroxyethylpiperazine- N'-2-ethanesulfonic acid
HPLC: High performance liquid chromatography
Log *S*: The aqueous solubility of a compound (estimated log *S* value is a unit stripped logarithm (base 10) of the solubility measured in mol/liter)
P_{app}: Apparent permeability coefficient
PBS: Phosphate buffer solution
PL: Polydatin
PMR: Polygonum Multiflorum Radix
PMRP: Polygoni Multiflori Radix Praeparata
PPRC: The Pharmacopoeia of the People's Republic of China
PR: Propranolol
RN: Rhein
RL: Resveratrol
R_t: Retention time
TCM: Traditional Chinese medicine
TEER: Transepithelium electrical resistance
TSG: 2,3,5,4′-Tetrahydroxystilbene-2-*O*-β-D-glucoside
TG: Triglyceride
NAFLD: Nonalcoholic fatty liver disease.

Conflict of Interests

The authors declare that there is no conflict of interests. They declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work. There is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in this paper.

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Research Article

Deciphering Molecular Mechanism Underlying Hypolipidemic Activity of Echinocystic Acid

Li Han, Peng Lai, and Jun-Rong Du

Department of Pharmacology, Key Laboratory of Drug Targeting and Drug Delivery Systems, Ministry of Education, West China School of Pharmacy and Translational Neuroscience Center, Sichuan University, Chengdu 610041, China

Correspondence should be addressed to Jun-Rong Du; dujr07@gmail.com

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Our previous study showed that a triterpene mixture, consisting of echinocystic acid (EA) and oleanolic acid (OA) at a ratio of 4 : 1, dose-dependently ameliorated the hyperlipidemia and atherosclerosis in rabbits fed with high fat/high cholesterol diets. This study was aimed at exploring the mechanisms underlying antihyperlipidemic effect of EA. Molecular docking simulation of EA was performed using Molegro Virtual Docker (version: 4.3.0) to investigate the potential targets related to lipid metabolism. Based on the molecular docking information, isotope labeling method or spectrophotometry was applied to examine the effect of EA on the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, acyl-CoA:cholesterol acyltransferase (ACAT), and diacylglycerol acyltransferase (DGAT) in rat liver microsomes. Our results revealed a strong affinity of EA towards ACAT and DGAT in molecular docking analysis, while low binding affinity existed between EA and HMG-CoA reductase as well as between EA and cholesteryl ester transfer protein. Consistent with the results of molecular docking, *in vitro* enzyme activity assays showed that EA inhibited ACAT and DGAT, with IC_{50} values of 103 and 139 μM, respectively, and exhibited no significant effect on HMG-CoA reductase activity. The present findings suggest that EA may exert hypolipidemic effect by inhibiting the activity of ACAT and DGAT.

1. Introduction

Hyperlipidemia is a key pathogenic factor for the development of cardiovascular and cerebrovascular diseases, such as atherosclerosis, hypertension, coronary heart disease, and brain stroke [1]. Pharmacotherapy is the primary way of ameliorating hyperlipidemia, among which statins and fibrate derivates are the most commonly used cholesterol- and triglyceride-lowering drugs [2]. However, a substantial number of patients treated with these lipid-lowering drugs fail to effectively improve dyslipidemia [3]. Moreover, several adverse effects such as hepatic dysfunction and muscle injury are reported with statin and fibrate therapy [4, 5]. In the recent years, with the advent of novel treatment targets for hyperlipidemia, numerous researches have been carried out in order to develop effective and safe lipid-lowering agents from natural products and synthetic compounds.

Phytochemical and pharmacological studies have demonstrated that triterpenoidal saponins are the main active constitutes of *G. sinensis* [6]. Our previous study demonstrated that oral administration of a pentacyclic triterpene mixture isolated from *G. sinensis* fruits (6 or 12 mg/kg/day), consisting of echinocystic acid (EA) and oleanolic acid (OA) at a ratio of 4 : 1, effectively improved the hyperlipidemia and subsequent atherosclerosis in rabbits fed with high fat/high cholesterol diets, suggesting the main constitute EA might be responsive to the hypolipidemic effect of triterpene extract from *G. sinensis* fruits *in vivo* [7]. It is reported that OA significantly inhibited the diacylglycerol acyltransferase (DGAT) from rat liver microsomes, lowered plasma cholesterol by inhibiting intestinal acyl-CoA:cholesterol acyltransferase (ACAT) activity in high-fat-fed hamsters, and protected against isoproterenol-induced myocardial ischemia in rats via antihyperlipidemic, antioxidative, and antiarrhythmic properties as well as its membrane-stabilizing action [8–10]. In addition, EA isolated from *G. sinensis* fruits prevented rat acute myocardial ischemia induced by isoproterenol and vasopressin [11].

Collectively, pentacyclic triterpenes, such as OA and EA, show potential therapeutic effects for cardiovascular diseases associated with dyslipidemia. However, the molecular mechanisms underlying the antihyperlipidemic effect of EA largely remain unclear.

A large body of studies has demonstrated that lipid profiles are governed by various enzymes and proteins, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, cholesteryl ester transfer protein (CETP), ACAT, and DGAT. HMG-CoA reductase acts as the rate-limiting enzyme of cholesterol biosynthesis pathway by catalyzing the conversion of HMG to mevalonate [4]. CETP promotes the transfer of cholesteryl esters from antiatherogenic high-density lipoprotein (HDL) to proatherogenic lipoproteins such as very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), and CETP inhibition or deficiency can effectively retard atherogenesis by increasing HDL and decreasing LDL [12]. ACAT catalyzes cholesterol esterification from cholesterol and fatty acyl coenzyme A, followed by subsequent cholesterol absorption, whereas ACAT inhibition is a therapeutic strategy for hypercholesterolemia and atherosclerosis through lowering cholesterol levels, diminishing the assembly and secretion of apolipoprotein B-containing lipoproteins such as VLDL, and inhibiting the formation of foam cells in the arterial walls [13]. DGAT catalyzes the formation of triglyceride in the final step of triglyceride biosynthesis via covalently linking a fatty acyl CoA with the free hydroxyl group of diacylglycerol, and DGAT inhibition is beneficial for the treatment of hypertriglyceridemia via decreasing serum triglyceride levels [14, 15]. Therefore, in order to explore the potential mechanisms of antihyperlipidemic effect of EA, the present study first performed the molecular docking of EA with HMG-CoA reductase, CETP, ACAT, and DGAT to predict the potential targets, and further investigated the effects of EA on the possible targets in *in vitro* rat liver microsomes.

2. Materials and Methods

2.1. Materials. Tris, phosphatidylserine, (R.S)-3-hydroxy-3-methylglutaryl coenzyme A [(R.S)-HMG-CoA], nicotinamide adenine dinucleotide phosphate (NADPH), 1,2-glyceryl dioleate, lecithin, and 3-oleic acid glycerol were purchased from Sigma-Aldrich (St. Louis, MO, USA). [1-^{14}C] oleoyl-CoA was purchased from New England Nuclear Corporation (Boston, USA). Scintillation solution was purchased from Lipoluma, Lumac Co (Clanton, USA). BCA protein assay kit was from Boster Biological Technology (Wuhan, China). Pravastatin was obtained from Bristol-Myers Squibb (Shanghai, China). Other reagents were obtained from commercial sources.

2.2. Isolation of Echinocystic Acid (EA). The fruits of *Gleditsia sinensis* Lam. (*G. sinensis*) were collected from Sichuan province in China, and the aqueous extract was prepared as we described previously [7], followed by isolation of echinocystic acid (EA) using high-performance liquid chromatography (HPLC). In brief, chromatographic separation was conducted on a column (40 × 500 mm) filled with reverse phase C18 silica gel. The mobile phase (MeOH-H_2O, 8 : 2, $v : v$) was conveyed to the column at a flow rate of 10 mL/min and the eluate was detected at 215 nm by diode array detector. EA ((3β,16α)-3,16-dihydroxyolean-12-en-28-oic acid, Figure 1) was collected and identified by spectral techniques including ^{1}H-NMR and ESI-MS and the purity of EA was examined based on the percentage of total peak areas by HPLC. In the present study, EA (purity > 98%) stock solution was prepared via mixing thoroughly 10 μL Tween 80, 20 μL polyethylene glycol 200, and 100 μL water with 1 mg EA and diluted with water prior to enzyme activity assay.

2.3. Molecular Docking Analysis. Molecular docking was performed by Molegro Virtual Docker (MVD) 4.3.0 tool adopting MolDock SE algorithm and Rerank scoring function as described previously [16]. The crystal structures of docked receptors (HMG-CoA reductase (PDB ID:1R31), CETP (PDB ID:2OBD), ACAT (PDB ID:1WL4), and DGAT (PDB ID: 1 K30)) were retrieved from RCSB Protein Data bank (http://www.rcsb.org/). The crystal structure of EA as docked ligand was available from NCBI's PCCompound database (http://pubchem.ncbi.nlm.nih.gov/).

Before docking, the receptor protein imported into the MVD software was preprocessed by removing cofactor, crystal water, and initial ligand, and then the free receptor protein was modified by adding the surface which shows the charge distribution of the receptor protein. Following the import of ligand EA into the MVD, molecular docking simulation of EA was performed to investigate the binding affinity of the receptor protein by computing the binding free energy between EA and the possible binding site of the receptor according to the software manual. The possible binding modes between EA and the ideal receptors were then analyzed according to the results of molecular docking. We conducted the docking of EA with HMG-CoA reductase, CETP, ACAT, and DGAT, receptively. The docking was done with the setting of the MVD as follows: (a) score: MolDock score [Grid]; (b) grid resolution (A): 0.30; (c) max iterations: 1500; (d) max population size: 50; (e) other parameters were the default setting. The pictures were prepared using MVD of 4.3.0 version.

2.4. Preparation of Rat Liver Microsomes. Male SPF Sprague-Dawley rats, 260~300 g, were purchased from Chongqing Tengxin Animal Center (Chongqing, China). The procedure of animal experiment was carried out following the institutional guidelines of Animal Care and Use Committee at Sichuan University.

Rat liver microsomes were prepared as described previously [17, 18]. In brief, the rats were killed by decapitation. Then, the liver was swiftly removed; rinsed by cold 0.9% NaCl solution; weighed,;cut finely into pieces with scissors; placed into 9 vol. of cold homogenization medium containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 100 mM sucrose, 10 mM EDTA, and 2 mM DTT; and homogenized in a Teflon homogenizer for 10 min. The homogenate was subsequently centrifuged at 12000 ×g for

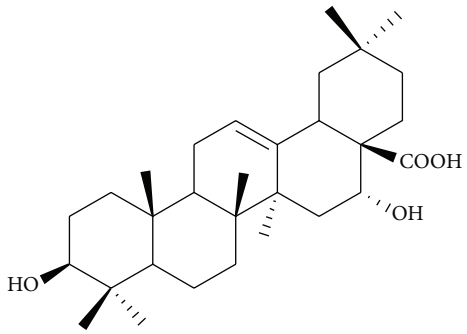


Figure 1: Chemical structure of echinocystic acid (EA).

20 min at 4°C (Beckmann refrigerated centrifuge TJ-6). The supernatant fraction was sucked out and centrifuged again at 100000 ×g for 60 min at 4°C after adding a certain amount of 1 M $CaCl_2$ solution (final concentration of $CaCl_2$: 8 mM). The precipitation fraction (microsomes) was acquired via the removal of the supernatant fraction. The prepared microsomes were, respectively, resuspended in KCl-Tris-HCl buffer solution (Tris-HCl: 10 mM, KCl: 100 mM, and pH 7.4) of 100–200 μL or 2.5 M sucrose solutions, mixed thoroughly, and then stored in −80°C for assays of microsomal enzyme activities.

2.5. Assay of Microsomal HMG-CoA Reductase Activity. Effect of EA on HMG-CoA reductase activity was tested via spectrophotometry using HMG-CoA and cofactor NADPH as described previously [19]. Briefly, the reactive mixture containing 100 μL of 0.2 mM NADPH, 600 μL of phosphate buffer (pH 6.8) (composed of 300 mM KCI, 240 mM potassium phosphate, 6 mM EDTA, and 15 mM DTT), 100 μL of the prepared microsome suspension (10 mg/mL protein), and 10 μL of the test sample EA (200 mM) or the positive control pravastatin (50 mM) was first monitored at 340 nm using ultraviolet spectrophotometer (UNICO) for HMG-CoA-independent oxidation of NADPH. The reaction was then initiated by adding 100 μL of 1 mM (R.S)-HMG-CoA. After 5 min of incubation at 37°C, the supernatant was sucked out and tested at 340 nm by spectrophotometer for HMG-CoA-dependent oxidation of NADPH. One unit of HMG-CoA reductase was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per gram of microsome protein. The protein concentration was measured by the method of BCA using BSA as the standard. The inhibitory effect of EA or pravastatin was calculated as a percentage of HMG-CoA reductase activity of control group, respectively.

2.6. Assay of Microsomal ACAT Activity. Effect of EA on ACAT activity was tested by the isotope labeling method as reported previously [20]. In brief, the prepared microsomes were unfrozen and dissolved at 37°C water bath. The reactive mixture, containing 10 μL of microsome suspension (10 mg/mL protein), 20 μL of 0.5 M potassium phosphate buffer (pH 7.4, 10 mM DTT), 10 μL of BSA (180 mg/mL), 2.0 μL of cholesterol in acetone (20 mg/mL), 130 μL of water, and 10 μL of the test sample EA at a concentration range of 0~400 μM, was preincubated for 30 min at 37°C. The reaction was started by adding 10 μL of [1-^{14}C] oleoyl-CoA (0.05 μCi: final concentration 10 μM). After 30 min incubation at 37°C, the reaction was terminated by adding 1.0 mL of *i*-PrOH-*n*-hexane (4 : 1, *v*/*v*) solution. A mixture of 0.6 mL of *n*-hexane and 0.4 mL of 0.1 M potassium phosphate buffer was subsequently added to the reaction mixture and mixed uniformly by vortexing. Standing for 2 min was allowed to separate the reaction mixture into aqueous and organic phases. The upper organic phase containing the radiolabeled cholesteryl ester products was sucked out. The radioactivity in 100 μL of the upper phase was determined using 4 mL of scintillation cocktail (Lipoluma, Lumac Co.) by a LS6000 Beckman Liquid Scintillation Counter (Beckman Inc). Data were presented as counts per minute (CPM) of [1-^{14}C] cholesteryl ester products and the readings were normalized to protein concentrations, which were measured by the method of BCA using BSA as the standard. Effect of EA on ACAT activity was calculated as the percentage of inhibition versus control group. Software Origin 7.5 (OriginLab, USA) was used to draw the relation curve of drug concentrations with the inhibition rate, and the 50% inhibitory concentration (IC_{50}) was calculated.

2.7. Assay of Microsomal DGAT Activity. Effect of EA on DGAT activity was tested by the isotope labeling method as reported previously [8]. In brief, EA at a concentration range of 0~400 μM was incubated 30 min at 37°C with the prepared microsome suspension (10 mg/mL protein), [1-^{14}C] oleoyl-CoA (0.05 μCi: final concentration 3 μM), 3 mM 1,2-glyceryl dioleate, and the incubation buffer that was composed of 200 μM $MgCl_2$, 1 mg/mL fatty acid-free BSA, 100 μM lecithin, 100 μM phosphatidylserine, and 5 mM Tris-HCl (pH 8). After 30 min of incubation, the reaction was stopped by adding chloroform-methanol (1 : 1, *v*/*v*) solution, chloroform and acidified sodium chloride solution (containing 17 mM NaCl and 1 mM H_2SO_4). The precooled unlabelled glyceryl trioleate was added to the above reaction mixture. The lipids in the mixture were extracted into the organic solvent by centrifugation at 2500 rpm for 10 min. The lower organic phrase containing lipids was recovered, dried under nitrogen, and redissolved in 100 μL of chloroform. The lipids were then separated by silica gel thin-layer chromatography plate in chloroform : diethyl ether : acetic acid (9 : 6 : 4, *v*/*v*/*v*). Triglyceride-specific bands were scraped after being verified by standards with exposure to I_2 vapor, and the radioactivity was measured by liquid scintillation counting. Data were presented as CPM of [1-^{14}C] triglyceride products and the readings were normalized to protein concentrations. Effect of EA on DGAT activity was calculated as the percentage of inhibition versus control group. Software Origin 7.5 was used to draw the relation curve of drug concentrations with the inhibition rate, and the IC_{50} was calculated.

2.8. Statistical Analysis. Data are presented as mean ± SD. Statistical analyses were performed with SPSS 16.0 software. The significance of data between the tested groups was

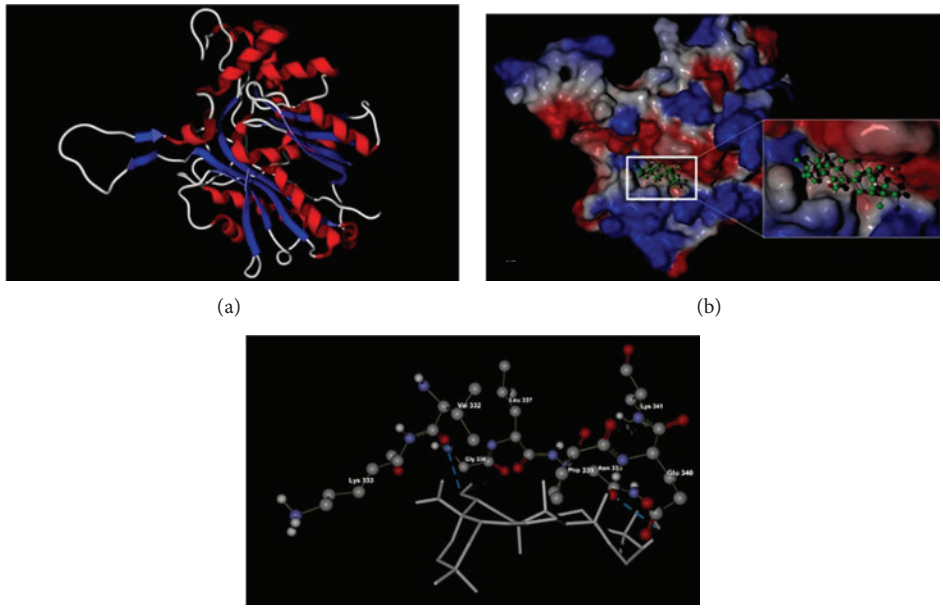


Figure 2: Molecular docking of echinocystic acid (EA) with ACAT in 3D diagram. (a) Three-dimensional structure of ACAT. (b) Optimized docking conformation of EA in the hydrophobic pocket of ACAT. The surface of ACAT was color-coded by electrostatic potential. Red, positive charge; white, neutral; blue, negative charge. (c) Detailed binding mode of EA with ACAT. Dotted blue lines display the hydrogen bonding between the carboxyl group and OH group of EA and amino acid residues Gly 336 and Glu 340 of ACAT.

determined by one-way ANOVA. The probability level for statistical significance was set at $P < 0.05$.

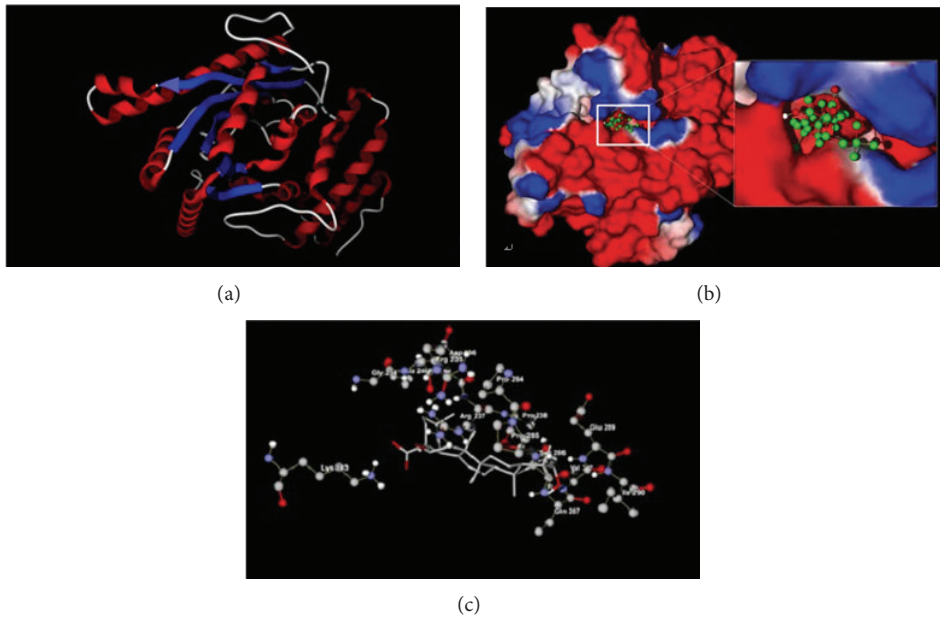
3. Results and Discussion

3.1. Molecular Docking. To predict the potential targets of lipid-lowering effects of EA, we performed the molecular docking of EA with HMG-CoA reductase, DGAT, ACAT, and CETP by MVD 4.3.0 tool using Rerank scoring function, respectively. As shown in Table 1, Rerank scores were recorded and used as the index of binding free energy between the ligand and the receptor protein, which is known to be negatively correlated with binding affinity. The docking results showed that EA exhibited a relatively strong binding affinity with ACAT and DGAT as inferred by their negative Rerank scores, −53 and −41, which indicate low binding free energy; the binding affinity between EA and HMG-CoA reductase was found to be very low, so was the binding affinity between EA and CETP, as evidenced by their positive Rerank scores, +4 and +12. These data indicate that EA has a strong binding affinity with ACAT and DGAT. In addition, it is reported that the negative free energy change (ΔG) values suggests a spontaneous interaction and correspond to a spontaneous binding process [21]. Therefore, the binding process between EA and HMG-CoA reductase and the binding between EA and CETP were probably not pontaneous, which implied that there was no specific binding ability of EA to HMG-CoA reductase and CETP. These molecular docking results suggest that ACAT and DGAT rather than HMG-CoA reductase and CETP are likely to be the potential targets of lipid-lowering effects of EA.

Table 1: Docking scores of echinocystic acid with the enzymes/protein related to lipid metabolism.

Enzymes	Rerank score
HMG-CoA reductase	+4
CETP	+12
ACAT	−53
DGAT	−41

Following the results mentioned above, we further analyzed the binding modes and interactions of EA with ACAT and DGAT, respectively. The results were shown in Figures 2 and 3. According to the results of MVD docking simulation, EA was most likely bound to ACAT's site within the hydrophobic pocket which is rich in hydrophobic amino acids such as Val, Leu, and Pro, as depicted in Figures 2(b) and 2(c). As a triterpenoid acid, EA has good hydrophobic property, which benefits from binding between the amino acid residues and small molecular compounds with hydrophobic property, so hydrophobic forces may well be one of the main interaction forces behind the binding of EA with ACAT. Furthermore, as seen in Figure 2(c), the OH group and carboxyl group of EA form two hydrogen bonds with residues Gly 336 and Glu 340 of ACAT, respectively. Therefore, the hydrogen bonds may be another interaction force behind the binding of EA and ACAT. As for DGAT, the MVD docking simulation revealed that it had a similar binding mode with EA towards ACAT. EA was also most probably going to bind with DGAT's site within the hydrophobic pocket (Figure 3(b)), which is likely due to the similar structure of DGAT to



(a) (b)

(c)

Figure 3: Molecular docking of echinocystic acid (EA) with DGAT in 3D diagram. (a) Three-dimensional structure of DGAT. (b) Optimized docking conformation of EA in the hydrophobic pocket of DGAT. The surface of DGAT was color-coded by electrostatic potential. Red, positive charge; white, neutral; blue, negative charge. (c) Detailed binding mode of EA with DGAT. Dotted blue line displays the hydrogen bonding between the carboxyl group of EA and residue Lys 193 of DGAT.

ACAT since both enzymes belong to the membrane-bound O-acyltransferase (MBOAT) family. However, the pocket, which is wealthy in residue Pro, was obviously not large enough to accommodate the whole structure of EA. It thus apparently decreases the hydrophobic interaction strength between EA and its surrounding residues, which, in turn, may decrease the inhibitory effect of EA on DGAT activity. Moreover, as shown in Figure 3(c), only one hydrogen bond was established between the carboxyl group of EA and residue Lys 193 of DGAT, which may be another reason why the binding affinity of EA with ACAT is stronger than that of EA with DGAT. These results are consistent with that obtained by Rerank scoring function which show that EA has a lower binding free energy and stronger binding affinity with ACAT compared to that with DGAT.

3.2. HMG-CoA Reductase Activity. HMG-CoA reductase is the rate-limiting enzyme of cholesterol biosynthesis pathway and thus is regarded as a major target for regulating hypercholesterolemia. In our previous study, 14-week treatment with a triterpene mixture consisting of 9.6 mg EA and 2.4 mg OA once daily (i.g.) decreased the total cholesterol levels in serum, aorta homogenates, and liver homogenates by 43%, 72%, and 75%, respectively, in hyperlipidemia and atherosclerosis rabbits fed with high fat/high cholesterol diets [7]. By contrast, however, the present molecular docking showed that the binding affinity between EA and HMG-CoA reductase was very low, suggesting that HMG-CoA reductase is not likely to be the potential target of cholesterol-lowering effect of EA. To validate this speculation, we assayed the effect of EA on the activity of HMG-CoA reductase in rat liver microsomes by spectrophotometry. As shown in Figure 4, EA showed no HMG-CoA reductase inhibitory activity even at 200 μM ($P > 0.05$), while pravastatin at a concentration of 50 μM exhibited a significant inhibition ($P < 0.05$) compared with controls. Taken together, the current results demonstrate that the other targets rather than HMG-CoA reductase is responsive to cholesterol-lowering activity of EA *in vivo*.

3.3. ACAT Activity. ACAT is regarded as a novel target for the treatment of hypercholesterolemia and atherosclerosis [13]. ACAT inhibitors, such as pactimibe, are reported to have cholesterol-lowering and antiatherosclerotic effects [22]. It is reported that OA at a concentration of 50 μM significantly inhibited ACAT activity in Caco-2 cells, a human intestinal cell line [9]. The present molecular docking showed that the binding affinity between EA and ACAT was much stronger than that between EA and HMG-CoA reductase, suggesting that ACAT is likely to be responsive for cholesterol-lowering effect of EA. Therefore, we investigated the effect of EA on ACAT activity in rat liver microsomes using the isotope labeling method. As shown in Figure 5, EA at a concentration range of 0~400 μM concentration-dependently reduced ACAT activity, with an IC_{50} value of 103 μM, suggesting that ACAT inhibition contributes to the potent cholesterol-lowering effect of EA/OA mixture *in vivo*.

3.4. DGAT Activity. Hypertriglyceridemia is known as a major risk factor of obesity and cardiovascular diseases [23]. DGAT, a key enzyme in triacylglycerol synthesis, is regarded as a potential target for the treatment of triglyceride metabolic disorders [14, 15, 24]. OA is reported to significantly inhibit

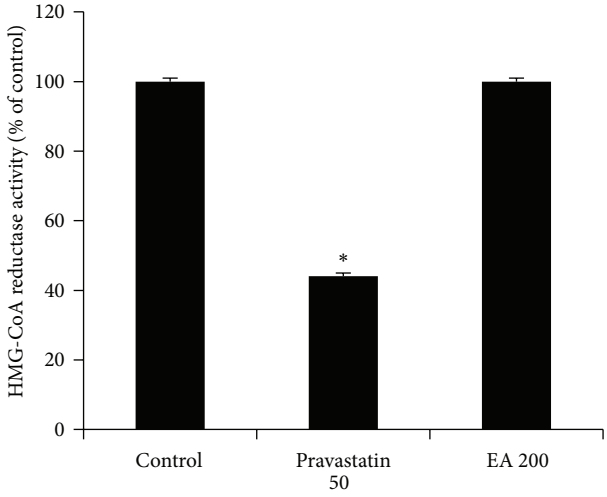


Figure 4: Effect of echinocystic acid (EA) on HMG-CoA reductase activity in rat liver microsomes. Pravastatin was used as a positive control. The inhibitory effect of 50 μM pravastatin (pravastatin 50) or 200 μM EA (EA 200) was calculated as the percentage of HMG-CoA reductase activity of control group, respectively. Data are expressed as mean ± SD ($n = 5$). $^*P < 0.05$ versus control group, determined by one-way ANOVA.

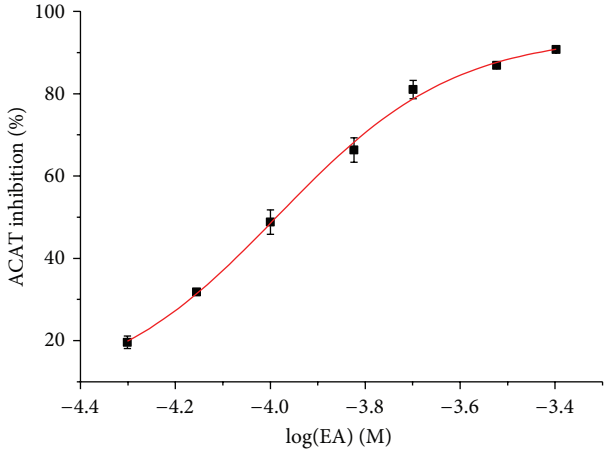


Figure 5: Effect of echinocystic acid (EA) on ACAT activity in rat liver microsomes. The inhibitory effect of EA was calculated as the percentage of inhibition versus control group. Data represent mean ± SD of three independent experiments.

DGAT from rat liver microsomes [8]. In our previous study, 14-week treatment with a triterpene mixture consisting of 9.6 mg EA and 2.4 mg OA once daily (i.g.) decreased the triacylglycerol levels in serum and aorta homogenates by 54.5% and 29%, respectively, in hyperlipidemia and atherosclerosis rabbits fed with high fat/high cholesterol diets [7]. The molecular docking results showed that EA exhibited a relatively strong binding affinity with DGAT, suggesting that DGAT inhibition is probably associated with triacylglycerol-lowering effect of EA. Therefore, we investigated the effect of EA on DGAT activity in rat liver microsomes using the

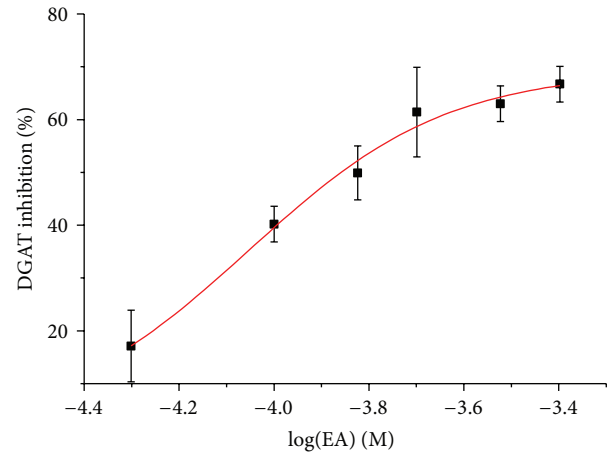


Figure 6: Effect of echinocystic acid (EA) on DGAT activity in rat liver microsomes. The inhibitory effect of EA was calculated as the percentage of inhibition versus control group. Data represent mean ± SD of three independent experiments.

isotope labeling method. As shown in Figure 6, EA at a concentration range of 0~400 μM concentration-dependently reduced DGAT activity in rat liver microsomes, with an IC_{50} value of 139 μM, suggesting that DGAT inhibition is responsive to triacylglycerol-lowering effect of EA/OA mixture *in vivo*.

Taken together, our current findings show, for the first time, that EA inhibits ACAT and DGAT with IC_{50} values of 103 and 139 μM, respectively, and exhibits no significant effect on the activity of HMG-CoA reductase. These results suggest that EA is a potential natural hypolipidemic agent by inhibiting ACAT and DGAT activity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Li Han and Peng Lai equally contributed to this study.

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Research Article

Effects of Preventive Acupuncture and Moxibustion on Fat Accumulation, Blood Lipid, and Uterus E_2 of Menopause Rats

Shi-Peng Zhu,[1] Yu-wei He,[2] Huan Chen,[1] Zhi-Fang Sun,[3] Na Ding,[4] Jie Mo,[5] Bing-Yan Cao,[1] Li Luo,[1] Qing-Qing Zhang,[1] Yang Wang,[1] Lu-Fen Zhang,[1] Xiao-Xuan Ren,[1] Meng-Wei Guo,[1] Ya-Fang Zhao,[1] Liang-Xiao Ma,[1] and Xiao-Hong Li[1]

[1] *School of Acupuncture, Moxibustion and Tuina, Beijing University of Chinese Medicine, Beijing 100029, China*
[2] *Huguosi Hospital Affiliated to Beijing University of Chinese Medicine, Beijing 100035, China*
[3] *Medical College of Hangzhou Normal University, Hangzhou 310000, China*
[4] *Yangzhou Maternal and Child Health Hospital, Yangzhou 225000, China*
[5] *Graduate School of Beijing University of Chinese Medicine, Beijing 100029, China*

Correspondence should be addressed to Liang-Xiao Ma; mlx@sina.com.cn and Xiao-Hong Li; 479633418@qq.com

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Objective. To observe the effect of preventive acupuncture and moxibustion on blood lipid of menopause rats. *Methods*. Seventy 10-month-old SD rats with estrous cycle disorders were divided into three control groups and four treatment groups ($n = 10$/group) and another ten 3.5-month-old female SD rats were chosen as young control group. Preventive acupuncture and moxibustion were applied at Guanyuan (CV 4). Body weight growth rate has been recorded. Plasma total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), and high density lipoprotein (HDL) levels and uterus E_2 level were measured. *Results*. Compared to young control group, plasma TC and LDL increased and uterus E_2 reduced significantly in 12-month-old control group. Compared to 12-month-old control group, plasma TC and LDL level and body weight growth rate decreased while HDL level increased remarkably in preventive acupuncture 12-month-old group. Compared to 14-month-old control group, plasma TC level and body weight growth rate decreased remarkably in preventive moxibustion 14-month-old group. *Conclusions*. Preventive acupuncture and moxibustion can significantly decrease the plasma TG and LDL, increase the plasma HDL, and prevent fat accumulation. Our finding suggests that preventive acupuncture and moxibustion have beneficial effects on blood lipid. Different treatment effects were found between preventive acupuncture and preventive moxibustion.

1. Introduction

Dyslipidemia, characterized by alterations in the levels and composition of blood lipids, is a potential risk for cardiovascular disease (CVD), stroke, and peripheral artery disease [1, 2]. Previous studies showed that menopause women have an adverse development of plasma lipoproteins, ischemic heart disease, stroke, and diabetes and occurred significantly more often than premenopause [3–5]. Meanwhile, some researchers found that the rapid reduction of estrogen in women at the age of menopause might be one of the most important causes for dyslipidemia [6].

Aggressive lipid-lowering strategy, especially lowering low-density lipoprotein (LDL) cholesterol levels, will reduce the rates of coronary heart disease and ischemic stroke [7, 8]. The latest ESC/EAS guidelines for management of dyslipidemia further highlighted the aggressive lipid-lowering strategy in subjects with documented CVD [9]. Statins are the first-line agents for treatment of most dyslipidemias, which can prevent atherosclerosis and reduce the morbidity and mortality of CVD by modulating dyslipidemia actively, especially lowering low-density lipoprotein cholesterol (LDL-C) [10]. However, the application of statins might be restricted

by the adverse effect on the liver function, especially in patients with old age and comorbidity.

Researches proved that traditional Chinese medicine (TCM) is an effective and safer alternative therapy for dyslipidemia. For example, Xuezhikang (an extract from red yeast rice) has been proved beneficial in the treatment of dyslipidemia by some systematic reviews and has been recommended in a guideline for China adult dyslipidemia prevention [11–13]. Acupuncture is another effective method to treat dyslipidemia. Several clinical trials demonstrated that acupuncture could remarkably decrease plasma TC, TG, and LDL levels [14–17].

Preventive acupuncture and moxibustion are important means for disease prevention in TCM. It refers to apply benign acupuncture or moxibustion stimulation on the body before the intrusion of diseases, in order to improve the resistance. Previous animal experiments proved that preventive acupuncture and moxibustion could adjust hormones [18], resist damages from free radicals [19], and regulate inflammatory cytokines [20] through the neuroendocrine immune network. Furthermore, it is widely accepted that acupuncture facilitates the release of certain neurotransmitters in the central nerve system and activates either sympathetic or parasympathetic nervous systems, which elicits profound psychophysical responses of immune and endocrine systems [21, 22]. Since the regulation of neuroendocrine immune network is shared mechanism of acupuncture and preventive acupuncture and moxibustion, we hypothesized that the latter may also have certain effects on dyslipidemia. In this study, we observed the effects of preventive acupuncture, and moxibustion on climacteric fat accumulation, dyslipidemia and uterus E_2 in natural aging climacteric rats.

2. Material and Methods

2.1. Animals Preparation. Clean female SD rats were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (License number: SCXK (Beijing) 200223). Experimental animals were raised in clean cabinets with free access to food and water. A controlled environment at a temperature of (20 ± 1)°C, humidity of 50%, and 12-hour light-dark cycle was maintained throughout the study. All procedures for animal experiments were conducted in accordance with World Health Organization's International Guiding Principles for Biomedical Research Involving Animals and were approved by the Animal Care and Use Committee at Beijing University of Chinese Medicine.

2.2. Grouping and Treatment

2.2.1. Grouping. The menopause was determined through vaginal smear method [23]. Histological changes of vaginal smears in 9.5-month-old female SD rats, stained with alkaline methylene blue solution, were observed daily under the microscope for three estrous cycles (15 d). According to cell morphology, type, and quantity, seventy 10-month-old female SD rats were screened in which estrous cycle disorders had begun to emerge (indicating the beginning of menopause), and were divided into seven groups (n = 10/group), namely, 10-month-old control group, 12-month-old control group, preventive acupuncture 12-month-old group, preventive moxibustion 12-month-old group, 14-month-old control group, preventive acupuncture 14-month-old group, and preventive moxibustion 14-month-old group. Besides, ten 3.5-month-old female SD rats were chosen as young control group.

2.2.2. Acupuncture and Moxibustion Method. Guanyuan (CV4) was located at the midpoint of the two hind legs roots. Acupuncture: the rats were supine on the table and fixed by the assistant's hand. The needle was directly pierced into CV4 about 0.5 cm and punctured upward. Then the needle was retained for 20 min. Moxibustion: rat hair in the region of 2 cm in diameter around CV4 was cut and the skin was exposed. The rats were supine on the table and fixed by the assistant's hand. A lit moxa cone was directly placed on the acupoint until it was burned out. Both acupuncture and moxibustion treatments were completed without anesthesia. Young control group and 10-month-old control group received no treatment. Preventive acupuncture 12-month-old group, preventive moxibustion 12-month-old group, preventive acupuncture 14-month-old group, and preventive moxibustion 14-month-old group received acupuncture or moxibustion treatment, from 10 months of age, twice a week for 8 weeks. Rats in the 12-month-old and 14-month-old control groups were only grabbed as those in the preventive acupuncture and moxibustion groups from 10 months of age without other treatments for 8 weeks (Figure 1).

2.3. Samples Obtaining. Materials were drawn from rats at corresponding age. Blood of rats in each group was immediately collected with rapid decapitation, placed in 2 mL anticoagulated tubes, and centrifuged (3 500 r/min, 15 min). The plasma was stored at −20°C. The above decapitated rats were placed on sterile ice. The uteri were rapidly removed, boiled in saline for 5 min, weighed, and placed in a glass homogenizer tube. One milliliter hydrogen chloride (1 mol/mL) was added. The tissues were homogenized on ice sufficiently, placed at room temperature for 100 min, and 0.8 mL sodium hydroxide (NaOH, 1 mol/mL) was added to neutralize to a certain pH. Then it was centrifuged (3 500 r/min, 15 min) and the supernatant was stored at −70°C.

2.4. Detection Indicators and Methods

2.4.1. Measure of Body Weight Growth Rate. Body weight of all rats was measured at the 10th month and before the sacrifice. Body weight growth rate = (weight at sacrifice − 10-month-old weight)/10-month-old weight.

2.4.2. Blood Lipids Assessment. These tests include plasma TC, TG, LDL, and HDL levels measurements. Biochemical methods were used in accordance with kit instructions.

2.4.3. Uterus E_2 Assessment. It was measured with radioimmunoassay. The process was guided strictly under instructions.

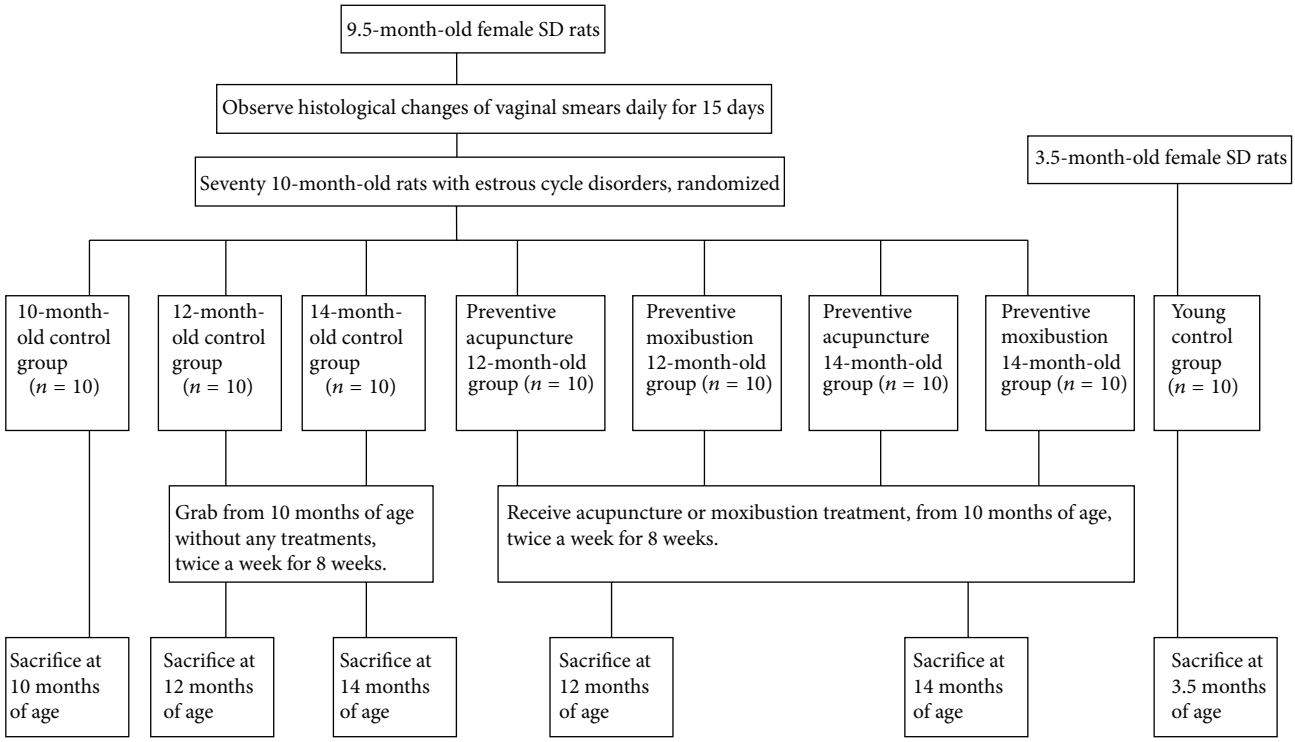

Figure 1: Experimental procedures.

2.5. Statistical Analysis. Data were presented as means ± standard deviation. Differences among groups were examined using one-way ANOVA, followed by Student-Newman-Keuls test. Analyses were performed with SPSS software version 13.0; $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of Preventive Acupuncture and Moxibustion on Body Weight Growth Rate. Preventive acupuncture 12-month-old group showed a remarkable decrease in body weight growth rate as compared to 12-month-old control group ($P = 0.005$, Figure 2(a)); body weight growth rate in preventive moxibustion 14-month-old group decreased significantly as compared to 14-month-old control group ($P = 0.013$, Figure 2(b)).

3.2. Effect of Preventive Acupuncture and Moxibustion on Blood Lipid

3.2.1. Plasma TC Levels. Compared to young control group, plasma TC level increased significantly in 12-month-old control group ($P = 0.002$) and preventive moxibustion 12-month-old group ($P = 0.003$). Preventive acupuncture 12-month-old group showed a remarkable decrease as compared to 12-month-old control group ($P = 0.001$, Figure 3(a)). Compared to young control group, plasma TC level slightly increased in 14-month-old control group. Plasma TC level remarkably decreased in preventive moxibustion 14-month-old group as compared to 14-month-old control group ($P = 0.018$, Figure 3(b)).

3.2.2. Plasma TG Levels. No significant difference was found among all the groups (Figures 4(a) and 4(b)).

3.2.3. Plasma HDL Levels. Compared to young control group, plasma HDL level showed a decreased trend in 12-month-old control group. Preventive acupuncture 12-month-old group showed a remarkable increase of HDL level as compared to 12-month-old control group ($P < 0.001$, Figure 5(a)). No significant difference was found among young control group, 14-month-old control group, preventive acupuncture 14-month-old group, and preventive moxibustion 14-month-old group (Figure 5(b)).

3.2.4. Plasma LDL Levels. Plasma LDL level increased remarkably in 12-month-old control group ($P = 0.001$) and preventive moxibustion 12-month-old group ($P < 0.001$) as compared to young control group; LDL in preventive acupuncture 12-month-old group showed a significant decrease as compared to 12-month-old control group ($P = 0.001$, Figure 6(a)). No significant differences existed among young control group, 14-month-old control group, preventive

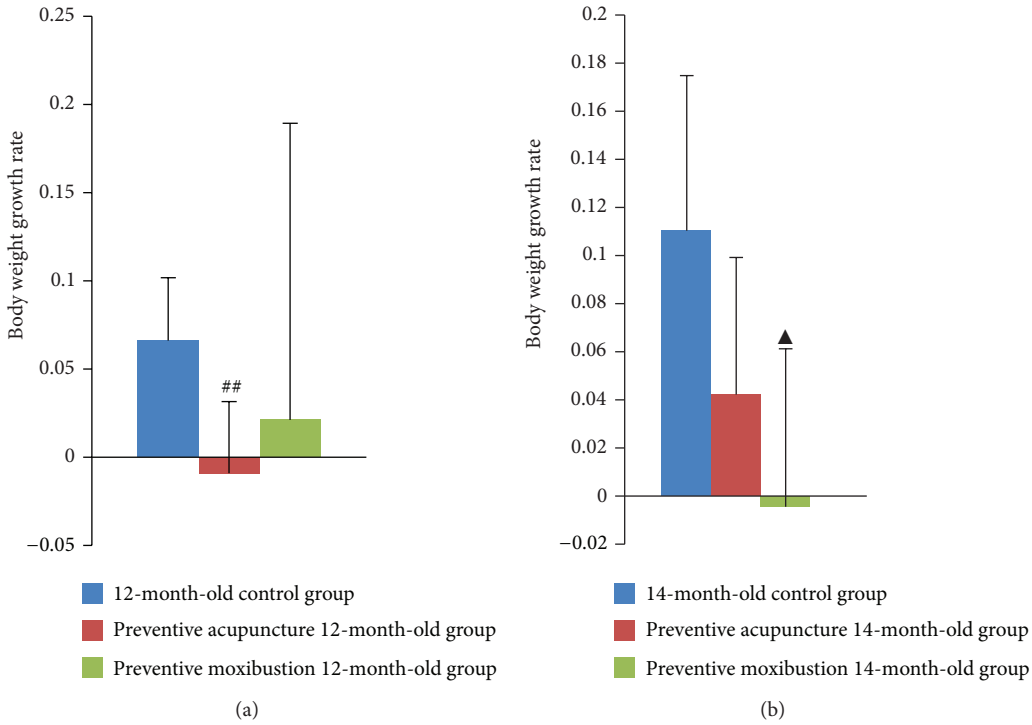

Figure 2: Effect of preventive acupuncture and moxibustion on body weight growth rate. Note: $^{\#\#}P < 0.01$, versus 12-month-old control group; $^{\blacktriangle}P < 0.05$, versus 14-month-old control group.

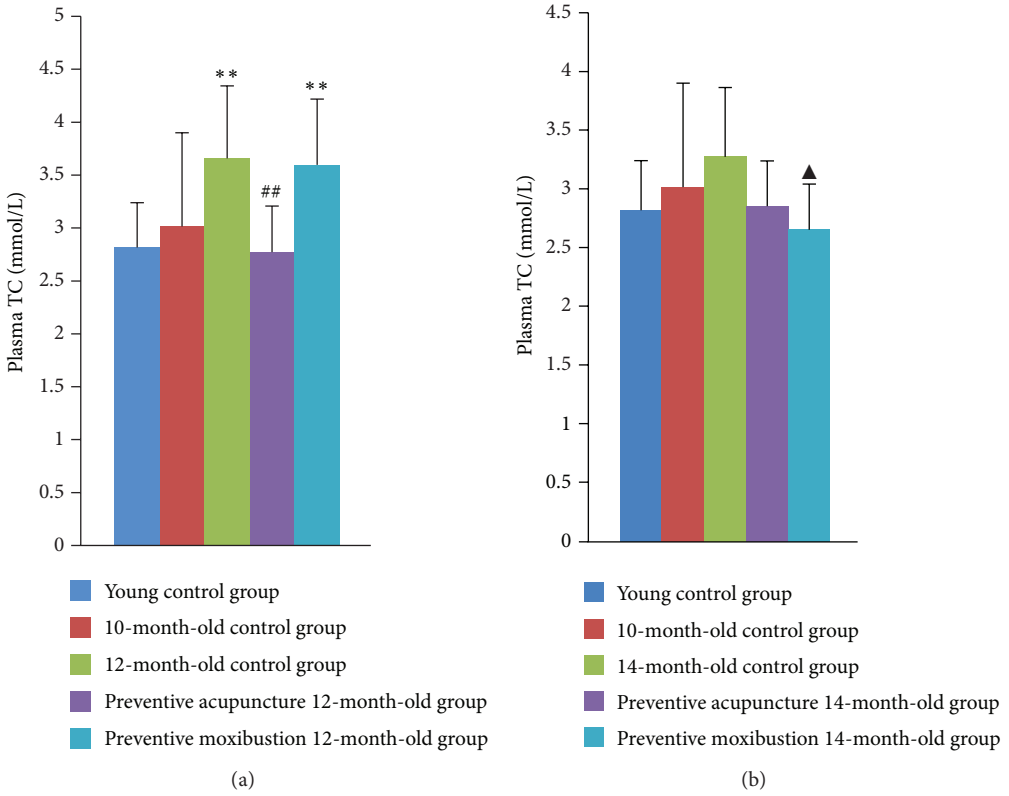

Figure 3: Effect of preventive acupuncture and moxibustion on plasma TC level. Note: $^{**}P < 0.01$, versus young control group; $^{\#\#}P < 0.01$, versus 12-month-old control group; $^{\blacktriangle}P < 0.05$, versus 14-month-old control group.

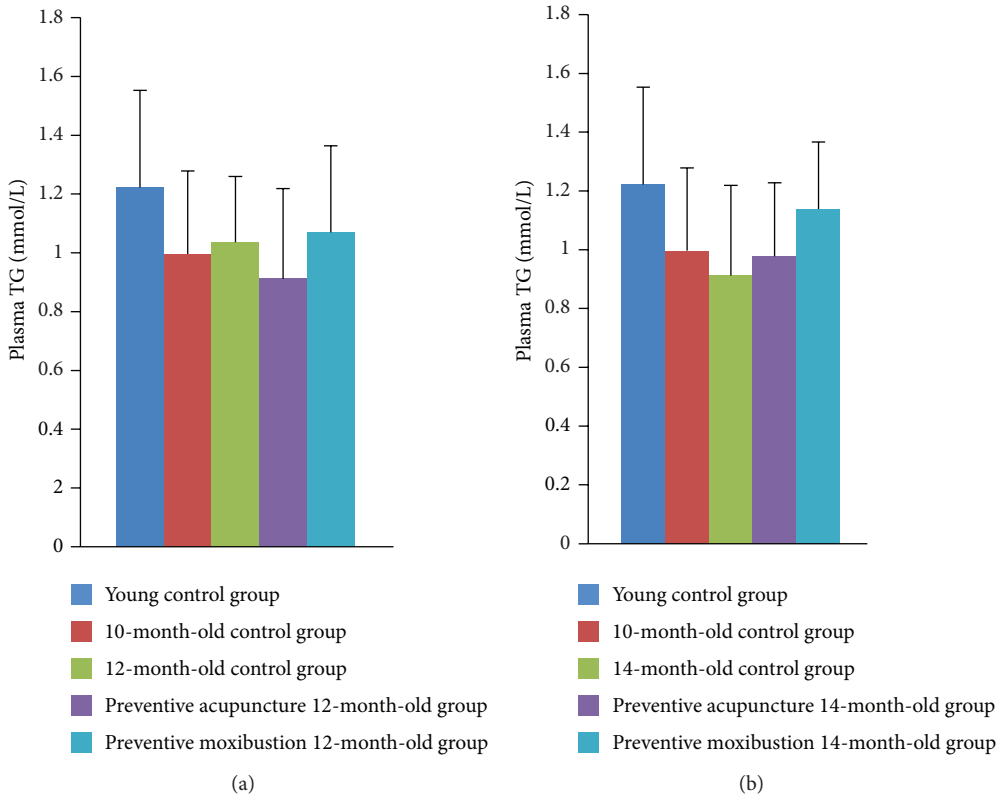

Figure 4: Effect of preventive acupuncture and moxibustion on plasma TG level.

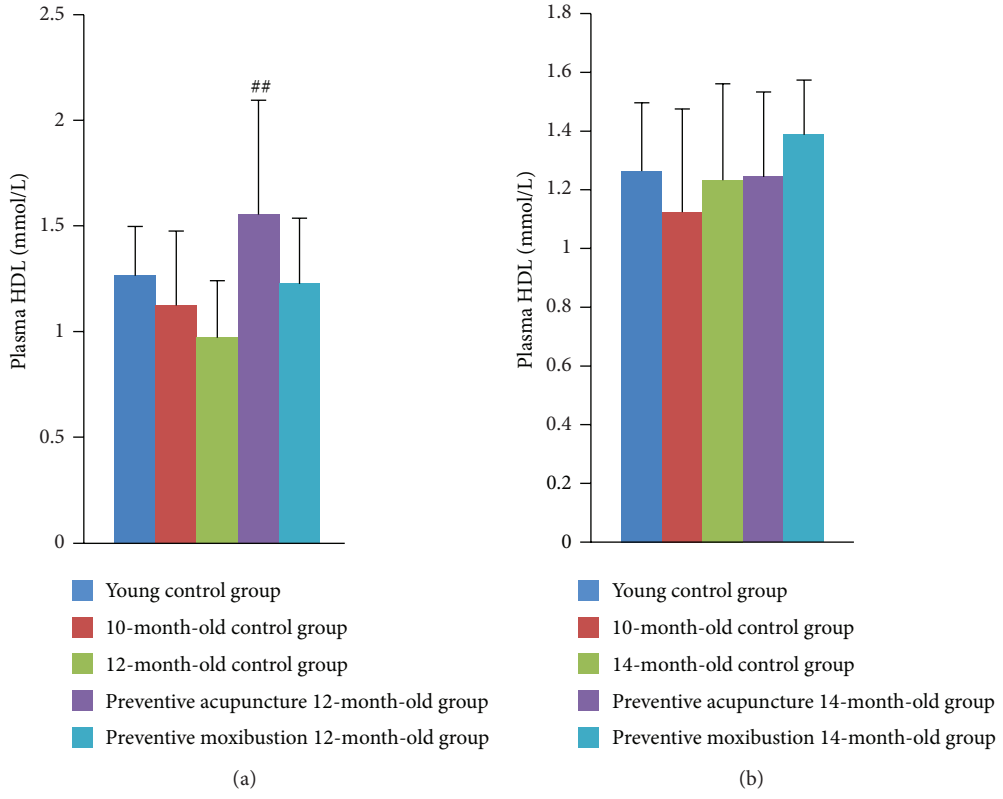

Figure 5: Effect of preventive acupuncture and moxibustion on plasma HDL level. Note: $^{\#\#}P < 0.01$, versus 12-month-old control group.

acupuncture 14-month-old group, and preventive moxibustion 14-month-old group (Figure 6(b)).

3.3. Effect of Preventive Acupuncture and Moxibustion on Uterus E_2 Level. Compared to young control group, uterus E_2 level decreased significantly in 12-month-old control group ($P = 0.049$), preventive acupuncture 12-month-old group ($P = 0.03$), preventive moxibustion 12-month-old group ($P = 0.03$), 14-month-old control group ($P = 0.024$), preventive acupuncture 14-month-old group ($P = 0.024$), and preventive moxibustion 14-month-old group ($P = 0.027$, Figures 7(a) and 7(b)). No significant differences were found among 12-month-old control group, preventive acupuncture 12-month-old group, and preventive moxibustion 12-month-old group (Figure 7(a)). No significant differences were found among 14-month-old control group, preventive acupuncture 14-month-old group, and preventive moxibustion 14-month-old group (Figure 7(b)).

4. Discussion

Preventive acupuncture and moxibustion refer to applying acupuncture and moxibustion treatment before the occurrence of diseases. It can stimulate meridian qi and reinforce body resistance. CV4, as an important point of the Conception Vessel, has invigorating effects on the immune system and can regulate the function of the genitourinary system according to TCM theory. In this study, we observed the effects of preventive acupuncture and moxibustion at CV4 on climacteric rats' fat accumulation, lipid metabolism disorder, and the content of uterus estrogen. The major finding of the present study is that preventive acupuncture and moxibustion at CV4 can prevent fat accumulation in natural aging climacteric rats, significantly decrease the concentrations of TC and LDL, and increase the concentration of HDL in plasma. Our results suggested that preventive acupuncture and moxibustion have beneficial influence on dyslipidemia in menopause period.

Menopause is a phase of life in women that signifies the end of their reproductive period. In this period, the kidney qi gradually declines and the internal environment of body changes, which result in multisystem dysfunction. Metabolic syndrome, manifested as dyslipidemia, hypertension, and central obesity, often occurred in menopause women. It is proved that menopause increased total body weight with central obesity as well as visceral (VAT) and subcutaneous abdominal fat (SAT) [24, 25]. In our study, it was found that, compared with rats in 10-month-old control group, natural aging climacteric rats tend to have increased body weight at the 12th and 14th months, which indicates fat accumulation during climacteric period. We also found that both preventive acupuncture and preventive moxibustion could prevent excessive weight gaining in climacteric rats. It is worthwhile to note that preventive acupuncture and preventive moxibustion have differential effects on body weight growth rate. Preventive acupuncture has a more rapid effect, which can be observed in the 12th month, while preventive moxibustion has cumulative effects that are more obvious in the 14th month. The reason may be that acupuncture and moxibustion are different forms of stimuli that could trigger different regulatory pathways from the central to the peripheral and finally lead to various therapeutic effects [26, 27].

Dyslipidemia is another important feature of menopausal metabolic syndrome. Several studies reported that women who had recently entered the menopause get increased concentrations of TC, TG, and LDL [4, 28]. Others demonstrated that menopausal women have comparatively lower HDL cholesterol levels compared with premenopausal women [29]. Results of this study showed that rats' blood lipid, especially plasma TC and LDL, had an adverse change along with the time. They started to increase at 10th month and increased significantly at the 12th month compared to young control group. At the 14th month, TC was still higher than that of the young control group, while LDL tended to decrease. Moreover, plasma HDL level decreased at the 12th month. Plasma TG showed no drastic changes along with the time. Therefore, we believe that natural aging climacteric rats experienced dyslipidemia. Clinical trials have proved that acupuncture has positive effects on blood lipid since it could decrease the levels of TC, TG, and LDL [14–16]. However, there are different results for acupuncture and moxibustion's effects on HDL. Some researchers found out that acupuncture and moxibustion can significantly increase HDL-C [15, 16] while others reported no significant effects [17]. In our study, we found that, compared to 12-month-old control group, rats in preventive acupuncture 12-month-old group were of significantly decreased plasma TG and LDL and increased HDL concentrations. This indicates that preventive acupuncture has a beneficial effect on climacteric dyslipidemia. However, the effect of preventive moxibustion is not found at the 12th month. Compared with 14-month-old group, both preventive acupuncture and preventive moxibustion reduced plasma TC concentration and the latter has more obvious effect. The differential effects of preventive acupuncture and moxibustion on dyslipidemia indicate that preventive acupuncture leads to a more quick reaction, while preventive moxibustion takes cumulative effect. Moreover, the data in this study shows that both preventive acupuncture and preventive moxibustion have no significant effect on plasma TG which is inconsistent with some previous researches [16, 17]. This may be explained by the time point of intervention (in our study we begin to intervene before the onset of the disease) and the application of different acupoints.

Although there are many reasons that contribute to menopausal metabolic syndrome, studies showed that signs and symptoms in menopausal transition are likely to be triggered by a progressive decrease of estrogenic secretion [30, 31]. Our research found that, compared with rats in the young control group, 12-month-old and 14-month-old rats had significantly decreased uterus estrogen (E_2). Our result demonstrated that climacteric females are of rapidly decreased E_2. We also found that preventive acupuncture and preventive moxibustion have a slight effect on uterus E_2 content. Compared to 12-month-old control group, uterus E_2 of preventive acupuncture 12-month-old rats and preventive moxibustion 12-month-old rats was even lower. However, uterus E_2 of preventive moxibustion 14-month-old rats tends

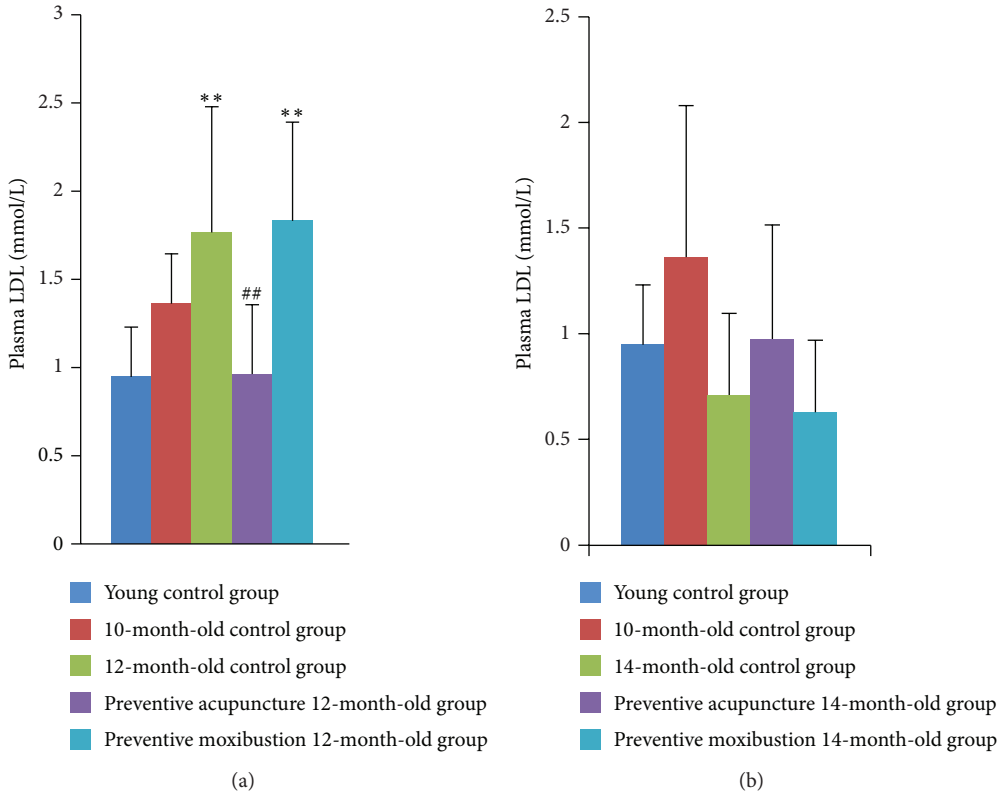

FIGURE 6: Effect of preventive acupuncture and moxibustion on plasma LDL level. Note: $^{**}P < 0.01$, versus young control group; $^{\#\#}P < 0.01$, versus 12-month-old control group.

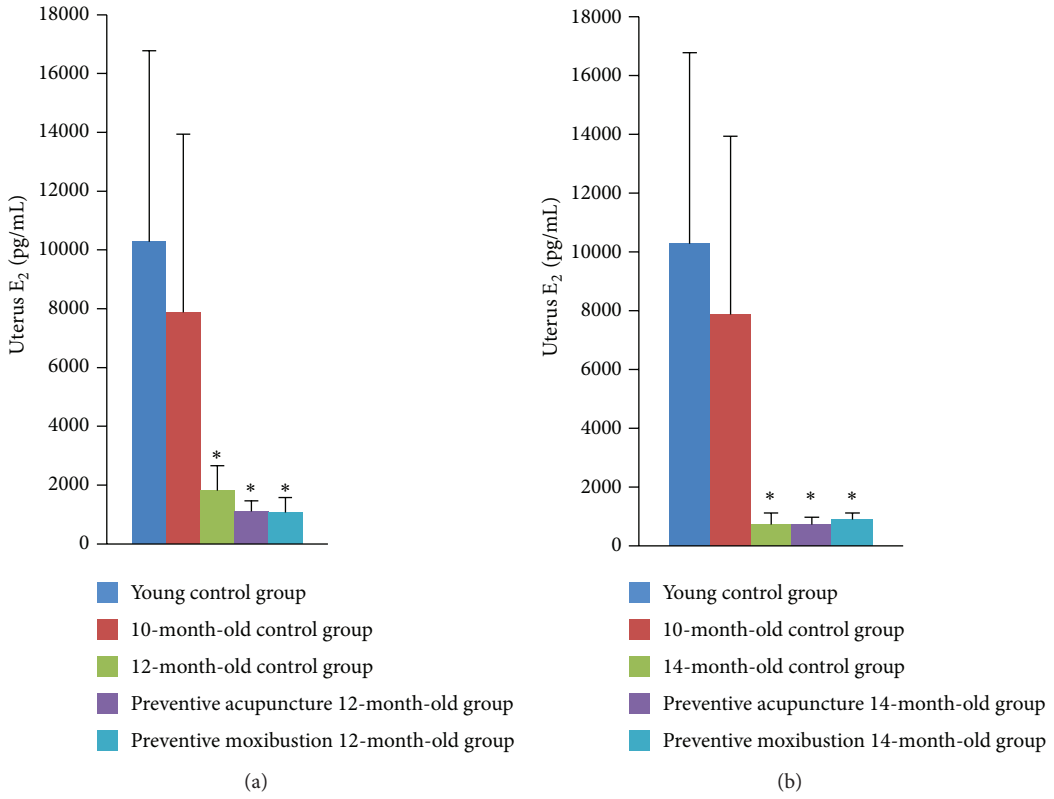

FIGURE 7: Effect of preventive acupuncture and moxibustion on uterus E_2 level. Note: $^{*}P < 0.05$, versus young control group.

to be increased compared with that of the 14-month-old control group. We speculate that 12-month-old rats have entered menopause period and are of internal milieu disorder. Although the preventive acupuncture and moxibustion are considered as a kind of beneficial prestimulations, they may further increase the burden of the organs leading to the decrease of uterus E_2. Since preventive moxibustion has cumulative effects, uterus E_2 in 14-month-old rats with preventive moxibustion tended to increase. The potential mechanism remains to be determined.

5. Conclusion

Preventive acupuncture and moxibustion have certain preventive effects on fat accumulation and dyslipidemia during climacteric period in rats. They can significantly decrease the concentrations of plasma TG and LDL and increase the plasma HDL. There are differences between the effects of preventive acupuncture and preventive moxibustion. The former has comparatively shorter time to achieve the effect, while the latter shows a cumulative effect.

Conflict of Interests

The authors report no conflict of interests.

Authors' Contribution

Shi-Peng Zhu, Yu-wei He, and Huan Chen contribute equally to this paper. Xiao-Hong Li and Liang-Xiao Ma are cocorrespondent authors of this paper.

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Research Article

Evaluation of the Effects of *Vaccinium arctostaphylos* L. Fruit Extract on Serum Lipids and hs-CRP Levels and Oxidative Stress in Adult Patients with Hyperlipidemia: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial

Rasool Soltani,[1] Mustafa Hakimi,[1] Sedigheh Asgary,[2] Syed Mustafa Ghanadian,[3] Mahtab Keshvari,[2] and Nizal Sarrafzadegan[2]

[1] *Department of Clinical Pharmacy and Pharmacy Practice, Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran*
[2] *Isfahan Cardiovascular Research Center, Isfahan Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran*
[3] *Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran*

Correspondence should be addressed to Sedigheh Asgary; sasgary@yahoo.com

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Background. Dyslipidemia produces atherosclerosis, which in turn results in coronary artery disease (CAD). Atherosclerosis is being considered as an inflammatory disease. *Vaccinium arctostaphylos* L. is a plant with fruits rich in anthocyanins. The aim of this study was to evaluate the effects of fruit extract of this plant on serum levels of lipids, hs-CRP, and malondialdehyde (MDA) as a marker of oxidative stress, in hyperlipidemic adult patients. *Methods.* In this randomized, double-blind, placebo-controlled clinical trial, 50 hyperlipidemic adult patients were randomly and equally assigned to receive either medicinal (*V. arctostaphylos* fruit extract) or placebo capsules twice daily for 4 weeks. Each medicinal capsule contained 45 ± 2 mg of anthocyanins. Fasting serum levels of total cholesterol, TG, LDL-C, HDL-C, hs-CRP, and MDA were obtained before and after the intervention and compared. *Results. V. arctostaphylos* fruit extract significantly reduced total cholesterol ($P < 0.001$), LDL-C ($P = 0.004$), TG ($P < 0.001$), and MDA ($P = 0.013$) compared to placebo but did not have any significant effect on HDL-C ($P = 0.631$) and hs-CRP ($P = 0.190$). *Conclusion.* Fruit extract of *Vaccinium arctostaphylos* has beneficial effects on serum lipid profile and oxidative stress in hyperlipidemic adult patients. Therefore, it could be considered as a supplement for treatment of dyslipidemia and prevention of atherosclerosis development.

1. Introduction

Dyslipidemia (one or more abnormalities of blood lipids) produces atherosclerosis, which in turn produces coronary heart disease (CHD) and coronary artery disease (CAD) including chronic stable angina, unstable angina, myocardial infarction, and ischemic cardiomyopathy [1]. Coronary atherosclerosis is the leading cause of death in developed countries [2]. Therefore, successful management of dyslipidemias alters the natural course of atherosclerosis and reduces CHD. Atherosclerosis is being considered as an inflammatory disease, since inflammatory processes play a key role in different stages of plaque development [3]. These include the activation of endothelial cells (ECs) leading to expression of adhesion molecules that attract inflammatory cells (e.g., neutrophils, T-cells, and monocytes) into the early atherosclerotic lesion. Within the plaque, smooth muscle cells (SMCs) and ECs secrete proinflammatory mediators that stimulate monocyte differentiation into macrophages. These macrophages further develop into foam cells upon

uptake of oxLDL and locally amplify the inflammatory response, thereby attracting more immune cells and inducing migration of SMCs into the plaque [4, 5].

High-sensitivity C-reactive protein (hs-CRP), a plasma protein synthesized by the liver, is a sensitive and dynamic systemic marker of inflammation [6]. CRP binds to LDL [7, 8] and is present in atherosclerotic plaques [9], so it has been proposed that CRP may have a causal role in coronary heart disease. A systematic review of 31 published prospective cohort studies has suggested that elevated CRP concentrations were associated consistently with increased CHD risk [10]. Therefore, it is possible that drugs with CRP-lowering property could reduce cardiovascular events in high-risk patients. In JUPITER trial, rosuvastatin, compared to placebo, reduced the combined primary endpoint of MI, stroke, arterial revascularization, hospitalization for unstable angina, or death from cardiovascular causes in patients with below average (<130 mg/dL) levels of LDL-C, and high levels of hs-CRP (>2 mg/dL) [11].

Oxidative stress, which occurs in response to an altered metabolic state, apoptosis, and lipid peroxidation, is additionally involved in the pathogenesis of atherosclerosis [12, 13]. Hyperlipidemia increases the production of reactive oxygen species (ROS) by endothelial cells, smooth muscle cells, and macrophages, leading to oxidative stress induction and low density lipoprotein (LDL) oxidation [13, 14]. The development of atherosclerosis is accompanied by an accumulation of oxidized LDL (oxLDL), one of the major oxidized lipids, in the arterial wall [12]. Therefore, phytochemicals and antioxidants that inhibit the production of ROS might have clinical value for treatment of atherosclerosis [15].

Vaccinium arctostaphylos L. (Caucasian Whortleberry) is a plant found in northern forests of Iran and commonly known as "Qaraqat." The fruits (berries) of this plant are rich in anthocyanins [16]. Anthocyanins are compounds with antioxidant, antiatherosclerotic, and antihyperlipidemic activities [17–21]. The aim of this study was to evaluate the effects of fruit extract of this plant on serum levels of lipids, hs-CRP, and malondialdehyde (MDA) as a marker of oxidative stress, in hyperlipidemic adult patients.

2. Materials and Methods

2.1. Plant Material and Extraction. Fresh ripe berries of *V. arctostaphylos* were collected from the forests of Asalem in the mountain chains of Alborz in the north of Iran in August 2012 and were identified by the Pharmacognosy Department of the Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran. After drying at room temperature (20–22°C), the berries were extracted by maceration with ethanol 70%. The extract was then filtrated, concentrated under vacuum, and standardized by spectroscopic determination of anthocyanin content.

2.2. Standardization of the Extract. Total anthocyanin content was determined by the pH differential method [22]. Experiments were carried out in two replicates and data were expressed as mmol cyanidin-3-glucoside equivalent. Briefly, two samples of 1 g dried extract were mixed with 10 mL of buffer solution with pH = 1 (125 mL of 0.2 M KCl and 375 mL of 0.2 M HCl) and 10 mL of buffer solution with pH = 4.5 (400 mL of 1 M sodium acetate, 240 mL of 1 M HCl, and 360 mL of water). Both solutions were diluted again 10 times with the buffers and the absorbance was read at 510 nm. Total anthocyanin content was determined by the following equation:

$$\text{anthocyanin content (\%)} = \left[\left(\text{Abs pH } 1.0 - \text{Abs pH } 4.5\right) \times 484.82 \times \frac{\text{DF}}{(24825 \times \text{Wt})}\right], \quad (1)$$

where 484.82 is the molecular mass of cyaniding-3-glicoside chloride, 24825 is molar absorptivity at 510 nm in pH = 1, DF is the dilution factor, and Wt is the sample weight.

2.3. Preparation of Medicinal and Placebo Capsules. The concentrated extract was mixed with tribasic calcium phosphate powder, granulated, and dried, and then its anthocyanin content was quantified. The medicinal capsules were filled with the mixed granules so that each contained 500 mg of dried granules equivalent to 45 ± 2 mg of total anthocyanin. The placebo capsules with shape, color, and size similar to medicinal ones were filled only with dried granulated tribasic calcium phosphate.

2.4. Patient Selection. All hyperlipidemic adult patients who met the following inclusion criteria were recruited in the study: (1) age ≥ 18 years, (2) serum levels of total cholesterol 200–300 mg/dL and/or triglyceride (TG) 150–199 mg/dL and/or LDL-C 130 130–190 mg/dL, (3) being non-smokers, (4) free of diseases affecting serum lipids (e.g., diabetes, thyroid disorders, and pancreatitis), (5) not using drugs or supplements affecting serum lipids (e.g., statins, fibrate derivatives, estrogens, progestins, beta-blockers, thiazide diuretics, and fish oil) within the last 3 months, (6) free of liver or kidney disease, and (7) nonpregnant, nonlactating women.

2.5. Study Design and Interventions. This was a randomized, double-blind, placebo-controlled clinical trial performed in Isfahan cardiovascular research center affiliated to Isfahan University of Medical Sciences, Isfahan, Iran, from September 2012 to January 2013. Informed consent was obtained from all participants and the study protocol was approved by the ethical committee (Research Ethics Committee of Isfahan Cardiovascular Research Center, Isfahan, Iran). Patients with inclusion criteria were randomly and equally assigned to either the study drug (*Vaccinium*) or placebo groups. Randomization was performed using random number table. Before intervention, the demographic characteristics (including BMI) and fasting serum levels of total cholesterol, TG, LDL-C, HDL-C, hs-CRP, and malondialdehyde (MDA) were recorded for all patients. Also, to detect any possible side effects of the drug on the liver and kidney, the serum levels of ALT, AST, BUN, and creatinine were obtained. The patients of drug and placebo groups used two medicinal and placebo

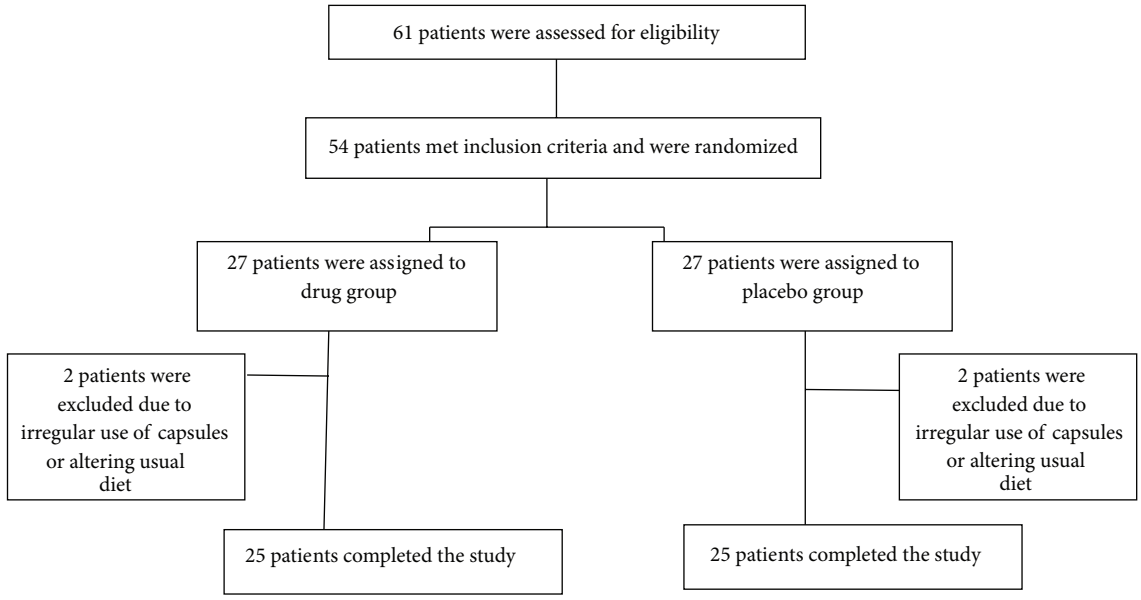

Figure 1: Flowchart of patient's enrollment in the study.

capsules, respectively, per day with food for 4 weeks. All patients were instructed to maintain their usual diet and physical activity and report any adverse effect during the study. The patients compliance was evaluated by counting their capsules at the end of use and their results were included in data analysis if they used more than 80% of their capsules. At the end of 4 weeks, all above-mentioned parameters were again measured and compared to baseline values. All participants, the physician, and laboratory personnel were blind to intervention type.

2.6. Statistical Analysis. SPSS 20.0 software was used for statistical analysis of resulting data. Kolmogorov-Smirnov test was performed to assess normal distribution of continuous data. Because of normal distribution of all continuous data, Student's t-test was used for comparisons. Paired-samples t-test was performed for comparison of measurements at the beginning and end of intervention within each group. General linear model (multivariate) analysis was used for comparing the mean changes of each parameter form baseline between drug (*Vaccinium*) and placebo groups. Chi-square test was performed for comparison of gender distribution in two groups. The level of significance was considered as $P < 0.05$.

3. Results

During the study, a total of 61 hyperlipidemic subjects were screened, with 54 found to be eligible according to the inclusion criteria; however, 2 subjects were excluded from each group during the trial because of either irregular use of capsules or altering their usual diet. Therefore, statistical analyses were performed for a total of 50 subjects (25 in each group) who fully completed the trial (Figure 1).

Table 1 shows baseline demographic and clinical characteristics of study patients. As shown, all subjects were matched regarding baseline values.

Table 2 presents changes of tested parameters from baseline after 4 weeks of intervention in *Vaccinium* and placebo groups and comparison of them. As shown, *Vaccinium* significantly reduced total cholesterol, LDL-C, TG, and MDA compared to placebo but had not any statistically significant effect on other tested parameters.

Table 3 shows the effects of *Vaccinium* and placebo on laboratory markers of liver and kidney function after 4 weeks of use. No significant changes were detected in these values during the study. Also, no complication or adverse effect was reported by patients of both groups.

4. Discussion

In the present study, we investigated the effects of fruit concentrated extract of *Vaccinium arctostaphylos* on serum lipid profile and serum levels of hs-CRP and MDA in hyperlipidemic adult patients. Only one previous study, published at the time of this writing, exists for the effects of *Vaccinium arctostaphylos* on lipid profile of hyperlipidemic patients [23]; however, several studies have been conducted for some other species of the genus Vaccinium. Our study showed cholesterol-, LDL-C-, and triglyceride-lowering effects of the berries of *Vaccinium arctostaphylos*, but no significant effect was observed on HDL-C. Similarly, in the above-mentioned recently published study conducted by Kianbakht et al., the use of hydroalcoholic extract of *V. arctostaphylos* (one 350 mg capsule every 8 hours) for 2 months, compared to placebo, significantly lowered the blood levels of total cholesterol ($P < 0.001$), TG ($P = 0.002$), and LDL-C ($P = 0.002$) [23]; however, in contrast to our result, the extract significantly increased the blood HDL-C levels ($P < 0.001$).

Table 1: Baseline demographic and clinical characteristics of study patients The values are presented as mean ± SD.

Parameter (unit)	Vaccinium ($n = 25$)	Placebo ($n = 25$)	P value
Age (years)	48.08 ± 16.39	46.36 ± 16.59	0.714
Gender (%male)	40.0	40.0	0.723
BMI (kg/m^2)	25.40 ± 1.75	25.21 ± 2.01	0.720
Total cholesterol (mg/dL)	226.48 ± 32.09	220.20 ± 45.76	0.577
LDL-C (mg/dL)	132.80 ± 23.76	121.08 ± 32.06	0.149
TG (mg/dL)	226.20 ± 96.99	191.36 ± 56.54	0.129
HDL-C (mg/dL)	45.76 ± 9.73	46.56 ± 10.52	0.781
hs-CRP (mg/L)	2.53 ± 2.33	2.80 ± 2.35	0.737
MDA (μmol/L)	0.738 ± 0.266	0.707 ± 0.212	0.653

Table 2: The effects of interventions on tested parameters after 4 weeks in study patients. The values are presented as mean ± SD.

Parameter (unit)	Vaccinium ($n = 25$)	Placebo ($n = 25$)	P-value
BMI (kg/m^2)			
Baseline	25.40 ± 1.75	25.21 ± 2.01	
End	25.06 ± 1.60	25.31 ± 2.07	0.062
Change	−0.33 ± 0.45	0.10 ± 0.45	
Total cholesterol (mg/dL)			
Baseline	226.48 ± 32.09	220.20 ± 45.76	
End	192.04 ± 28.81	223.52 ± 42.31	<0.001
Change	−34.44 ± 22.44	3.32 ± 15.47	
LDL-C (mg/dL)			
Baseline	132.80 ± 23.76	121.08 ± 32.06	
End	121.36 ± 27.46	124.36 ± 30.29	0.004
Change	−11.44 ± 3.28	3.28 ± 16.04	
TG (mg/dL)			
Baseline	226.20 ± 96.99	191.36 ± 56.54	
End	156.56 ± 46.76	198.56 ± 63.30	<0.001
Change	−69.64 ± 76.86	7.20 ± 27.51	
HDL-C (mg/dL)			
Baseline	45.76 ± 9.73	46.56 ± 10.52	
End	45.60 ± 9.72	45.68 ± 9.72	0.631
Change	−0.16 ± 6.36	−0.88 ± 3.85	
hs-CRP (mg/L)			
Baseline	2.53 ± 2.33	2.80 ± 2.35	
End	1.99 ± 1.60	3.04 ± 2.34	0.190
Change	−0.54 ± 2.83	0.23 ± 0.49	
MDA (μmol/L)			
Baseline	0.738 ± 0.266	0.707 ± 0.212	
End	0.648 ± 0.16	0.769 ± 0.26	0.013
Change	−0.09 ± 0.23	0.06 ± 0.18	

Table 3: The effects of interventions on the liver and kidney function tests after 4 weeks. The values are presented as mean ± SD.

Parameter (unit)	Vaccinium ($n = 25$)			Placebo ($n = 25$)		
	Baseline	Week 4	P-value	Baseline	Week 4	P-value
ALT (U/L)	22.48 ± 10.88	20.88 ± 11.51	0.193	24.72 ± 9.12	24.24 ± 8.18	0.643
AST (U/L)	21.60 ± 7.77	20.60 ± 7.94	0.246	23.20 ± 7.48	23.68 ± 8.48	0.580
BUN (mg/dL)	14.28 ± 3.27	13.96 ± 4.60	0.616	14.60 ± 4.41	13.36 ± 4.76	0.075
Creatinine (mg/dL)	0.96 ± 0.15	0.92 ± 0.14	0.166	0.96 ± 0.23	0.93 ± 0.22	0.513

This difference could be due to higher dose of consumed extract and longer duration of this study. The lipid-lowering effects of some other species of Vaccinium have been demonstrated in several animal studies [24–26] and clinical trials [27, 28]. In the study of Lee et al., the use of cranberry (*Vaccinium macrocarpon*) extract with the daily dose of 1500 mg for 12 weeks by patients with type 2 diabetes caused significant reduction in serum levels of LDL-C and total cholesterol and total cholesterol/HDL cholesterol ratio [28]. In contrast to our results, in a study of patients with features of metabolic syndrome, daily consumption of 400 g fresh bilberries (*Vaccinium myrtillus*) for 8 weeks had not any significant effect on serum lipids [29]. In another study performed by Basu et al., the use of cranberry (*Vaccinium macrocarpon*) juice for 8 weeks did not significantly affect lipid profile in patients with metabolic syndrome [30]. The observed lipid-lowering effects of *V. arctostaphylos* in the present study may be due to its anthocyanin content. Also, water-soluble fibers in the berries could be responsible for reduction of serum cholesterol through binding to bile salts in the intestine leading to inhibition of their enterohepatic cycle [31]. It is noteworthy that the administration of our tested extract for a longer period of time might be more effective at lowering the serum lipids.

In the present research, *V. arctostaphylos* had not any significant effect on the serum level of hs-CRP in hyperlipidemic subjects. In the study of Kolehmainen et al., daily intake of 400 g fresh bilberries (*Vaccinium myrtillus*) for 8 weeks significantly reduced serum hs-CRP and some other inflammatory markers [29]. Also, in the study of Karlsen et al., supplementation with 330 mL bilberry juice/day for 4 weeks resulted in significant decrease in plasma concentrations of CRP [32]. Conversely, others have shown no significant effects [33–35]. Considering a slight decrease in hs-CRP by *V. arctostaphylos* (in contrast to placebo) observed in our study, the use of higher doses of this extract for longer periods may have more significant effect on this inflammatory marker.

In our research, *V. arctostaphylos* fruits extract showed significant effect in reduction of MDA as a marker of oxidative stress compared to placebo ($P = 0.013$). Consistently, in the study of Basu et al., daily use of cranberry juice for 8 weeks caused a significant decrease in both ox-LDL and MDA versus placebo treatment (−33% versus −17% and −50% versus +7%, resp.) [30]; however, in this study, the baseline MDA values were higher than those of our subjects and the duration of intervention was longer. Our results show antioxidant activity of the fruit extracts of *V. arctostaphylos*. This suggests that it can be considered as a nutritional supplement for prevention of atherosclerosis in high-risk patients including hyperlipidemic subjects as increasing evidence indicates inhibition of LDL oxidation through attenuation of oxidative stress is beneficial in preventing the development of atherosclerosis [12]. The reduction of plasma MDA concentrations in response to *V. arctostaphylos* is likely to be attributable to its polyphenolic compounds as they exert a potent antioxidant activity [36, 37].

In conclusion, daily consumption of the fruit extract of *Vaccinium arctostaphylos* significantly reduces the serum levels of total cholesterol, LDL-C, and triglyceride (TG) and oxidative stress in hyperlipidemic patients. Therefore, this extract could be considered as a potential agent for treatment of dyslipidemia and prevention of atherosclerosis development. However, more studies with higher doses and longer periods of time are mandatory.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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