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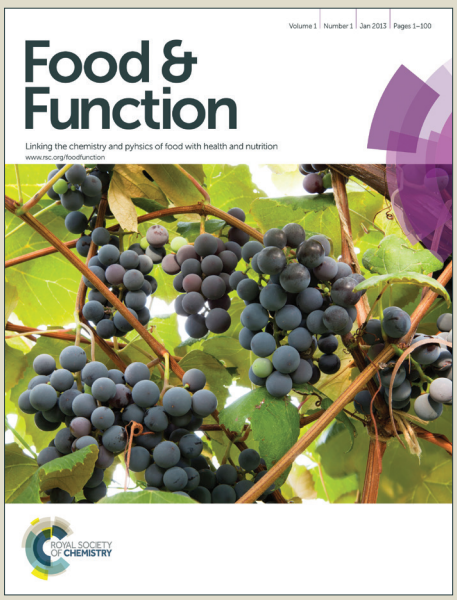

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ARTICLE TYPE

Physicochemical properties, antioxidant activities and protective effect against acute ethanol-induced hepatic injury in mice of foxtail millet (*Setaria italica*) bran oil

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This study was designed to investigate physicochemical characterization of the oil extracted from foxtail millet bran (FMBO), and the antioxidant and hepatoprotective effects against acute ethanol-induced hepatic injury in mice. GC-MS analysis revealed that unsaturated fatty acids (UFA) accounts for 83.76% of the total fatty acid, especially the linoleic acid (C18:2) is predominant PUFA, and the compounds of squalene and six phytosterols (or phytostanols) were identified in unsaponifiable matter of FMBO. Antioxidant activity examination *in vitro* of FMBO showed highly ferric-reducing antioxidant power and scavenging effects against DPPH·, HO·. Furthermore, the protective effect of FMBO against acute hepatic injuries induced by ethanol was verified in mice that intragastric administration with different dosages of FMBO in mice ahead of acute ethanol administration could observably antagonize the ethanol-induced increases in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and the hepatic malondialdehyde (MDA) levels, respectively, along with enhanced hepatic superoxide dismutase (SOD) levels, relative to the control. Hepatic histological changes were also observed and confirmed that FMBO is capable of attenuating ethanol-induced hepatic injury.

1. Introduction

As one of the important cereal crop, foxtail millet (*Setaria italica*) is now cultivated in semi-arid areas all over the world (1). In China, foxtail millet is continues planted as one of the major grain sources and the total productions approach 5,000 thousand ton annually. The millet bran, which consists of pericarp layer, aleurone layer and cereal germ, makes up about 8%-10% of millet quality and is the main by-product during the millet processing from the foxtail millet seed. Foxtail millet bran is extensively used as animal feed in China in the preceding years but recently has attract attention due to the millet bran oil proved to be rich in polyunsaturated fatty acids, especially linoleic acid and tocopherol, phytosterol compositions of millet bran oil are also available (2, 3).

It is well established that some kind edible oils such as olive oil, rice bran oil and tea seed oil, which are rich in unsaturated fatty acid and special antioxidants, have shown functional effects against several degenerative pathologies, including cardiovascular diseases, hepatic injury and cancer (4-6). Antioxidants and phytochemicals in these oils were intake as oxygen radical scavengers due to their abilities to neutralize the actions of free radicals which can bind to macromolecules, such as proteins, lipids, and DNA, resulting in physiologic dysfunction (7, 8). According to the free radical theory, blocking or retarding the chain reaction of oxidation is one of the practicable strategies to preventing oxidative stress-induced damage. In the liver protection study, tea seed oil diet has been proved to protect the liver against CCl_4-induced oxidative damage in rats and the hepatoprotective effects was considered presumably be correlated with its antioxidant and free radical scavenger effects (9). Another study showed that olive oil ingestion by rats could protect the liver from ethanol-induced oxidative damage by affecting the cellular redox potential (10).

Several studies regarding antioxidant activity from edible flours, proteins and insoluble fibers of foxtail millet seeds have been reported (11-13). However, specific research has not been reported on antioxidant activities of their bran oil *in vitro* or *vivo*.

The objective of this investigation was initiated to obtain and evaluate the antioxidant properties of the foxtail millet bran oil (FMBO). Supercritical fluid extraction was used to extract the FMBO and the chemical characterization of fatty acids compositions, unsaponifiable matters were analyzed. Free radical scavenging activities were investigated by HO·and DPPH·radicals quenching techniques and ferric-reducing

antioxidant power was also essayed. In addition, the hepatoprotective effects of FMBO on acute ethanol-induced liver damage in mice were examined.

2. Materials and methods

2.1.Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co.(St. Louis, MO, USA). Potassium ferricyanide [$K_3Fe(CN)_6$] were purchased from Sangon (Sangon, Shanghai). Silica G used for TLC plate preparation was obtained from Qingdao Ocean Chemical Factory (Qingdao, China). Test kits of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and serum triglycerides (TG) and diagnostic kits of malondialdehyde (MDA), total-superoxide dismutase (T-SOD) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The organic solvent used for GC-MS analysis were of HPLC grade purchased from Guoyao Chemical Reagent Co., Ltd.(Chengdu, China). All other chemicals and reagents were of highest grade and commercially available.

2.2 Foxtail millet bran material and oil (FMBO) preparation

Foxtail millet bran was obtained from an oil plant in Baishui county (Shaanxi, China) and oil was obtained by supercritical fluid extraction (SFE). The SFE of FMBO was performed on an HA121-50-01C device (Hua'an Supercritical Fluid Extraction corp., Nantong, China), described in detail by Wei (14), using carbon dioxide as solvent. Carbon dioxide (purity 99.9%) was purchased from Henglong Gas Corp (Hefei, China). Foxtail millet bran samples (150g), with the chosen particle size and water content, were loaded into the extraction vessel. Carbon dioxide from a cylinder was passed through a chiller kept at 2℃ and pumped into the extractor by a high pressure pump. The pressure and temperature were controlled to an accuracy of ± 0.5MPa and ± 0.5℃, respectively. The flow rate of CO_2 was controlled at 20kg/h for all experiments. After each extraction, the oil was collected in the first separator while water and volatile components were recovered in the second one.

2.3. Assay for chemical characterization of FMBO

2.3.1 Physicochemical Property Assays for the Crude FMBO

Important physicochemical properties of the crude FMBO, concerning specific gravity, refractive index, saponification value, and acid value, peroxide value, phospholipid content were characterized according to the AOCS methods (15). The color of the oils was determined by lovibond tintometer (Shanghai Technologies, China). Three replicates were done for each analysis.

2.3.2 Fatty Acid Composition of FMBO

The fatty acid composition of FMBO was analyzed according to AOCS method (15).A Shimada GC-MS-QP2010 (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (DB-wax, 30.0 m×0.25mm, 0.25μm film thickness, Agilent Technologies Co., Ltd.) was used. The GC parameters were: the carrier gas, high purity helium; injector temperature, 250℃; detector temperature, 320℃; the split ratio, 20: 1; column flow rate, 1 mL /min; inject volume, 1μL. The column temperature was programmed to increase to 230℃ from the initial 180℃ at the rate of 2℃/min. The mass spectrometer was operated in electron impact ionization (70eV), full scan (40-800 *m/z*) mode. The MS parameters were: scan speed, 1666; inter scan, 0.5s; source temperature, 250℃; interface temperature 285 ℃. Compounds were identified by comparison of their retention indices and mass spectra with the mass spectra library.

2.3.3 *Sn*-2 Fatty Acid Distribution of the Triacylglycerols

The *Sn*-2 position fatty acid distribution in FMBO was analyzed as previously described (16, 17) with some modifications. The triglycerides of FMBO were firstly separated by TLC method and the spot of triglycerides was scratched and extracted by hexane. About 100mg triglycerides of FMBO were diluted in 2 mL tris buffer (pH 8.0) and heated at 37℃ for 2 min. Then, 0.2 mL of $CaCl_2$ (220 mg/mL), 0.5 mL of sodium cholate (2 mg/mL), and 20 mg of pancreatic lipase were added. After the mixture incubating at 37℃ for 25 min, reaction was then stopped with 1 mL of 6M HCl and 2 mL of diethyl ether was added to extract the hydrolyzate. Thin layer chromatography (TLC) was used to separate hydrolyzate on the system of hexane/diethyl ether/methanoic acid (v/v/v=70/30/1) as developing solvent. The *Sn*-2 monoacylglycerol (*Sn*-2-MAG) spot was scratched and extracted by hexane. The obtained MAG was methylated and the resulting fatty acid methyl ester was subjected to GC analysis.

2.3.4 Determination of unsaponifiable matter of FMBO

An amount of 1.0 g of FMBO was saponificated with 10 mL 0.5 M KOH-C_2H_5OH solution and the unsaponifiable matter was recovered and then was diluted with 2 mL of hexane and injected into the GC-MS instrument. A Shimada GC-MS-QP2010 (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (HP-5, 30.0 m×0.32mm, 0.25μm film thickness, Agilent) was used. The GC parameters were: the carrier gas, high purity helium; injector temperature, 250℃; detector temperature, 320℃; the split ratio, 20: 1; column flow rate, 1 mL /min; inject volume, 1μL. The column temperature was programmed to increase to 285℃ from the initial 200℃ at the rate of 5℃/min and then maintained for 10 min. The mass spectrometer was operated in electron impact ionization (70 eV), full scan (40-800 m/z) mode. The MS parameters were: scan speed, 1666; inter scan, 0.5s; source temperature, 250℃; interface temperature 285 ℃. Compounds were identified by comparison of their retention indices and mass spectra with the mass spectra library. The relative content of each phytosterol (or phytostanol) peak was detected by the normalization method of peak area.

2.3.5 Fourier Transform Infrared Spectrum (FT-IR) Analysis

A Nicolet 6700 spectrometer (Nicolet Instrument Corp., Madison, USA) with a DTGS detector was used for FT-IR analysis. A film of the oil sample was deposited between two disks of KBr. A total of 32 scans were collected for each sample at a resolution of 4 cm^{-1} in the range between 400 and 4000 cm^{-1}.

2.4. Determination of *in vitro* antioxidant activity of FMBO

2.4.1 Scavenging activity on DPPH radicals

The determination of radicals-scavenging activity of FMBO

against DPPH·was determined according to Lee (18) with some modifications. Various concentrations (0-50 mg/mL) sample was mixed with 3.0 mL of aqueous methanol containing 0.1 mM DPPH·. The mixed solutions was shaken vigorously and immediately placed in the dark for 30 min at room temperature. The absorbance was monitored at 517 nm using a spectrophotometer (Fullerton, CA, USA). The experiments were performed in triplicate. DPPH· scavenging activity from method was expressed in tea-seed oil (TSO) as equivalents. The percentage of DPPH·radicals-scavenging activity was calculated with the following formula: scavenging activity (%) = ($A_{blank} - A_{sample}$)/ A_{blank}×100, Where A_{blank} and A_{sample} are the absorbance values of the blank and the tested samples, respectively.

2.4.2 Measurement for ferric-reducing antioxidant power

The reducing power of FMBO was determined according to previously described (19) with some modifications. 1.0 mL of FMBO solution at gradient concentrations (20-140 mg/mL) was added with 2.5 mL of 1% $K_3Fe(CN)_6$ and 2.5 mL of phosphate buffer (pH 6.6). Mixture was incubated at 50 ℃water bath for 15 min, and then 2.5 mL of 10% TCA was added to each test tube, followed by centrifuging at 2300g for 15 min. Absorbance was measured at 700 nm and an increased absorbance of the reaction mixture indicates greater reducing power. Ferric-reducing antioxidant power was expressed in tea-seed oil (TSO) as equivalents.

2.4.3 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of FMBO was determined by the method described by Zhang et al. (20) with some modifications. Both 0.5 mL 1, 10-phenanthroline (0.75 mM) and 0.5 mL FeSO4 (0.75 mM) were dissolved in 1 mL phosphate buffer (pH 7.4) and mixed thoroughly. 0.5 mL H2O2 (0.01%) and various concentrations (0-50 mg/mL) sample were then added. The mixture was incubated at 37 ℃for 60 min, and the absorbance value was measured at 536 nm. Hydroxyl radical scavenging activity (%) = ($A_{blank} - A_{sample}$)/ A_{blank}×100, Where A_{blank} and A_{sample} are the absorbance values of the blank and the tested samples, respectively.

2.5 Animal experiments

Kunming male mice (body weight 20 ± 2 g) were purchased from Laboratory Animal Center of Anhui Medical University (Anhui, China). They were allowed free access to tap water and a standard laboratory rodent food. All animals maintained in a controlled environment at 25±1℃ and 60±5% relative humidity with 12h dark / light cycle and acclimatized for one week prior to use.

The mice were randomly divided into six groups (10 animals in each group). In the normal and ethanol-intoxicated model groups, animals were given normal saline (0.9%, 3.75mL/kg·bw) once a day. The mice in test groups received intragastric doses of 2.50, 3.75, 5.00 mL/kg·bw /day of millet bran oil as group FMBO1, FMBO2, FMBO3, respectively. In positive tea seed oil (TSO) group, mice received 3.75mL/kg·bw /day reference tea seed oil. All the administrations were conducted between nine and ten o' clock in the morning for four consecutive weeks, and dosage were adjusted according to the body weight once a week. After 6 h of the last intragastric administration of FMBO and TSO, all the groups except the normal group received a ethanol (50%, v/v) intragastric administration at the dose of 12mL/kg·bw, and the normal group received same dosage saline (0.9%) administration. Later, all the mice were fasted for 16 h but water was supplied as usual, and then all of the mice were weighted.

Blood was sampled from mice eyes and blood serum was separated and collected by centrifugation and serum was stored at -20℃ for subsequent analysis. Meanwhile, the mice sacrificed via cervical dislocation and liver tissues were carefully excised and washed with ice-cold normal saline, part of the dissected liver of each mice was immediately fixed in 10% formaldehyde for specimen preparation and rest liver were prepared to 10% liver homogenate refrigerated at -80 ℃ immediately for assessment of biochemical parameters. Hepatosomatic index (HI) was worked out in accordance with the subjacent expression: HI = liver weight/body weight ×100%.

2.6. Assays of AST, ALT, TG activities in serum

The enzymatic activities of serum aspartate aminotransferase AST and alanine amino transferase ALT were used as biochemical makers for acute liver injury and assessed by using commercially available diagnostic kit. In addition, the levels of TG was determined in the serum of animals using relevant test kits. The results were expressed as U/L for the activities of AST and ALT, and mmoL/L for levels of TG, severally.

2.7. Measurement for hepatic antioxidant capacity

Hepatic lipid peroxide was estimated by measuring the formed malondialdehyde (MDA) using thiobarbituric acid reactive substances method and superoxide dismutase (SOD) activity in liver homogenate was also assayed. All of these biochemical makers were measured by using commercial kits according to instructions. The MDA and T-SOD activity were expressed as nmol/mg protein and U/mg protein, respectively.

2.8. Morphological measurements in liver tissues

Histopathological assessment was used to complete the research of liver damage. A portion of liver tissue from the left lobe were fixed in 10% neutral buffered formalin. After 48h fixation, liver tissues were embedded in paraffin, and cut into slices (4-7μm thick) sections and then strained with hematoxylin and eosin (H&E). Histopathological changes in the slices were observed microscopically and photographed under a light microscope for the evaluation of pathological change analysis.

2.9. Statistical analysis

The results were expressed as means ± SD and all statistical analysis was done with SPSS 18.0 statistical software package. Data were analyzed statistically by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussions

3.1 Physicochemical Properties of FMBO

The physical and chemical properties of crude FMBO are presented in Table 1. The specific gravity (20 ℃), refractive index at 20 ℃, saponification value and iodine value were

0.9199±0.0006 g/cm^3, 1.4706±0.0001, 176.89 ± 0.33 mg KOH/g, and 103.16±1.90 gI_2/100g, respectively. The saponification value showed differences from the previous work that the saponification value of millet oil were 192-197 mg KOH/g (21) and 186.29±0.51(2). Unsaponifiable matter and phospholipid content of FMBO were 3.58±0.23 (%) and 0.188 mg/g, respectively.

Table 1 Physical and chemical properties of crude FMBO

Characteristic	Crude FMBO
Specific gravity	0.9199±0.0006
Refractive index (20℃)	1.4706±0.0001
Color (Lovibond, 1 in.)	Y35, R2.0
Acid value (mg KOH/g)	9.2 ±0.35
Peroxide value (mmol/kg)	2.33±0.03
Iodine value (gI_2/100g)	103.16±1.90
Saponification value (mg KOH/g)	176.89±0.33
Unsaponifiable matter (%)	3.58±0.23
Phospholipid (mg/g)	0.188

The FT-IR spectrum of FMBO was presented in Fig. 1. Data showed that tiacylglycerol was the main component in millet bran oil. The strong absorption band of ester carbonyl functional group –C=O of TG was around 1710 cm^{-1}.The stretching vibrations of –CH_3 and –CH_2 appeared at 2980-2930 cm^{-1} and 2950–2850 cm^{-1}, whereas the bending vibrations of these groups appeared at 1470 cm^{-1}, 1280 cm^{-1}, and 723 cm^{-1}, respectively. Stretching vibration at 3010 cm^{-1} was attributed to the absorbance of unsaturated bond C–H, indicated that the higher content of linoleic acid in millet bran oil than sunflower oil, which contain higher linoleic acid and show strong absorbance at 3009 cm^{-1} (*22*)**.** FT-IR technology here has been proved to be effective to analyze millet bran oil since that oil's differences in composition, length, and unsaturated degree of the fatty acids as well as their positions in the chain leads to different absorbance in the FT-IR spectrum(*23-26*).

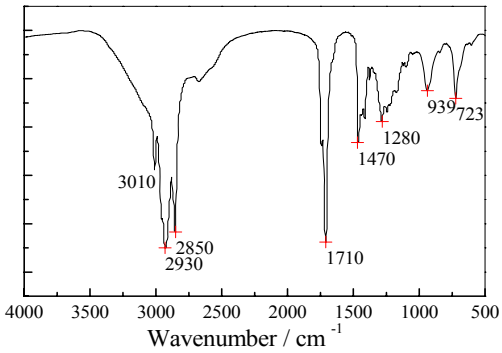

Fig.1 FT-IR spectrum of FMBO in the frequency range 4000-500 cm^{-1}

3.2 Fatty acids composition and positional distribution of the tiacylglycerol of FMBO

As shown in Table 2, eight main components of fatty acids, four saturated fatty acids (SAFA), two monounsaturated fatty acids (MUFA) and two polyunsaturated fatty acids (PUFA) were identified. Among them, palmitic acid C16:0 (10.23%) and stearic acid C18:0 (3.73%) were the most predominant SAFA, oleic acid C18:1 19.65%) was the principal MUFA, and linoleic acid C18:2 (58.85%) as well as the linolenic acid C18:3 (3.96%) were the main PUFA. These values are close to the previous report but a slight differences in fatty acid compositions might due to different cultivars and districts and other processing treatments (2). PUFA have been recommended over the last few years as a dietary change to lower serum cholesterol, and assist in preventing lipoprotein structural alterations (27, 28). For being rich in unsaturated fatty acids especially the high content of linoleic acid, FMBO can be considered to have the same beneficial effects as other nutritive and healthy vegetable oils such as olive oil (29), rice bran oil(30), tea seed oil (31) and germ oil (32).

Table 3 presented that the major fatty acid in the *sn*-2 position of triacylglycerols are completely different from the total fatty acid composition of FBMO. The content of palmitic acid C16:0 and stearic acid C18:0 were 46.20% and 33.93% and the unsaturated fatty acids oleic acid and linoleic acid account for only 19.87% in the *sn*-2 position. This is totally different from the previous studies that about 71.17% linoleic acid was obtained in the *sn*-2 distributions of triacylglycerols of FMBO(*2*) and the results seemed not in compliance with the general law for fatty acid distribution of natural triacylglycerols that the unsaturated fatty acids occupied almost in the *sn*-2 position of glycerol backbone. The high content of palmitic acid in the *sn*-2 position gave a more possible nutrition and function of FMBO since the *sn*-2 palmitic acid served several particular functions as described (33).

Table 2 Fatty acid composition of FMBO

Fatty acids	Content (%)[a]
Palmitic acid (C16:0)	10.23
Stearic acid (C18:0)	3.73
Oleic acid (C18:1)	19.65
Linoleic acid (C18:2)	58.85
Linolenic acid (C18:3)	3.96
Arachidic acid (C20:0)	1.52
Behenic acid (C20:1)	1.30
Docosenoic acid (C22:0)	0.76
Saturated fatty acid(SAFA)	16.24
Unsaturated fatty acid(UFA)	83.76
U/S	5.15

[a] Mean of triplicated determinations

Table 3 Fatty acids composition of *Sn*-2 position of the triacylglycerols of FMBO

Fatty acids	Content (%) [a]
Palmitic acid (C16:0)	46.2
Stearic acid (C18:0)	33.93
Oleic acid (C18:1)	7.96
Linoleic acid (C18:2)	11.91
Saturated fatty acid(SAFA)	80.13
Unsaturated fatty acid(UFA)	19.87

[a] Mean of triplicated determinations

3.3 Analysis of unsaponifiable matter

GC-MS was utilized to investigate unsaponifiable matter of FMBO. Squalene was found due to its principal fragment ions at m/z 69, 81, 95 in the mass spectrum. The total phytosterols amount to 1.55% and the chemical type of phytosterols (or phytostanols) were identified as campesterol, ergostanol, stigmasterol, β-sitosterol, stigmastanol and fucosterol due to the characteristic ions. A detailed characteristic fragment ions and ionization mass spectra of approximate pytosterols were listed in Table 4.

Squalene is proved to be a kind of lipid unsaponification substance with a variety of biological functions (34) and phytosterols play major roles in pharmaceuticals and nutriology since they are known as cholesterol lowing agents as well as anti-inflammatory, anti-oxidation, anti-cancer functions (35, 36). Compositions of unsaponifiable matter supplied FMBO potential functions of healthy biology activities.

3.4 Antioxidant activity of FMBO *in vitro*

As shown in Fig. 2, the antioxidant potential of FMBO *in vitro* can be appraised with the conventional DPPH·, OH· and ferric-reducing antioxidant power assay. The scavenging activity of FMBO on DPPH· was 25.5%, 37.4%, 49.6%, 59.1% and 68.1% at various concentrations of 10, 25, 30, 40 and 50 mg/mL while the scavenging activity of tea-seed oil (TSO) on DPPH· was 11.16%, 23.37%, 35.16%, 45.37% and 53.05% (Fig. 2A). Fig. 2B displayed a preferable degree of ferric-reducing antioxidant power in a dose-dependent manner of FMBO than tea-seed oil. Meanwhile, FMBO also observably showed the OH· scavenging effects of 87%, 94.01%, 94.68%, 94.72%, 94.83% against OH· at the tested concentrations 10-50 mg/mL (Fig.2C). The overall antioxidant capacity of FMBO was shown to be superior to that of TSO at the same concentrations (Fig. 2), with IC_{50} values of 30.05 mg/mL and 3.52 mg/mL for scavenging DPPH·and OH·, whereas the IC_{50} of TSO was 46.8 mg/mL and 22.5 mg/mL, respectively.

3.5 Effects of FMBO administration on body weight, liver weight and HI in mice

The effects of FMBO on body weight and liver weight of treated mice were presented in Table 5. Results showed that treated mice with FMBO gained equivalent body weight to normal group after 4 week's continuous intragastric administration. The mean relative liver weight of ethanol-treated model group was 1.38±0.21g against 1.21±0.22 g of normal group ($p<0.05$). Pretreatment of ethanol-induced mice by FMBO intragastric intake tended to ameliorate the relative liver weight growth performance. As the dosage at 3.75 mL/kg·bw and 5.0mL/kg·bw of FMBO, the relative liver weight both decreased significantly, which was 1.16±0.28g and 1.12±0.31 ($p<0.05$, when compared with model group) respectively.

Table 4 Chemical type and characteristic fragment ions of phytosterols (or phytostanols) of FMBO (GC/ (EI) MS; 70 eV)

Chemical type	Content (%)[a]	Characteristic fragment ions *m/z* (relative abundance)
Campesterol	15.91	145(47), 213(47), 255(31), 315(50), 382(45), 400(80)
Ergostanol	2.97	215(100), 233(80), 276(17), 387(39), 402(49)
Stigmasterol	5.58	213(23), 255(46), 300(21), 351(20), 394(5), 412(49)
β-Sitosterol	56.26	213(53), 255(39), 303(38), 329(56), 396(48), 414(84)
Stigmastanol	15.12	215(100), 233(91), 257(6), 401(41), 416(55)
Fucosterol	4.16	229(45), 271(10), 281(41), 299(23), 314(100), 412(6)

[a] Mean of triplicated determinations

Table 5 Effects of FMBO on body weight, liver weight and hepatosomatic index (HI) of treated mice

Treatments [a]	Body weight (g)	Liver weight (g)	HI(%)
normal	26.92±1.78	1.21±0.22	4.56±0.45
Model	27.49±1.62	1.38±0.21#	5.02±0.78#
FMBO1(2.50mL/kg·bw)	27.94±1.45	1.29±0.35	4.93±0.36
FMBO2(3.75mL/kg·bw)	26.49±1.87	1.16±0.28*	4.69±0.63*
FMBO3(5.0mL/kg·bw)	26.76±2.02	1.12±0.31*	4.82±0.45*
TSO(3.75mL/kg·bw)	27.48±1.98	1.08±0.19*	4.01±0.96*

[a] Values are expressed as means±SD of 10 mice in each group

# $p < 0.05$, vs the normal group

*$p < 0.05$, vs model group

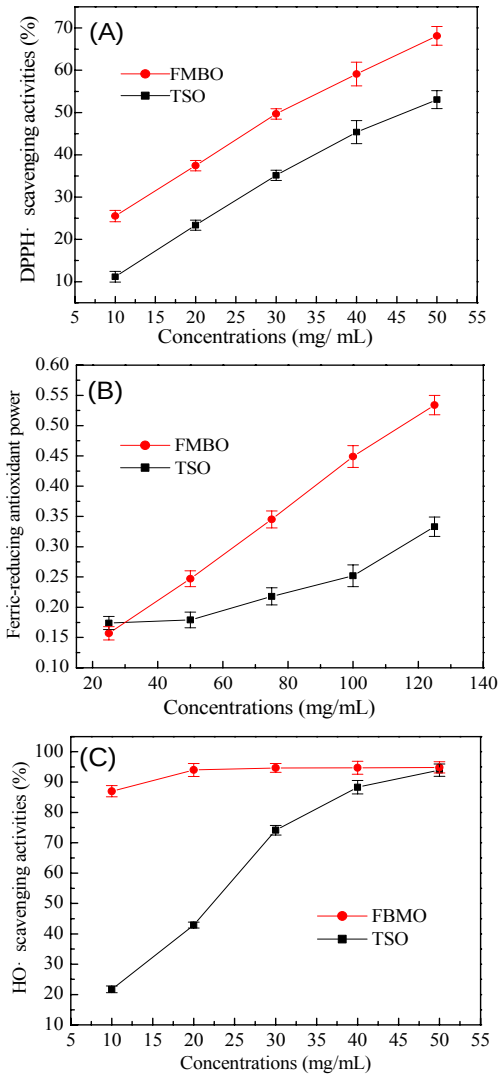

Fig.2 *In vitro* antioxidant effects of FMBO and positive tea-seed oil (TSO). (A) DPPH·-scavenging activities of FMBO and TSO. (B) Ferric-reducing antioxidant power of various concentrations of FMBO and TSO. (C) HO·-scavenging effects of FMBO and TSO

3.6 Serum biochemical markers levels

The effects of pretreatment with FMBO on the ethanol-induced elevation of serum ALT and AST activities were shown in Fig.3, A and B, respectively. Acute ethanol caused hepatotoxicity in mice, as indicated by the increases in serum ALT and AST levels after ethanol administration. FMBO and TSO treated group prevented the ethanol-induced elevation of serum ALT and AST levels in a dose-dependent manner (Fig.3, A, B). Ethanol administration also induced significant accumulation of TG in the serum and liver. FMBO and TSO pretreatment obviously inhibited the increase of the serum and hepatic TG levels, and the effects of FMBO are comparable to TSO (Fig.3, C). These results indicated that FMBO might be mitigate ethanol-induced pathological changes in the liver of mice.

Ethanol-induced hepatic damage is characterized by release of hepatic marker enzymes such as AST, ALT into the circulatory system. The elevation of these enzymes in serum indicates cellular leakage and loss of functional integrity of cell membranes in the liver (37). The results of reduced AST, ALT and TG on ethanol -induced liver damage in rats received FMBO can be attributed to the relatively high content of unsaturated fatty acids. It has been reported that oleic acid is an efficient antioxidant against a variety of oxidative stressors (38). Tocopherols are known to have significant antioxidant activity (39). Obviously, FMBO enriched with oleic acid (19.65%), linoleic acid (58.85%), tocopherols (65mg/100 g) (*2*) and a number of minor components such as squalene, phytosterols, and phenolic compounds. Diets rich in these compounds can decrease blood pressure, prevent oxidative stress and maintain body weight in humans.

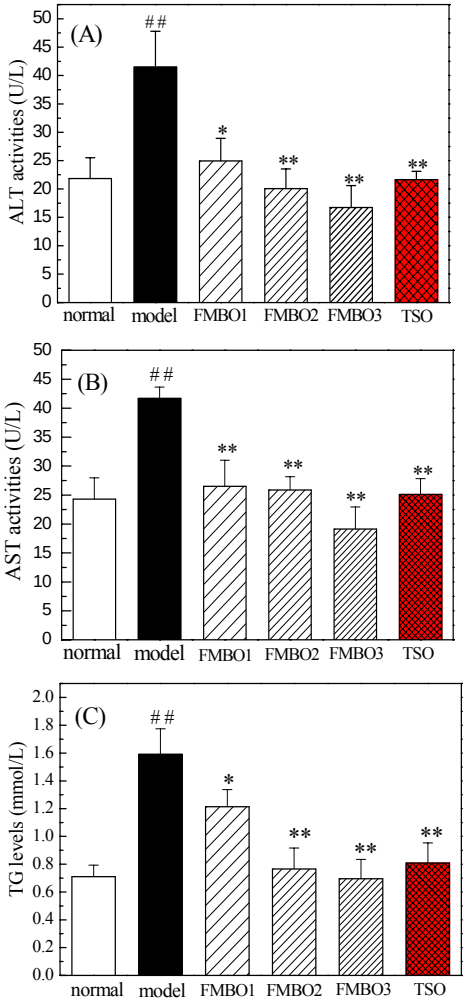

Fig.3 The effects of FMBO on serum ALT (A), AST (B), TG (C) levels in acute ethanol-induced mice. Mice were received intragastric administration of 2.50mL/kg·bw dose of FMBO1, 3.75mL/kg·bw dose of FMBO2, 5.0mL/kg·bw dose of FMBO3, and 3.75mL/kg·bw dose of TSO once daily for four consecutive weeks and subsequently subjected to intragastric single injection of ethanol (50%, v/v, 12mL/kg·bw), and serum biomarkers were measured immediately. Values are presented as means ± SD for 10 mice in each group. ##$p < 0.01$, vs the normal group.* $p < 0.05$, and **$p < 0.01$, vs ethanol-induced group

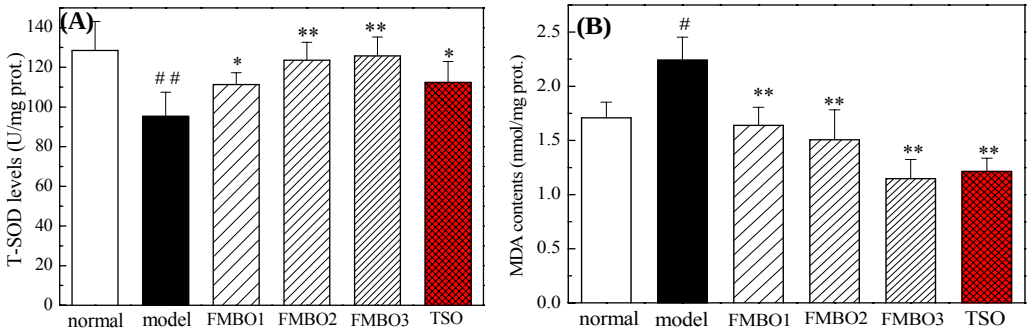

Fig. 4 The effects of FMBO on the levels of hepatic T-SOD (A), MDA (B) after administration of ethanol in mice. Mice were received intragastric administration of 2.50mL/kg·bw dose of FMBO1, 3.75mL/kg·bw dose of FMBO2, 5.0mL/kg·bw dose of FMBO3, and 3.75mL/kg·bw dose of TSO once daily for four consecutive weeks and subsequently subjected to intragastric single injection of ethanol (50%, v/v, 12mL/kg·bw). Values are presented as means ± SD for 10 mice in each group. ##$p < 0.01$, vs the normal group.* $p < 0.05$, and **$p < 0.01$, vs ethanol-induced model group

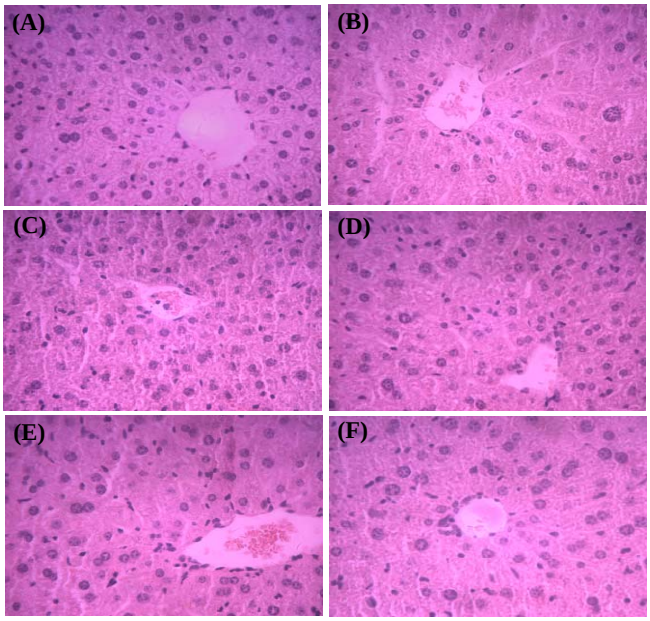

Fig. 5 Effects of FMBO on histopathological changes of liver hepatocytes stained with H&E in acute ethanol -induced mice. Images were obtained from each test group (magnification, 40×10). (A) The normal liver, showing no hepatic damage. (B) the model group, acute ethanol-induced liver, showing seriously and broadly hepatocellular necrosis. (C) Low-dose of FMBO1 (2.50mL/kg·bw) + ethanol. (D) Medium-dose of FMBO2 (3.75mL/kg·bw) + ethanol. (E) High-dose of FMBO3 (5.0mL/kg·bw) + ethanol. (F) TSO (3.75mL/kg·bw) +ethanol

3.7 Hepatic antioxidant enzyme activities and lipid peroxide

Lipid peroxide is a major parameter which can be included as a marker of oxidative damage and MDA is widely used as a marker of lipid peroxidation (40). Antioxidant enzyme such as SOD provides protection against oxidative stress since it is involved in the antioxidant defense mechanism by converting superoxide anions to H_2O_2 (41) .To evaluate the effect of FMBO pretreatment on ethanol-induced liver lipid peroxidation, MDA levels and the hepatic T-SOD were monitored to evaluate the oxidative damage of lipid peroxidation. As shown in Fig.4 B, MDA production in the ethanol-treated group significantly increased compared to the normal group ($p < 0.01$). Briefly, in mice pretreated with FMBO (2.50, 3.75, 5.0 mL/kg·bw) plus ethanol, the MDA levels were significantly reduced ($p < 0.01$) compared with those of solely ethanol-treated model group (Fig.4, B,). Fig.4 A indicated that ethanol treated mice exhibited significant decreases in hepatic SOD activities compared to control mice ($p < 0.01$). Pretreatment with the dose (3.75, 5.0 mL/kg·bw) of FMBO significantly inhibited the SOD depletion induced by ethanol ($p < 0.01$) (Fig.4, A). Consistent with the serum levels of ALT, AST and TG, FMBO pretreatment significantly decreased the ethanol-induced hepatic lipid peroxidation.

3.8 Histopathological examination of mice liver

Histopathological studies of the liver provided supportive evidence for the biochemical analysis. In the normal group, liver

slices showed typical hepatic cellular architecture with well-preserved cytoplasm, clear nucleus, and central vein (Fig. 5A). The ethanol-treated model group revealed faint microvesicular steatosis, lobular inflammation and hepatocellular swelling (Fig.5 B). Acute ethanol exposure caused degenerative morphological changes exhibited by fat droplets in liver sections in the liver. Fig.5 C and Fig.5 D showed a decrease of hepatocytes with fatty change in the tissue and with the increase of dosage, histopathological hepatic damage were nearly absent in the group FMBO3 treated in a dosage of 5.0 mL/kg·bw, as presented in Fig. 5 E. Meanwhile, it was also observed that the TSO group at dose 3.75 mL/kg·bw , can also achieve the protective effect of liver damage(Fig.5 F). With respect to the histological examination, pre-treatment with FMBO suppressed the acute hepatic damage and was consistent with an improvement in the serum biological parameters of hepatotoxicity.

4 Conclusions

In conclusion, results of this study showed that pre-treatment with FMBO is effective in the prevention of ethanol-induced hepatic damage in rats and the hepatoprotective effects may be due to several constituents with potential healthy biological properties, such as unsaturated fatty acids, tocopherols and other lipid accompaniments. The mechanisms of hepatoprotection included the inhibition of lipid peroxidation processes and an increase in antioxidant enzyme activity. These results combined with liver histopathology demonstrated that foxtail millet bran oil has potent hepatoprotective effects, and could be utilized as a functional food for the therapy and prevention of liver damage.

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ARTICLE TYPE

Physicochemical properties, antioxidantactivitiesand protective effect against acute ethanol-induced hepatic injuryin mice of foxtail millet (*Setariaitalica*)bran oil

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This study was designed to investigate physicochemical characterization of the oil extracted from foxtail millet bran (FMBO), and the antioxidant and hepatoprotective effects against acute ethanol-induced hepatic injury in mice. GC-MS analysis revealed that unsaturated fatty acids (UFA) accounts for 83.76% of the total fatty acid, especially the linoleic acid (C18:2) is predominant PUFA, and the compounds of squalene and six phytosterols (or phytostanols) were identified in unsaponifiable matter of FMBO. Antioxidant activity examination *in vitro* of FMBO showed highly ferric-reducing antioxidant power and scavenging effects against DPPH·, HO·. Furthermore, the protective effect of FMBO against acute hepatic injuries induced by ethanol was verified in mice that intragastric administration with different dosages of FMBO in mice ahead of acute ethanol administration could observably antagonize the ethanol-induced increases in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and the hepatic malondialdehyde (MDA) levels, respectively, along with enhanced hepatic superoxide dismutase (SOD) levels, relative to the control. Hepatic histological changes were also observed and confirmed that FMBO is capable of attenuating ethanol-induced hepatic injury.

1. Introduction

As one of the important cereal crop, foxtail millet (*Setariaitalica*) is now cultivated in semi-arid areas all over the world(1). In China, foxtail millet is continues planted as one of the major grain sources and the total productions approach 5,000 thousand ton annually. The millet bran, which consists of pericarp layer, aleurone layer and cereal germ, makes up about 8%-10% of millet quality and is the main by-product during the millet processing from the foxtail millet seed. Foxtail millet bran is extensively used as animal feed in China in the preceding years but recently has attract attention due to the millet bran oil proved to be rich in polyunsaturated fatty acids, especially linoleic acid and tocopherol, phytosterol compositions of millet bran oil are also available(2, 3).

It is well established that some kind edible oils such as olive oil, rice bran oil and tea seed oil, which are rich in unsaturatedfatty acid and special antioxidants, have shown functional effects against several degenerative pathologies, including cardiovascular diseases, hepatic injury and cancer(4-6). Antioxidants and phytochemicals in these oils were intake as oxygen radical scavengers due to their abilities to neutralize the actions of free radicals which can bind to macromolecules, such as proteins, lipids, and DNA, resulting in physiologic dysfunction(7, 8). According to the free radical theory, blocking or retarding the chain reaction of oxidation is one of the practicable strategies to preventing oxidative stress-induced damage. In the liver protection study, tea seed oil diet has been proved to protect the liver against CCl_4-induced oxidative damage in rats and the hepatoprotective effects was considered presumably be correlated with its antioxidant and free radical scavenger effects(9). Another study showed that olive oil ingestion by rats could protect the liver from ethanol-induced oxidative damage by affecting the cellular redox potential(10).

Several studies regarding antioxidant activity from edible flours, proteins and insoluble fibers of foxtail millet seeds have been reported (11-13). However, specific research has not been reported on antioxidant activities of their bran oil *in vitro* or *vivo*.

The objective of this investigation was initiated to obtain and evaluate the antioxidant properties of the foxtail millet bran oil (FMBO). Supercritical fluid extraction was used to extract the FMBO and the chemical characterization of fatty acids compositions, unsaponifiable matters were analyzed. Free radical scavenging activities were investigated by HO·andDPPH·radicals quenching techniques and ferric-reducing antioxidant power was

also essayed. In addition, the hepatoprotective effects of FMBO on acute ethanol-induced liver damage in mice were examined.

2. Materials and methods

2.1.Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co.(St. Louis, MO, USA). Potassium ferricyanide [$K_3Fe(CN)_6$] were purchased from Sangon (Sangon, Shanghai). Silica G used for TLC plate preparation was obtained from Qingdao Ocean Chemical Factory (Qingdao, China). Test kits of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and serum triglycerides (TG) and diagnostic kits of malondialdehyde (MDA), total-superoxide dismutase (T-SOD) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The organic solvent used for GC-MS analysis were of HPLC grade purchased from Guoyao Chemical Reagent Co., Ltd.(Chengdu, China). All other chemicals and reagents were of highest grade and commercially available.

2.2 Foxtail millet bran material and oil (FMBO) preparation

Foxtail millet bran was obtained from an oil plant in Baishuicounty (Shaanxi, China) and oil was obtained by supercritical fluid extraction(SFE). The SFE of FMBO was performed on an HA121-50-01C device (Hua'an Supercritical Fluid Extraction corp., Nantong, China), described in detail by Wei (14), using carbon dioxide as solvent. Carbon dioxide (purity 99.9%) was purchased from Henglong Gas Corp (Hefei, China). Foxtail millet bran samples (150g), with the chosen particle size and water content, were loaded into the extraction vessel. Carbon dioxide from a cylinder was passed through a chiller kept at 2□ and pumped into the extractor by a high pressure pump. The pressure and temperature were controlled to an accuracy of±0.5MPa and±0.5□, respectively. The flow rate of CO_2 was controlled at 20kg/h for all experiments. After each extraction, the oil was collected in the first separator while water and volatile components were recovered in the second one.

2.3. Assay for chemical characterization of FMBO

2.3.1 Physicochemical Property Assays for the Crude FMBO

Important physicochemical properties of the crude FMBO, concerning specific gravity, refractive index, saponification value, and acid value, peroxide value, phospholipid content were characterized according to the AOCS methods (15). The color of the oils was determined by lovibondtintometer (Shanghai Technologies, China). Three replicates were done for each analysis.

2.3.2 Fatty Acid Composition of FMBO

The fatty acid composition of FMBO was analyzed according to AOCS method (15).A Shimada GC-MS-QP2010 (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (DB-wax, 30.0 m×0.25mm, 0.25μm film thickness, Agilent Technologies Co., Ltd.) was used. The GC parameters were: the carrier gas, high purity helium; injector temperature, 250□; detector temperature, 320□; the split ratio, 20: 1; column flow rate, 1 mL /min; inject volume, 1μL. The column temperature was programmed to increase to 230□ from the initial 180□ at the rate of 2□/min. The mass spectrometer was operated in electron impact ionization (70eV), full scan (40-800 *m/z*) mode. The MS parameters were: scan speed, 1666; inter scan, 0.5s; source temperature, 250□; interface temperature 285 □. Compounds were identified by comparison of their retention indices and mass spectra with the mass spectra library.

2.3.3 *Sn*-2 Fatty Acid Distribution of the Triacylglycerols

The *Sn*-2 position fatty acid distribution in FMBO was analyzed as previously described (16, 17) with some modifications. The triglycerides of FMBO were firstly separated by TLC method and the spot of triglycerideswas scratched and extracted by hexane.About 100mg triglycerides of FMBO were diluted in 2 mL tris buffer (pH 8.0) and heated at 37□ for 2 min. Then, 0.2 mL of $CaCl_2$ (220 mg/mL), 0.5 mL of sodium cholate (2 mg/mL), and 20 mg of pancreatic lipase were added. After the mixture incubating at 37□ for 25 min, reaction was then stopped with 1 mL of 6M HCl and 2 mL of diethyl ether was added to extract the hydrolyzate. Thin layer chromatography (TLC) was used to separate hydrolyzate on the system of hexane/diethyl ether/methanoic acid (v/v/v=70/30/1) as developing solvent. The *Sn*-2 monoacylglycerol (*Sn*-2-MAG) spot was scratched and extracted by hexane. The obtained MAG was methylated and the resulting fatty acid methyl ester was subjected to GC analysis.

2.3.4 Determination of unsaponifiable matter of FMBO

An amount of 1.0 g of FMBO was saponificated with 10 mL 0.5 M KOH-C_2H_5OH solution and the unsaponifiable matter was recovered and then was diluted with 2 mL of hexane and injected into the GC-MS instrument. A Shimada GC-MS-QP2010 (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (HP-5, 30.0 m×0.32mm, 0.25μm film thickness, Agilent) was used. The GC parameters were: the carrier gas, high purity helium; injector temperature, 250□; detector temperature, 320□; the split ratio, 20: 1; column flow rate, 1 mL /min; inject volume, 1μL. The column temperature was programmed to increase to 285□ from the initial 200□ at the rate of 5□/min and then maintained for 10 min. The mass spectrometer was operated in electron impact ionization (70eV), full scan (40-800 m/z) mode. The MS parameters were: scan speed, 1666; inter scan, 0.5s; source temperature, 250□; interface temperature 285 □. Compounds were identified by comparison of their retention indices and mass spectra with the mass spectra library.The relative content of each phytosterol (or phytostanol) peak was detected by the normalization method ofpeak area.

2.3.5 Fourier Transform Infrared Spectrum (FT-IR) Analysis

A Nicolet 6700 spectrometer (Nicolet Instrument Corp., Madison, USA) with a DTGS detector was used for FT-IR analysis. A film of the oil sample was deposited between two disks of KBr. A total of 32 scans were collected for each sample at a resolution of 4 cm^{-1} in the range between 400 and 4000 cm^{-1}.

2.4. Determination of *in vitro* antioxidant activity of FMBO

2.4.1 Scavenging activity on DPPH radicals

The determination of radicals-scavenging activity of FMBO against DPPH·was determined according to Lee (18) with some

modifications. Various concentrations (0-50 mg/mL) sample was mixed with 3.0 mL of aqueous methanol containing 0.1 mM DPPH·. The mixed solutions was shaken vigorously and immediately placed in the dark for 30 min at room temperature. The absorbance was monitored at 517 nm using a spectrophotometer (Fullerton, CA, USA). The experiments were performed in triplicate. DPPH·scavenging activity from method was expressed in tea-seedoil (TSO) as equivalents. The percentage of DPPH·radicals-scavenging activity was calculated with the following formula: scavenging activity (%) = ($A_{blank} - A_{sample}$)/ A_{blank}×100, Where A_{blank} and A_{sample} are the absorbance values of the blank and the tested samples, respectively.

2.4.2 Measurement for ferric-reducing antioxidant power

The reducing power of FMBO was determined according to previously described (19) with some modifications. 1.0 mL of FMBO solution at gradient concentrations (20-140 mg/mL) was added with 2.5 mL of 1% $K_3Fe(CN)_6$ and 2.5 mL of phosphate buffer (pH 6.6). Mixture was incubated at 50 □water bath for 15 min, and then 2.5 mL of 10% TCA was added to each test tube, followed by centrifuging at 2300g for 15 min. Absorbance was measured at 700 nm and an increased absorbance of the reaction mixture indicates greater reducing power. Ferric-reducing antioxidant power was expressed in tea-seedoil (TSO) as equivalents.

2.4.3 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of FMBO was determined by the method described by Zhang et al. (20) with some modifications. Both 0.5 mL 1, 10-phenanthroline (0.75 mM) and 0.5 mL FeSO4 (0.75 mM) were dissolved in 1 mL phosphate buffer (pH 7.4) and mixed thoroughly. 0.5 mL H2O2 (0.01%) and various concentrations (0-50 mg/mL) sample were then added. The mixture was incubated at 37 □for 60 min, and the absorbance value was measured at 536 nm. Hydroxyl radical scavenging activity (%) = ($A_{blank} - A_{sample}$)/ A_{blank}×100, Where A_{blank} and A_{sample} are the absorbance values of the blank and the tested samples, respectively.

2.5 Animal experiments

Kunming male mice (body weight 20 ± 2 g) were purchased from Laborary Animal Center of Anhui Medical University (Anhui, China). They were allowed free access to tap water and a standard laboratory rodent food. All animals maintained in a controlled environment at 25±1□ and 60±5% relative humidity with 12h dark/light cycle and acclimatized for one week prior to use. This study was carried out in strict accordance with the internationally accepted guidelines for laboratory animal use and care. All experimental protocol used in this study was approved by the Committee on Care and Use of Laboratory Animals of Anhui Medical University, China.

The mice were randomly divided into six groups (10 animals in each group). In the normal and ethanol-intoxicated model groups, animals were given normal saline (0.9%, 3.75mL/kg·bw) once a day. The mice in test groups received intragastric doses of 2.50, 3.75, 5.00 mL/kg·bw /day of millet bran oil as group FMBO1, FMBO2, FMBO3, respectively. In positive tea seed oil (TSO) group, mice received 3.75mL/kg·bw /day reference tea seed oil. All the administrations were conducted between nine and ten o' clock in the morning for four consecutive weeks, and dosage were adjusted according to the body weight once a week.

After 6 h of the last intragastric administration of FMBO and TSO, all the groups except the normal group received a ethanol(50%, v/v) intragastric administration at the dose of 12mL/kg·bw, and the normal group received same dosage saline (0.9%) administration. Later, all the mice were fasted for 16 h but water was supplied as usual, and then all of the mice were weighted.

Blood was sampled from mice eyes and blood serum was separated and collected by centrifugation and serum was stored at -20□ for subsequent analysis. Meanwhile, the mice sacrificed via cervical dislocation and liver tissues were carefully excised and washed with ice-cold normal saline, part of the dissected liver of each mice was immediately fixed in 10% formaldehyde for specimen preparation and rest liver were prepared to 10% liver homogenate refrigerated at -80 □ immediately for assessment of biochemical parameters. Hepatosomatic index (HI) wasworked out in accordance with the subjacent expression: HI = liver weight/body weight ×100%.

2.6. Assays of AST, ALT, TG activities in serum

The enzymatic activities of serum aspartate aminotransferase AST and alanine amino transferase ALT were used as biochemical makers for acute liver injury and assessed by using commercially available diagnostic kit. In addition, the levels of TG was determined in the serum of animals using relevant test kits. The results were expressed as U/L for the activities of AST and ALT, and mmoL/L for levels of TG, severally.

2.7. Measurement for hepatic antioxidant capacity

Hepatic lipid peroxide was estimated by measuring the formed malondialdehyde (MDA) using thiobarbituric acid reactive substances method and superoxide dismutase (SOD) activity in liver homogenate was also assayed. All of these biochemical makers were measured by using commercial kits according to instructions. The MDA and T-SOD activity were expressed as nmol/mg protein and U/mg protein, respectively.

2.8. Morphological measurements in liver tissues

Histopathological assessment was used to complete the research of liver damage. A portion of liver tissue from the left lobe were fixed in 10% neutral buffered formalin. After 48h fixation, liver tissues were embedded in paraffin, and cut into slices (4-7μm thick) sections and then strained with hematoxylin and eosin (H&E). Histopathological changes in the slices were observed microscopically and photographed under a light microscope for the evaluation of pathological change analysis.

2.9. Statistical analysis

The results were expressed as means±SD and all statistical analysis was done with SPSS 18.0 statistical software package. Data were analyzed statistically by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussions

3.1 Physicochemical Properties of FMBO

The physical and chemical properties of crude FMBO are presented in Table 1. The specific gravity (20 ℃), refractive index at 20 ℃, saponification value and iodine value were 0.9199±0.0006 g/cm^3, 1.4706±0.0001, 176.89 ± 0.33 mg KOH/g, and 103.16±1.90 gI_2/100g, respectively. The saponification value showed differences from the previous work that the saponification value of millet oil were 192-197 mg KOH/g (21) and 186.29±0.51(2). Unsaponifiable matter and phospholipid content of FMBO were 3.58±0.23 (%) and 0.188 mg/g, respectively.

The FT-IR spectrum ofFMBO was presented in Fig. 1. Data showed that tiacylglycerol was the main component in millet bran oil. The strong absorption band of ester carbonyl functional group –C=O of TG was around 1710 cm^{-1}.The stretching vibrations of –CH_3 and –CH_2 appeared at 2980-2930 cm^{-1} and 2950–2850 cm^{-1}, whereas the bending vibrations of these groups appeared at 1470 cm^{-1}, 1280 cm^{-1}, and 723 cm^{-1}, respectively. Stretching vibration at 3010 cm^{-1} was attributed to the absorbance of unsaturated bond C–H, indicated that the higher content of linoleic acid in millet bran oil than sunflower oil, which contain higher linoleic acid and show strong absorbance at 3009 cm^{-1} (*22*)**.** FT-IR technology here has been proved to be effective to analyze millet bran oil since that oil's differences in composition, length, and unsaturated degree of the fatty acids as well as their positions in the chain leads to different absorbance in the FT-IR spectrum(*23-26*).

3.2 Fatty acids composition and positional distribution of the tiacylglycerol of FMBO

As shown in Table 2, eight main components of fatty acids, four saturated fatty acids (SAFA), two monounsaturated fatty acids (MUFA) and two polyunsaturated fatty acids (PUFA) were identified. Among them, palmitic acid C16:0 (10.23%) and stearic acid C18:0 (3.73%) were the most predominant SAFA, oleic acid C18:1 19.65%) was the principal MUFA, and linoleic acid C18:2 (58.85%) as well as the linolenic acid C18:3 (3.96%) were the main PUFA. These values are close to the previous report but a slight differences in fatty acid compositions might due to different cultivars and districts and other processing treatments(2). PUFA have been recommended over the last few years as a dietary change to lower serum cholesterol, and assist in preventing lipoprotein structural alterations(27, 28). For being rich in unsaturated fatty acids especially the high content of linoleic acid, FMBO can be considered to have the same beneficial effects as other nutritive and healthy vegetable oils such as olive oil(29), rice bran oil(30), tea seed oil(31) and germ oil(32).

Table 3 presented that the major fatty acid in the *sn*-2 position of triacylglycerols are completely different from the total fatty acid composition of FBMO. The content of palmitic acid C16:0 and stearic acid C18:0 were 46.20% and 33.93% and the unsaturated fatty acids oleic acid and linoleic acid account for only 19.87% in the *sn*-2 position. This is totally different from the previous studies that about 71.17% linoleic acid was obtained in the *sn*-2 distributions of triacylglycerols of FMBO(*2*) and the results seemed not in compliance with the general law for fatty acid distribution of natural triacylglycerols that the unsaturated fatty acids occupied almost in the *sn*-2 position of glycerol backbone. The high content ofpalmitic acid in the *sn*-2 position gave a more possible nutrition and function of FMBO since the *sn*-2 palmitic acid served several particular functions as described (33).

3.3 Analysis of unsaponifiable matter

GC-MS was utilized to investigate unsaponifiable matter of FMBO. Squalenewas found due to its principal fragment ions at m/z 69, 81, 95 in the mass spectrum. The total phytosterols amount to 1.55% and the chemical type of phytosterols (or phytostanols) were identified as campesterol, ergostanol, stigmasterol, β-sitosterol,stigmastanol and fucosterol due to the characteristic ions. A detailed characteristic fragment ions and ionization mass spectra of approximatepytosterols were listed in Table 4.

Squalene is proved to be a kind of lipid unsaponification substance with a variety of biological functions (34) and phytosterols play major roles in pharmaceuticals and nutriology since they are known as cholesterol lowing agents as well as anti-inflammatory, anti-oxidation, anti-cancer functions (35, 36). Compositions of unsaponifiable matter supplied FMBO potential functions of healthy biology activities.

3.4 Antioxidant activity of FMBO *in vitro*

As shown in Fig. 2, the antioxidant potential of FMBO *in vitro* can be appraised with the conventional DPPH·, OH·and ferric-reducing antioxidant power assay. The scavenging activity of FMBO on DPPH· was 25.5%, 37.4%, 49.6%, 59.1% and 68.1% at various concentrations of 10, 25, 30, 40 and 50 mg/mL while the scavenging activity of tea-seed oil (TSO) on DPPH·was 11.16%, 23.37%, 35.16%, 45.37% and 53.05% (Fig. 2A). Fig. 2B displayed a preferable degree of ferric-reducing antioxidant power in a dose-dependent manner of FMBO than tea-seed oil. Meanwhile, FMBO also observably showed the OH·scavenging effects of 87%, 94.01%, 94.68%, 94.72%, 94.83% against OH· at the tested concentrations 10-50 mg/mL (Fig.2C). The overall antioxidant capacity of FMBO was shown to be superior to that of TSO at the same concentrations (Fig. 2), with IC_{50} values of 30.05 mg/mL and 3.52 mg/mL for scavenging DPPH·and OH·, whereas the IC_{50} of TSO was 46.8 mg/mL and 22.5 mg/mL, respectively.

3.5 Effects of FMBO administration on body weight, liver weight and HI in mice

The effects of FMBO on body weightand liver weight of treated mice were presented in Table 5. Results showed that treated mice with FMBO gained equivalent body weight to normal group after 4week's continuous intragastric administration.The mean relative liver weight of ethanol-treated model group was 1.38±0.21g against 1.21±0.22 g of normal group ($p<0.05$). Pretreatment of ethanol-induced mice by FMBOintragastric intake tended to ameliorate the relative liver weight growth performance. As the dosage at 3.75 mL/kg·bw and 5.0mL/kg·bw of FMBO, the relative liver weight both decreased significantly, which was 1.16±0.28g and 1.12±0.31 ($p<0.05$, when compared with model group) respectively.

3.6 Serum biochemical markers levels

The effects of pretreatment with FMBO on the ethanol-induced elevation of serum ALT and AST activities were shown in Fig.3, A and B, respectively. Acute ethanol caused hepatotoxicity in mice, as indicated by the increases in serum ALT and AST levels after ethanol administration. FMBO and TSO treated group prevented the ethanol-induced elevation of serum ALT and AST levels in a dose-dependent manner (Fig.3, A, B). Ethanol administration also induced significant accumulation of TG in the serum and liver. FMBO and TSO pretreatment obviously inhibited the increase of the serum and hepatic TG levels, and the effects of FMBO are comparable to TSO (Fig.3, C). These results indicated that FMBO might be mitigateethanol-induced pathological changes in the liver of mice.

Ethanol-induced hepatic damage is characterized by release of hepatic marker enzymes such as AST, ALT into the circulatory system. The elevation of these enzymes in serum indicates cellular leakage and loss of functional integrity of cell membranes in the liver(37). The results of reduced AST, ALT and TG on ethanol -induced liver damage in rats received FMBO can be attributed to the relatively high content of unsaturated fatty acids. It has been reported that oleic acid is an efficient antioxidant against a variety of oxidative stressors (38). Tocopherols are known to have significant antioxidant activity (39). Obviously, FMBO enriched with oleic acid (19.65%), linoleic acid (58.85%), tocopherols (65mg/100 g) (*2*) and a number of minor components such as squalene, phytosterols, and phenolic compounds. Diets rich in these compounds can decrease blood pressure, prevent oxidative stress and maintain body weight in humans.

3.7 Hepatic antioxidant enzyme activities and lipid peroxide

Lipid peroxide is a major parameter which can be included as a marker of oxidative damage and MDA is widely used as a marker of lipid peroxidation (40). Antioxidant enzyme such as SOD provides protection against oxidative stress since it is involved in the antioxidant defense mechanism by converting superoxide anions to H_2O_2 (41) .To evaluate the effect of FMBO pretreatment on ethanol-induced liver lipid peroxidation, MDA levels and the hepatic T-SOD were monitored to evaluate the oxidative damage of lipid peroxidation. As shown in Fig.4B, MDA production in the ethanol-treated group significantly increased compared to the normal group ($p < 0.01$). Briefly, in mice pretreated with FMBO (2.50, 3.75, 5.0 mL/kg·bw) plus ethanol, the MDA levels were significantly reduced ($p < 0.01$) compared with those of solely ethanol-treated model group (Fig.4, B,). Fig.4A indicated that ethanol treated mice exhibited significant decreases in hepatic SOD activities compared to control mice ($p < 0.01$). Pretreatment with the dose (3.75, 5.0 mL/kg·bw) of FMBO significantly inhibited the SOD depletion induced by ethanol ($p < 0.01$) (Fig.4, A). Consistent with the serum levels of ALT, AST and TG, FMBO pretreatment significantly decreased the ethanol-induced hepatic lipid peroxidation.

3.8 Histopathological examination of mice liver

Histopathological studies of the liver provided supportive evidence for the biochemical analysis. In the normal group, liver slices showed typical hepatic cellular architecture with well-preserved cytoplasm, clear nucleus, and central vein (Fig. 5A). The ethanol-treated model group revealed faint microvesicularsteatosis, lobular inflammation and hepatocellular swelling (Fig.5B). Acute ethanol exposure caused degenerative morphological changes exhibited by fat droplets in liver sections in the liver. Fig.5C and Fig.5 D showed a decrease of hepatocytes with fatty change in the tissue and with the increase of dosage, histopathological hepatic damage were nearly absent in the group FMBO3 treated in a dosage of 5.0 mL/kg·bw, as presented in Fig. 5 E. Meanwhile, it was also observed that the TSO group at dose 3.75 mL/kg·bw , can also achieve the protective effect of liver damage(Fig.5F). With respect to the histological examination, pre-treatment with FMBO suppressed the acute hepatic damage and was consistent with an improvement in the serum biological parameters of hepatotoxicity.

4 Conclusions

In conclusion, results of this study showed that pre-treatment with FMBO is effective in the prevention of ethanol-induced hepatic damage in rats and the hepatoprotective effects may be due to several constituents with potential healthy biological properties, such as unsaturated fatty acids, tocopherols and other lipid accompaniments. The mechanisms of hepatoprotection included the inhibition of lipid peroxidation processes and an increase in antioxidant enzyme activity. These results combined with liver histopathology demonstrated that foxtail millet bran oil has potent hepatoprotective effects, and could be utilized as a functional food for the therapy and prevention of liver damage.

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Notes

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Table captions

Table 1 Physical and chemical properties of crude FMBO

Table 2 Fatty acid composition of FMBO

Table 3 Fatty acid composition of *Sn*-2 position of the triacylglycerols of FMBO

Table 4 Chemical type and characteristic fragment ions of phytosterols (or phytostanols) of FMBO (GC/ (EI) MS; 70 eV)

Table 5 Effects of FMBO on body weight, liver weight and hepatosomatic index (HI) of treated mice

Table 1 Physical and chemical properties of crude FMBO

Characteristic	Crude FMBO
Specific gravity	0.9199±0.0006
Refractive index (20□)	1.4706±0.0001
Color (Lovibond, 1 in.)	Y35, R2.0
Acid value (mg KOH/g)	9.2 ±0.35
Peroxide value (mmol/kg)	2.33±0.03
Iodine value (gI_2/100g)	103.16±1.90
Saponification value (mg KOH/g)	176.89±0.33
Unsaponifiable matter (%)	3.58±0.23
Phospholipid (mg/g)	0.188

Table 2 Fatty acid composition of FMBO

Fatty acids	Content (%)[a]
Palmitic acid (C16:0)	10.23
Stearic acid (C18:0)	3.73
Oleic acid (C18:1)	19.65
Linoleic acid (C18:2)	58.85
Linolenic acid (C18:3)	3.96
Arachidic acid (C20:0)	1.52
Behenic acid (C20:1)	1.30
Docosenoic acid (C22:0)	0.76
Saturated fatty acid(SAFA)	16.24
Unsaturated fatty acid(UFA)	83.76
U/S	5.15

[a] Mean of triplicated determinations

Table 3 Fatty acids composition of *Sn*-2 position of the triacylglycerols of FMBO

Fatty acids	Content (%) [a]
Palmitic acid (C16:0)	46.2
Stearic acid (C18:0)	33.93
Oleic acid (C18:1)	7.96
Linoleic acid (C18:2)	11.91
Saturated fatty acid(SAFA)	80.13
Unsaturated fatty acid(UFA)	19.87

[a] Mean of triplicated determinations

Table 4 Chemical type and characteristic fragment ions of phytosterols (or phytostanols) of FMBO (GC/ (EI) MS; 70 eV)

Chemical type	Content (%)[a]	Characteristic fragment ions *m/z* (relative abundance)
Campesterol	15.91	145(47), 213(47), 255(31), 315(50), 382(45), 400(80)
Ergostanol	2.97	215(100), 233(80), 276(17), 387(39), 402(49)
Stigmasterol	5.58	213(23), 255(46), 300(21), 351(20), 394(5), 412(49)
β-Sitosterol	56.26	213(53), 255(39), 303(38), 329(56), 396(48), 414(84)
Stigmastanol	15.12	215(100), 233(91), 257(6), 401(41), 416(55)
Fucosterol	4.16	229(45), 271(10), 281(41), 299(23), 314(100), 412(6)

[a] Mean of triplicated determinations

Table 5 Effects of FMBO on body weight, liver weight and hepatosomatic index (HI) of treated mice

Treatments [a]	Body weight (g)	Liver weight (g)	HI(%)
normal	26.92±1.78	1.21±0.22	4.56±0.45
Model	27.49±1.62	1.38±0.21#	5.02±0.78#
FMBO1(2.50mL/kg·bw)	27.94±1.45	1.29±0.35	4.93±0.36
FMBO2(3.75mL/kg·bw)	26.49±1.87	1.16±0.28*	4.69±0.63*
FMBO3(5.0mL/kg·bw)	26.76±2.02	1.12±0.31*	4.82±0.45*
TSO(3.75mL/kg·bw)	27.48±1.98	1.08±0.19*	4.01±0.96*

[a] Values are expressed as means±SD of 10 mice in each group

# $p < 0.05$, vs the normal group

*$p < 0.05$, vs model group

Figure captions

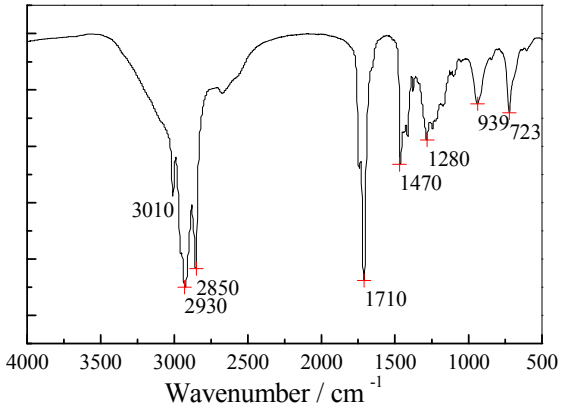
Fig. 1 FT-IR spectrum of FMBO in the frequency range 4000-500 cm^{-1}

Fig. 2 *In vitro* antioxidant effects of FMBO and positive tea-seed oil (TSO). (A) DPPH·-scavenging activities of FMBO and TSO. (B) Ferric-reducing antioxidant power of various concentrations of FMBO and TSO. (C) HO·-scavenging effects of FMBO and TSO

Fig. 3 The effects of FMBO on serum ALT (A), AST (B), TG (C) levels in acute ethanol-induced mice. Mice were received intragastric administration of 2.50mL/kg·bw dose of FMBO1, 3.75mL/kg·bw dose of FMBO2, 5.0mL/kg·bw dose of FMBO3, and 3.75mL/kg·bw dose of TSO once daily for four consecutive weeks and subsequently subjected to intragastric single injection of ethanol (50%, v/v, 12mL/kg·bw), and serum biomarkers were measured immediately. Values are presented as means ± SD for 10 mice in each group. ##$p <$ 0.01, vs the normal group.* $p < 0.05$, and **$p < 0.01$, vs ethanol-induced group

Fig. 4 The effects of FMBO on the levels of hepatic T-SOD (A), MDA (B) after administration of ethanol in mice. Mice were received intragastric administration of 2.50mL/kg·bw dose of FMBO1, 3.75mL/kg·bw dose of FMBO2, 5.0mL/kg·bw dose of FMBO3, and 3.75mL/kg·bw dose of TSO once daily for four consecutive weeks and subsequently subjected to intragastric single injection of ethanol (50%, v/v, 12mL/kg·bw). Values are presented as means ± SD for 10 mice in each group. ##$p < 0.01$, vs the normal group.* $p < 0.05$, and **$p < 0.01$, vs ethanol-induced model group

Fig. 5 Effects of FMBO on histopathological changes of liver hepatocytes stained with H&E in acute ethanol-induced mice. Images were obtained from each test group (magnification, 40×10). (A) The normal liver, showing no hepatic damage. (B) the model group, acute ethanol-induced liver, showing seriously and broadly hepatocellular necrosis. (C) Low-dose of FMBO1 (2.50mL/kg·bw) + ethanol. (D) Medium-dose of FMBO2 (3.75mL/kg·bw) + ethanol. (E) High-dose of FMBO3 (5.0mL/kg·bw) + ethanol. (F) TSO (3.75mL/kg·bw) +ethanol



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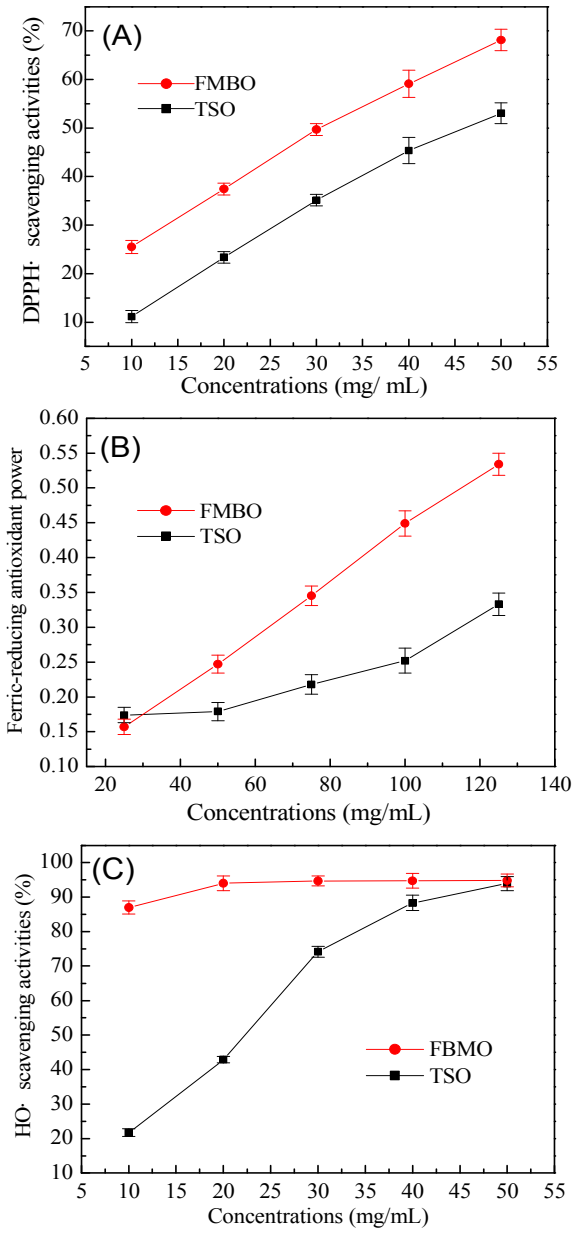

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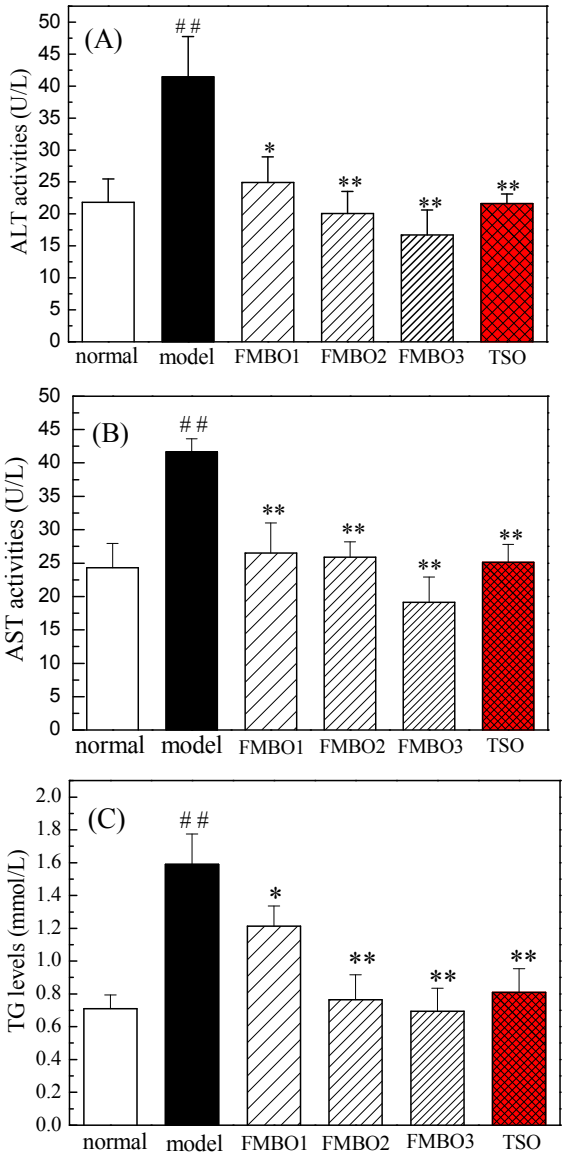

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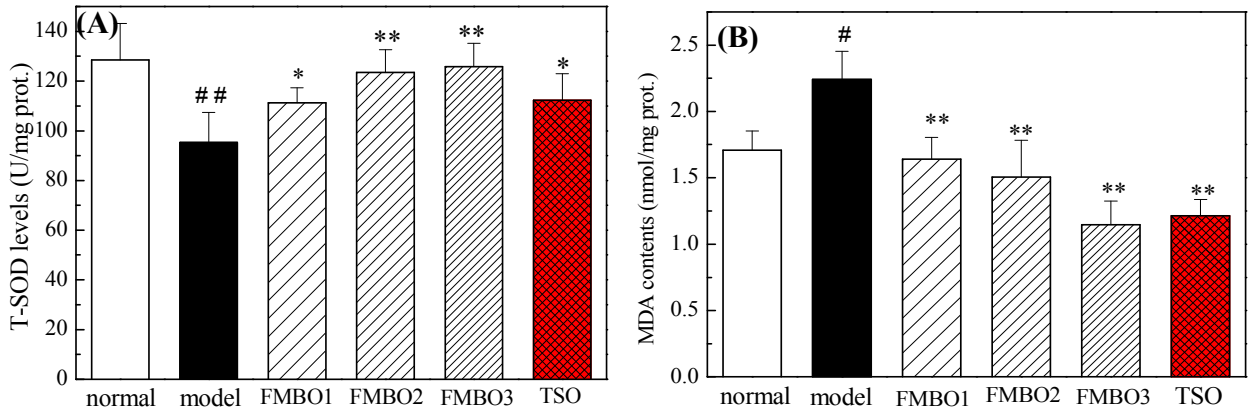

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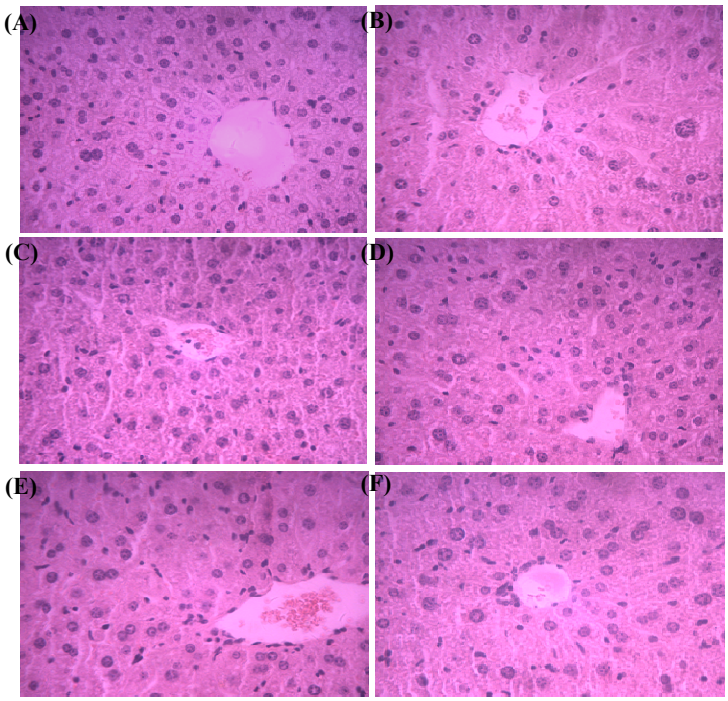

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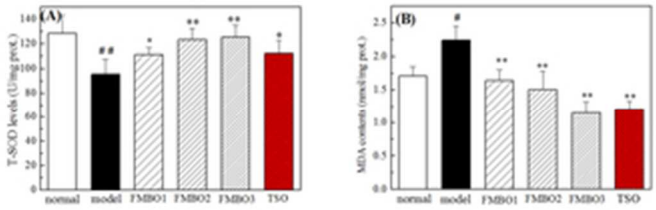

Fig. 4The effects of FMBO on the levels of hepatic T-SOD (A), MDA (B) after administration of ethanol in mice. Mice were received intragastric administration of 2.50mL/kg•bw dose of FMBO1, 3.75mL/kg•bwdose of FMBO2, 5.0mL/kg•bw dose of FMBO3, and 3.75mL/kg•bw dose of TSO once daily for four consecutive weeks and subsequently subjected to intragastric single injection of ethanol (50%, v/v, 12mL/kg•bw). Values are presented as means ± SD for 10 mice in each group. ##p< 0.01, vs the normal group.* p < 0.05, and **p< 0.01, vs ethanol-induced model group
27x10mm (300 x 300 DPI)

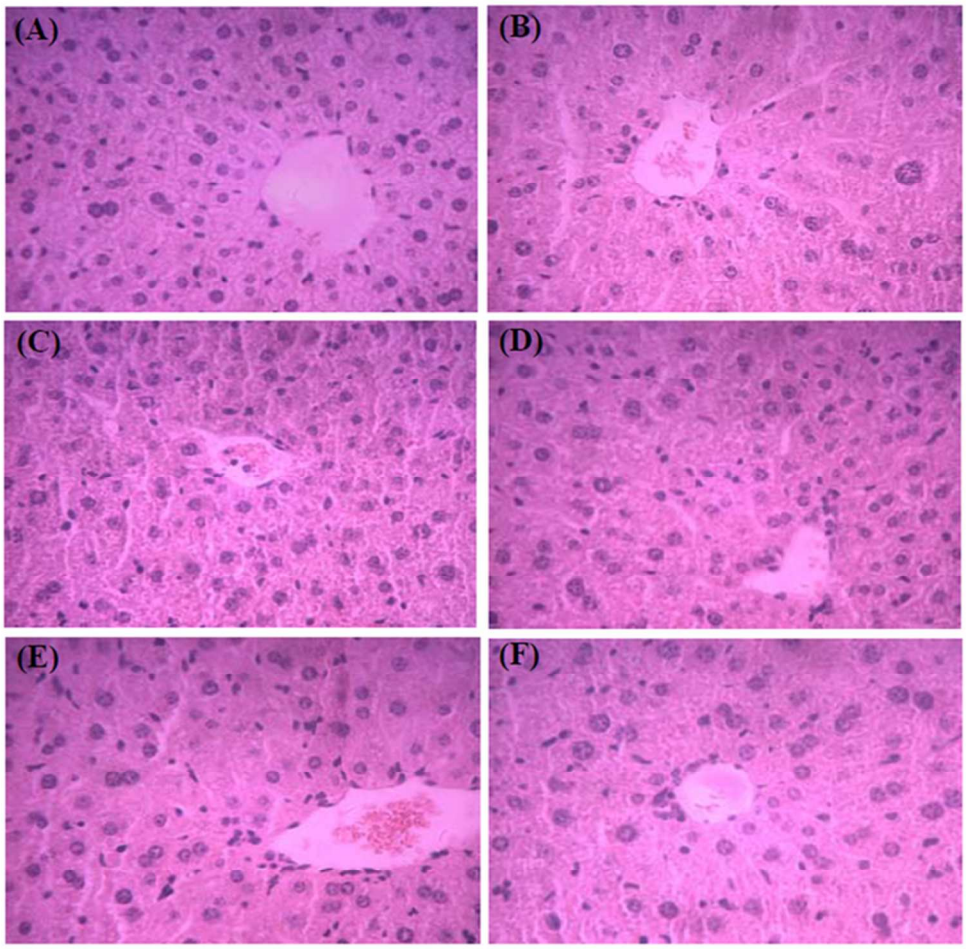

Fig. 5Effects of FMBO on histopathological changes of liver hepatocytes stained with H&E in acute ethanol-induced mice. Images were obtained from each test group (magnification, 40×10). (A) The normal liver, showing no hepatic damage. (B) the model group, acute ethanol-induced liver, showing seriously and broadly hepatocellular necrosis. (C) Low-dose of FMBO1 (2.50mL/kg•bw) + ethanol. (D) Medium-dose of FMBO2 (3.75mL/kg•bw) + ethanol. (E) High-dose of FMBO3 (5.0mL/kg•bw) + ethanol. (F) TSO (3.75mL/kg•bw) +ethanol
61x60mm (300 x 300 DPI)

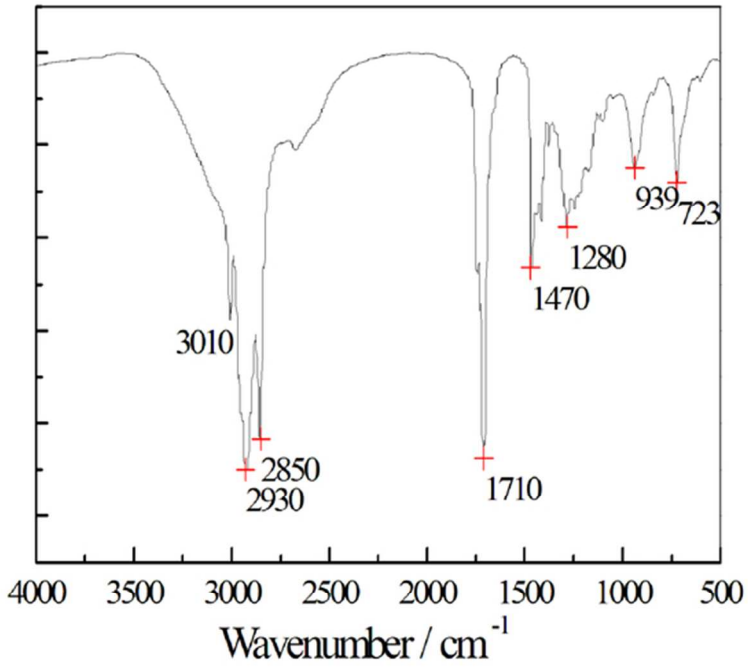

Fig. 1 FT-IR spectrum of FMBO in the frequency range 4000-500 cm-1
61x47mm (300 x 300 DPI)

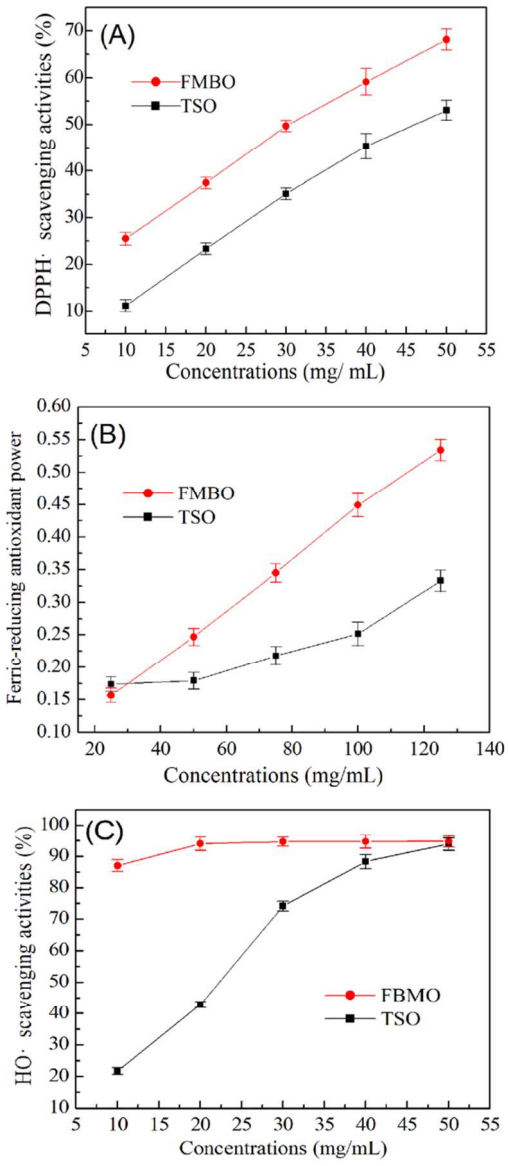

Fig.2In vitro antioxidant effects of FMBO and positive tea-seed oil (TSO). (A) DPPH•-scavenging activities of FMBO and TSO. (B) Ferric-reducing antioxidant power of various concentrations of FMBO and TSO. (C) HO•-scavenging effects of FMBO and TSO
68x147mm (300 x 300 DPI)

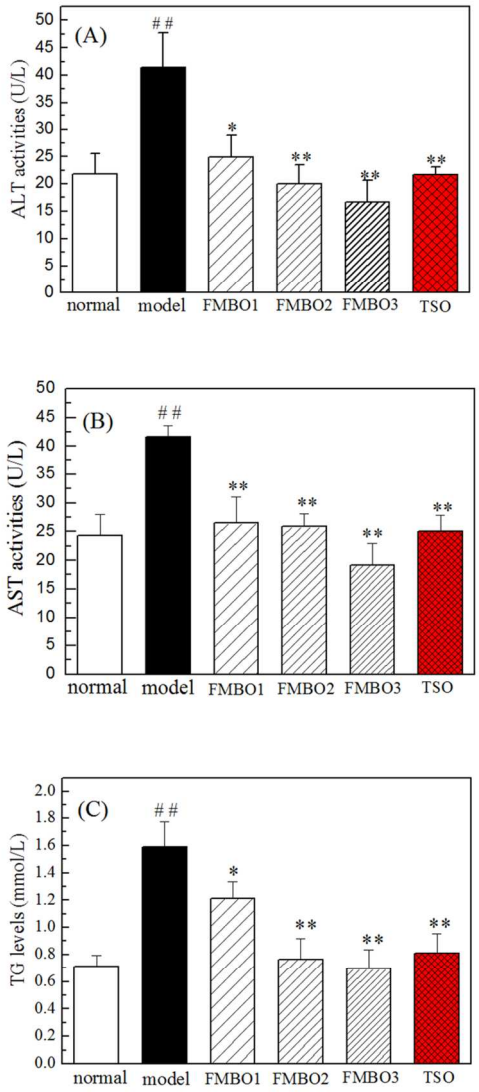

Fig.3The effects of FMBO on serum ALT (A), AST (B), TG (C) levels in acute ethanol-induced mice. Mice were received intragastric administration of 2.50mL/kg•bw dose of FMBO1, 3.75mL/kg•bwdose of FMBO2, 5.0mL/kg•bw dose of FMBO3, and 3.75mL/kg•bw dose of TSO once daily for four consecutive weeks and subsequently subjected to intragastric single injection of ethanol (50%, v/v, 12mL/kg•bw), and serum biomarkers were measured immediately. Values are presented as means ± SD for 10 mice in each group. ##p <0.01, vs the normal group.* p <0.05, and **p < 0.01, vs ethanol-induced group
81x170mm (300 x 300 DPI)

Millets are good sources for nutraceuticals. Oil extracted from foxtail millet bran proved to be a nutrient by the antioxidant activities *in vitro* and ameliorating effects against ethanol-induced hepatic injury in mice