Hepatoprotective Effect of Seed Coat of *Euryale ferox* Extract in Non-alcoholic Fatty Liver Disease Induced by High-fat Diet in Mice by Increasing IRS-1 and Inhibiting CYP2E1

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Abstract: Non-alcoholic fatty liver disease (NAFLD), a common chronic liver disease characterized by hepatic steatosis, affects 30-40% of the population in the world. The seed of *Euryale ferox* salisb. possesses several pharmacological actions, including metabolic syndrome. However, the seed coat of *E. ferox* was usually discarded as waste, which contains comparatively abundant polyphenols, and its biological activity has been rarely investigated. In this work, we evaluate the hepatoprotective effect of *E. ferox* seed coat extract (EFSCE), in NAFLD mice induced by high-fat diet (HFD). The HPLC-MS analysis indicated that the main components of EFSCE were polyphenols. And then, mice were treated with HFD for 4 weeks to induce NAFLD. The result showed that the body weight, weight of adipose tissue, the ratio of liver to body weight in NAFLD mice increased compared with control group. In addition, blood lipids parameters including total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) also increased in NAFLD mouse model. It was showed that, after treated with EFSCE (15 and 30 mg/kg/day) for 4 weeks, the body weight, lipids deposition in the liver and blood lipids in HFD-induced NAFLD mice markedly reduced. Compared with NAFLD mice, EFSCE administration could also prevent malondialdehyde (MDA) overproduction and strengthen Superoxide Dismutase (SOD) activity to counteract oxidative stress. Moreover, EFSCE was also found effective in reducing alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in HFD-induced NAFLD model, which indicated liver injury in NAFLD. Therefore, EFSCE (rich in polyphenols) is indicated as bioactive nature product for HFD-induced NAFLD treatment, by eliminating lipid accumulation and oxidative stress via regulation of IRS-1 and CYP2E1.

Key words: seed coat of *Euryale ferox*, non-alcoholic fatty liver disease, oxidative stress, high-fat diet, IRS-1, CYP2E1
huge demand for exploring effective medical treatment for NAFLD.

Traditional Chinese Medicine (TCM) provides abundant sources of biologically effective substances. Many studies revealed that traditional Chinese herbal extracts, natural products and herb formulas have significant effect on fighting against NAFLD. Euryale ferox salis. is a type of large floating-leaf aquatic plant that belongs to family Nymphaeaceae. The seed of E. ferox is widely used in TCM, and has been applied in the treatment of kidney failure, diarrhea, spleen dysfunction and myocardial ischemic reperfusion injury. Preliminary work also showed that extract of E. ferox seed exhibited antidiabetic, antihyperlipidemic effect and antioxidant effect in streptozotocin induced diabetic rats. But the seed coat of E. ferox was usually discarded in large quantities after the seeds had been harvested, which contains comparatively abundant polyphenols. In preliminary experiment, polyphenols in seed coat of E. ferox displayed antioxidant and anti-fatigue activity in vitro and in vivo. However, the efficacy of E. ferox seed coat extract (EFSCE) in reducing hepatic lipid accumulation and oxidant stress in NAFLD mice model has not yet been evaluated.

Therefore, in this study, we aim to investigate if EFSCE, a fractionation extract from seed coat of E. ferox improves hepatic lipid accumulation and oxidative stress, in a high-fat diet (HFD)-induced mouse model of NAFLD, meanwhile, its related mechanism was also demonstrated. Results from this study may provide a reference for the development of EFSCE as a natural drug for the treatment of HFD-induced NAFLD.

2 Materials and Methods

2.1 Chemicals, Reagents and Antibodies

The chemicals and solvents used for the mobile phase were shown as follows: methanol (LC-MS grade) was purchased from Fisher scientific (Schwert, Germany), formic acid (HPLC grade) was purchased from Acros Organics (Geel, Belgium). Water (LC-MS grade) was generated by a Milli-Q reagent water system (Millipore, USA) freshly. The other solvents and reagents used for preparation and isolation were obtained from Merck (Darmstadt, Germany) in analytical grade purity.

Protein extraction kit and BCA protein assay kit were purchased from KeyGen Biotechnology (Nanjing, China). The commercial kits for quantifying total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

The following primary antibodies were used for the Western blotting (WB) assay: CYP2E1 (ab28146, 1:1000) was purchased from Abcam (Cambridge, MA, USA). β-actin (#3700, 1:1000), IRs-1 (#2382, 1:1000) and p-IRS-1 (Tyr895) (#3070, 1:1000) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit IgG, HRP-linked Antibody (#0724, 1:3000) and Anti-mouse IgG, HRP-linked Antibody (#7076, 1:3000) were used as secondary antibody, which were purchased from Cell Signaling Technology.

2.2 Plant material

The fresh fruit of E. ferox was collected in the summer of 2017 at Suzhou, China (N31°10’ 35.59”, E120°26’ 33.54”), and identified by the corresponding author. The voucher specimen was deposited at the Herbarium of the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (voucher number: 2018I107). The seed coat was dried overnight, separated and processed into powder for future use.

2.3 Preparation and analysis of EFSCE

Extract of E. ferox seed coat was prepared according to the published method of Wu et al. in previous study with some modifications. Briefly, dried powder (about 100 g) of E. ferox seed coat was extracted three times with 1000 mL 80% ethanol in a water bath at 90°C for 2 times, 1 h each time. The ethanol extract was filtered and lyophilized. The resulting powder was dissolved in distilled water, centrifuged and applied to a macroreticular resin XAD16 column (The Dow Chemical Company, 2 cm × 80 cm) equilibrated with distilled water. The column was eluted with distilled water, 50% and 95% ethanol solution in turn. The 50% ethanol fraction was concentrated and dried in a vacuum to obtain EFSCE (brown powder).

Polyphenols content in EFSCE was determined by the Folin-Ciocalteu reagent according to the published method. Briefly speaking, the EFSCE samples were diluted by 50% ethanol to the appropriate concentration, which was used as test solutions. Test solutions were incubated with Folin-Ciocalteu phenol reagent (Sigma-Aldrich). And then, Na2CO3 (20%) was added to develop color. Finally, the absorbance at 755 nm was evaluated using a Molecular Devices Spectra Max Plus automatic plate reader (Molecular Device, Sunnyvale, CA, USA). Gallic acid was used as a standard for the calibration curve, and final content of polyphenols in EFSCE is expressed as gallic acid equivalent based on the calibration curve.

Furthermore, EFSCE was dissolved in methanol at 2 mg/mL and filtered for HPLC-MS analysis. Isolation was carried out with a reversed phase Agilent ZORBAX SB-C18 column (1.8 µm, 4.6 × 100 mm; Waldbronn, Germany). Methanol (A) and 0.1% formic acid (B) were used as the mobile
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To obtain serum, liver and adipose tissues were disected quickly on ice. Serum samples were stored at -80°C prior to analysis.

2.4 Animals experiment

Male ICR (Institute of Cancer Research) mice (Sino-British SIPPR/BK Lab Animal Co., Ltd., Shanghai, China; certificate No. SCXK2013-0016) weighing 20-25 g were fed in a laboratory environment room with 12:12 h light-dark cycle, 25 ± 2°C temperature and 60 ± 5% relative humidity. All the animals received commercial food and sterile water.

After the mice were acclimatized for 1 week, at age of 6 weeks, mice were treated with regular diet (XieTong Organism Inc., China), or high fat diet (HFD) for 4 weeks according to previous study. Mice were divided into 4 groups (10 animals of each group) and treated as follows: regular diet-fed mice as CON group, high-fat diet-fed mice as HFD group, HFD + 15 mg/kg EFSC treated group, HFD + 30 mg/kg EFSC treated group. They were gavaged once daily with control (equal volume saline) and EFSC (15 mg/kg and 30 mg/kg). All mice were administrated for 28 days. All protocols used in the study were conducted in compliance with the Guide for the Care and Use of Laboratory Animals stated by China Pharmaceutical University.

At the end of the treatment period (8 weeks), the mice were fasted for 12 h after the last drug administration. All mice were sacrificed under chloral hydrate anesthesia. Blood samples were collected from the abdominal vein and were immediately placed into ice-chilled tubes for 15 min. Then blood samples were separated at 3000 rpm for 15 min at 4°C to obtain serum. Liver and adipose tissues were dissected quickly on ice. Serum samples were stored at -80°C prior to analysis.

2.5 Biochemical parameters analysis

A Molecular Devices Spectra Max Plus automatic plate reader (Molecular Device, Sunnyvale, CA, USA) was used to determine the serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations by commercial kits according to the manufacturer’s instructions. The commercial kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). To determine hepatic superoxide dismutase (SOD) and malondialdehyde (MDA) content, the frozen liver tissues were homogenized in phosphate buffer solution (PBS). SOD and MDA contents in the supernatants were detected using commercial kits. All experiments were analyzed in triplicate, and the average values are presented.

2.6 Histopathological Examination

Parts of fresh liver samples from mice in the control, HFD and HFD + EFSC (15 mg/kg and 30 mg/kg) groups were fixed in 4% paraformaldehyde overnight, paraffin embedded and sectioned (4 μm). The liver tissue sections were stained with hematoxylin & eosin (H&E) according to standard instruction. Images were captured under an Axio Zoom.V16 Zoom Microscope from Carl Zeiss (Carl Zeiss, Oberkochen, Germany) at 200 × magnification. Each section was examined by a specialist who had no knowledge about the sample information.

2.7 Western blotting

Fresh liver tissues were homogenized and lysed using a commercial protein extraction kit. According to the manufacturer’s protocol the tissue lysate samples were centrifuged at 12,000 rpm at 4°C for 5 min. The supernatants were collected and the lysates protein concentrations were measured by BCA protein assay. Denatured proteins (25 μg) were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a PVDF membrane (Millipore, MA, USA). The membrane was washed in Tris-buffered saline (TBS) and blocked with 5% skimmed milk powder solution at room temperature for 2 h. The membranes were incubated with primary antibody overnight at 4°C, and then incubated with secondary antibody at room temperature for 2 h. Membrane-bound antibodies were detected by an enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Luminescent signal was captured with a Fuji medical X-ray film (Fujifilm, Tokyo, Japan). β-actin was used as a loading control for total protein content.

2.8 Statistical analysis

All data were obtained from three separate experiments and expressed as the mean ± standard error (SE). Data analysis was done by using GraphPad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA, USA). A one-way ANOVA was used to perform multiple group parametric data. Statistical significance was considered when \( p<0.05 \).

3 Results

3.1 Polyphenols content and analysis in EFSC

The most abundant compound in the E. ferox seed coat was polyphenols, the content of which was accounting for
94.7 ± 1.3% (gallic acid equivalents). Total ion chromatogram of EFSCE in negative mode was shown in Fig. 1, and the compounds characterization is presented in Table 1.

### 3.2 Effect of EFSCE on the body, liver and adipose tissue weights in NAFLD mice

As can be seen from Fig. 2A-C, HFD treatment for 4 weeks could induce NAFLD. NAFLD mice had obviously increased in body, adipose tissue and liver weight compared to the negative controls \((p<0.001)\). These parameters were prevented by the different dose of EFSCE (15 mg/kg/day and 30 mg/kg/day) \((p<0.001)\). Compared with control group, the ratios of liver to body weight \((\%\) in the NAFLD mice increased dramatically \((p<0.001)\). After treated with high dose of EFSCE, the ratio significantly decreased compared with NAFLD group \((p<0.001)\) (Fig. 2D). The results indicated that treated with EFSCE inhibited the progression of fat gain in the body, and also reduced weight gain in the liver tissue.

### 3.3 Histopathological examination

In Fig. 3A presents, control group livers were shown in deep red color, the tissue were glossy and resilient. Meanwhile, the livers in NAFLD mice were enlarged, lost luster and had yellow necrosis foci on the surface. Different dose of EFSCE treatment (15 mg/kg and 30 mg/kg) improved liver appearance in a dose-dependent manner.

The liver histology photo sections (H&E staining, 200 × magnification) were shown in Fig. 3B. Tissues of normal diet treated mice (control group) showed well-arranged liver lobular structure; neither lipid droplets nor hepatocyte swelling was observed. However, treated with HFD could dramatically increase disorder of hepatic lobule structures, and lipid droplets were observed in the most of liver cells. After treated with EFSCE, hepatocyte swelling, volumes and quantities of lipid droplet were significantly reduced compared with that in NAFLD animals. Morphology of liver lobular structure recovered to nearly normal status, especially in mice which received 30 mg/kg EFSCE treatment. We also examined the appearance of adipose tissue (Fig. 3C), and found that HFD treatment induced fat accumulation, after treated with EFSCE, it decreased.

### 3.4 EFSCE ameliorates Hepatic Steatosis in NAFLD mice

To assess the impacts of EFSCE on lipid metabolism in NAFLD, relevant parameters were detected. The serum TC, TG, HDL and LDL content showed significant eleva-
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Fig. 2  Body weight (A), weight of adipose tissue (B), liver weight (C) and ratio of liver to body weight (%) (D). Data are presented as mean ± SE (n = 10). * * * * p < 0.001 vs. the CON group; *** p < 0.001 vs. the HFD group.

Fig. 3  Appearance of liver tissue (A), histological analysis of liver tissues (magnification, ×200) (B) and appearance of adipose tissue (C).

3.5 EFSCE reduces oxidative stress and liver injury in NAFLD mice

Figure 5A and B showed that serum samples from NAFLD group displayed less abundant SOD and excessive accumulation of MDA in relation to the control group, which indicated that the balance between anti-oxidative capacity and oxidative stress was broken in HFD-induced...
NAFLD mice. Following treatment with EFSCE partially rectified this imbalance by increasing SOD content and reducing MDA level (Fig. 5A and B). Compared with control group, superabundant ALT and AST contents were observed in NAFLD mice. EFSCE treatment offered significant protection against HFD induced liver injury in mice by reducing ALT and AST elevation ($p<0.05$; Fig. 5C and D).
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3.6 EFSCE changes pathways involved in insulin resistance and oxidative stress

To further elaborate the underlying mechanism of EFSCE treat HFD-induced NAFLD, we continued to verify whether EFSCE achieved protective function by regulating IRs-1 and CYP2E1 in liver tissue. As showed in Figs. 6 and 7, the phosphorylation of IRs-1 was significantly attenuated in the liver of mice after HFD treatment \( (p < 0.001) \); meanwhile, CYP2E1 dramatically increased \( (p < 0.001) \). EFSCE administration repaired HFD induced inhibition of IRs-1 in a dose-related manner \( (p < 0.01) \). In addition, EFSCE treatment brought a significant decrease of CYP2E1 expression at a high dose.

4 Discussion

The current study found that, treated mice with high fat diets could establish NAFLD model. In this model, the mice revealed lipid metabolism disorder, oxidative stress and liver damage in the liver. Administration of EFSCE directly reduces the lipid accumulation and oxidative in NAFLD with a dose-dependent manner.

The seed of *Euryale ferox*, which is widely used in TCM, has extensive biological activity such as in the treatment of kidney failure, diarrhea, spleen dysfunction and myocardial ischemic reperfusion injury. Extracts of seed of *E. ferox* also have shown therapeutic effects on diabetes, hyperlipidemia, obesity and oxidant stress in diabetic mice induced by streptozotocin. A study revealed that seed coat of *E. ferox* displayed antioxidant and anti-fatigue activity *in vitro* and *in vivo*, among which, polyphenols are effective substances. According to this statistics, the content of polyphenols in the seed coat of *E. ferox* is as high as 62.57 ± 1.68. Based on the extraction and separation method in above study, we improved the method to obtain polyphenols in EFSCE, whose content achieved 94.7 ± 1.3% (expressed as gallic acid equivalents). HPLC-MS analysis indicated that the main the polyphenols in EFSCE were gallic acid, 1,6-digalloyl glucose, 1,2,3-trigalloyl glucose, 1,2,6-trigalloyl glucose, corilagin, ethyl gallate and 1,2,3,6-tetragalloyl glucose. However, the biological effect of EFSCE has been rarely reported. NAFLD was refered as metabolic syndrome in the liver, the biological activity of EFSCE on HFD-induced NAFLD was further investigated.
widely distributed in hepatic cells of the HFD-treated mice, it also found disorder of hepatic lobule structures in NAFLD animals. TC, TG, HDL and LDL levels in the serum increased markedly. The results above indicated the existence of hepatic fat accumulation in NAFLD. EFSCE treatment could effectively prevent the disease process of NAFLD by decreasing weight gain of body, liver and adipose tissue. EFSCE also produced significant therapeutic effects on plasma TC, TG, HDL and LDL levels, the results revealing that EFSCE may ameliorate NAFLD by reducing fat deposition; in addition, pathological analysis also accordant with these results. Studies have suggested that lipid metabolism is regulated by the insulin signaling pathway mainly\(^8,10\), and hepatic lipid accumulation is strongly associated with insulin resistance (IR)\(^11\). As a protein phosphorylated by insulin receptor tyrosine kinase, insulin receptor substrate-1 (IRs-1) is responsible for the transduction of insulin signaling. Impaired insulin signaling leading to IR, which typically occurs at the level of IRs-1, has been observed in metabolic disorders including NAFLD\(^12,13\). Restoration of IRs-1 is essential for proper insulin signaling, which might be beneficial for attenuating NAFLD\(^14\). In this study, in HFD induced NAFLD model, EFSCE regulating IRs-1 pathway produced pharmaceutical effects on excessive lipids accumulation (including TC, TG, HDL and LDL), which was associated with IR. This might be part of the mechanisms underlying HFD-induced NAFLD improvement by EFSCE.

On the one hand, oxidative stress was considered as "second hit" after "first hit" in NAFLD progression\(^6\). Oxidative stress was considered as a disturbance in the balance between production of free radical production and antioxidant defenses. Oxidative stress can induce oxidant damage in lipids, cell membranes, proteins, and DNA, which is participated in the progression of NAFLD\(^6\). MDA is one of the low-molecular-weight end products of fatty acid peroxidation, and it is a good indicator of the degree of oxidative stress. The product of lipid peroxidation can be eliminated by enzymatic antioxidant defense systems. SOD belongs to enzymatic antioxidant defense systems, which can help cells defense toxic effects induced by free radicals. SOD is also a physiological antioxidant, which can prevent subsequent lipid peroxidation\(^10\). In NAFLD condition, the imbalance between MDA generation and SOD activity was observed\(^4,6,17\). These findings are in concurrence with our study. After treated with EFSCE, the imbalance condition got reversed. Besides this, the fatty liver index often accompanied with elevation of ALT and AST, they are also important biomarkers of NAFLD, which indicate liver injury\(^18\). Serum levels of ALT and AST dramatically increased in mice treated with HFD, but this increase was blocked by EFSCE, and the liver injury parameters got recovered.

As the member of the oxidoreductase cytochrome family, Cytochrome P450 2E1 (CYP2E1) is responsible for oxidizing a variety of small molecule substrates including fatty acids\(^6,19\). In NAFLD, the capacity of CYP2E1 to generate ROS is critical to induced oxidative stress\(^20\). Many studies showed that increasing expression of CYP2E1 makes a significant contribution to oxidative stress in NAFLD by increasing MDA and decreasing SOD; absence or blocking of CYP2E1 may exert beneficial effects on NAFLD by inhibiting oxidative stress\(^6\). The data suggested that HFD-induced over expression of CYP2E1 in NAFLD, and EFSCE could significantly prevent the decline in SOD and reduce the MDA levels by inhibiting CYP2E1 expression. This revealed the therapeutic effect on oxidative stress of EFSCE in HFD-induced NAFLD. These results suggested that EFSCE might be a natural product which could alleviate HFD-induced NAFLD associated oxidative stress via inhibiting CYP2E1.

5 Conclusions

In summary, we provide evidence that administration of *Euryale ferox* seed coat extract (EFSCE) significantly improved HFD-induced NAFLD by reducing lipids deposition and ameliorating oxidative stress. The mechanisms may be attributed to IRs-1 activation and CYP2E1 inhibition. These findings indicated hepatoprotective effect of EFSCE extract from *Euryale ferox* seed coat in HFD-induced NAFLD probably due to the abundant of the phenolic ingredients. It could be a nutraceutical to prevent HFD-induced metabolic syndrome, however, this requires further investigation.

Conflict of interest

The authors declare no conflict of interest.

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