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Protective effects of *Sapindus mukorossi* Gaertn against fatty liver disease induced by high fat diet in rats

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ABSTRACT

Objectives: Study the effects of alcohol extract of *Sapindus mukorossi* Gaertn (AESM) on the metabolism of blood fat, morphology of fenestrated liver sinusoidal endothelial cells (LSEC), and the ultrastructure of liver cells of the rats with non-alcoholic fatty liver disease (NAFLD).
Methods: Divide SD rats into control group, model group, simvastatin (7.2 mg/kg) group, and *S. mukorossi* Gaertn group with high dosage (0.5 g/kg), moderate dosage (0.1 g/kg), and low dosage (0.05 g/kg). After feeding with fat-rich nutrients for 3 weeks and establishing the model of hepatic adipose, conduct intragastric administration and provide the rats with fat-rich nutrients at the same time. At the 43rd day, take blood sample and measure aminotransferase and different indexes of blood fat; take hepatic tissue for pathological section, and observe the hepatic morphological patterns under light microscope; obtain and fix the hepatic tissue after injecting perfusate into the body, and observe the changes of fenestrated LSEC under scanning electron microscope; observe the ultrastructure of liver cells under transmission electron microscope.
Results: High-dosage alcohol extracts of *S. mukorossi* Gaertn can alleviate the AST, ALT, TC, TG, LDL, γ-GT, and ALP level, as well as raise the HDL and APN level in the serum of NAFLD-rat model. In addition, through the observation from light microscope and electron microscopes, the morphology of the hepatic tissue and liver cells as well as the recovery of the fenestrated LSEC in the treatment group has become normal.
Conclusions: Alcohol extracts of *S. mukorossi* Gaertn can regulate the level of blood fat and improve the pathological changes of the hepatic tissues in NAFLD-rat model, which demonstrates the effects of down-regulating fat level and protecting liver.

1. Introduction

With the improvement of life and changes in diet structure, the incidence of non-alcohol fatty liver disease (NAFLD) in Asian Pacific area is gradually increasing, which has reached 15% in Shanghai, China [1]; in Western countries, the incidence among ordinary people is 20–30%, and the population bearing obesity or diabetes has reached 70–90% [2]. NAFLD, a problem threatening the health of human beings, has become more and more aware of, and some scholars have proposed to change the name of NAFLD to "metabolic disorders of the liver" [3,4], to adapt to the understanding of the disease as well as the updates of the clinical decisions.

The in-depth studies of the mechanism of NAFLD have promoted changes in the principle of treatment, which has shifted from simply focusing on the hepatic fat content towards the preventing and treating the risk of the metabolism in the body caused by the excess of fat [5,6]. Pravastatin can improve the extent of the pathological changes in hepatic histology of the patients with NAFLD [7]; Atorvastatin can improve the ultrasonic appearance of hepatic adipose degeneration as well as down-regulate the level of liver enzymes, but the function of improving the extent of pathological changes in hepatic histology remains unknown [8], fibrates are beneficial for improving the hepatic adipose degeneration. However, the security of the above medications has always been concerned in clinical field.

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Wu Huan Zi (*Sapindus mukorossi* Gaertn), as a traditional Chinese medicine, has been employed for clinical uses for a long time. The studies from Chinese scholars have found the skin of *S. mukorossi* Gaertn is rich in saponin [9], and can regulate metabolism of blood fat as well as protect endothelial cells of blood vessels to some degree [10,11], but there is no in-depth report describing the research about *S. mukorossi* Gaertn in preventing and treating fatty liver yet. To further clarify the effects of *S. mukorossi* Gaertn in down-regulating blood fat and preventing and treating fatty liver, as well as to make a better use of such a rich source of Chinese herbal medicine, the group has conducted research in the effects of the alcohol extracts of *S. mukorossi* Gaertn on the blood fat and hepatic tissues of the rats with NAFLD, so as to provide theoretical evidence for the use of *S. mukorossi* Gaertn in preventing and treating fatty liver.

2. Materials and methodology

2.1. Animal model and fat-rich nutrient

48 SD rat, SPF-level, half males and half females, weight ranging from 180 to 220 g (Quality Certification: 0086083); fat-rich nutrient (12% lard oil, 2% cholesterol, 0.2% propylthiouracil, 0.5% bile salt No. 3, 85.3% ordinary feed powder, quality certification: 0087730). All materials were provided by Guangdong Medical Lab Animal Center, license: SCXK (Guangdong) 2008-0002.

2.2. Reagents and equipments

10% Chloral hydrate, 10% formalin, perfusate (50% PBS, 20% of 4% paraformaldehyde, 25% of 10% glutaraldehyde, 20% distilled water). All reagents were purchased from Southern Medical University Reagent Center. Simvastatin (Merck Sharp & Dohme Limited U.K., Lot No. 100613), *S. mukorossi* Gaertn (purchased from Yulin, Guangxi, China, certificate of quality: K20111205); CircuLexTM Rat Adiponectin ELISA Kit (CycLex Co. Ltd, Cat#CY-8049); PINGFAN TDZ5-WS Centrifuge, BECKMAN COULTER CX9 Automatic Biochemical Analyzer, PerkinElmer1420 Multilabel Counter VICTOR3V Multi-functional Microplate Reader, LEICA RM2135 Microtome, NIKON ECLIPSE Ti Microscope, HITACHI S-3000N Scanning Electron Microscope, HITACHI H-7650 Transmission Electron Microscope, Shimadzu 20A High Performance Liquid Chromatography (Shimadzu, Japan).

2.3. Methodology

2.3.1. Group division

The 48 SD rats were divided into 6 groups randomly, with 8 rats in each group, and the males and females were separated by cages. Models were established after regular feeding for 1 week. Weights were measured weekly.

2.3.2. Preparation of ethanol extracts from S. mukorossi Gaertn

The dry skins of *S. mukorossi* Gaertn were firstly immersed in 4:1 (w/w) 85% alcohol for 30 min, followed by 2-h reflux extraction, and the extracted solution was collected; secondly, a 1.5-h reflux extraction was conducted in 3:1 (w/w) 85% alcohol. After filtration, both extracted solutions were combined and concentrated to 1 g/ml, and was stored at 4 °C for further use.

2.3.3. Establishment of animal model and treatment

The control group was given ordinary feed, and the rest five groups were continuously fed with fat-rich nutrient for 6 weeks. From the fourth week, intragastric administration was conducted for the rats from treatment groups, using simvastatin (7.2 mg/kg), *S. mukorossi* Gaertn with high dosage (0.5 g/kg), moderate dosage (0.1 g/kg), and low dosage (0.05 g/kg). Same aliquots of distilled water were given to the control and model group via intragastric administration. At the night before the ending of the experiment, the rats underwent fasting but were allowed to drink.

2.3.4. Preparation of serum sample

After the rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g), the blood was collected from abdominal aorta, and the serum was separated (3000r, 10 min) and delivered for measurement of the biochemical parameters (TC, TG, HDL-C, LDL-C, AST, ALT, γ-GT, and ALP) in Clinical Laboratory, Hospital of Traditional Chinese and Western Medicine, Southern Medical University, and the APN index was measured using Adiponectin (Rat) ELISA Assay Kit.

2.3.5. Preparation of pathological hepatic specimen for light microscope

After the livers were taken from the rats, the wet weight was measured, the liver index was calculated, and a piece of the hepatic tissue was cut off horizontally with 1 cm away from the edge of the right lobe (about 3 mm × 3 mm × 10 mm). The tissue was fixed in 10% neutral formalin, dehydrated conventionally, and stained using HE. The criteria for evaluating the severity of fatty liver were diagnosed according to international CMS diagnostic criteria.

2.3.6. Preparation of hepatic specimen for scanning electron microscope

One rat was selected randomly from each group. Intubation was conducted from hepatic portal vein, and after washing by using 0.9% physiological saline, the perfusate was dripped until blood flowed out from the incision of inferior vein and the redness disappeared from the tissue. A tissue sample (same size and position as is described in 2.3.5) was fixed through immersion in electron microscopy-purpose glutaraldehyde and stored at 4 °C. The sample was delivered to Electron Microscopic Laboratory, Southern Medical University for gradient dehydration, drying at the critical point of CO_2, and coating with Platinum.

Table 1
Comparsion of liver index in different treated NAFLD rats. Data are expressed as mean ± SD, $N = 6$.

Group	Dosage (g/kg)	Weight of rat (g)	Weight of live (g)	Liver index (%)
Control	–	293.97 ± 65.53	6.21 ± 0.47	2.11 ± 0.10
NAFLD model	–	203.37 ± 20.19##	7.70 ± 1.10#	3.83 ± 0.76##
Simvastatin	0.0072	195.08 ± 22.11	5.91 ± 0.89*	3.02 ± 0.17*
High dose AESM	0.5	194.82 ± 23.92	6.09 ± 1.39*	3.10 ± 0.42
Middle dose AESM	0.1	188.82 ± 16.63	6.04 ± 0.98*	3.19 ± 0.33
Low dose AESM	0.05	213.30 ± 19.75	5.76 ± 0.67**	2.70 ± 0.15**

# $P < 0.05$ 01 vs control group.
$P < 0.01$ vs control group.
* $P < 0.05$ vs NAFLD model group.
** $P < 0.01$ vs NAFLD model group.

Table 2
Effects of EASM on blood-lipid indexes in NAFLD rats. Data are expressed as mean ± SD, $N = 6$.

Group	Dosage (g/kg)	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control	–	0.86 ± 0.04	0.42 ± 0.26	0.76 ± 0.21	0.38 ± 0.07
NAFLD model	–	2.96 ± 0.68##	1.19 ± 0.37##	0.56 ± 0.07#	1.81 ± 0.23##
Simvastatin	0.0072	1.90 ± 0.45**	0.21 ± 0.02**	0.90 ± 0.08**	0.88 ± 0.24**
High dose AESM	0.5	1.97 ± 0.73*	0.43 ± 0.15**	0.91 ± 0.05**	1.38 ± 0.44*
Middle dose AESM	0.1	2.04 ± 0.84	0.66 ± 0.19*	0.79 ± 0.07**	1.31 ± 0.42*
Low dose AESM	0.05	2.30 ± 0.18	0.74 ± 0.44	0.70 ± 0.13*	1.25 ± 0.43*

# $P < 0.05$ 01 vs control group.
$P < 0.01$ vs control group.
* $P < 0.05$ vs NAFLD model group.
** $P < 0.01$ vs NAFLD model group.

Table 3
Effects of EASM on serum biochemical parameters of liver function in NAFLD rats. Data are expressed as mean ± SD, $N = 6$.

Group	Dosage (g/kg)	AST (U/L)	ALT (U/L)	γ-GT (U/L)	ALP (U/L)	APN (ng/mg)
Control	–	61.33 ± 26.98	11.67 ± 3.93	0.82 ± 0.50	53.93 ± 20.53	14.82 ± 4.81
NAFLD model	–	167.76 ± 74.91##	51.17 ± 30.50##	3.22 ± 1.11##	184.85 ± 57.29##	3.65 ± 1.05##
Simvastatin	0.0072	93.83 ± 13.47**	19.17 ± 2.14**	2.12 ± 1.01	127.40 ± 16.93**	10.48 ± 4.60**
High dose AESM	0.5	82.83 ± 10.36*	17.33 ± 1.21*	2.15 ± 0.95	133.55 ± 44.17	12.61 ± 1.99**
Middle dose AESM	0.1	88.83 ± 9.91*	21.50 ± 5.50*	2.57 ± 1.04	210.00 ± 59.43	13.81 ± 4.98**
Low dose AESM	0.05	96.00 ± 15.49*	23.17 ± 3.66*	2.17 ± 0.91	143.68 ± 37.22	13.56 ± 4.85**

$P < 0.01$ vs control group.
* $P < 0.05$ vs NAFLD model group.
** $P < 0.01$ vs NAFLD model group.

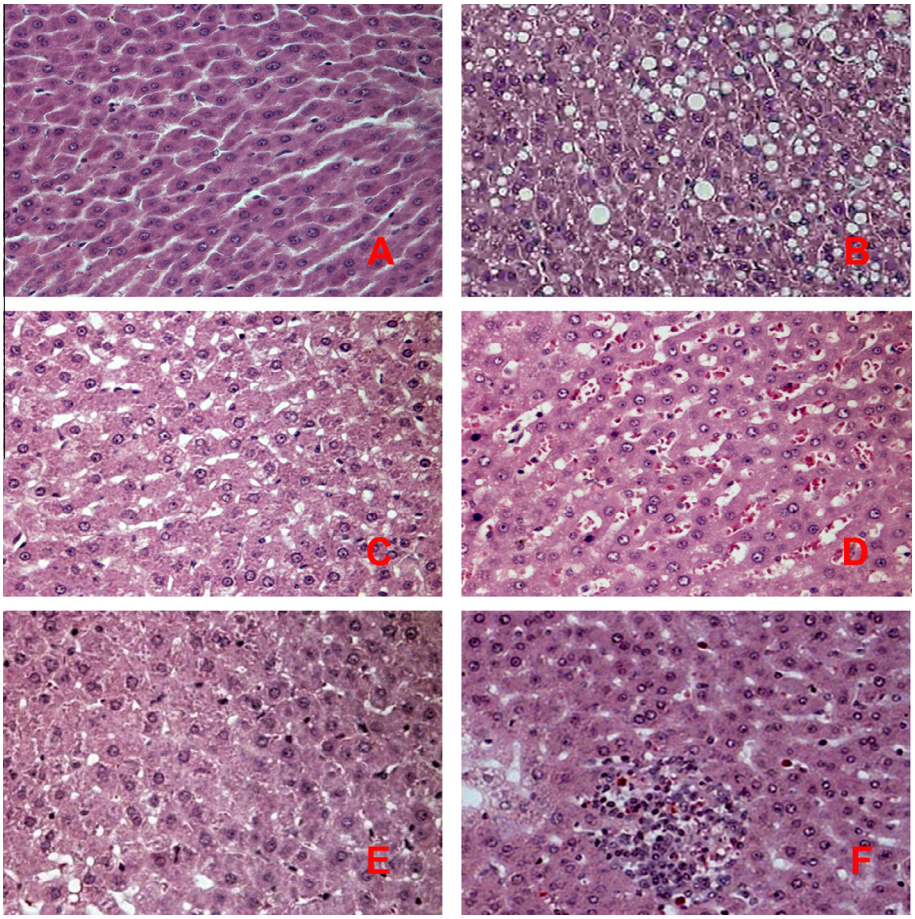

Fig. 1. The pathological change (HE staining ×400) in hepatic tissue of NAFLD rats. Normal control rats (A), NAFLD model rats (B), simvastatin treated rats (C), low-dose of AESM rats (D), middle-dose of AESM treated rats(E) and high-dose of AESM rats (F).

2.3.7. Preparation of hepatic specimen for transmission electron microscope

One rat was selected randomly from each group. The liver tissue was isolated (about 1 mm^3), immersed in electron microscopy-purpose glutaraldehyde, and stored at 4 °C. The sample was delivered to Electron Microscopic Laboratory, General Hospital of PLA Guangzhou Military Area for gradient dehydration, covering by Resin 812, and slicing into ultrathin layers.

2.3.8. Statistical analysis

All the results were analyzed using SPSS 13.0 software. The measurement data were processed through One-Way Analysis of Variance (ANOVA) and expressed in terms of $\overline{X} \pm S$, and the data were regarded as statistically significant when $P < 0.05$. The ranked data were processed through non-parametric K Independent Samples, and the data were regarded as statistically significant when $P < 0.05$.

3. Results

3.1. Effects of AESM on liver index in NAFLD rats

NAFLD model was successfully established indicated by liver index firstly. Compared with control group, the liver index in NAFLD model rats were significantly increased to 3.83 ± 0.76% ($P < 0.01$). Hepatic index of low-dose AESM-treated group decreased dramatically compared with that of model group ($P < 0.01$) (Table 1). These results demonstrate that AESM is an potential agent for the prevention of NAFLD.

Table 4
Effects of AESM on liver histopathological grading in NAFLD rats.

Group	Severity score of fatty liver					*P* value	Severity score of liver cell necrosis				*P* value
	F_0	F_1	F_2	F_3	F_4		G_0	G_1	G_2	G_3	
Control	5	1	0	0	0	0.025	6	0	0	0	0.047
NAFLD model	0	1	2	2	1		1	1	1	3	
Simvastatin	2	2	2	0	0		3	1	2	0	
High dose AESM	2	1	2	1	0		4	1	1	0	
Middle dose AESM	3	1	1	1	0		3	1	2	0	
Low dose AESM	1	1	2	1	1		2	2	2	0	

Fatty liver, $\chi^2 = 12.82$; liver cell necrosis $\chi^2 = 11.24$.

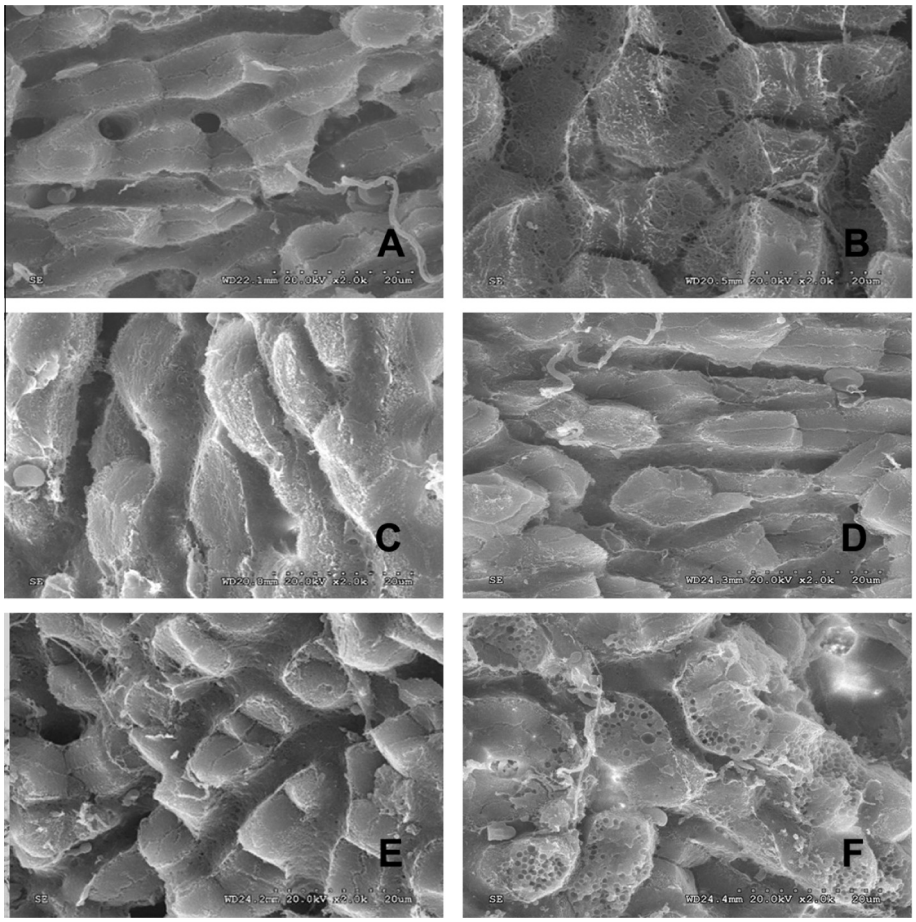

Fig. 2. The ultrastructure of transmission electron microscope change (EM ×2000) of hepatic tissue in NAFLD rats. Normal control rats (A), NAFLD model rats (B), simvastatin treated rats (C), low-dose of AESM rats (D), middle-dose of AESM treated rats(E) and high-dose of AESM rats (F).

3.2. Effects of AESM on serum lipid and lipoprotein in rats

NAFLD induced by high fat and cholesterol diet provoked a significant increment of TC and TG activities ($P < 0.01$) compared with control group (Table 2), which indicates the successful establishment of the NAFLD model in rats. Results showed that high dose of AESM could both significantly decrease TC and TG content when compared with those in NAFLD-model group ($P < 0.01$). Similarly, serum LDL-C was significantly increased in model group compared with control group ($P < 0.01$) and significantly decreased in AESM-treatment group compared with that in model group ($P < 0.05$). In contrast, HDL-C serum level was markedly decreased at the end of experiment, and AESM treatment could significantly improved HDL-C level compared with that in NAFLD-model group ($P < 0.05$).

3.3. Effects of AESM on serum biochemical parameters of liver function in NAFLD rats

Serum level of AST and ALT were markedly increased at the end of experiment. Treatment with AESM reduced AST and ALT levels ($P < 0.05$), but there were no statistical significance in the levels of γ-GT and ALP. Moreover, AESM dramatically increased APN level compared with that in NAFLD-model group ($P < 0.01$) (Table 3).

3.4. Pathological changes in hepatic tissue

The histological change of the hepatic tissue was examined by light microscope. The photomicrographs of the HE stain showed that the structure of rat's liver lobule and liver sinusoid were obvious, and no obvious fat vacuoles were found in the hepatic cells of control group. Hepatic fat in model group rats deposit was increased, fat-storing cells (FSCs) actively proliferated and the deposit of IV-collagen was gradually increased in rat liver in comparison to the control group (Fig. 1A and B). The images of HE staining also displayed macrovesicular steatosis for many single large droplets had displaced the nucleus and ballooning degeneration caused conspicuous swelling of the cell and cytoplasmic vacuolation (Fig. 1B). In the treatment groups, especially in high-dose of AESM group, lipid degeneration and inflammatory response were significantly alleviated compared with the model group. Liver cell volume became smaller, hepatic lobules were clearly delineated, and the fat droplets were reduced (Fig. 1F). Furthermore, ASEM or simvastatin administration significantly ameliorated the HFD induced NAFLD animal when compared with that in model group, the average scores for severity score of fatty liver and liver cell necrosis in AESM-treated rats were markedly reduced in a dose-dependent manner (Table 4).

In addition, the ultrastructure of liver tissues was obtained by scanning electron microscope. Normally shaped orifice (Φ0.05–0.2 μm) arranged in clumps and evenly distributed on LSEC surface in the control group (Fig. 2A). The rats in model group had developed ultrastructural abnormalities of the mitochondria, including apparent disruptions in the cristae, hypodense matrix, and mitochondrial swelling/rounding. Besides, almost all orifice (Φ0.3–1.2 μm) exhibited a fused growth and each orifice was remarkably dilated as well as the body of LSEC damaged (Fig. 2B). While in simvastatin group, the shape, size and relative quantity of the orifice (Φ0.05–0.2 μm) reach the level of the comparison group

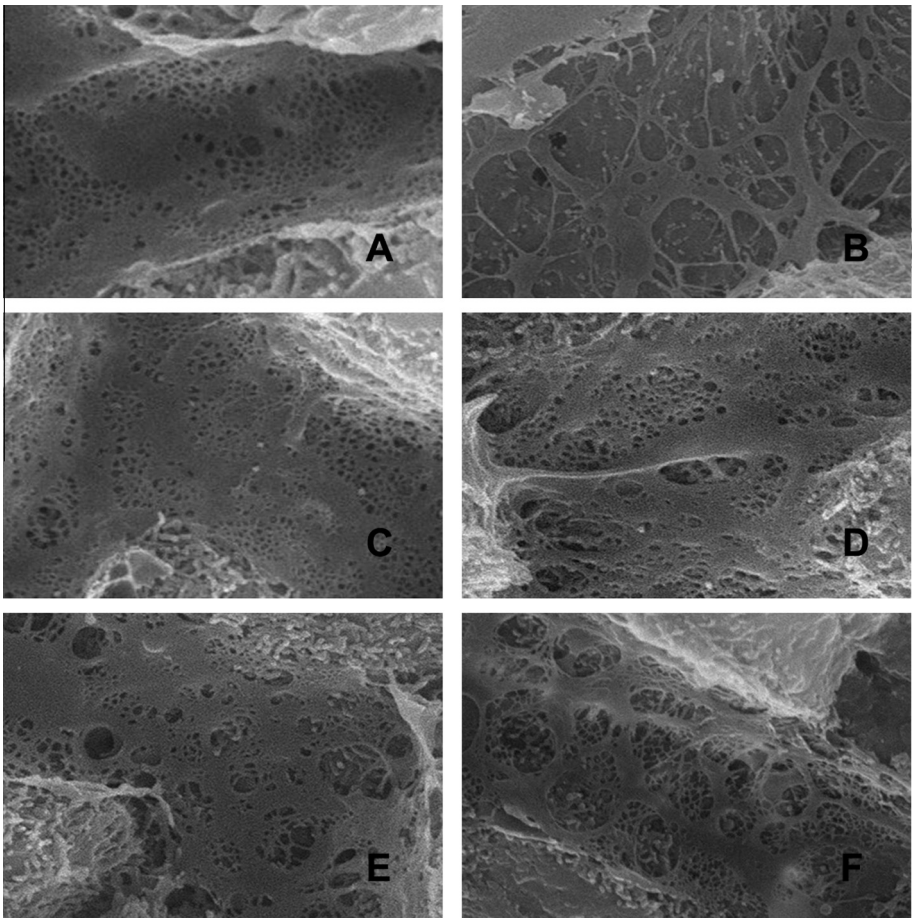

Fig. 3. The ultrastructure of scanning electron microscope change (EM ×2000) of hepatic sinusoidal endothelial cells in NAFLD rats. Normal control rats (A), NAFLD model rats (B), simvastatin treated rats (C), low-dose of AESM rats (D), middle-dose of AESM treated rats(E) and high-dose of AESM rats (F).

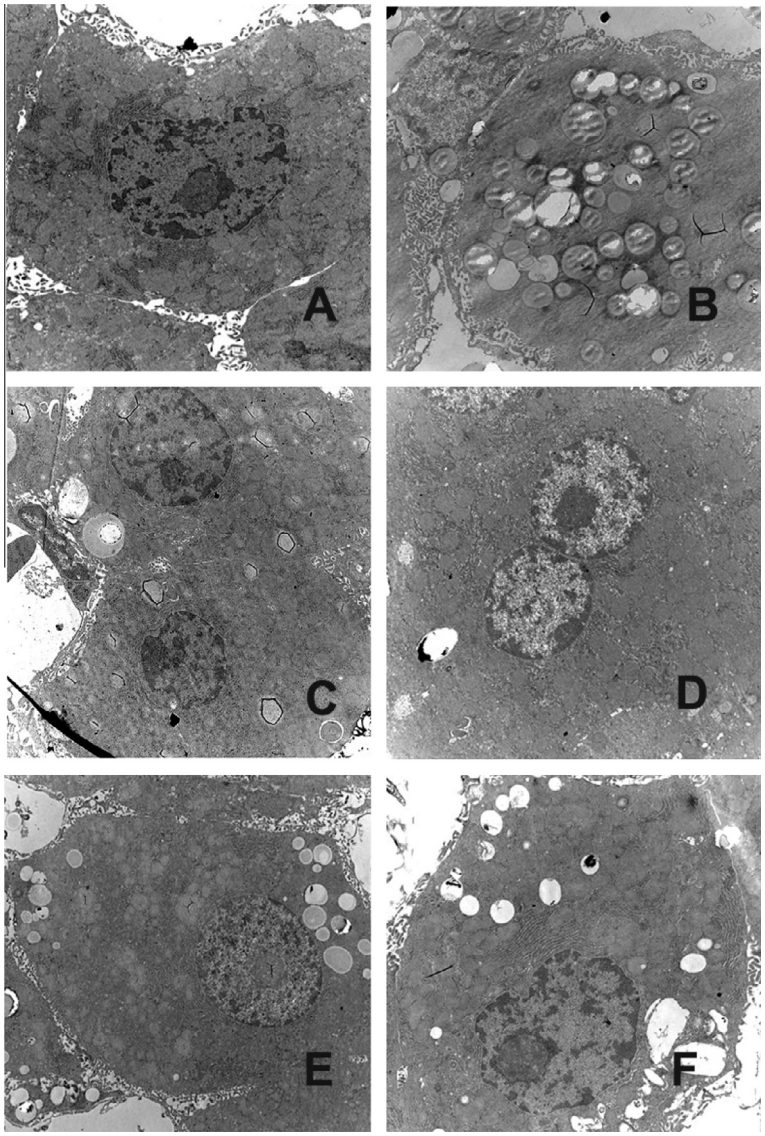

Fig. 4. The ultrastructure of transmission electron microscope change(EM × 2000) of hepatic cells in NAFLD rats. Normal control rats(A), NAFLD model rats (B), simvastatin treated rats (C), low-dose of AESM rats (D), middle-dose of AESM treated rats(E) and high-dose of AESM rats (F).

(Fig. 2C). Compared with control group, The orifice in high-dose (Φ0.1–0.5 μm), middle-dose (Φ0.1–0.8 μm) and low-dose (Φ0.2–1 μm) ASEM groups were expand slightly and the normal morphology were maintained (Fig. 2D–F).

The ultrastructure of hepatic cells were examined by transmission electron microscope, and the results showed that the structure of liver cell was normal, membrane well defined, chromatin was evenly distributed in control group. There was no fat droplet in the mitochondria, endocytoplasmic reticulum and liver cell, The cell nucleus are round or overall in shape (Fig. 3A). Obviously, liver cells of model group had many fat droplets and formation vacuoles, as well as hepatic collagen and reticulin proliferation, swollen mitochondria, lipid deposit, rough endoplasmic reticulum dilatation could be observed in some of the liver cells (Fig. 3B). Notably, the morphology of liver cells in treated group rats was similar to that of control group, containing a few fat droplets and chromatin was evenly distributed (Fig. 3).

4. Discussion

HFD induced NAFLD animal models require a lengthy feeding period, but these models resemble the pathophysiology observed in human NAFLD closely, including induced obesity, insulin resistance, and hepatic steatosis in mice or rats [12]. In terms of traditional medicine theory, the etiology of NAFLD is poor diet or emotional disorders, with the key points of blood stasis and phlegm (fluid overflowing), which are related to "liver", "spleen" and "kidney" [13]. The pathogenesis for the disease based virtual real standard of evidence, in the deficiency of liver and kidney, ecchymosis, sputum. The methods of liver-kidney-tonifying, promoting blood circulation to remove meridian obstruction, reducing phlegm and removing dampness are effective to treat NAFLD. Currently, despite the huge effort put in the prevention and treatment of NAFLD from researchers and clinicians, there are few options to retard or even reverse the progression of this disease. Therefore, it is important to search a novel agent to delay the progression of pathogenesis in NAFLD. The aim of this study was to explore the use of Chinese herb in the prevention of HFD induced NAFLD rat model (see Fig. 4).

It is reported that pretreatment with AESM, an herbal medicine used in TCM, traditionally used for detoxification and clearing excessive heat in the body. It is commonly prescribed for treating various infections and hypertension, is also used as an antimicrobial herb by Chinese medicine practitioners [14,15]. Previous

research has shown that *Sapindus*-saponin is the main components of *S. mukorossi* peel extract, including oleanane tetracyclic triterpenoids (monodesmoside, bisdesmoside), dammarane-type and tirucallane-type triterpenoid saponins and Sesquiterpene oligoglycoside [16]. Five kind of dammarane-type triterpenoid saponins, sapinmusaponins A–E, were got from the fruits of *S. mukorossi* at the first times by Kuo [17]. Four new oleanane-type saponins and two new dammarane-type saponins, along with seven known saponins were isolated by Huang [18]. This study adopts a method of organic solvent extraction, the chemical composition of AESM was found to consist of mostly saponins (65%), polysaccharides and vitamins (30%), flavonoid and tannin (less than 5%).

To investigate the effect AESM on blood fat reducing, we reproduced a rat model with HFD. In model group, it is demonstrated that the liver index was significantly increased, serum TG and TC level were increased; serum LDL-C level and ALT and AST activities were markedly increased; HDL-C were markedly reduced and hepatic tissue steatosis and mitochondrial damage. We found AESM is able to prevent the elevation of ALT and AST, and to decreased LDL-C level, which consistent with the efficacy of simvastatin in NAFLD. According to the previous study and our investigations, we review and summarize the function and mechanism of AESM in the treatment of NAFLD as follows.

First, The Chinese medicinal herbs exert their pharmacological effects through a multi-component and multi-target way [19,20]. AESM is a mixture of various component, the main ingredient of saponin, plays the liver-protect effects by multi-target way could well be relates to increases the expression of LDLR of liver, so as to decreasing the serum level of LDL, TC and TG. Second, HL, LCAT and LPL are key enzymes during lipoprotein metabolism, not only hydrolyze the CM and surplus TG, but also regulate lipid metabolism by promoting the metabolic products of CM and VLDL to convert into HDL. On the other hand, AESM may reduces the level of ingredient in lipid synthesis and decreases the endogenous lipid synthesis by improve the activities of the crucial rate limiting enzymes of cholesterol synthesis or reduces acetyl-CoA. Lastly, according to the Chinese materia medica, *S. mukorossi* are loaded with cold yin energy and thus are potent in eliminating phlegm by cooling. It had been found that some of clearing-heat drugs may promoting cholesterol to convert into bile acid in liver, increasing the excretion of bile acid and inhibiting bowel loops, and then reducing absorption of bile acid so as to lowered the blood fat. It is maybe the mechanism involve in the effect of AESM in NAFLD.

In conclusion, our results demonstrated AESM exhibits the liver-protect propertie through multiple ways during the development of NAFLD in a rat model, it is reveal that AESM is a promising agent for the prevention of NAFLD. However, the effective components in AESM and the underlying molecular mechanism needs to be further investigated.

Author contributions

Designed the study: WX.X., ZX.M., and HB.C. Coordinated the study and finalized the manuscript: Q.Z., QX.P., and WX.X. Read and approved the manuscript: M.S., Q.F., HW.Z., YK.Z., and X.L. Performed the experiments: Q.Z., QX.P., and WX.X. Analyzed the data: QZ. Wrote the paper: Q.Z. and QX.P.

Competing financial interests

The authors declare no competing financial interests.

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