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High Anti-oxidative and Lipid-lowering Activities of Flavonoid Glycosides-rich Extract from the Leaves of *Zanthoxylum bungeanum* in Multi-system

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Abstract Epidemiological studies observed that *Zanthoxylum bungeanum* leaf, a popular vegetable in China, could interfere progression of cardiovascular diseases, especially hyperlipidemia. In the work, the pharmacological properties of *Z.bungeanum* leaves extract (ZLE) containing isovitexin, vitexin, hyperoside, rutin, isoquercitrin, foeniculin, trifolin, quercitrin, astragalin, and afzelin were investigated using multi-system such as mice organ tissue, HepG2 cell and apoE-/- mice. ZLE exhibited a stronger activity of scavenging free radicals. ZLE also appeared to inhibit lipid peroxidation of mice organ tissues including the heart, liver, spleen, lung, kidney, brain and pancreas. ZLE could lower lipid accumulation in HepG2 cells induced by free fatty acids (FFAs, an inducer of lipid peroxidation and free radicals production) in a dose-dependent manner. Compared with fenofibrate, a commercial product popularly used in clinical, the treatment of ZLE in apoE-/- mice had a stronger anti-hyperlipidemia without any detectable histopathological damage. This is the first report that ZLE containing high content of flavonoids with satisfying safety could significantly inhibit the development of hyperlipidemia by preventing the oxidative damage induced by free radicals and lipid peroxidation.

Keywords: *Zanthoxylum bungeanum, Hyperlipidemia, Flavonoid glycosides, HepG2, apoE-/- deficient mice*

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1. Introduction

Several lines of evidence indicate that reducing the lipid-levels is an effective method to treat atherosclerosis and hyperlipidemia [1,2,3]. At present, fenofibrate and atorvastatin are the primary drugs of the choice in prevention of metabolic disorders. But they have the long-term side effects such as leg pain, rhabdomyolysis, diabetes, which limit the use of them [4,5]. The natural products containing flavonoids in human diet and food had been reported to be able to decrease the incidences of cardiovascular and other chronic diseases, especially hyperlipidemia, due to its antioxidant and free radical scavenging activities [6,7]. These natural products as alternative sources are gaining much more attention in the pharmaceutical industry to develop better and safe drugs with low side effect [8].

Zanthoxylum bungeanum leaf, commonly called "Huajiao" leaf in Chinese, is widely used as a vegetable and seasoning in China [9]. Previous study had also found that Z. *bungeanum* leaves extract, especially the *Z. bungeanum* ethyl acetate extract from 70% MeOH extract (hereafter defined as ZLE) might be a potential source of natural antioxidants because of its high content of flavonoids including isovitexin, vitexin, hyperoside, rutin, isoquercitrin, foeniculin, trifolin, quercitrin, astragalin and afzelin [10]. However, the pharmacological properties about the anti-hyperlipidemia and the action mechanism of ZLE are unclear.

The present work was to investigate antioxidant and lowering lipidemic effects of ZLE containing the qualifying ten flavonoids in vivo and in vitro and exploit the action mechanism of ZLE on anti-hyperlipidemia. The antioxidant activity of ZLE was evaluated using DPPH and TBA assay, and the lowering lipidemic activity was determined using HepG2 cells and apoE-/- mice.

2. Materials and Methods

2.1. Sample Preparation

Dried leaves of Z. *bungeanum* (200 g) purchased from Hanyuan was ground into fine powder and extracted with 1000 mL of 70% methanol by continuous stirring at room temperature for 24 h. After centrifugation at 5000 rpm for 10 min, the supernatants were collected. After filtration,

the filtrates were evaporated to dryness at 45°C under vacuum and a total of 48.6 g crude extract was obtained. An amount of 38 g of the crude extract was suspended in 150 mL of distilled water and successively re-extracted with ethyl acetate (500 mL). The solvents were removed at reduce pressure to give ethyl acetate (2.28 g) fractions. The lyophilized product was kept in deep freezer and used in this experiment. Total phenolics and flavonoids contents were determined according to the previous study [10]. Briefly, the total phenolics were quantified by using Folin-Ciocalteu method [11]. An amount of 0.1 mL of ZLE with different dilution was mixed with 2 mL of Na_2CO_3 (20 mg/mL) for 2 min, and then 0.9 mL of Folin-Ciocalteu's reagent (previously diluted 2-fold with distilled water) was added and the mixture was incubated for 30 min at room temperature. The absorbance of reaction was measured at 750 nm by using the MAPADA V-1100D spectrophotometer (Xinke Instruments Co., Ltd., Sichuan, China). Total phenolics contents were calculated as gallic acid from a calibration curve: $y = 0.8116x - 0.0018$, $R^2 = 0.999$, where y was the absorbance and x was the gallic acid equivalent (mg gallic acid/g extract).

And the total flavonoids were determined by using a modified spectrophotometrical method [12]. In brief, 0.1 mL of ZLE with different dilution and 2 mL of distilled water were mixed with 0.1 mL of 5% $NaNO_2$ for 6 min, and then 0.2 mL of 10% $AlCl_3$ was added and mixed for 5 min. The total volume was made up to 3 mL with distilled water. The absorbance of reaction was measured at 420 nm against a prepared blank by using the MAPADA V-1100D spectrophotometer. Total flavonoid contents were calculated as rutin from a calibration curve: $y = 0.352\ x - 0.0221$, $R^2 = 0.999$, where y was the absorbance and x was the rutin equivalent (mg rutin/g extract).

The results showed that ZLE contains 614.74 mg gallic acid/g extract of total phenolics and 998.70 mg rutin/g extract of flavonoids, including Isovitexin (62.4), vitexin (71.7), hyperoside (1070), rutin (456), isoquercitrin (684), foeniculin (55), trifolin (230), quercitrin (255), astragalin (145) and afzelin (70.5) ($P < 0.05$), which was expressed as micrograms per gram (mg/100 g) dry weight of *Zanthoxylum bungeanum* leaves [10]. The extraction and determination of samples were repeated 3 times.

2.2. Detection of Radical Scavenging Assay

The DPPH radical-scavenging activity was different from the previous study and assayed according to the method described by Brand-Williams, Cuvelier, and Berset (1995), converted into a micromethod [13]. Briefly, a stock methanolic solution (1 mg/mL) of ZLE was diluted with EtOH to prepare samples ranging from 0.625 to 20 μg/mL. Then, 50 μL of each sample was pipetted into 96-well plates in triplicate and was added in each well 50 μL of DPPH solution (0.5 mM in absolute ethanol). Plates were placed in dark for 30 min at room temperature and then the absorbance was measured at 510 nm. The results were plotted as the percentage of losing DPPH (%IDPPH) against the concentration (μg/mL) of the samples added.

$$\%I(DPPH) = \{1 - (\frac{A_{sample}}{A_{blank}})\} \times 100 \quad (1)$$

A_{sample} = absorbance of the sample, A_{blank} = absorbance of the blank.

Results are expressed as inhibitory concentration (IC50), which corresponds to extract concentration (μg/mL) required to quench 50% of the initial DPPH radicals under the given experimental conditions.

2.3. Determination of Lipid Peroxidation Assay in Vivo and in Vitro

Lipid peroxidation assay was performed as previously reported with a minor modification [14]. Tissue homogenate including heart, liver, spleen, lung, kidney, brain and pancreas were prepared as lipid-rich media. Briefly, 0.1 mL of ZLE (Final concentration was 2.5, 5, 10, and 20 μg/mL) in ethanol was thoroughly mixed with 0.5 mL of tissue homogenate (10%, v/v, diluted with pure water) and made up to 1 mL with pure water. Ferrous sulfate (50 μL, 70 mM) was added to induce lipid peroxidation, and the mixture was incubated for 30 min at 37.5°C. Afterward, 1.5 mL of 20% acetic acid (v/v, pH 3.5, diluted with pure water) and 1.5 mL of 0.8% (w/v) thiobarbituric acid (TBA) in 1.1% sodium dodecyl sulfate (w/v, diluted with pure water) were added, and the resulting mixture was vortexed and heated at 95°C for 60 min. After cooling, each tube was centrifuged at 5000×g for 15 min. The organic upper layer was collected and measured spectrophotometrically at 532 nm using a microplate reader. The inhibition of lipid peroxidation was calculated as follows: Inhibition (%) = (1 − sample/control) ×100. control was considered the absorbance of the control (i.e., ethanol, instead of the sample). The determination of lipid peroxidation in vitro is conducted as follows. Male KM mice, weighing 24±2 g, were provided by the Sichuan University, animals were housed at 22±2°C under a 12/12 h light/dark cycle. The experimental animals were divided into two groups of five animals each. Firstly, each group received orally administered ZLE in a dose of 250 mg/kg during seven days and then the mice were anesthetized with pentobarbital sodium, and tissue was collected for the determination lipid peroxidation according to the method in vivo.

2.4. Cell Culture and Treatments

The cultured human hepatoma HepG2 cell line was grown in DMEM supplemented with 10% FBS, 100 units/mL penicillins and 100 μg/mL streptomycin as previously described. Cells were incubated in a humidified atmosphere of 5% CO2 at 37°C.

HepG2 cells were cultured in complete medium with 10% FBS to 80% cell confluence and subjected to assays after overnight serum depletion. ZLE dissolved in DMSO were added to the medium. The final concentration of DMSO did not exceed 0.1%, which did not affect cell viability. A cell model for fatty acids–induced accumulation of hepatic lipids was used by exposing HepG2 cells to a FFA-free bovine serum albumin (oleate [OA] / palmitate [PA], 2:1) and the mixture was added to medium for 24 h to a final concentration of 1 mM as described previously [15]. Briefly, HepG2 cells were quiesced in serum-free DMEM overnight and incubated in

DMEM containing either a normal medium or a FFA-free bovine serum albumin medium for 24 h.

2.5. Cytotoxicity of ZLE on HepG2 Cells

Samples were dissolved in DMSO and diluted with DMEM medium to different concentrations and incubated with HepG2 cells for 24 h. The cytotoxicity of samples in 0.1% DMSO (final DMSO concentration in medium) was tested using MTT assay. In brief, cells were washed once with PBS carefully, incubated with 0.2 mL of serum-free DMEM medium containing 0.05% MTT for 4 h. After that, the culture medium was removed and 0.15 mL of DMSO was added to solubilise the formed formazan. The absorbance of each well was measured at 490 nm with a microplate reader. We compared the absorbance of the treated cells with the control cells, which were considered as the 100% viability value.

2.6. Determination of Lipid Levels in HepG2 Cells

To test the lipid levels induced by FFAs in cells, HepG2 cells at 80% confluence were exposed to 1 mM FFAs with 1% BSA in a 12-well plate. The ZLE samples were added with the FFAs-BSA complex and incubated for a further 24 h. After 24 h in culture, FFAs-containing medium was removed from wells and cells were washed twice with PBS. Finally, we collected the supernatants of different groups to determine the TG/TC levels in the cell lysates according to the manufacturer's instructions (Triglyceride/ Cholesterol Quantification Colorimetric/ Fluorometric Kit, Bivision USA). Oil red O staining was used to specifically stain the intracellular lipid drops. The HepG2 cells were treated with 1 mM of FFAs together with ZLE (125, 250, 500 μg/mL) for 24 hours. Cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min. After being washed with Isopropyl alcohol (60%), cells were incubated with Oil red O dyeing liquid for 4 h, re-suspended in water and the photos were taken using optical microscope (Olympus BX51, Japan). To quantify Oil Red O content levels, isopropyl alcohol was added to each sample to extract lipid drops at room temperature for 20 min, the density of samples were read at 510 nm on a spectrophotometer. The results were expressed as the percentage of remaining lipid contents which corresponds to the lipid-lowing effect of ZLE.

2.7. Effect of ZLE Treatment for 60 days on Serum Lipid of apoE-/- Mice

2.7.1. Animals and Experimental Protocols

Male homozygous apoE-/- mice (16 weeks of age) weighing 20-25 g were purchased from the Animal Center, Health Science Center Beijing University (Beijing, China, NO:11400700019702). The mice were maintained on a high-diet chow and given free access to both food and water in a temperature- and light-controlled animal facility with a light/dark cycle of 12 A.M. to 12 P.M. After 1 week of acclimatization, the apoE-/-mice were randomly divided into three groups (n=8): model group (B), ZLE group (C), fenofibrate group (D) compared with the control group (A) using KM mice with a normal diet. Mice were orally administered ZLE in a dose of 250 mg/kg, which was dissolved in 0.5% CMC. Control and model animals received the same dosage compared with the ZLE group. Positive control animals were daily intragastrically administered fenofibrate (100 mg/kg) dissolved in the 0.5% CMC. The body weight of each mouse was measured every 2 days in order to adjust the dosages of drugs. After 8 weeks the mice were killed, blood and the main viscera samples were obtained from animals subjected to biochemical analyses. All experiments were conducted according to the Institutional Animal Care and Use Committee Guidelines.

2.7.2. Determination of Serum Lipid Concentrations

At the end of this experiment, whole blood samples were obtained from the orbital vein of mice, and serum samples were prepared by centrifuging the whole blood for 15 min at 1600×g at 4°C and preserved at -80°C for lipid analysis. Serum lipoproteins levels were measured enzymatically by 126 Laboratory (West China Hospital, China).

2.7.3. Histopathological Examinations of apoE-/- Mice Organ Tissues

Tissues recovered from the necropsy were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) for histological examination using standard techniques. After hematoxylin-eosin staining, the slides were observed and photos were taken using optical microscope (Olympus BX51, Japan). All the identity and analysis of the pathology slides were blind to the pathologist.

2.8. Data and Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) and expressed as means ± S.E.M. Inter-group difference was detected by Dunnett's test using SPSS 19.0 software. Comparison between two groups was done by using Student's t-test. The difference was considered significant when $P < 0.05$.

3. Results and Discussion

3.1. DPPH Radical Scavenging Assay

Oxidative damage in the human body is a crucial etiological factor contributing to several chronic diseases such as hyperlipidemia, atherosclerosis, diabetes mellitus [16,17,18]. Flavonoids, as secondary plant metabolites, are widespread in nature and are abundant in our diets such as green vegetables, onions, citrus fruits, apples, berries, green tea, coffee and red wine [17]. They have been reported to scavenge free radicals (an important index to reflect antioxidant capacity) and reduce incidence of atherosclerosis and cardiovascular disease owing to the antioxidative properties of flavonoids such as rutin, hyperoside and isoquercitrin containing in the extracts [18,19]. Several reports also provided the evidence that rutin, hyperoside and isoquercitrin monomers could significantly scavenge free redicals and protect against lipid peroxidation and treat hyperlipidemia [20,21].

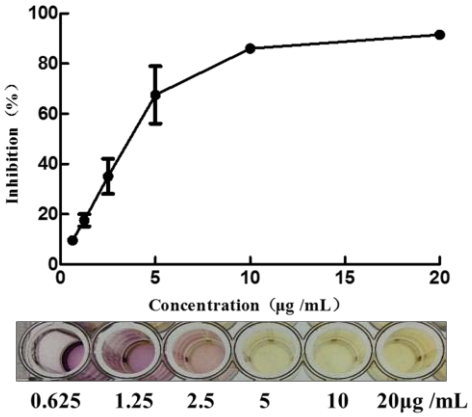

Figure 1. DPPH radical scavenging activity of ZLE. The absorbance of the DPPH at 510 nm treated with different concentrations of ZLE (A) and a microplate showed the reaction of DPPH with different concentrations of ZLE (B), 2.5 μg/mL (dark purple), 5 μg /mL (pink purple),10 μg /mL (yellowish), 20 μg/mL (light yellowish)

In this work, the antioxidant property of ZLE was firstly determined by DPPH assay. It may be noted in Figure 1 that ZLE has the stronger ability to reduce the radical levels in a dose-dependent manner. The inhibition rate raised from 9.5% to 91.5% with increasing ZLE concentration from 0.625 to 20 μg/mL, appearing a 3.8 μg/mL of IC50 (Figure 1). The results indicated that ZLE with a high content of flavonoids had a strong DPPH radicals scavenging capacity, which was positively correlated with flavonoids, especially hyperoside, rutin and isoquercitrin containing in ZLE. This finding was agreement with a previous report that anti-oxidative ability is attributed to content of flavonoids in grains [19,20,21].

3.2. Effect of ZLE on Lipid Peroxidation of Mice Organ Tissues in Vitro and Vivo

Oxygen free radical and lipid peroxidation play an important role in body's metabolism process. The oxygen free radicals can be contributed to the reaction of unsaturated fatty acid lipid peroxidation, leading to tissue damage and diseases happening [22,23]. As shown in Table 1, ZLE could inhibit lipid peroxidation of mice organ tissues both in vivo and vitro, especially liver, which in responds to a good antioxidant property in vitro determined by DPPH.

In our previous study, ZLE was quantified to have ten flavonoids, including isovitexin, vitexin, hyperoside, rutin, isoquercitrin, foeniculin, trifolin, quercitrin, astragalin and afzelin [10], especially hyperoside, rutin and isoquercitrin with a higher content in ZLE. Hyperoside, rutin and isoquercitrin had previously been reported to act as oxygen-free radical scavengers and protect against lipid peroxidation and were extensively used for the clinical treatment of anti-oxidation [20,21,24,25]. In addition, a number of evidences confirmed that flavonoids compounds such as rutin and hyperoside, particularly from vegetables, fruits and some herbs, have been proposed to prevent and treat oxidative stress and hyperlipidemia induced by free radicals and lipid peroxidation [26].

It can be concluded from the current study that bioactive components of ZLE especially hyperoside, rutin and isoquercitrin have the ability to inhibit the lipid peroxidation and repair organ injuries induced by oxidative stress, thus providing scientific evidence in favor of its pharmacological use in treating hyperlipidemia. Our present work could also explain the protective effect of ZLE on lipid peroxidation which probably results from the antioxdative role of these flavonoids including rutin, isoquercitrin, hyperoside, quercitrin, astragalin, which supported the results reported by Zhang et. al [26].

Table 1. Effect of the treatment with ZLE on lipid peroxidation in vivo and vitro (n=5)

	Inhibition rate (%)			
	In vivo	In vitro		
	250 mg/kg	2.5 μg /mL	5 μg /mL	10 μg /mL
Heart	18%	2%	2%	4%
Liver	89%	17%	34%	56%
Spleen	0%	0%	0%	5%
Lung	31%	0%	8%	3%
Kidney	27%	17%	21%	21%
Brain	0%	13%	14%	27%
Pancreas	2%	17%	20%	29%

3.3. Effect of ZLE on Cell Proliferation and Lipid Levels in HepG2 Cells

Studies had shown that the occurrence and development of atherosclerosis is closely connected with lipid peroxidation damage, and the decrease of lipid-levels in plasma is an effective method to treat atherosclerosis, as well as hyperlipidemia [27]. Liver is the major organ in lipid metabolism, which plays a key role in synthesizing the fatty acid and lipid circulation [28]. Our work showed that ZLE with ten flavonoids had a good antioxidant property in both previous and present studies, and a good inhibition on lipid peroxidation of mice organ tissues, especially for liver. In the work, we further study the lipid lowing effect of ZLE using HepG2 cells. As shown in Figure 2B, the varying concentrations from 100 to 1000 μg/mL of ZLE did not influence HepG2 cell survival after 24 h exposure. The 500 μg/mL and 1000 μg/mL of ZLE stimulated the cell proliferation without a significant morphological change (Figure 2A), suggesting ZLE concentrations using in the experiment are safe for HepG2 cell.

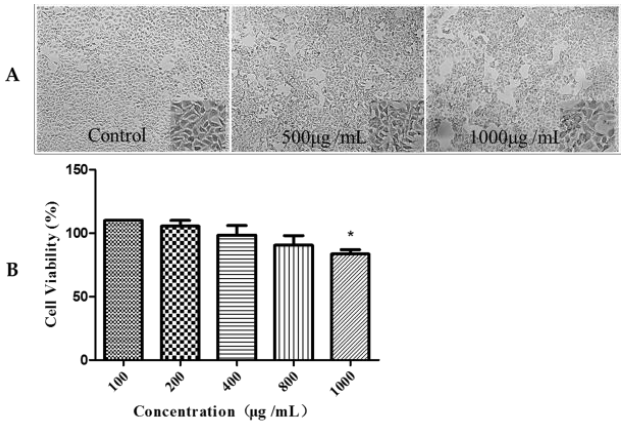

Figure 2. ZLE had no detected cytotoxic effects on HepG2 cells. Cell morphology treated with different concentration of ZLE for 24 h (A) andCell viability of ZLE was detected by MTT assay (B). ZLE had no detected inhibition on proliferation of HepG2 cells in concentration of 100-1000 μg/mL. Values are presented as mean ± SE of three experiments. $P < 0.05$, vs control group

The intracellular absorption of FFAs, an inducer of lipid peroxidation and free radicals production, is of utmost importance in understanding accumulation of fatty acids in liver cells [29]. Thus in our study, HepG2 cell was used to study lipid peroxidation and lipid accumulation induced by FFAs. Compared with the control group, the HepG2 cells exposing to 1 mM of FFAs for 24 h could increase lipid accumulation by 3 folds (Figure 3A, B, G, H). Compared with the FFAs group cells, the lipid content of the cells significantly decreased after treated with fenofibrate (100 μM, a commercial procduct of the lowing lipid) and ZLE. The lipid lowering effect of ZLE appeared in a dose-dependent manner (89.5%, 75.5% and 48.5% (P < 0.05)) (Figure 3A-F, measured by Oil red O assay; Figure 3 G and H, examined by a microplate and expressed as the lipid content in HepG2 cells induced by FFAs). The total amounts of TG level of FFA-induced HepG2 cells treated with 125, 250 and 500 μg/mL of ZLE was reduced to 98%, 69%, 32%, respectively, while TC level was reduced to 96%, 95%, 83% respectively. Compared with FFAs group, the TC and TG levels in fenofibrate (100 μM) group were reduced to 95% and 82% (P < 0.05) (Figure 4 I) respectively. It may be noted that ZLE could cause a significant decrease of lipid in a concentration-dependent manner, and was much effective than fenofibrate on lipid lowering activity.

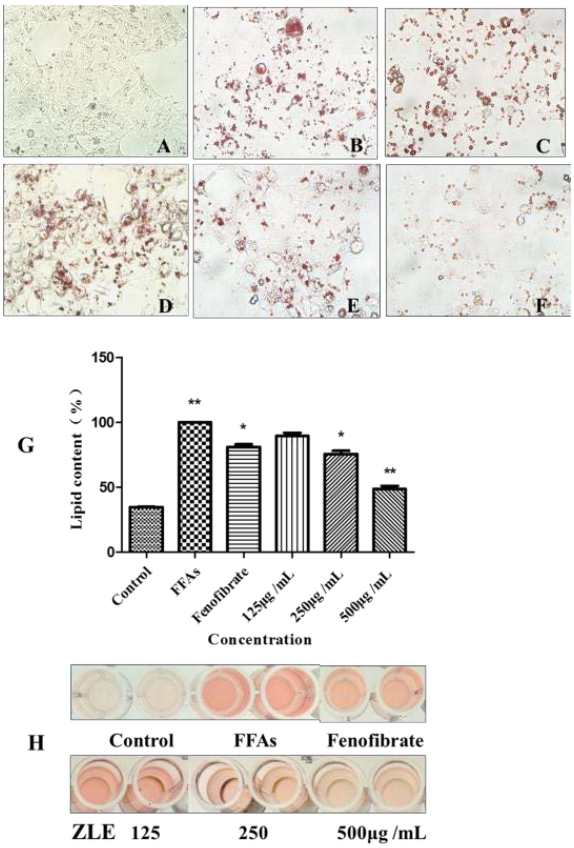

Figure 3. ZLE decreased the lipid accumulation induced by FFAs in a dose-dependent manner. (A) Control group: cells treated with DMEM for 24 h; (B) FFAs group: cells treated with 1 mM FFAs for 24 h; (C) Cells treated with fenofibrate in a dose of 100 μM for 24 h; (D,E,F) Cells were treated with ZLE in a various concentration of 125, 250, 500 μg /mL for 24 h. The cells were stained with Oil red O for measuring intracellular lipid accumulation and examined by light microscopy. Amicroplate showed the lipid content in different groups (G., H); Results are representative of 3 independent experiments (n=3)

ZLE was a rich source of flavonoids. The daily intake of flavonoids in humans is estimated to be as much as 1 g [30]. Flavonoid consumption has been documented to be negatively associated with coronary heart disease, such as hyperlipidemia [31,32]. Rutin has been widely documented to process anti-hyperlipidemia and anti-atherosclerosis activity owing to its high antioxidant ability [24]. Hyperoside, isoquercitrin, rutin and quercitrin also have a dose-dependent protecting activity to a dyslipidemia rats induced by high fat [23,25]. Zhang et al. reported that hawthorn fruits extract detected with high content flavonoids (rutin, quercitrin, isoquercitrin et. al) could regulate cardiovascular and involved the protection of oxidative stress induced by lipid peroxidation [26]. The present work suggested that this lipid lowering action of ZLE in vitro maybe mainly associated with the high content of flavonoids, particularly the presence of hyperoside, isoquercitrin, rutin and quercitrin.

3.4. Effect of ZLE on Serum Lipids in apoE-/- Mice

Since ZLE could reduce lipid levels in vitro, we further employed the apoE-/- deficient mice for confirming the lipid lowering effect of ZLE. The apoE-/- deficient mice have extremely high lipid levels and develop serious atherosclerotic lesions in response to a high-fat diet, which is identical to that in humans system [33]. Figure4 revealed that compared with the model group fed on a high fat diet, ZLE at a dose of 250 mg/kg could decrease LDL-C, TG and TC by 25%, 45%, 72% respectively, and increase HDL-C by 72% significantly (P < 0.05) (Figure4 II). The result was consistent with the previous study reported by Ma, et al [34]. It may be noted that ZLE treatment could attenuate the serum triglyceride level more than cholesterol level, and was efficient than fenofibrate treatment. In addition, we also found a significant decrease in the level of atherosclerosis-index (AI) in mice treated with ZLE (Figure4 II), compared with model group (P < 0.05). Many reports have shown that the AI was the most reliable indicator of an increased risk with cardiovascular disease (CVD) [35,36]. Our study demonstrated that ZLE containing high content of flavonoids could be a good source against the deterioration of atherosclerosis and the accumulation of lipids.

The present work suggested that ZLE could not only protect against the procession of oxygen free radicals and lipid peroxidation, but also lower the lipid levels both in vivo and vitro as a good antioxidant (P<0.05), which was consistent with the previous studies [16,23,24]. Furthermore, ZLE could significantly up-regulated the levels of GSH-Px and SOD in vivo (Data are not shown). In physiological processes, there is a balance between oxidant stress and the ability of antioxidant defense (including the enzymes SOD, glutathione peroxidase (GPx) and catalase, as well as hydrophilic antioxidants) in organism [37].The results of our study proved that ZLE could improve antioxidant defense against oxidant stress, and regulate lipids levels in multi systems including cells, organs and animals. A series of evidences have shown flavonoids including hyperoside, isoquercitrin and rutin have capacity of antioxidant effect, which was clearly associated with its anti-hyperlipidemia activity [25,26].

ZLE had been studied to have the similar activity and mainly due to its high content of flavonoids, especially hyperoside, isoquercitrin and rutin.

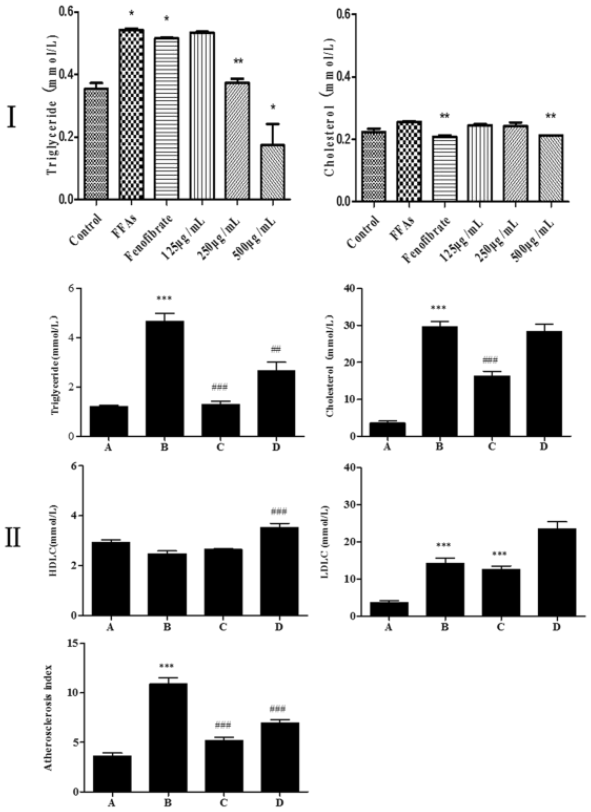

Figure 4. ZLE decreased the lipid levels in a dose-dependent manner. The triglyceride and cholesterol levels in HepG2 cells were measured with a Colorimetric/Fluorometric Kit; Results are representative of 3 independent experiments (n=3), *$p < 0.05$ vs FFAs group. The effect of ZLE on serum lipids in apoE-/- mice were showed in Fig 4 Ⅱ. Group A: Control group, use 0.5% CMC; Group B: Model group given 0.5% CMC fed with a high diet. Group C: ZLE group given a dosage of 250 mg/(kg.d); and Group D are positive group given a dosage of fenofibrate (100 mg/kg.d). The experiment last 2 months, lipid levels in model group were significantly higher than those in the control group, and treatment with ZLE (250 mg/kg.d) had great effect on the level of lipids compared with fenofibrate. *$p < 0.05$ vs Control group; # $p < 0.05$ vs Model group

3.5. Effect of ZLE on Histology in apoE-/- Mice

Nowadays, various drugs are used in the treatment of hyperlipidemia, such as statins and fibrates. Although fibrates are effective and used as the first line therapies, they associated adverse reactions such as nausea, diarrhea, and rhabdomyolysis, limits the clinical uses. In the study, we found that ZLE could prevent and treat oxidative stress as a good antioxidant and inhibit lipid metabolism disorders induced by free radicals and lipid peroxidation. In order to determine the toxicity of ZLE on these organ tissue, histopathological examination of apoE-/- mice treated with or without ZLE was evaluated by HE staining. Figure5 revealed that the liver, lung and kidney of mice fed with ZLE for 8 weeks were not observed to have a significant histological change, suggesting that ZLE was safety for treating hyperlipidemia in a long term administration.

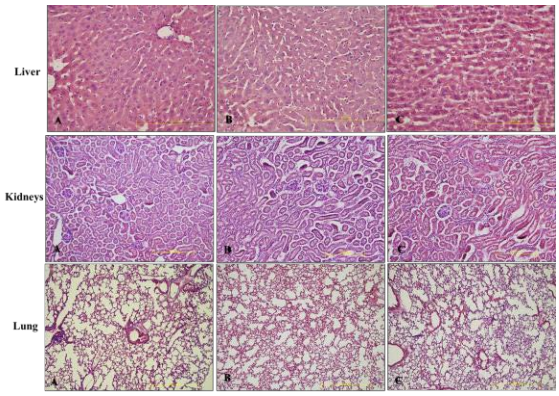

Figure 5. Effect of ZLE **on histology changes in apoE-/- mice.** Higstoppathology of liver, kidney and lung from apoE-/- mice (HE staining: original magnification ×20). The mice were treated with 0.5% CMC (A), 250 mg/kg ZLE (B), 100 mg/kg fenofibrate (C) for 8 weeks. The main organs were collected and processed for histological analysis. The sections were stained with hematoxylin-eosin. No detectable injures were examined, suggesting that the ZLE had no distinct toxicity on liver, kidney and lung and well tolerated

4. Conclusion

Z. bungeanum leaf is a popular vegetable in China. In the previous work, we investigated the mainly active components as flavonoids in *Z. bungeanum*'s leaves, and found the positive relation of the flavonoid content and antioxidative activity. In this work, we confirmed the ability of *Z. bungeanum* leaf extract (ZLE) on scavenging free radicals and anti-hyperlipidemia in multi system such as mice organ tissue, HepG2 cell and apoE-/- mice. The data of our study indicated that ZLE with satisfying safety could significantly inhibit the development of hyperlipidemia by preventing the oxidative damage induced by free radicals and lipid peroxidation. Moreover, we found that ZLE enriched flavonoids could attenuate hyperlipidemia, which is stronger than fenofibrate, a commercial drug. The results suggest the potential utility of the leaf of ZLE as a natural source of antioxidant and anti-lipid accumulation against hyperlipidemia and atherosclerosis.

The further studies are required to investigate the molecular mechanism of anti-hyperlipidemia of Z. bungeanum leaf extract. In preliminary study, we found that ZLE treatment would result in a decrease in the ATP level in HepG2 cells (Data are not shown). Evidences have showed that ATP is closely linked with the regulation of hepatocellular lipids [38,39]. The lipid lowering actions of ZLE related to ATP pathways needs to be confirmed in the future work.

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