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Original Research Article

Extract of *Zanthoxylum bungeanum* Maxim Seed Oil Reduces Hyperlipidemia in Hamsters Fed High-Fat Diet via Activation of Peroxisome Proliferator-Activated Receptor γ

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Abstract

Purpose: *To investigate the anti-hyperlipidaemic effect of extract of Zanthoxylum bungeanum Maxim. seed oil (EZSO) on high-fat diet (HFD)-induced hyperlipidemic hamsters.*
Methods: *Following feeding with HFD for 30 days, hyperlipidemic hamsters were intragastrically treated with EZSO for 60 days. Serum levels of triglyceride (TG), total cholesterol (TC), low-density-lipoprotein-cholesterol (LDL-C), nitric oxide (NO) and malondialdehyde (MDA) were analyzed. Protein expression and location of peroxisome proliferator-activated receptor γ (PPARγ) in liver were determined by Western blot and immunohistochemical assays, respectively.*
Results: *EZSO at 5 and 10 g/kg significantly reduced levels of TG by 26 and 23 % ($p < 0.05$), TC by 19 % ($p < 0.01$) and 13 % ($p < 0.01$), LDL-C by 18 % ($p < 0.05$) and 21 % ($p < 0.01$), NO by 15 % ($p < 0.01$) and 31 % ($p < 0.01$), and MDA by 16 % ($p < 0.05$) and 30 % ($p < 0.01$), respectively, in serum of hyperlipidemic hamsters. However, EZSO did not show significant effect on HDL-C level in serum. Furthermore, EZSO at 5 and 10 g/kg markedly promoted protein expression of PPARγ by 71 % ($p < 0.05$) and 102 % ($p < 0.01$) in liver tissue of hyperlipidemic hamsters. EZSO also notably increased the content of PPARγ protein in the nucleus of liver cells of hyperlipidemic hamsters.*
Conclusion: *These findings suggest that EZSO can reduce hyperlipidemia and improve oxidative stress in hyperlipidemic hamsters through activation of PPARγ, and that EZSO is a promising novel hypolipidemic health product.*

Keywords: *Zanthoxylum bungeanum, Peroxisome proliferator activated receptor γ, Hyperlipidemia, Hamster, High-fat diet*

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INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death and a major economic burden for the health care system of the developed countries all over the world. Preventing the occurrence of CHD with dietetic interventions is a promising strategy that has attracted a lot of research attention [1].

There is a great number of scientific evidence from human trials that n-3 polyunsaturated fatty acids (PUFAs) from fish or fish oil supplements include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can significantly

reduce risk factors for heart disease [2,3]. For alpha-linolenic acid (ALA), the relation to cardiovascular health is contradictory. Some evidence has been demonstrated that dietary ALA has significant cardio-protective effects [4]. However, another study found that most cardiovascular risk markers, including triglyceride (TG), total cholesterol (TC) and low-density-lipoprotein-cholesterol (LDL-C), did not appear to be affected after treatment with ALA [5]. Further researches in human and animal are both needed to clarify the cardioprotective efficacy of ALA.

Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor and acts as intracellular lipid and glucose sensor to regulate lipid and lipoprotein metabolism, glucose homoeostasis and inflammatory response [6]. The n-3PUFAs are potent activators of PPARγ [7]. Thus, dietary supplementation with ALA or EPA plus DHA should induce expression of key proteins involved in cardiac lipid and lipoprotein metabolism through activating PPARγ [8].

In the present study, we investigated the effect of extract of *Zanthoxylum bungeanum* Maxim. seed oil (EZSO), which is rich in ALA, on serum lipid level, oxidation status and underlying mechanism in hyperlipidemic hamsters. These findings may provide insight into the cardioprotective effect of EZSO and help guide the exploration of utilization of *Zanthoxylum bungeanum* Maxim. seeds.

EXPERIMENTAL

EZSO preparation

Zanthoxylum bungeanum Maxim. was purchased from Hangcheng Xiongdi Pepper Co., Ltd. (Shangxi, China) and was identified by Dr. Jifen Zhang, College of Pharmaceutical Sciences, Southwest University (Chongqing, China). It was authenticated using thin-layer chromatography with reference to methods recommended by the Chinese Pharmacopoeia (Chinese Pharmacopoeia, 2010). A voucher specimen number 2012-112 was deposited at the Herbarium of the Chongqing Key Laboratory of Nature Medicine Research, Chongqing Technology and Business University. EZSO was prepared and its compositions analyzed as described elsewhere [9].

Animals and high-fat diet

Male golden Syrian hamsters weighing 100-120 g were purchased from Chinese Academy of Sciences (Beijing, China). All hamsters were housed in a temperature-controlled room (23 ± 2 °C) under a light/dark cycle with lights on from 7:00 am to 7:00 pm. They were allowed food and water *ad libitum*. The animals were adapted to experiment environment for 1 week before experiments were carried out. All animal procedures were approved by the Ethical Committee in Animal Research of Chongqing Technology and Business University (Ethics No. 2011-3-19/CTBU). The high-fat diets (HFD) were purchased from Vital River Laboratories (Beijing, China). The diet contained 10 % fat, 1 % cholesterol, 10 % egg yolk powder, 0.2 % sodium cholate.

Animal groups and treatment

Hamsters were fed on HFD for 30 days to develop hyperlipidemia. Hyperlipidemic hamsters were randomly divided into six groups with 10 hamsters in each group: (1) Control group; (2) Hyperlipidemia (HPL) group; (3) Simvastatin (SIM, Hangzhou Merck Sharp & Dohme Pharmaceutical Co., Ltd., China); (4) 2.5 g/kg EZSO group; (5) 5 g/kg EZSO group; (6) 10 g/kg EZSO group. Hamsters of groups 2 to 6 were fed on HFD during treatment. There were no significant differences in TG levels among groups 2 to 6 before treatment. Hamsters in groups 3, 4, 5 and 6 were intragastrically treated with SIM or EZSO once a day. Hamsters in groups 1 and 2 were intragastrically treated with an equal volume of saline once a day. All the hamsters received treatment for 60 days.

Biochemical analysis

Hamsters were fasted for 12 h and sacrificed under anesthesia. Blood was collected from hearts and serum was obtained by centrifuge at 3000 rpm for 20 min after blood coagulated. The serum was kept at -20 °C until analysis. TG, TC, HDL-C, LDL-C, NO and MDA levels in serum were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Assays were performed according to the manufacturer's instructions.

Immunohistochemical assay

The liver of hamsters was removed and fixed in 4 % paraformaldehyde for 24 h at 4 °C. The tissues were embedded in paraffin, then serial paraffin sections (5 μm) were stained using a two-step polymer (non-biotin) detection kit (GBI Labs, USA). The slides were de-paraffinized and blocked for 10 min with 3 % hydrogen peroxide at room temperature. Antigen retrieval with Tris/EDTA buffer (10 mM Tris-HCl, 1 mM EDTA,

0.05 % Tween 20, pH 9.0) was performed by microwave for 10 min. The sections were incubated with rabbit polyclonal PPARγ (Santa Cruz, USA) overnight at 4 °C with dilution of 1:100. After washing in TBS (0.5 M Tris-HCl, 0.15 M NaCl, pH 7.6), the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 min at room temperature. After washing in TBS, the complex was visualized using 3, 3'-diaminobenzidine for 5 minutes. The sections were counterstained with hematoxylin and observed under a light microscope to calculate the number of positive-stained cells ($PPARg^+$ cells) per area (·100).

Western blot assay

Frozen liver tissue was homogenized in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, pH 7.6) and EDTA-free protease inhibitor cocktail (Roche, Switzerland). Equal amounts of protein samples were separated by SDS-PAGE electrophoresis and transferred onto PVDF membranes. After blocked with 5 % defatting milk in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween 20, pH 7.6) for 1 h at room temperature, PVDF membranes were incubated with anti-PPAR · (Santa Cruz, USA) or anti-β-actin antibody at 4 °C overnight. Then the membranes were incubated for 1 h at room temperature with appropriate HRP-conjugated secondary antibody. The protein bands were detected with enhanced chemiluminescence reagents (Milipore, USA). Chemiluminescent signals were detected and analyzed using the ChemiDoc XRS imaging system (Bio-Rad, USA).

Statistical analysis

All data were presented as mean ± standard error (SEM). To analyze immunohistochemical data, Mann-Whitney U test was used. Statistical comparison of other parameters was evaluated by ANOVA using SPSS 16 software. Results were considered significant at $p < 0.05$.

RESULTS

Effects of EZSO on TG, TC, LDL-C and HDL-C levels in serum

Serum levels of TG, TC, LDL-C and HDL-C of HPL group hamsters significantly increased after 30 days on HFD relatively to those of Control group hamsters (all $p < 0.001$). Doses of 5 g/kg and 10 g/kg EZSO significantly reduced the serum levels of TG by approximately 26 % ($p < 0.05$) and 23 % ($p < 0.05$), TC by approximately 19 % ($p < 0.01$) and 13 % ($p < 0.01$), LDL-C by approximately 18 % ($p < 0.05$) and 21 % ($p < 0.01$) in hyperlipidemic hamsters. SIM markedly elevated the serum level of HDL-C by approximately 67 % ($p < 0.01$) compared with HPL group. However, all the doses of EZSO did not show significant effect on the serum level of HDL-C (Table 1).

Effects of EZSO on MDA and NO levels in serum

Serum levels of MDA and NO in HPL group hamsters were significantly higher than Control group hamsters, as showed in Table 3 (both $p < 0.001$). Doses of 5 g/kg and 10 g/kg EZSO significantly decreased the levels of MDA by approximately 16 % ($p < 0.05$) and 30 % ($p < 0.001$), NO by approximately 15 % ($p < 0.01$) and 31 % ($p < 0.001$) (Table 2).

Effects of EZSO on PPAR · expression and location in liver cells

In the immunohistochemical assay, PPAR · expression was significantly higher in the liver of the HPL group hamsters than that of Control group hamsters ($p < 0.01$), and PPAR · was detected both in the cytoplasm and nucleus. SIM markedly promoted the expression of PPAR · in the liver by approximately 200 % compared with the HPL group ($p < 0.01$). Doses of 5 g/kg and 10 g/kg EZSO significantly increased the expression of PPAR · in the liver by approximately 101 % ($p < 0.01$) and 142 % ($p < 0.01$) compared with the HPL group. Furthermore, in SIM, 5 and 10 g/kg EZSO groups, more PPAR · was detected in the nucleus than in the cytoplasm (Figure 1).

Effect of EZSO on PPAR · expression in liver tissues

In Western blot assay, the PPAR · protein was significantly increased by approximately 120 % in the liver tissues of the HPL group compared with the Normal group ($p < 0.01$). SIM markedly promoted the expression of PPAR · in the liver tissues by approximately 120 % ($p < 0.01$) compared with the HPL group. Doses of 5 g/kg and 10 g/kg EZSO significantly increased the expression of PPAR · in liver tissues by approximately 70 % ($p < 0.05$) and 100 % ($p < 0.01$) compared with the HPL group (Figure 2).

Table 1: Effect of EZSO on the serum levels of TG, TC, HDL-C and LDL-C in hyperlipidemic hamsters

Group	Dose	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control		1.93 ± 0.81	1.98 ± 0.43	1.20 ± 0.23	0.93 ± 0.32
HPL		7.60 ± 2.53*	9.35 ± 2.30*	2.54 ± 0.41*	7.84 ± 1.61*
SIM	4 mg/kg	3.34 ± 1.64$^{\#\#}$	6.54 ± 1.55$^{\#\#}$	4.24 ± 1.20$^{\#\#}$	2.87 ± 0.98$^{\#\#}$
EZSO	2.5 g/kg	7.58 ± 2.75	8.67 ± 2.44	2.51 ± 0.46	7.67 ± 2.21
	5 g/kg	5.59 ± 1.55$^{\#}$	7.61 ± 1.62$^{\#\#}$	2.45 ± 0.41	6.45 ± 1.58$^{\#}$
	10 g/kg	5.87 ± 1.45$^{\#}$	8.11 ± 0.97$^{\#\#}$	2.54 ± 0.70	6.21 ± 0.86$^{\#\#}$

Data are presented as mean ± SEM (n = 10); $^{*}p < 0.001$ vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. HPL group

Table 2: Effects of EZSO on serum levels of MDA and NO in hyperlipidemic hamsters

Groups	Dose	MDA (μmol/L)	NO (μmol/L)
Control		4.86 ± 0.87	11.31 ± 2.97
HPL		20.69 ± 4.39*	62.87 ± 7.11*
SIM	4 mg/kg	9.65 ± 1.35$^{\#\#}$	21.27 ± 5.19$^{\#\#}$
EZSO	2.5 g/kg	19.80 ± 3.32	61.78 ± 7.88
	5 g/kg	17.37 ± 2.36$^{\#}$	53.46 ± 6.25$^{\#\#}$
	10 g/kg	14.41 ± 2.64$^{\#\#}$	44.06 ± 6.48$^{\#\#}$

Data are presented as mean ± SEM (n = 10). $^{*}p < 0.01$ vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. HPL group

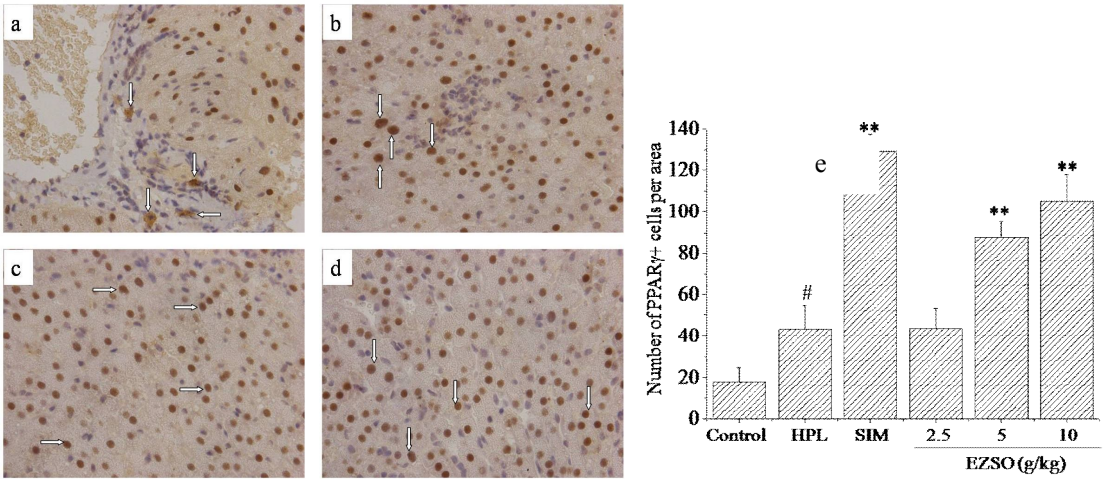

Fig 1: Effect of EZSO on PPAR・ expression and location in the liver cells of hamsters by immunohistochemical assay. A: Expression and location of PPAR・ protein in the liver cells of hamsters were analyzed by immunohistochemical assay. The results presented were representative of immunohistochemical analysis (・100). (a) HPL group; (b) SIM group; (c) 5 g/kg EZSO; (d) 10 g/kg EZSO, (e) Bar charts show quantitative evaluation of the number of PPAR・$^{+}$ cells per area (・100). $^{\#}p < 0.01$ compared with Control group; $^{**}p < 0.01$ compared with HPL group

DISCUSSION

The present study demonstrates that EZSO, which is rich in ALA, significantly decreases the levels of TG, TC and LDL-C in serum of HFD-induced hyperlipidemic hamsters. However, the hypotriglyceridemic and hypocholesterolemic effects were not dose-dependent. The mean TG and TC concentrations of hamsters treated with 5 g/kg EZSO were similar to those of 10 g/kg EZSO. This data implies that the low dose of EZSO may provide cardioprotective effects similar to that of higher doses in this animal model.

In this study we used the hamsters to evaluate the effects of EZSO on levels of serum lipids. Previous studies have used rats for the research of hypolipidemic agents. However, rats may not

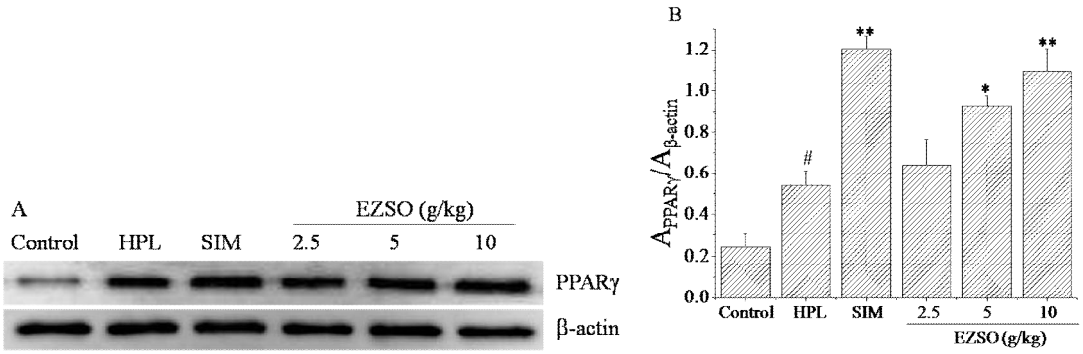

Fig 2: Effect of EZSO on PPAR expression in the liver tissues of hamsters. A: Expression of PPAR protein in the liver tissues of hamsters was analyzed by Western blot assay. β-actin is used as loading control. The result presented was representative of four independent experiments; B: Bar charts show quantitative evaluation of PPAR bands by densitometry from four independent experiments; $^{\#}p < 0.01$ compared with Control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with HPL group

be a good model for lipid studies as they are associated with some inherent deficiencies including high HDL to LDL ratios, lack of gallbladders, require high cholesterol diets with supplemental bile acids and thiouracil to develop hypercholesterolemia, and the organ contribution of cholesterol synthesis varies significantly from humans [10]. The plasma lipoprotein profile, bile acid pool composition, and metabolic responses to changes in dietary cholesterol make the hamsters much more comparable to humans. Hence, hamsters represent a better animal model for the evaluation of lipid-lowing agents [11]. In this study we found that TG, TC, HDL-C and LDL-C in hamster serum increased about 4-, 5-, 2- and 8-fold, respectively, after continuous feeding with HFD for 30 days. These results suggest that hamsters are a good animal model for hyperlipidemia.

The cardioprotective action of n-3 PUFAs have been demonstrated by studies of animal models and human trails [3]. EZSO is rich in ALA, where, ALA may be the main component of EZSO responsible for its hypolipidemic effect. Much evidence support a role for ALA and its metabolic products in improving many cardiovascular risk factors including TG, TC and LDL-C in both animals and humans, reducing the risk of CHD [12,13]. It has been reported that in rats, flaxseed, which is rich in ALA, reduced plasma TC, TG and LDL-C by 33 %, 23 % and 67 %, respectively [14]. Study showed that ALA might alter serum lipid concentrations when fed at physiologically relevant concentrations [15]. A study in Costa Rica found that high adipose tissue ALA content was associated with a lower risk of non-fatal acute myocardial infarction, which suggested that consumption of vegetable oils rich in ALA confers protection against cardiovascular disease [16].

However, some researchers arrived at different conclusions. In a 12-week trial involving 86 healthy Canadian men and women, flaxseed oil (ALA dose 1 g/d) had no effects on levels of serum TC, TG, LDL-C, HDL-C, LDL oxidation, platelet aggregation, or inflammatory markers [17]. In another randomized trial in 62 healthy men in the United States, flax oil (ALA doses of 1.2, 2.4, or 3.6 g/d), did not alter plasma TC, TG, HDL-C [18]. Much of the data variability on serum lipids in human or animal studies is attributed to complex contributions of dietary cholesterol, fat and other compositions of the diet, animal gender, drug dosage, medication time, route of administration and human or animal genetic background. To minimize the effect of these confounders in this study, only hamsters with the same age, sex and weight were used, and hamsters were not administered by the admixture with diets and drugs which may cause the dosage inconsistent, but by gastric lavage daily, and simvastatin, a widely used hypolipidemic drug in clinical practice, was used as a positive control drug. We found in this study that EZSO markedly reduced the levels of TG by 26 %, TC by 19 % and LDL-C by 18 % in serum of hyperlipidemic hamsters. Although the hypolipidemic efficiency of EZSO was less than that of simvastatin, these data indicated that EZSO might improve the cardiovascular risk markers. Interestedly, we found that EZSO did not statistically change the content of serum HDL-C in hyperlipidemic hamsters. Morise *et al* reported that ALA (linseed oil) were not significantly decreased the serum HDL-C in hyperlipidemic hamsters [19], which was consistent with our experimental results.

Since currently the protection against CVD of ALA has been identified and the lipid-lowering results are not consistent, other underlying

mechanisms may be associated with the cardiovascular protective effects of ALA. Oxidative stress is involved in the pathogenesis of CHD. Excess oxidative stress is caused by an imbalance between pro- and anti-oxidants, leading to an overproduction of free radicals, which may damage cellular components and functions. Hypertriglyceridemia or hypercholesterolemia may increase activity of the oxidant producing enzymes to induce the overproduction of free radicals and NO, which may damage the vascular structure and functions [20]. In this study, we found that EZSO decreased the contents of two markers of oxidative stress in hyperlipidemia in a dose-dependent manner, suggesting that anti-oxidative stress might be involved in the cardioprotective action of EZSO.

PPARγ is a member of the nuclear hormone receptor super family, which forms heterodimers with the retinoid X receptor. After the nuclear translocation, PPARγ regulates genes expression by binding to a PPAR-responsive element (PPRE) in the promoter region of a variety of target gene [6]. Activation of the PPARγ can improve insulin sensitivity and reduce hyperlipidemia, hyperglycemia and inflammation [7]. In this study, we found that EZSO increased the expression of PPARγ in a dose-dependent manner using immunohistochemical assay and Western blot assay. We also found that more PPARγ was detected in the nucleus than in the cytoplasm after EZSO treatment. These results suggest that EZSO may induce the nuclear translocation and transcriptional activation of PPARγ.

CONCLUSION

In this study, we found that EZSO significantly decreased the levels of TG, TC, LDL-C, NO and MDA in serum of hyperlipidemic hamsters. This indicates that EZSO shows potential hypolipidemic and anti-oxidative effects in hyperlipidemic hamsters. These findings suggest that EZSO would be a promising candidate as a novel hypolipidemic health product.

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