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The effects of *Zanthoxylum bungeanum* extract on lipid metabolism induced by sterols

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ABSTRACT

Variant pharmacological activities of *Zanthoxylum bungeanum* were determined before. The aim of this study was to assess whether *Z. bungeanum* could regulate lipid metabolism. The cholesterol over-loading HepG2 cells induced by sterols were used as *in vitro* model to study lipid-lowering activities of the *n*-butanol (BuOH) fraction isolated from *Z. bungeanum* (ZBBu). Male apolipoprotein E knockout (apoE-KO) mice with high fat diet were used as *in vivo* model. We firstly demonstrated ZBBu had effects on reversed lipid accumulation, decreased apoB and enhanced apoA1 secretion. It increased the amount of low density lipoprotein receptor (LDLR) protein, also significantly inhibited the expression of SREBP-1 and SREBP-2's target molecule (hydroxy methylglutaryl coenzyme A reductase, HMGCR), which might be active in stimulation of RCT. And the expression of genes involved in RCT, such as CYP27A1, LXR-α, ABCG1, was promoted by ZBBu. Furthermore, ZBBu could reduce serum TC, TG levels in apoE-KO mice. Our study indicated that ZBBu could regulate the lipid metabolism through increasing the amount of low density lipoprotein receptor (LDLR) and inducing the expression of genes involved in RCT.

1. Introduction

Atherosclerosis, the leading cause of death in modern society, is a disease of lipid metabolism disorder as well as chronic inflammation. The lipid homeostasis results from the balance between synthesis and elimination. During the process of lipid biosynthesis SREBP-1 plays an essential role in fatty acid and TG synthesis, while SREBP-2 regulates the genes involved in cholesterol biosynthesis (1), such as LDLR and HMGCR, a main rate-limiting enzyme of the mevalonate pathway that produces cholesterol and other iso-prenoids. Low density lipoprotein cholesterol (LDL-c) has been considered to be "bad cholesterol" which results in an increased risk of atherosclerosis. When in excess it can deposit in artery and initiates atherosclerosis. And its clearance can be mediated by hepatic LDLR (2). Differ from LDL-c, high density lipoprotein

cholesterol (HDL-c) may owe the protection against atherosclerosis not only to reverse cholesterol transport, but also to provide anti-oxidant enzymes (3). During the process of lipid elimination, ATP-binding cassette (ABC) transporters play important roles in exporting free cholesterol (FC) to extracellular apolipoproteins, such as apoB and apoA1 (4), which is called RCT. The interplay of those factors makes cholesterol stay balanced.

Zanthoxylum bungeanum belongs to the rutaceae family and has been widely used as aromas in China and reported to possess a broad diversity of structures and functions. In modern research, cardiac protective activity of *Z. bungeanum* fruit has been reported for its essential oil (5). Ethanol extract (6) is considered to be useful in circulation. The antioxidant activity of methanol extract from *Z. bungeanum* is also found by Yamazaki et al. (7). But there still are not any reports about the effects of *Z. bungeanum* on lipid meta-bolism, which is what we investigated in our study.

Since the regulation of hepatic LDLR and HMGCR activity can be observed in HepG2 cells, previous literatures always use HepG2 cells to study lipid metabolism (8). The role of exogenous lipid as modulator of hepatic cholesterol synthesis and apoB containing lipoprotein secretion has been well established. Intracellular lipid or cholesterol ester accumulation and apoB secretion are not only

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significantly affected by cholesterol alone, but also can be increased by addition of 25-hydroxycholesterol (9). Incubated with 50 μg/ml cholesterol and 4 μg/ml 25-hydroxycholesterol for 24 h, HepG2 accumulates approximately 2.0-fold greater apoE and apoB as compared to BSA-treated cells, while reduces mRNA and activity of LDL receptor (10). So combination of cholesterol and 25-hydroxycholesterol were applied to establish HepG2 cells cholesterol over-loading model. And the study of Yucun Niu gave us an evident that this model might be used to screen and assess functional factors for lowering lipids (11). In our present study, cholesterol and 25-hydroxycholesterol in cultured cells were shown to significantly elevate lipid accumulation, increase apoB secretion, as well as repress genes encoding proteins involved in cholesterol biosynthesis by feedback (12). Hence we undertook the present model to investigate how ZBBu affects lipid metabolism in normal condition and reverse lipid accumulation caused by exogenous sterols.

In our previous study, we compare the effects of three fractions isolated from *Z. bungeanum* (petroleum ether fraction, ethyl acetate fraction and *n*-butanol fraction) on regulating the cholesterol metabolism in HepG2 cells and find ZBBu is best effective; we also has demonstrated its pharmacological activity in reducing oxidative stress and inflammation *in vivo* (13). Assays including thin-layer chromatography (TLC) and high-performance liquid chromatography/mass spectrometry (HPLC–MS) have been used to investigate its chemical compositions, which revealed the presence of many flavone glycosides. Dietary flavonoids show the protective activity in inhibiting the thrombus formation, overcoming damaging effects of cardiovascular risk factors and delaying the onset of atherosclerosis (14,15). In the context of these evidences we predicted ZBBu might exert biological effects on pathogenesis of atherosclerosis.

2. Methods

2.1. Materials

HepG2 cells were obtained from American Type Culture Collection (ATCC). *Z. bungeanum* fruit (Family: Rutaceae) was purchased from Sichuan province, China. The standard substances (Rutin, Hyperin) were purchased from Shanghai R&D Center for Standardization of Traditional Chinese Medicines (Shanghai, China). 25-hydroxycholesterol and cholesterol were obtained from Sigma (MO, USA). Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (CA, USA). Total RNA extraction reagent, reverse transcription kit, and SYBR Premix Ex Taq (Tli RNaseH plus) were purchased from TAKARA (Japan). Antibodies against SREBP-1, HMGCR and LDLR were acquired from Santa Cruz (TX, USA), Abcam (MA, USA) and R&D (MN, USA) respectively. Intracellular TC, FC, FFA and TG quantification kits were obtained from Applygen Technologies Inc. (Beijing, China). Biochemical reagents assessing TC, TG levels in the serum samples were purchased from NanJing JianCheng Bioengineering Institute (Nanjing, China). Human apoB and apoA1 ELISA kit were purchased from Assaypro (MO, USA) and Shanghai Hufeng Biotechnology Company (Shanghai, China) respectively. BCA protein assay kit was purchased from Shanghai Biocolor BioScience & Technology Company (Shanghai, China).

2.2. Extraction

Z. bungeanum fruit was extracted with 95% ethanol by 50 °C heating reflux method for 3 times (16). The extractions were dried in a vacuum rotary evaporator to a yield of 33.3%, which was then suspended in H_2O and partitioned with petroleum ether (PE), ethyl

acetate (EtOAc) and *n*-butanol (BuOH) successively (17). These three fractions were dried by vacuum at 40 °C and the yield of PE, EtOAc and BuOH were 2.7%, 16.7%, and 12%. We have valued the activities of these three fractions, and found the fraction of BuOH was the most prospective, which was used for further experiment.

2.3. Chromatographic fingerprint

In our previous study, we found rutin and hyperin presented in ZBBu. And preliminary HPLC study of ZBBu and comparison with standards (rutin, hyperin) also indicated the presence of these compounds in the extract. The dried extract of ZBBu was dissolved in methanol at the concentration of 0.5 mg/ml and filtered through a 0.2-μm Millipore filter. 2 μl of methanol solution was used for HPLC analysis (Agilent 1260). We applied agilent eclipse plus C18 (3.5 μm × 2.1 mm × 100 mm) to separate the ZBBu fraction with a column temperature of 29 °C. The mobile phase comprised a binary eluent of 0.05% aqueous formic acid (A) and acetonitrile (B) using a liner gradient program of 5%–18% B, 0–3 min; 18% B, 3–10 min; 18%–63% B, 10–20 min; 63% B, 20–25 min. It was delivered at a flow rate of 0.3 ml per min with detection at 254 nm.

2.4. Cell culture

All of the testing solutions were dissolved with ethanol as stock solution, which were diluted to the needed concentration with DMEM. The HepG2 was maintained in DMEM supplemented with 10% FBS in the presence of 100 U/ml penicillin and 100 U/ml streptomycin, and incubated at 37 °C under the atmosphere of 5% CO_2. 24 h after seeding, DMEM was removed. HepG2 cells were washed twice with PBS and incubated in serum-free DMEM supplemented with different concentrations of ZBBu, 100 μM hyperin and 40 μM rutin for 12 h. Then 1×10^4 μmol/L Cholesterol $+ 1 \times 10^3$ μmol/L 25-hydroxycholesterol were dissolved in ethanol, diluted with DMEM to 10 μmol/L Cholesterol + 1 μmol/L 25-hydroxycholesterol and added to cells. The same volume of ethanol was added to the control cells. To validate this model, simvastatin (1 μM), a well-established drug to treat atherosclerosis, was used as a positive control. After another 24 h, monolayers were washed three times with PBS and harvested. Under present condition, the final concentration of ethanol did not exceeded 0.1%. Exogenous cholesterol and 25-hydroxycholesterol did not affect the growth of HepG2 cells.

2.5. Animals

Male C57BL/6 mice and male apoE-knockout (apoE-ko) mice (6–8 weeks) in the C57BL/6 background were purchased from Beijing HFK Bio-Technology Co. Ltd. and maintained in SPF room with 12 h light/dark cycle. C57BL/6 mice as normal control group were fed a chow diet. apoE KO mice were given high fat diet and randomly divided into 4 groups with 6 animals each: the model group (high fat diet only), positive control group (simvastatin, 3 mg/kg/d), low dose group (ZBBu 50 mg/kg/d) and high dose group (ZBBu 200 mg/kg/d), administered by oral gavage for 4 weeks. At the end, blood was collected after overnight fasting and then centrifuged to get serum (3000 rpm, 10 min, 4 °C). All mice used were handled according to the guidelines of the Instituted Animal Care and Use Committee of Fudan University for the care and use of laboratory animals.

2.6. Determination of intracellular TC, FC, FFA and TG

The harvested cells were incubated with lysis buffer and centrifuged to get the supernatant. Then the contents of intracellular TC, FC, FFA and TG were determined using standard enzymatic

kit. Results were expressed as a ratio to control. The protein concentration was measured using BCA kit.

2.7. Secreted apoB and apoA1

Levels of apoB, apoA1 in supernatant of HepG2 were determined according to manufacturer's instruction with an automatic biochemical analyzer (Au5600 OLYMPUS). After the incubation, the culture medium was collected and added into 96-well plate coated with an anti-human ApoB/ApoA1 polyclonal antibody (purchased from Assaypro) and incubated for 2 h at room temperature. The 96-well plate was washed five times with wash buffer. Then biotinylated human ApoB antibody was added and incubated for 1 h. The 96-well plate was washed five times before incubated with streptavidin–peroxidase conjugate for another 30 min, then chromogenic substrate and stop solution were added successively. Absorbance was read at a wavelength of 450 nm.

2.8. RNA isolation and real-time RT-PCR

Total RNA was isolated from HepG2 cells with TRI-zol reagent and reverse-transcribed using Real-time PCR master mix. Real-time PCR reaction was performed using Bio-Rad™ iQ5 real-time PCR optical detection system. Final results were standardized to the expression of β-actin, and values were expressed relatively to the average values for negative control, which was set to 1.0. Real-time PCR primer sequences were as below:

β-actin: forward, 5′-AGCCATGTACGTAGCCATCC-3′, reverse, 5′-CTCTCAGCTGTGGTGGTGAA-3′; SREBP-2: forward, 5′-GCAACAACA GACGGTAATGATC-3′, reverse, 5′-GACTTGAGGCTGAAGGACTTG-3′; HMGCR: forward, 5′-ACATTGTCACCGCCATCTACATTGC-3′; reverse, 5′-GGCTTGCTGAGGTAGTAGGTTGGT-3′; LDLR: forward, 5′-CTGTCT CTGTTGCGGATACCAAGG-3′, reverse, 5′-GCGAGTAGATGTCCACAC CATTCA-3′; LXR-α: forward, 5′-ATGGACACCTACATGCGTC-3′, reverse, 5′-CTTCAGGCGGATCTGTTCTT-3′; ABCG1: forward, 5′-TGC

AATCTTGTGCCATATTTGA-3′, reverse, 5′-CCAGCCGACTGTTCT GATCA-3′; CYP27A1: forward, 5′-AGATGCAGCTACTCCTCGCAA-3′, reverse, 5′-AGGCCCACTTTCTTATTGGGA-3′.

2.9. Western blotting analysis

After incubation, cells were washed with PBS and then treated with lysis buffer containing PMSF. After protein quantitation, protein samples were equally loaded onto 8% poly-acrylamide gel, electrophoresed, and transferred onto a PVDF membrane. Membranes were blocked with 5% defatted milk in TBS containing 0.1% Tween 20 at room temperature for 1 h, incubated overnight at 4 °C with HMGCR, LDLR and SREBP-1 primary antibody, and then with horseradish peroxidase conjugated secondary antibody. The immunoreactivity was visualized using the ECL chemiluminescence kit according to the manufacturer's instructions, recorded by Alpha gel imaging system and quantitated by Image J.

2.10. Determination of TC, TG levels in serum

Serum TC, TG levels were assessed using the biochemical reagent kits according to manufacturer's instructions.

2.11. Statistical analysis

Data was expressed as means ± SD. Significance differences were assessed by one-way ANOVA and student t-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. HPLC analysis

The Analysis of ZBBu by HPLC revealed rutin and hyperin were existed in ZBBu, which was identified by their retention
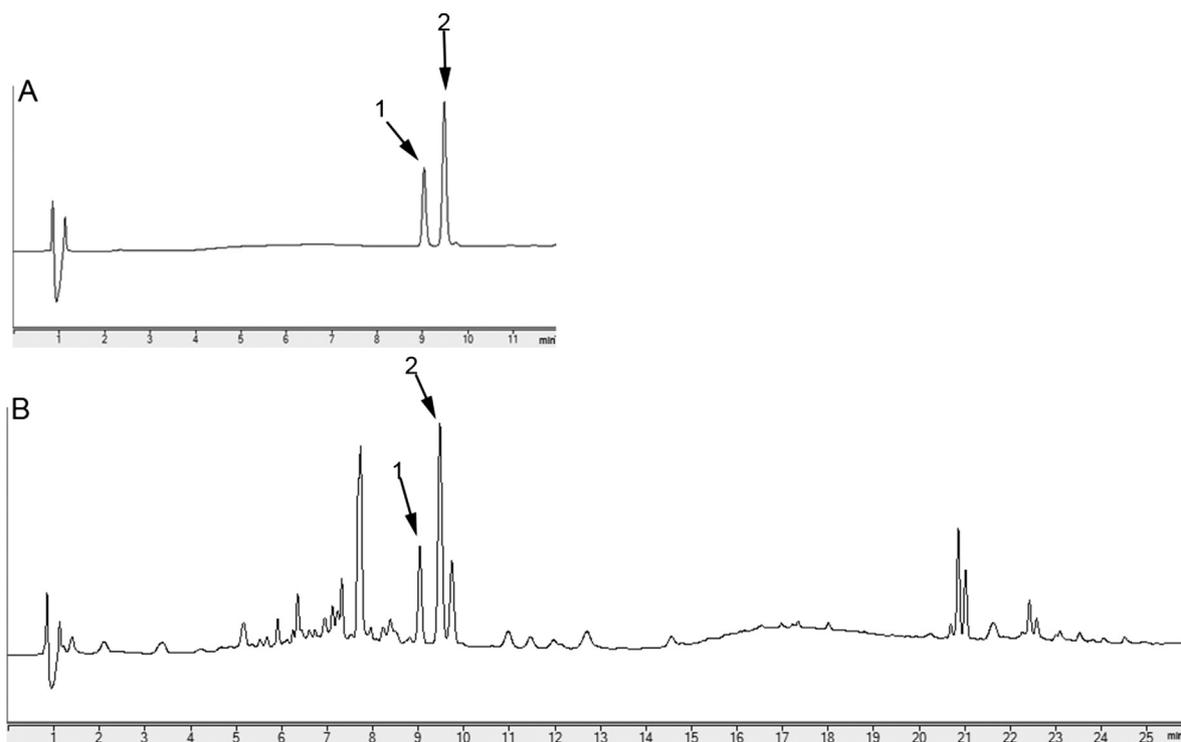
Fig. 1. Chromatogram of standard substances and ZBBu. A: the chromatogram of standard substances. B: the chromatogram of ZBBu. 1, Rutin; 2, Hyperin.

time of 9.04 min and 9.48 min (Fig. 1). Content of rutin and hyperin presenting in ZBBu were approximately 12.12% and 31%.

3.2. ZBBu affects intracellular TC, FC, FFA and TG

To study ZBBu further, HepG2 cells were initially maintained in serum-free DMEM to create a reduced-sterol environment, and then pretreated with 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml ZBBu or vehicle. In previous study, it was demonstrated that addition of 25-hydroxycholesterol was highly effective in altering the

concentration of cellular lipids (9). As expected, with this treatment the intracellular TC level was increased significantly (Fig. 2A), as well as TG (Fig. 2C) and FC (Fig. 2D), but we found no increase in FFA (Fig. 2B). After exposure to ZBBu, TG could be dose-dependently decreased both in the absence and presence of sterols ($P < 0.01$). Similarly, TC, FC and FFA were also reduced. Higher dosage of ZBBu in current experiment status showed similar lipid-lowing effect to simvastatin. We also valued the effect of hyperin and rutin. Our data indicated that 100 μM hyperin and 40 μM rutin could decrease intracellular TC (Fig. 2E) and FC (Fig. 2F), which might account for the effect of ZZBu.
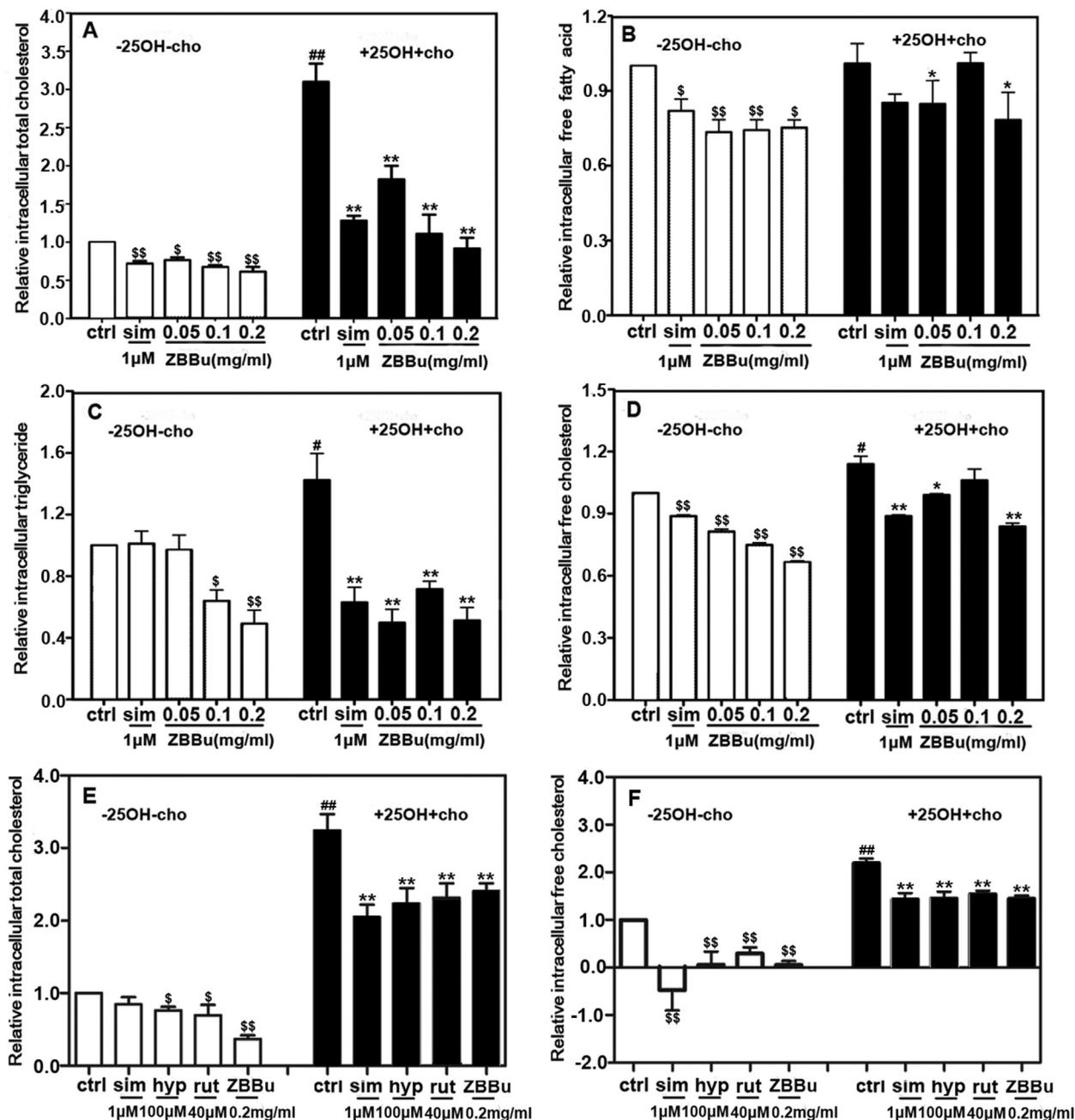
Fig. 2. Effects of ZBBu on intracellular TC, FFA, FC and TG. HepG2 cells, were pretreated with different concentrations of ZBBu or 100 μM hyperin, 40 μM rutin for 12 h. Then cells were exposed to 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol or only vehicle for 24 h. TC (A, E), FFA (B), TG (C) or FC (D, F) was evaluated. ZBBu could decrease the intracellular level of TC, FFA, TG, FC. 0.2 mg/ml ZBBu had similar effect to Rutin and hyperin (E, F). Data are normalized to total protein, expressed as a ratio to the control without treatment of sterols and presented as means ± SD. The mean of at least 3 independent experiments is shown. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ versus normal group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group treated with 25OH + cho. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared these two control groups. Ctrl: control. Sim: simvastatin. 25OH: 25-hydroxycholesterol. Cho: cholesterol.
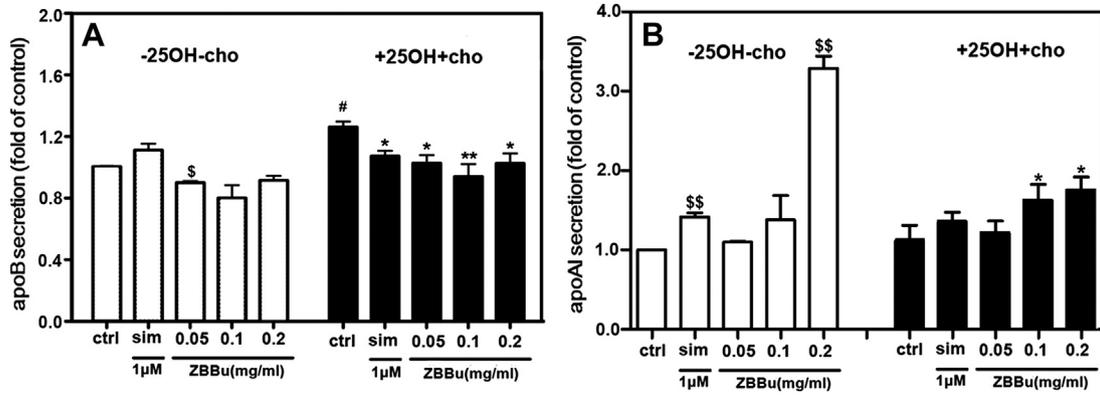
Fig. 3. Effects of ZBBu on apoB and apoA1 secretion. HepG2 cells were pretreated with 0.05 mg/ml−0.2 mg/ml ZBBu for 12 h and stimulated with sterols for 24 h. After the treatment, apoB and apoA1 in supernatants were evaluated by ELISA. ZBBu could reverse the increased level of apoB induced by 25OH + cho (A). And ZBBu promoted the secretion of ApoA1 (B). Data are expressed as ratios to the control without treatment of sterols and presented as means ± SD. The mean of at least 3 independent experiments is shown. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ versus normal group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group treated with 25OH + cho. $^{\#}P < 0.05$ compared these two control groups. Ctrl: control. Sim: simvastatin. 25OH: 25-hydroxycholesterol. Cho: cholesterol.
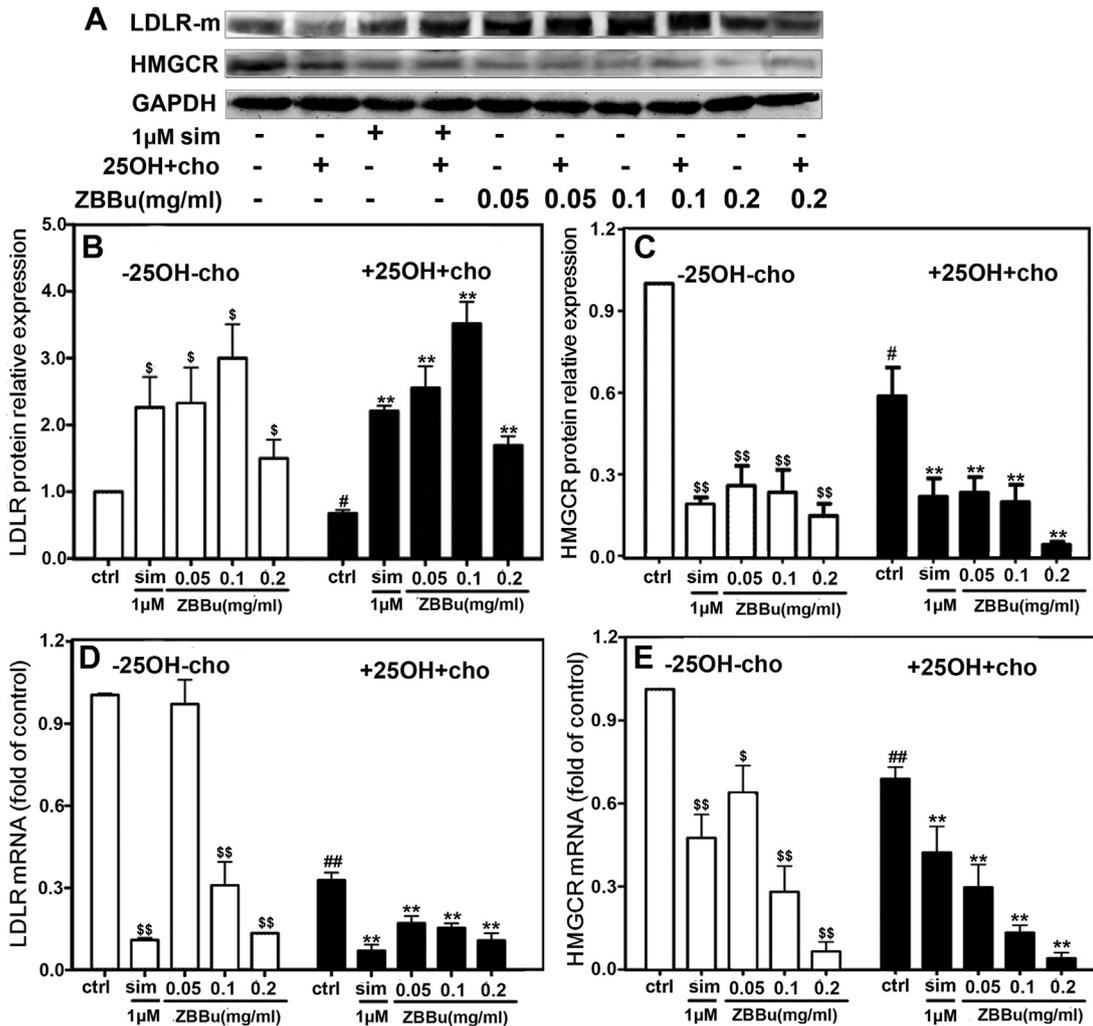
Fig. 4. Effects of ZBBu on mRNA and protein expression of HMGCR and LDLR. Western blotting of LDLR (mature form) and HMGCR protein (A) ZBBu treatment increased LDLR protein expression (B) while inhibited LDLR mRNA (D) abundance. It also showed down-regulation of HMGCR both on protein level (C) and mRNA level (E) in HepG2 cells. Data are expressed as ratios to the control without treatment of sterols, and presented as means ± SD. The mean of at least 3 independent experiments is shown. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ versus normal group. $^{**}P < 0.01$ versus control group treated with 25OH + cho. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared these two control groups. Ctrl: control. Sim: simvastatin. 25OH: 25-hydroxycholesterol. Cho: cholesterol.

3.3. ZBBu affects apoB and apoA1 secretion in HepG2 cells

Apolipoprotein B (apoB) is a structural protein of LDL and very low density lipoprotein (VLDL) (18). We firstly studied the ability of ZBBu affecting apoB secretion in HepG2 cells in normal physiological status. HepG2 cells pretreated with ZBBu had an inhibitory effect on apoB secretion, although it wasn't shown to be dose-dependent. The amount of apoB secreted by HepG2 cells was elevated upon addition of exogenous sterols. Both treatment with ZBBu and simvastatin attenuated the induction of apoB secretion (Fig. 3A).

We also investigated whether ZBBu affected the production of apoA1, the major structural protein of HDL. Overexpression of HDL or apoA1 can reduce the risk of atherosclerosis (19). Fig. 3B has shown to us that 0.2 mg/ml ZBBu and simvastatin led to an increase of apoA1 in the absence of sterols. After exposure to cholesterol and 25-hydroxycholesterol, ZBBu also exerted a dose-dependent stimulatory effect on apoA1 secretion ($P < 0.05$), which was more effective than positive control drug.

3.4. Effects of ZBBu on genes and proteins expression of cholesterol biosynthesis enzymes

In consideration of the notable changes in intracellular lipids and secretion of apoB and apoA1 accompanying ZBBu treatment, we tried to find out how it affected the lipid homeostasis. The results in Fig. 4C and E showed that ZBBu treatment decreased HMGCR protein and mRNA at a dose-dependent manner in depletion of sterols. When sterols were supplemented to the cells, which were condition to inhibit endogenous cholesterol synthesis by negative feedback, HMGCR protein and mRNA expressions were suppressed. However, treated with ZBBu, HMGCR protein and mRNA were further reduced.

Hepatocyte LDLR can regulate apoB secretion by controlling the uptake of newly secreted particles and degradation of nascent apoB (20). The potential of ZBBu inhibiting apoB secretion leaded us to investigate the expression of LDLR mRNA and protein. A lower LDLR gene expression in ZBBu treated groups was observed as compared to the control group (Fig. 4D). Sterols decreased LDLR
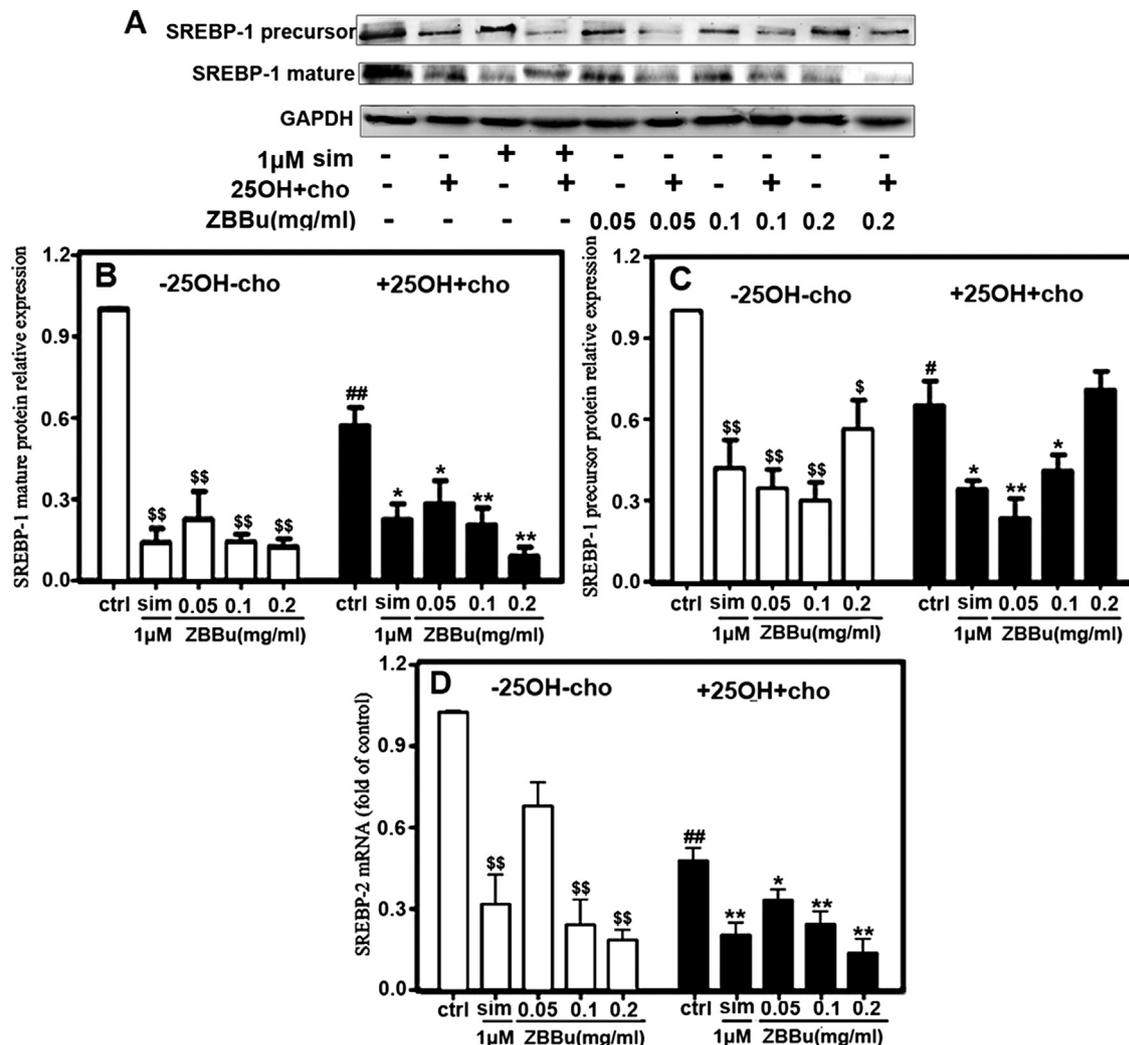
Fig. 5. ZBBu inhibited SREBP-1 and SREBP-2 expression. Western blotting of SREBP-1 protein (precursor and mature form) (A), and quantification after normalizing by GADPH (B), (C). D, abundance of SREBP-2 mRNA. Data are expressed as ratios to the control without treatment of sterols, and presented as means ± SD. The mean of at least 3 independent experiments is shown. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ versus normal group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group treated with 25OH + cho. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared these two control groups. Ctrl: control. Sim: simvastatin. 25OH: 25-hydroxycholesterol. Cho: cholesterol.

mRNA abundance and addition of ZBBu down regulated it further. In agreement with the changes in LDLR mRNA abundance, sterols also suppressed LDLR protein expression. However, ZBBu treatment significantly increased LDLR protein expression ($P < 0.01$), although there was no great difference among the three groups (Fig. 4B).

Because of the effects on HMGCR and LDLR, we predicted that ZBBu might cause some changes in the expression of their regulatory factors such as SREBP-1 and SREBP-2. The result of present study showed that the active SREBP-1 mature protein was dose-dependently decreased with the pretreatment of ZBBu (Fig. 5B). After exposure to sterols for 24 h, the expression of SREBP-1 was restrained comparing with vehicle group. Similar to simvastatin, treatment of ZBBu significantly further lowered expression of SREBP-1 (both precursor and mature form). SREBP-2, which regulates HMCGR, is affected by intracellular and extracellular cholesterol. Fig. 5D showed SREBP-2 mRNA abundance was down regulated by ZBBu in both normal condition and the presence of exogenous sterols.

3.5. Effects of ZBBu on the expression of cholesterol efflux related factors in HepG2 cells

To explore whether ZBBu influenced the gene involved in RCT, we used real-time PCR to assess relative changes in the expression of gene LXRα, ABCG1 and CYP27A1. The results showed that pretreatment of ZBBu dose-dependently enhanced the mRNA abundance of CYP27A1, LXRα and ABCG1 both in the presence and absence of sterols (Fig. 6).

3.6. Effects of ZBBu on serum lipid levels in apoE-ko mice

To confirm the hypolipidemic effect of ZBBu *in vivo*, we used apoE KO mice fed with high fat diet to induce animal hyperlipidemia model. After 4 weeks high fat diet, the serum TC and TG levels in the model group were up-regulated, indicating that the animal hyperlipidemia model was successfully established. ZBBu application could significantly inhibit the hyperlipidemia effects (increased serum TC and TG level) caused by the high fat diet, which was similar with the effect of Simvastatin, as shown in Fig. 7.

4. Discussion

In consideration of anti-inflammation or anti-septic activities of *Z. bungeanum* have been studied before, here we extracted the BuOH fraction from ethanol extract of *Z. bungeanum* and identified that ZBBu effectively regulated lipid metabolism and worked through a similar way to simvastatin.

In this study, we demonstrated the hypolipidemic effects of ZBBu both *in vitro* and *in vivo*. Many flavonoid glycosides, such as rutin and hyperin were found in the component of ZBBu. As a member of polyphenols, flavonoid glycoside could regulate lipid metabolism through mediating lipoprotein lipase expression (21), suppress progression of atherosclerosis *in vivo* (22) and alter cholesterol homeostasis by increasing the expression of LDLR (23). *In vitro*, rutin lowers the HMGCR activity to reduce the cholesterol metabolism (24,25). The effects of rutin and hyperin on lipid metabolism are also proved *in vivo* (26–28). So the flavonoid glycoside existing in ZBBu would account for the regulation function of ZBBu on cholesterol homeostasis.
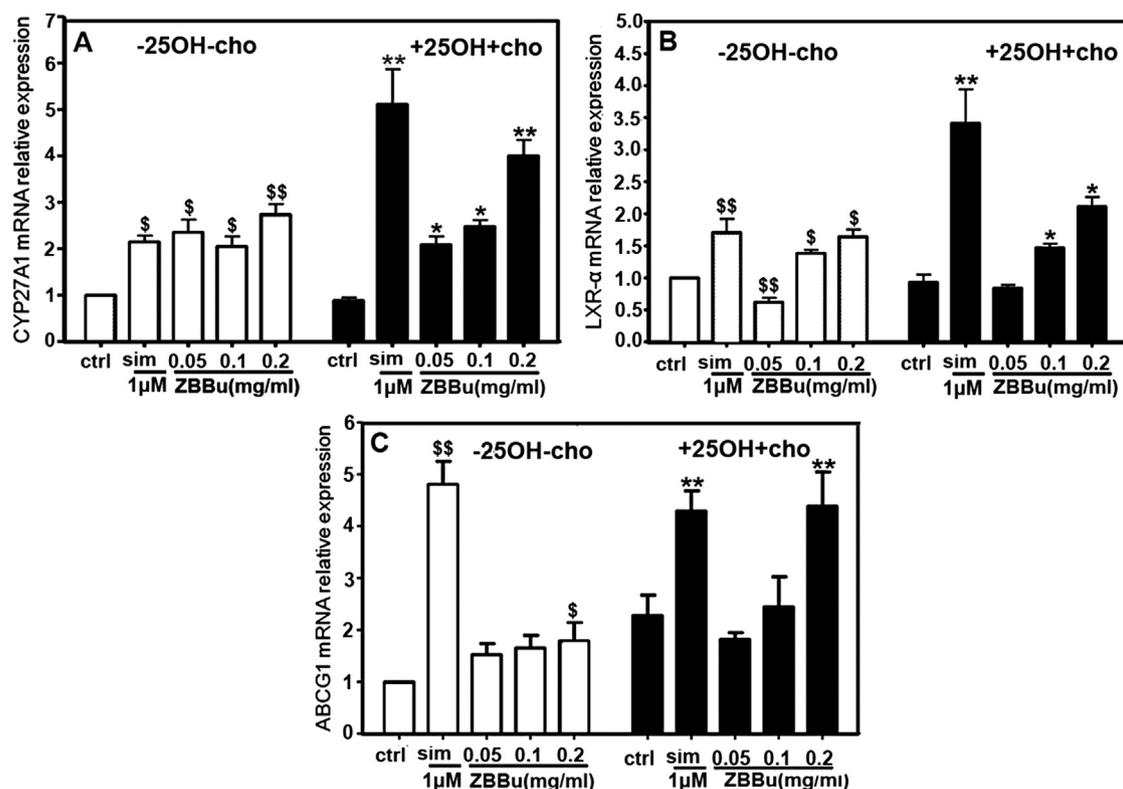
Fig. 6. Effects of ZBBu on genes' expression involved in RCT. ZBBu increased the mRNA expression of CYP27A1 (A). Activation of CYP27A1 stimulated the mRNA abundance of LXR-α (B) and subsequently ABCG1 (C). Data are expressed as ratios to the control without treatment of sterols, and presented as means ± SD. The mean of at least 3 independent experiments is shown. $^{$}P < 0.05$, $^{$$}P < 0.01$ versus normal group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group treated with 25OH + cho. Ctrl: control. Sim: simvastatin. 25OH: 25-hydroxycholesterol. Cho: cholesterol.
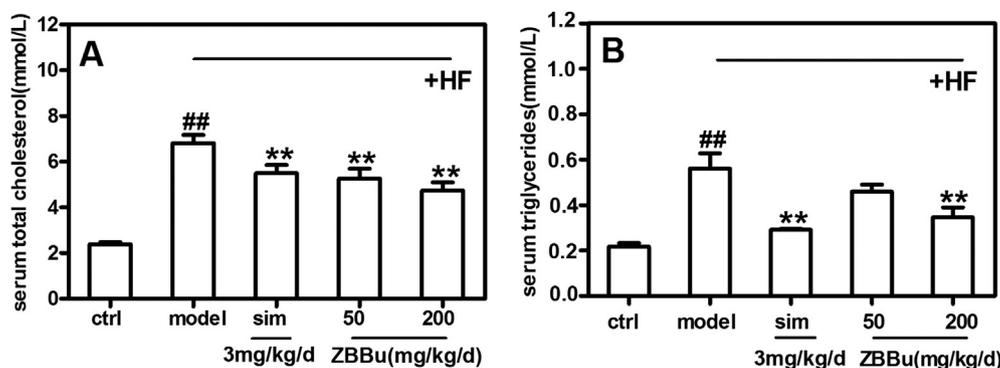
Fig. 7. Effects of ZBBu on serum lipid levels *in vivo*. High fat diet increased the serum lipid levels in model group significantly. Treated with ZBBu, the increased levels of serum TC (A) and TG (B) were attenuated in high fat group. Data are presented as means ± SD ($n = 6$). $^{**}P < 0.01$ versus model group. $^{##}P < 0.01$ versus control group. Ctrl: control. Sim: simvastatin. HF: high fat diet.

SREBP-1 stimulates the transcription of genes encoding hepatic fatty acid synthesis. SREBP-2 is primarily responsible for regulating the cholesterol biosynthesis genes (2,29). Using the cell model above, the results showed that ZBBu down-regulated the expression of SREBP-1, SREBP-2 and HMGCR, which could explain the fact of lower intracellular TC, TG, FG and FFA levels. LDLR is one of another genes regulated by SREBPs. Interestingly, ZBBu stimulated the protein abundance of LDLR in HepG2 cells, leading to the observation of decreased apoB secretion. The down-regulation of LDLR on cell surface enhanced levels of LDL-c in the plasma (30). Elevated plasma LDL-c has a great association with the development of coronary atherosclerosis (31). In the arterial intima, LDL-c can be oxidatively modified by oxygen species forming Ox-LDL, which can initiate the atherosclerotic process (32). So the effect of ZBBu on LDLR could inhibit the process of atherosclerotic.

What's more, we recognized the ZBBu might affect other pathways involved in lipid metabolism. Liver X receptor-alpha (LXRα) has been proposed to be an important regulator involved in HDL-c metabolism and bile acid (33). Its agonists promote cholesterol efflux, accelerated RCT or anti-atherosclerosis (34). And several important genes for lipid metabolism, such as ABCA1 and ABCG1, have been shown to be responsible to LXRα. Accordingly, the therapies focused on RCT becomes a target to treat atherosclerosis. LXRα can be stimulated by CYP27A1. The importance of CYP27A1 for bile acid synthesis has long been documented. Liver can eliminate cholesterol by CYP27A1 mediating formation of cholestenoic acid. Being exposed to ZBBu, CYP27A1, LXRα and ABCG1 mRNA levels in HepG2 cells were increased. ABCG1 promotes the efflux of cholesterol from cells to particles containing lipid-poor apoA1 (35). Regulated by LXRα, ABCG1 was further affected by CYP27A1. Activation of these genes has favorable effects on chronic metabolism of lipids (36). In the RCT, excess cellular cholesterol from peripheral tissues could efflux to mature HDL particles mediated by ABCG1. Furthermore, cholesterol, which was transported by the HDL particles, could return to the liver for excretion from bile and ultimately feces (37). Modification of RCT and cholesterol efflux may provide new therapeutic approaches to cardiovascular disease, which plays a major role in anti-atherogenesis (38). Considering the influence of ZBBu on RCT, ZBBu may further suppress the process of atherosclerosis.

As the effects of ZBBu *in vitro* had been proved, we wanted to figure out whether it could work on lipid metabolism *in vivo*. ApoE, as a ligand of LDLR, has an important role in lipid metabolism. And apoE deficiency is associated with dyslipidemia (39). Then we chose apoE KO mice which were fed with high fat diet as animal model. It was a widely used hyperlipidemia model. As expected, the results of animal experiment demonstrated that ZBBu could also

influence the lipid metabolism *in vivo*, reducing serum TC and TG dose-dependently. Further study will be carried out to determine the pathologic change and molecular mechanism undergoing *in vivo*.

In conclusion, decreased lipid biosynthesis *in vivo* and *in vitro*, increased LDLR protein expression and genes involved in RCT *in vitro* could account for the hypolipidemic effects of flavonoids-rich ZBBu. Therapies targeting the interplay between lipid metabolism and inflammation have potential in the treatment of atherosclerosis. In the context of the evidences, we predicted the ZBBu might exert biological effects on the pathogenesis of atherosclerosis.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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