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Antioxidant and anti-hyperlipidemic effects of mycelia zinc polysaccharides by *Pleurotus eryngii* var. *tuoliensis*

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ABSTRACT

The aims of this work were designed to investigate the hepatoprotective and antioxidant effects of acidic- and alkali-extractable mycelia zinc polysaccharides (AcMZPS, AlMZPS) from *Pleurotus eryngii* var. *tuoliensis* on high-fat-high-cholesterol emulsion-induced hyperlipidemic mice. The *in vivo* experiments demonstrated that both AcMZPS and AlMZPS had potential hepatoprotective effects by significantly decreasing the levels of LDL-C, VLDL-C, TC, TG, ALT, AST, ALP, MDA and LPO, and remarkably increasing the HDL-C, SOD, GSH-Px, and CAT in serum lipid/liver homogenate, respectively. In addition, four polysaccharide fractions of AcMZPS-1, AcMZPS-2, AlMZPS-1, and AlMZPS-2, purified from AcMZPS and AlMZPS using DEAE chromatography, respectively, were subjected to monosaccharide composition analysis and valuated for the *in vitro* antioxidant activity. The results obtained in present study suggested that AcMZPS, AlMZPS and their purified fractions could be used as functional foods and natural drugs in preventing the hyperlipidemia and non-alcoholic fatty liver.

1. Introduction

In recent years, both experimental and clinical studies have given more evidence on demonstrating that the oxidative stress or impaired antioxidant defense show adverse effects on human health [1,2]. It is generally known that the reactive oxygen species (ROS), such as superoxide anion radicals (O_2^{-} •), hydroxyl radicals (•OH), DPPH radicals *etc.* [1–3], were considered to be the cause of oxidative damage on the liver and the loss of body functions. Previous literatures have indicated that the hyperlipidaemia, the most common lipid metabolic disturbance disease, has been incriminated as a fatal factor in inducing fatty liver, hypertension, atherosclerosis and cerebrovascular disease, which were accompanied with these ultra-production of oxygen free-radicals [4]. The liver is the major site of xenobiotic metabolism, and the metabolism of these xenobiotics will induce the generation of ROS in hepatocytes and result in hepatic damage, gross cellular change and cell death causing hepatotoxicity and liver damage [5]. Notoriously, clinical studies have demonstrated that taking too much high-fat or high-cholesterol food/diet will result in metabolic dysfunctions of lipid, resulting in hyperlipidemia, liver cirrhosis and even hepatocellular carcinoma [1,4,6].

Interestingly, many evidences has indicated that natural polysaccharides from edible and medicinal mushrooms exhibit potential antioxidant and pre-oxidant properties, suggesting that the polysaccharides have promising activities in antagonizing the hepatic toxicity induced by high-fat-high-cholesterol emulsion (HFHCE) [4,6]. However, since the cell wall of mycelium are hard-degradation, the traditional extraction method with many insufficient problems of long extraction time, high extraction temperature, high energy consumption and low extraction yield is unacceptable [7]. Thus, some assistant methods, such as acidic- and

Abbreviations: AcMZPS, acidic-extractable polysaccharides; AlMZPS, alkalic-extractable polysaccharides; ALP, alkaline phosphatase; ALT, alamine aminotransferase; Ara, arabinose; AST, aspertate aminotransferase; CAT, catalase; GC, gas chromatography; GSH-Px, GSH peroxide; HDL-C, high-density lipoprotein cholesterol; HFHCM, high-fat-high-cholesterol emulsion; LDL-C, low density lipoprotein cholesterol; LPO, lipid peroxidation; MC, model control; MDA, malondialdehyde; NC, normal control; O_2^{-}•, superoxide anion; PC, positive control; prot, protein; ROS, reactive oxygen species; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; TC, total cholesterol; TG, triglyceride; VLDL-C, very low-density lipoprotein cholesterol.

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alkalic-assisted extraction, are considered to be emerging technologies in the polysaccharides extraction with many advantages such as high extraction yield, high reproducibility, lower investment costs, lower energy requirements, and simplified manipulations [7,8]. Nevertheless, as far as we are concerned, scare literature about acidic- and alkalic-assisted method applying in extraction has been published. For this reason, there is a desirable need for exploring effective methods in extracting polysaccharides. Up to now, not only microorganism but also plant have deeply studies on both acidic- and alkalic-extractable polysaccharides. Among the most important metabolites obtained from mushrooms, polysaccharides have gained much attention owing to their demonstrated bioactivities, and have also been confirmed in various experiments as having antioxidative and renoprotective [9], anti-diabetic [10], and immunoregulatory effects [11].

The polysaccharides extracted from *Pleurotus eryngii* var. *tuoliensis*, an edible and medicinal mushroom classified in the family *Pleurotaceae* of the phylum *Basidiomycota*, have shown various biological activities including antioxidant and hepatoprotective activities [12,13]. However, scare publication information about the antioxidant effects of polysaccharides extract from the mycelium of *P. eryngii* var. *tuoliensis* is available. Furthermore, zinc is an essential trace element in the human body and it participates in various pathways of metabolism [14]. It has been proved by our previous report that, after submerged fermentation with zinc-compound (zinc acetate); zinc-enriched polysaccharides exhibit significantly superior antioxidant activities than the regular polysaccharides [15]. Hence, it is quite necessary and significative to explore the natural acidic- (AcMZPS) and alkalic-extractable (AlMZPS) polysaccharides from the zinc-enriched mycelia of *P. eryngii* var. *tuoliensis* in preventing the hyperlipidemia induced by HFHCM.

In this work, the AcMZPS and AlMZPS from *P. eryngii* var. *tuoliensis* were isolated and purified by DEAE-52 chromatography. Biological antioxidant activities, hepatoprotective properties, and the monosaccharide compositions were also investigated.

2. Materials and method

2.1. Fermentation and chemicals

The strain of *P. eryngii* var. *tuoliensis* used in this experiment was provided by Fungi Institute of Academy of Agricultural Sciences (Taian, China). The diagnostic kit for the total antioxidant activity (T-AOC) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Standard monosaccharide samples were purchased from Sigma Chemicals Company (St. Louis, USA). DEAE-52 cellulose was purchased from Pharmacia Co. (New Jersey, USA). All other chemicals used in present work were analytically grade and purchased from local chemical suppliers.

2.2. Preparation of AcMZPS and AlMZPS

The liquid fermentation technology was applied to produce *P. eryngii* var. *tuoliensis* mycelia, with zinc acetate (3 g/L) for supplying the zinc. The obtained dried mycelia powder was extracted with proper volumes of hydrochloric acid (1 M), sodium hydroxide (1 M) for 6 h at 40 °C (1:5, w/v). After centrifugation (3000 × *g*, 10 min), the supernatant homogenate was precipitated with three volumes of ethanol (95%, v/v) overnight (4 °C). The precipitates were collected by centrifugation (3000 × *g*, 10 min), and deproteinated by the method of Sevag [16], respectively. Finally, the purified precipitates, considered as AcMZPS and AlMZPS, were collected by lyophilization for further experiments.

2.3. Anti-hyperlipidemic activities in vivo

2.3.1. Design of animal experiment

The Kunming strain mice (male, 20 ± 2 g) were purchased from Taibang Biological Products Ltd. Co. (Taian, China). The mice were housed in cages under controlled conditions of 12 h light/dark cycles at 22 ± 2 °C and 60–65% humidity with free access to water and standard food. The experimental protocol was approved by the animal ethics guidelines of Institutional Animal Ethics Committee, and in accordance with the guidance of Shandong Agricultural University Committee.

After a 7-day acclimatization period, all animals were randomly distributed into six dosage groups (ten in each group) including six dosage groups of AcMZPS groups (500, 300, 100 mg/kg bw) and AlMZPS groups (500, 300, 100 mg/kg bw), as well as three control groups of normal control group (NC), model control group (MC), and positive control group (PC). The hyperlipidemia was induced by gavage of HFHCE, which was composed of oil phase of cholesterol (10 g), liquid lard oil (25 g), methylthiouracil (1 g) and of Tween-80 (25 mL), and water phase of distilled water (30 mL), propylene glycol (20 mL) and sodium deoxycholate (2 g) [6]. During the experiment procedure, dosage groups of HFHCM and polysaccharides were processed every other day, using distilled water in NC groups, HFHCE in MC groups and simvastatin (200 mg/kg bw) in PC groups, respectively. The whole treatment was lasted for 40 consecutive days.

2.3.2. Biochemical assays

At the end of the experiment, all mice were overnight fasting, and then sacrificed by exsanguinations under diethyl ether anesthesia. The serum was separated from blood by centrifugation (12,000 × *g*, 10 min, 4 °C). The alanine aminotransferase (ALT) activities, alkaline phosphatase (ALP) activities, aspartate transaminase (AST) activities, high-density lipoprotein cholesterol (HDL-C) levels, low-density lipoprotein cholesterol (LDL-C) levels, very low-density lipoprotein cholesterol (VLDL-C) levels, total cholesterol (TC) levels, and triacylglycerols (TG) levels were measured using an automatic biochemical analyzer (ACE, USA).

The tissue of liver was immediately excised, weighed and homogenized (1:9, g/mL) in phosphate buffer solutions (0.2 M, pH 7.4). After centrifugation (3000 × *g*, 4 °C) for 20 min, the supernatants were collected for further biochemical analysis.

The SOD activity was measured according to the method of Hong et al. [17] with slight modifications. The mixture, including 0.5 mL supernatant, 2 mL PBS (0.05 M, pH 7.8), 0.5 mL methionine (130 mM), 0.5 mL nitroblue tetrazolium (NBT, 750 μM), 0.5 mL EDTA (100 μM) and 0.5 mL riboflavin (20 μM), was reacted for 40 min under the illumination (4,000 lx). The absorbance was measured at 550 nm against the mixture in dark as a blank. The SOD activity was calculated by the following formula and 1 U/mg prot was expressed as 50% inhibition of photochemical reduction of NBT in relative units per milligram protein.

$$\mathrm{SOD}activities(\mathrm{U/mg}prot) = \frac{A_0 - A_s}{A_0} \times \frac{1}{2} \times N$$

Where A_0 was the absorbance of PBS, A_s was the absorbance of samples, and N was the dilution ratio, respectively.

The CAT activity was measured by the method of ammonium molybdate colorimetry [18] with slight modifications. The substrate solution was mixed with sodium-potassium phosphate buffer (pH 7.4, 60 mM) and hydrogen peroxide (65 μM, pre-incubated for 5 min at 30 °C) with a proportion of 1:1 (v/v). Then the reaction systems including the measuring tubes of 0.5 mL samples and 2.0 mL substrate solution, the standard tubes of 0.5 mL buffer solution and 2.0 mL substrate solution, and the control tubes of 2.5 mL buffer solution (0.05 M, pH 7.8) were all reacted at 30 °C

for 60 s. Immediately, the reactions were ended by adding 2.0 mL ammonium molybdate (32.4 mM). After standing by for 10 min, all tubes were absorbed at 410 nm. The CAT activity was calculated by the following formula.

$$CATactivities(\mathrm{U/mg}prot) = \frac{A_1 - A_2}{A_0} \times \frac{60 \times 2}{0.5}$$

Where A_1 was the absorbance of measuring tubes, A_2 was the absorbance of control tubes, and A_0 was the absorbance of standard tubes, respectively.

The GSH-Px activity was analyzed by a reported method of Flohé and Günzler [19] with slight modifications. The reaction mixture contained 0.4 mL PBS (50 mM, pH 7.0), 0.5 mM of edetate disodium, 1 mM of sodium azide, 0.15 mM of triphosphopyridine nucleotide, 1 mM of GSH, and 2.4 U/mL of glutathione reductase (GR, 2.4 U/mL in 0.1 M potassium phosphate buffer, pH 7.0). After addition by H_2O_2 (0.15 mM, 0.2 mL), the reaction was incubated at 37 °C for 10 min and the rate of NADPH consumption was recorded at 340 nm. The 1 U/mg prot of GSH-Px was expressed as mM of NADPH oxidized per minute per milligram of protein.

The T-AOC activities were analyzed using commercial reagent kits according to the instructions.

The LPO contents were measured referring to the method of Lai with a slight modification [20]. The mixture of supernatant (0.5 mL) and TBA (2.68 g/L, 2.5 mL) were kept warming in boiled water for 30 min, and the 7 mL *n*-butyl alcohol was added immediately after the reaction was cooled in cold water. Five minutes for standing, the absorbance was measured at 520 nm, using distilled water as blank. The positive control was performed with 1,1,3,3-tetraethoxypropane (TEP, 40 mM) instead of samples. The contents of LPO (mmol/g prot) were calculated using the following formula.

$$LPOcontents(\mathrm{mmol/g}prot) = \frac{A_i}{A_j} \times 40$$

Where A_i was the absorbance of samples, and A_j was the absorbance of TEP.

The content of MDA was measured by the method of Zhao et al. [21] with slight modifications. The measuring tubes, including 1.5 mL deionized water, 0.5 mL of sample and 2.0 mL TBA (0.6%, w/v), were heated in boiling water for 15 min. After cooling rapidly, the mixture was centrifuged (3000 × *g*, 10 min), and the supernatant was absorbed at 532, 600 and 450 nm. The content of MDA was calculated by the following formula.

$$MDAcontents(\mathrm{mmol/g}prot) = 6.45 \times (OD_{532nm} - OD_{600nm}) - 0.56 \times OD_{450nm}$$

2.3.3. Histopathological observation

The fresh liver tissue masses were fixed in formaldehyde solution (pH 7.4, 4%) for over 24 h, embedded in paraffin, cut in slices (4–5 μm thickness) and stained with hematoxylin-eosin. Each section was photo-graphed under microscope showing the histopathological changes (×400 magnifications).

2.4. Purification of AcMZPS and AlMZPS

The lyophilized powered of AcMZPS and AlMZPS (500 mg) were dissolved in distilled water (50 mL), loaded onto a DEAE-52 cellulose anion-exchange column (1.6 cm × 20 cm), and eluted with distilled water and then with gradient solutions (0.1, 0.3, 0.5 and 1 mol/L NaCl) at a flow of 5 mL/tube. The eluents were collected, and determined by phenol-sulfuric acid method, using glucose as standard. The major fractions were collected and dried for further studies.

2.5. Antioxidant activity in vitro

2.5.1. Reducing power

The reducing power was determined according to the method with slight modifications established by Oyaizu [22]. The reaction system, contained 1 mL polysaccharide samples, 2.5 mL PBS (pH 6.6, 0.2 M) and 1.0 mL potassium ferricyanide solution (1%, w/v), was water-bath heated for 20 min at 50 °C. The reaction was terminated by adding 2.0 mL of trichloroacetic acid (10%, *w/v*). After centrifugation (3000 × *g*, 10 min), the supernatant (6.5 mL) was collected and mixed with 1.2 mL ferric chloride (0.1%, w/v), subsequently. The absorbance was measured at 700 nm by spectrophotometer.

2.5.2. Scavenging abilities on hydroxyl radicals

The hydroxyl radical scavenging abilities were measured according to the method of Koksal et al. [23]. The reaction mixture, contained 1 mL ferrous sulfate (9 mM), 1 mL salicylic acid (9 mM), 1 mL hydrogen peroxide (8.8 mM, v/v), and 1 mL polysaccharide samples, was incubated at 37 °C for 30 min. The absorbance was measured at 510 nm, and the hydroxyl radical scavenging ability was calculated by the following formula.

$$Scavengingabilities(\%) = (1 - \frac{A}{A_0}) \times 100\%$$

Where A was the absorbance of the polysaccharide samples, and A_0 was the absorbance of the blank.

2.5.3. Scavenging abilities on DPPH scavenging assay

The scavenging activity on DPPH radicals was measured according to the method of Brand-Williams et al. [24] and Kong et al. [25] with minor modifications. The reaction mixture contained 2 mL polysaccharide sample and 2.0 mL DPPH solution (0.2 mM) or ethanol (95%, w/v). After standing for 30 min in the dark, the absorbance was determined at 517 nm, and the scavenging ability was calculated by following formula.

$$Scavengingabilities(\%) = (1 - \frac{A}{A_0}) \times 100\%$$

Where A was the absorbance of mixture contained samples and DPPH, and A_0 was the absorbance of mixture contained samples and ethanol.

2.6. Monosaccharide composition analysis

The determination of monosaccharide compositions was analyzed by gas chromatography (GC-2010, Shimadzu) equipped with a capillary column of Rtx-1 (30 mm × 0.25 mm × 0.25 μm) according to a reported method of Sheng et al. [26] with slight modifications. Briefly, the samples were hydrolyzed with trifluoroacetic acid (TFA, 2 M, 110 °C) for 4 h. The hydrolyzed products were acetylated with hydroxylamine hydrochloride and pyridine. The supernate (1 μL) was injected into the column and equipped with flame ionization detector. Monosaccharide components were analyzed by processing the comparison with monosaccharide standards of xylose, arabinose, glucose, rhamnose, galactose, ribose, inositol, and mannose, and the relative molar ratios were calculated by the area normalization method.

2.7. Statistical analysis

The data were expressed as the means ± standard deviations (S.D.) and were statistically analyzed by one-way ANOVA. Significant differences were defined when $P < 0.05$.

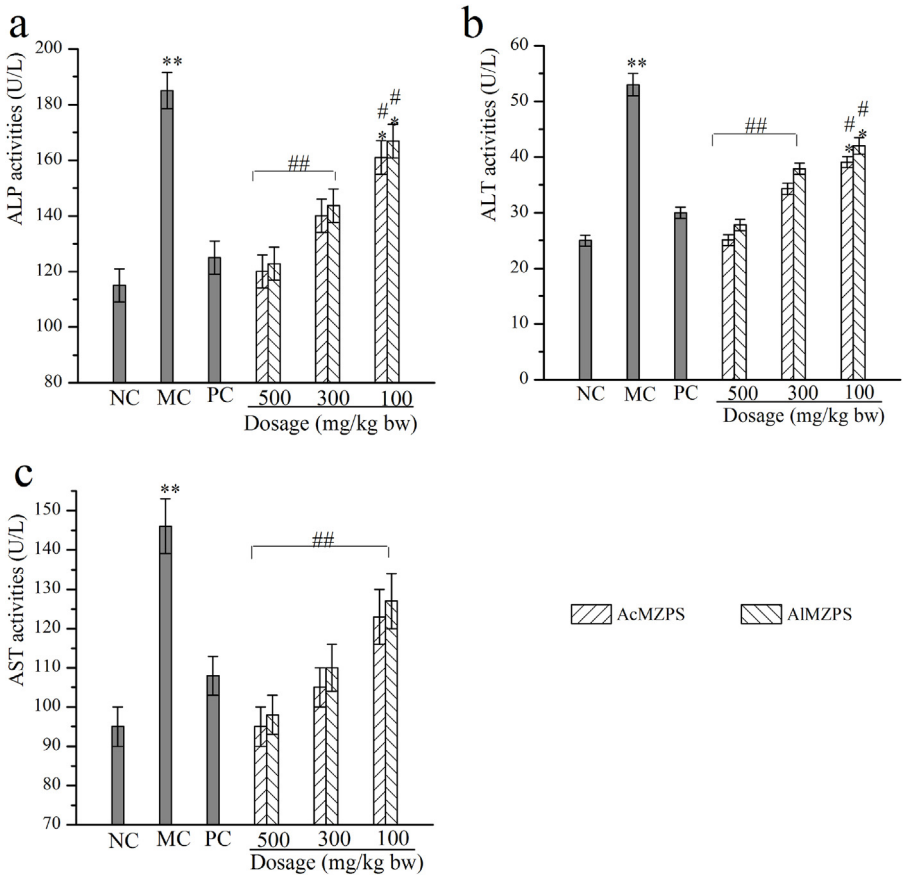

Fig. 1. Effects of AcMZPS and AlMZPS on serum enzyme activities of (a) ALP, (b) ALT, and (c) AST in hyperlipidemic mice. (**) $P<0.01$ and (*) $P<0.05$ compared with NC groups; (##) $P<0.01$ and (#) $P<0.05$ compared with MC groups.

Table 1
Effects of AcMZPS and AlMZPS on body weights (g) and liver index (%) in high-fat-high-cholesterol emulsion induced hyperlipidemic mice.

Groups	Body weight (g)		Liver index (%)
	Initial	Final	
NC	30.30 ± 0.46	33.70 ± 0.64	4.82 ± 0.26
MC	31.30 ± 0.50	41.80 ± 0.67**	7.56 ± 0.34**
PC	30.10 ± 0.47	35.04 ± 0.72	4.98 ±0.30
AcMZPS			
500 mg/kg bw	31.31 ± 0.46	34.32 ± 0.67##	4.92 ± 0.27##
300 mg/kg bw	30.59 ± 0.51	34.79 ± 0.71##	5.41 ± 0.71##
100 mg/kg bw	31.30 ± 0.37	36.11 ± 0.48#,*	5.91 ± 0.51#,*
AlMZPS			
500 mg/kg bw	30.12 ± 0.49	33.72 ± 0.37##	4.98 ± 0.32##
300 mg/kg bw	31.09 ± 0.57	36.59 ± 0.71#,*	5.38 ± 0.47#,*
100 mg/kg bw	30.78 ± 0.64	37.08 ± 0.53#,*	6.02 ± 0.78#,*

The values were reported as the mean ± S.D. of ten mice per group
** $P<0.01$ compared with NC groups.
* $P<0.05$ compared with NC groups.
$P<0.01$ compared with MC groups.
# $P<0.05$ compared with MC groups.

3. Results

3.1. *Capacity of anti-hyperlipidemic activities* in vivo

3.1.1. *Effects of AcMZPS and AlMZPS on body weight and liver index*

As shown in Table 1, significant difference between the initial and the final body weight could be remarkably observed ($P=0.0009$). After 40 days of experiment, the body weight in MC group (41.80 ± 0.67 g) was significantly higher than that in NC group (33.70 ± 0.64 g, $P=0.0001$), indicating that the mice had suffered obesity. However, this pathological obesity could be postponed by the administration of AcMZPS and AlMZPS at different doses, indicating that both AcMZPS and AlMZPS had potential contributions in suppressing the weight gain induced by HFHCE and the effects of AcMZPS was superior to that of AlMZPS.

Observably, the liver index of the mice in MC groups (7.56 ± 0.34%) expressed a significant increase after the HFHCE treatment when compared with that in the NC group (4.82 ± 0.26%, $P=0.0012$), reflecting the serious liver damage occurred in the mice (Table 1). However, this increase could be mitigated by the treatment with AcMZPS and AlMZPS at their dose concentrations (500, 300, and 100 mg/kg bw). Compared with the MC group, at the end of the experiment, the liver index of mice treated with Ac-MZPS and Al-MZPS at three doses of 500, 300, and 100 mg/kg bw reduced by 34.92 ± 0.54, 28.44 ± 0.26, 21.83 ± 0.24, 34.12 ± 0.34, 28.84 ± 0.51 and 20.37 ± 0.18%, respectively ($P<0.05$ or $P<0.01$). Similar results was also be observed after the treatment with the simvastatin (PC groups, 4.98 ± 0.31%) at a dosage of 200 mg/kg bw.

3.1.2. *Biochemical assays in blood serum*

Several enzyme activities in serum were commonly used for the investigation of early liver damage. As displayed in Fig. 1, significant increase of ALP, ALT, and AST activities were observed in mice after the gavage with HFHCE (MC groups), indicating that liver damage had been occurred. Interestingly, treatment with AcMZPS and AlMZPS could dramatically suppress the elevations in these indexes ($P<0.01$ or $P<0.05$) when compared with that in MC groups. As shown in Fig. 1a, the ALT activities reached

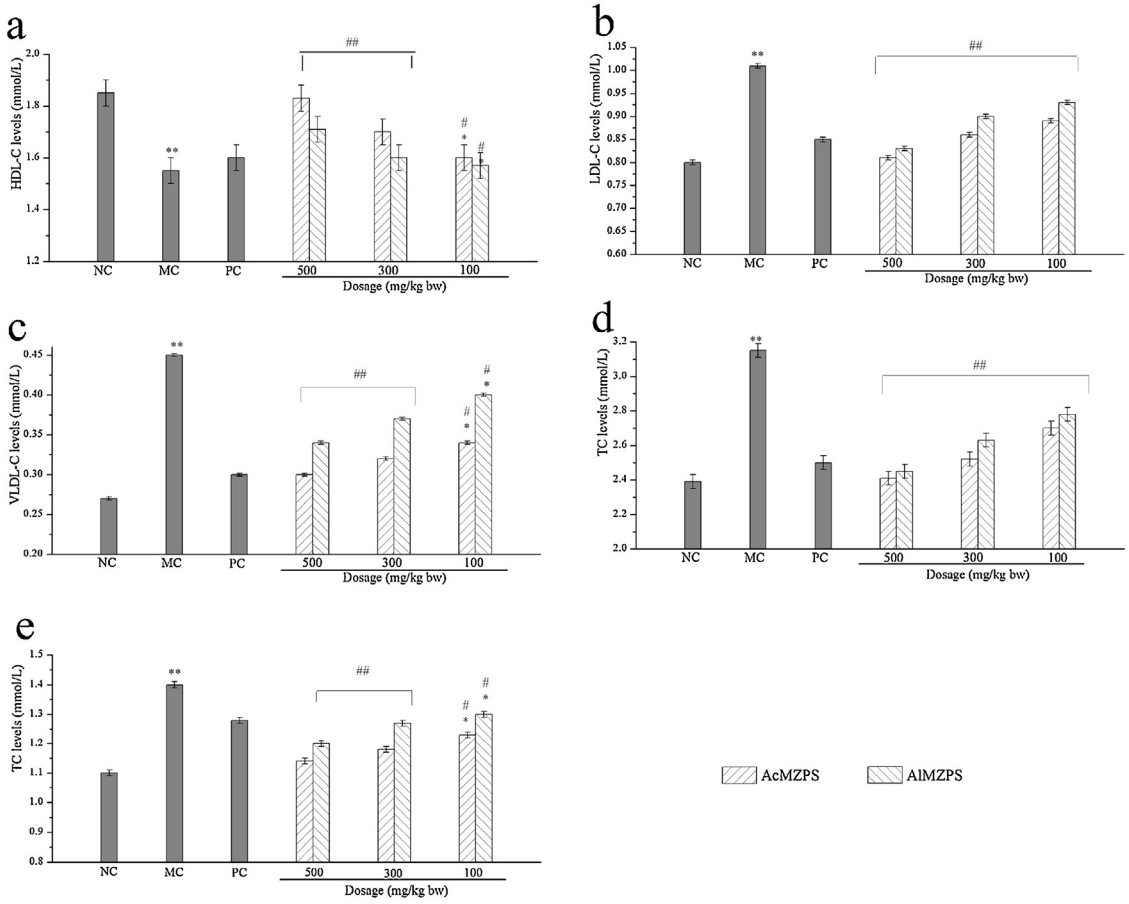

Fig. 2. Effects of AcMZPS and AlMZPS on serum lipid levels of (a) HDL-C, (b) LDL-C, (c) VLDL-C, (d) TC and (e) TG in hyperlipidemic mice. (**) $P < 0.01$ and (*) $P < 0.05$ compared with NC groups; (##) $P < 0.01$ and (#) $P < 0.05$ compared with MC groups.

25.09 ± 2.86 U/L in AcMZPS groups at the dosage of 500 mg/kg bw, which were 52.64 ± 4.13% lower than that in MC groups ($P = 0.0004$), but 9.71 ± 0.73% higher than that in AlMZPS groups (27.80 ± 4.15 U/L, $P > 0.05$) at the same dosage. As shown in Fig. 1b and c, it showed that the AST and ALP activities were 95.01 ± 3.56, 120.1 ± 9.87, 98.0 ± 2.85 and 122.9 ± 4.19 U/L in dosage groups of AcMZPS and AlMZPS at 500 mg/kg bw, which were 34.93 ± 6.91, 35.08 ± 5.32, 37.93 ± 2.16, 37.88 ± 3.14% lower than that in MC groups ($P = 0.0029$), respectively. These results testified that both AcMZPS and AlMZPS had protective effects against HFHCE in liver.

As described in Fig. 2, the mice of MC groups treated with HFHCE showed early dysregulation of lipid metabolism as evidenced by significant decrease of HDL-C levels ($P = 0.0012$), and remarkable increase of LDL-C, VLDL-C, TC, and TG levels when compared with that in NC groups (with all $P < 0.01$). However, these pathologic changes could be remitted by the treatment with AcMZPS and AlMZPS ($P < 0.01$ or $P < 0.05$). The VLDL-C, TC, and TG levels in dosage groups of Ac-MZPS at the dosage of 500 mg/kg bw reached 0.30 ± 0.01 (Fig. 2c), 2.41 ± 0.05 (Fig. 2d), and 1.01 ± 0.03 mmol/L (Fig. 2e), which were 33.33 ± 0.03, 23.49 ± 0.01, and 28.57 ± 0.07% lower than that in MC groups (with all $P < 0.01$), and 11.76 ± 0.06, 1.63 ± 0.01, 15.83 ± 0.03% lower than that in the dosage groups of AlMZPS (with all $P > 0.05$), while the LDL-C levels reached 0.81 ± 0.03 mmol/L (Fig. 2b), which were 19.80 ± 0.17% lower than that in MC groups (1.01 ± 0.02 mmol/L, $P = 0.0016$), but 2.41 ± 0.02% higher than that in AlMZPS groups at the same concentration ($P > 0.05$). Meanwhile, the HDL-C levels was 1.83 ± 0.01 mmol/L at the dosage of 500 mg/kg bw, with 1.08 ± 0.01% significant higher than that in MC groups (1.55 ± 0.02 mmol/L), but 1.94 ± 0.03% non-significant lower than that treated with AlMZPS (1.71 ± 0.03 mmol/L) (Fig. 2a). These results indicated that both AcMZPS and AlMZPS had potential effects on the improvement of lipid profiles, and the favorable effects of AcMZPS was more potent than simvastatin at a dosage of 200 mg/kg bw ($P < 0.05$).

3.1.3. *Biochemical assays in liver*

The enzyme activities of SOD, CAT and GSH-Px and non-enzyme activities of T-AOC were commonly used as biochemical marker for monitoring early oxidative damage *in vivo*. As shown in Fig. 3, significant decreases in the hepatic activities of SOD, CAT, GSH-Px and T-AOC were observed in HFHCE-induced hyperlipidemia mice as compared to the NC group ($P = 0.0016$), respectively, indicating that serious oxidative damage had been occurred in liver. Briefly, in the dosage groups of 500 mg/kg bw treated with AcMZPS and AlMZPS, the SOD activities reached 11.80 ± 0.76 and 10.1 ± 0.57 U/mg prot, with 31.36 ± 4.12, 19.8 ± 2.31% higher than that of the MC group (8.09 ± 0.43 U/mg prot, $P = 0.0012$). Furthermore, at the dosage of 500 mg/kg bw the hepatic SOD activities treated with AcMZPS and AlMZPS were higher than that in the NC groups (Fig. 3a).

Fig. 3b shows that the CAT activities in the mice treated with AcMZPS and AlMZPS at the dosage of 500 mg/kg bw reached 22.60 ± 1.54 and 19.30 ± 1.45 U/mg prot, which were significant higher than that in MC groups (12.07 ± 0.44 U/mg prot, $P < 0.01$), and were almost approximate to the PC groups (19.99 ± 2.31 U/mg prot).

In present study, the hepatic GSH-Px activities of dosage groups treated with AcMZPS and AlMZPS at dosage of 500 mg/kg bw

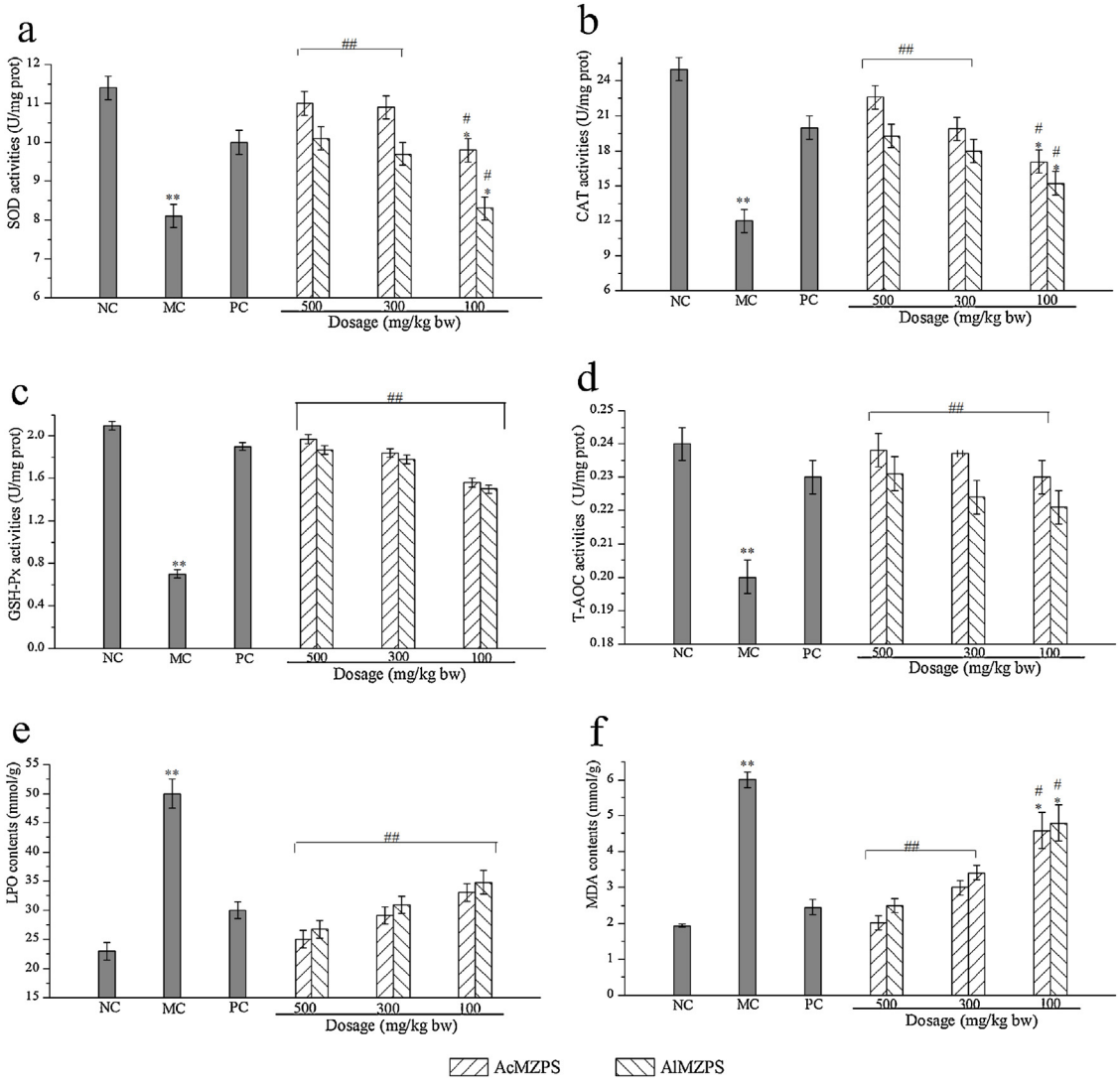

Fig. 3. Effects of AcMZPS and AlMZPS on hepatic activities of (a) SOD, (b) CAT, (c) GSH-Px, and (d) T-AOC, and contents of (e) LPO and (f) MDA in hyperlipidemic mice. (**) $P < 0.01$ and (*) $P < 0.05$ compared with NC groups; (##) $P < 0.01$ and (#) $P < 0.05$ compared with MC groups.

reached 1.97 ± 0.09 and 1.89 ± 0.07 U/mg prot, respectively, which were 64.46 ± 5.14 and 62.96 ± 3.98% higher than that in the MC group (0.7 ± 0.11 U/mg prot, $P = 0.0005$) (Fig. 3c). In particular, the GSH-Px activity in the mice treated with Ac-MZPS at the dosage of 500 mg/kg bw was almost approximate to the NC groups (2.13 ± 2.74 U/mg prot), even better than the PC group (1.90 ± 0.24 U/mg prot, $P < 0.05$)

For analysis of the T-AOC activities in dosage groups treated with AcMZPS and AlMZPS, as illustrated in Fig. 3d, it reached the maximum of 0.238 ± 0.03 U/mg prot and 0.231 ± 0.02 U/mg prot at the highest doses (500 mg/kg bw), with 15.97 ± 1.33% and 13.41 ± 1.12% higher than that of the MC group (0.2 ± 0.01 U/mg prot, $P = 0.0012$).

Besides all the dosage groups, when tested at a dosage of 200 mg/kg bw, simvastatin-treated mice also manifested significant decline of the SOD, CAT, GSH-Px and T-AOC activities in HFHCM induced oxidative damage.

The products of lipid peroxidation (LPO and MDA) were also investigated. As illustrated in Fig. 3, the gavage of HFHCE significantly enhanced LPO and MDA contents in MC groups compared with that in NC group (all $P < 0.01$). However, after the treatment with AcMZPS and AlMZPS at the dosage of 500 mg/kg bw, the hepatic LPO contents reached 26.0 ± 2.21 and 27.10 ± 3.87 mmol/g prot, which were 48.0 ± 5.32 and 46.01 ± 3.13% lower than that of MC groups (Fig. 3e, $P = 0.0007$), while the hepatic MDA contents reached 2.01 ± 0.01 and 2.49 ± 0.05 mmol/g prot, which were 66.53 ± 5.26 and 58.51 ± 4.61% lower than that of MC groups (Fig. 3f, $P = 0.0003$), respectively, indicating that both AcMZPS and AlMZPS had potential anti-hyperlipidemia effects by alleviating the oxidative stress.

3.1.4. Histopathological observations of mice livers

Macroscopically, the livers of normal control group had dark red color, sharp edges and tough textures, whereas the livers of model control group had cream yellow color, blunt edges, friable textures and showed significant accumulations of fat (data were not shown). As for microscopic examination, displayed in Fig. 4a, the liver slices of mice in the NC groups showed typical hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus and visible central veins, showing no symptoms of fat degeneration (Fig. 4a). However, in contrast, the HFHCE-induced mice (MC groups) showed clinical hyperlipemic symptoms including extreme swelling, large fat vacuoles, increased cell volume, hepatic steatosis, vesicular degeneration, as well as disappearance of nuclei. Interestingly, as depicted in Fig. 4d–i, changes on the structures of the hepatic lesions caused by HFHCE were markedly

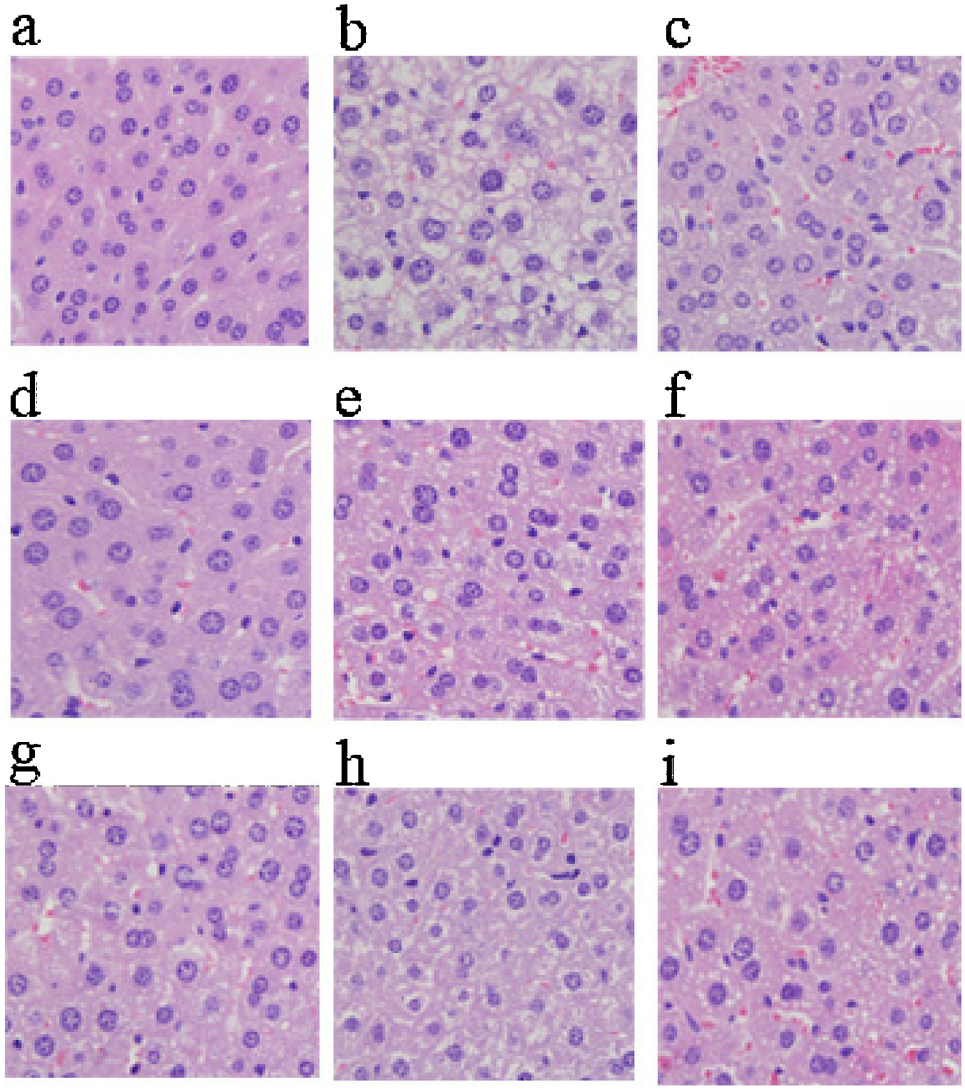

Fig. 4. Optical micrographs of mice liver tissues slice (magnification 400×). (a) liver of mice in NC groups, (b) liver of mice in MC groups, (c) liver of mice in PC groups, (d and g) liver of mice fed with AcMZPS and AlMZPS at dosage of 500 mg/kg bw showing almost normal histology similar to control mice, (e and h) liver of mice fed with AcMZPS and AlMZPS at dosage of 300 mg/kg bw showing mild architectural damage with few showing abnormal structure, and (f and i) liver of mice fed with AcMZPS and AlMZPS at dosage of 100 mg/kg bw showing mild architectural damage.

ameliorated by the pretreatment with AcMZPS and AlMZPS at different dosages (100, 300 and 500 mg/kg bw), indicating that both AcMZPS and AlMZPS had latent effects in liver recovering abilities. Meanwhile, the simvastatin (200 mg/kg bw) treatment also had obviously protective effects against HFHCM-induced liver damage (Fig. 4c).

3.2. Purification of AcMZPS and AlMZPS

The chromatography results of AcMZPS and AlMZPS by DEAE cellulose column chromatography were illustrated in Fig. 5. Obviously, the elution curves showed two relatively symmetrical peaks of AcMZPS (named as AcMZPS-1 and AcMZPS-2, Fig. 5a), and AlMZPS (named as AlMZPS-1 and AlMZPS-2, Fig. 5b), respectively, indicating their heterogeneous profiles. Both crude polysaccharides and their fractions (AcMZPS, AcMZPS-1, AcMZPS-2, AlMZPS, AlMZPS-1, and AlMZPS-2) were chosen for further characterizations and *in vitro* antioxidant investigations.

3.3. *Antioxidant capacities assaying* in vitro

In this experiment, to analyze the *in vitro* antioxidant capacities of AcMZPS, AlMZPS, and their purified fractions, three parameters including reducing power, as well as scavenging abilities on DPPH and hydroxyl radicals were investigated and the results were shown in Fig. 6.

As shown in Fig. 6a, the reducing power performed a concentration-dependent manner within the concentration range of 0–1000 mg/L. Apparently, at the concentration of 1000 mg/L, the reducing power of AcMZPS-1 reached 0.879 ± 0.07, which was 9.875 ± 0.06, 22.6 ± 0.74, 30.6 ± 1.06, 38.5 ± 1.24 and $46.7 \pm 2.15\%$ ($P < 0.01$) higher than that of AcMZPS-2 (0.80 ± 0.01), AcMZPS (0.68 ± 0.03), AlMZPS-2 (0.61 ± 0.02), AlMZPS-1 (0.54 ± 0.03), and AlMZPS (0.412 ± 0.02), respectively.

It could be seen from Fig. 6b that the DPPH radicals scavenging abilities dose-dependently increased with the increasing concentrations of the components. All of the components had strong radical scavenging abilities, and the scavenging ability of AcMZPS-1 reached $49.8 \pm 1.87\%$ at the concentration of 1000 mg/L, with

8.77 ± 0.02, 12.6 ± 0.06, 24.5 ± 0.12, 33.3 ± 0.18, and 36.8 ± 0.23% ($P < 0.01$) higher than that of AcMZPS-2 (51.8 ± 1.78%), AlMZPS (49.8 ± 1.52%), AlMZPS-2 (43.0 ± 1.89%), AlMZPS-1(37.8 ± 1.26%), and AlMZPS (36.0 ± 1.07%), respectively. The DPPH scavenging results revealed that AcMZPS, AlMZPS and their purified components probably contained substances of hydrogen donors, which could convert free radicals to stable diamagnetic molecules.

As displayed in Fig. 6c, when the polysaccharides concentrations were raised from 0 to 1000 mg/L, the AcMZPS-1showed a considerably stronger effective ($P < 0.01$) on scavenging hydroxyl radicals (20.3 ± 3.79%) when compared to AcMZPS-2 (19.3 ± 3.26%), AcMZPS (18.8 ± 2.65%), AlMZPS-1 (18.0 ± 2.16%), AlMZPS2 (17.1.0 ± 1.76%) and AlMZPS (14.9 ± 1.52%), respectively. All the polysaccharide fractions were found to have higher scavenging ability on hydroxyl radicals, indicating that the zinc mycelia of *P. nebrodensis* significantly affects the scavenging of the hydroxyl radical.

3.4. Monosaccharide composition analysis

As identified by comparing the retention time of standards (Fig. 7a), the gas chromatography analysis showed that there were three peaks in the AcMZPS (Fig. 7b), The mine peak were mannose, the major peaks were, galactose and glucose with a ratio of 4.0:5.97:1.9 and contents of 33.70, 50.29 and 16.01% respectively, while AcMZPS-1 was composed of xylose, mannose, galactose and glucose with a mass percentage of 6.72, 24.23, 32.37 and 36.58% in a molar ratio of 3.3:1.0:1.33:1.5 (Fig. 7c), and AcMZPS-2 contained four monosaccharides of xylose, mannose, galactose and glucose in a mass percentage of 1.13, 5.6, 8.4, and 84.87% with a molar ratio of 1.9:12.8:4.0:2.9 (Fig. 7d); and no xylose was monitored in AcMZPS, indicating that xylose may play important roles in possessing the strongest antioxidant capacities. As could be seen from Fig. 7e, the

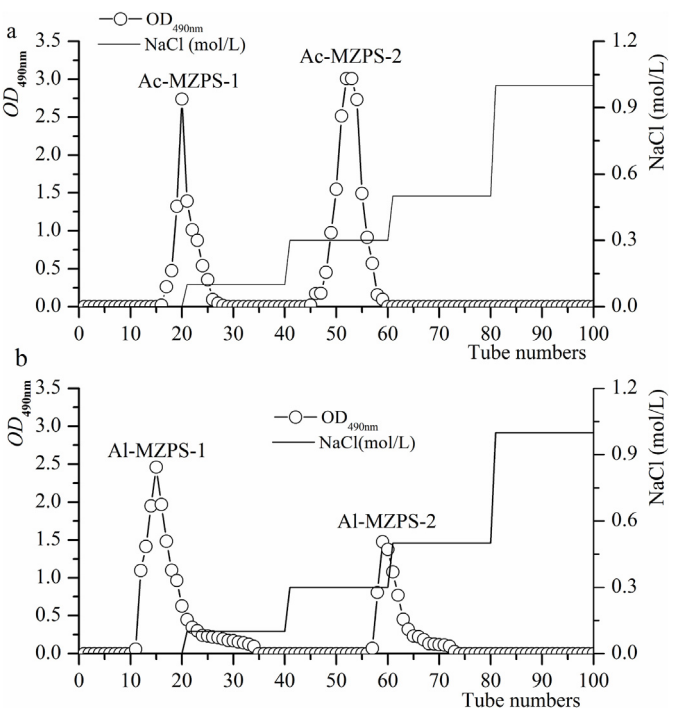

Fig. 5. Elution profiles on DEAE-52 chromatography with gradient of NaCl solutions (0, 0.1, 0.3, 0.5 and 1.0 mol/L). (a) AcMZPS, and (b) AlMZPS.

AlMZPS contained two kinds of monosaccharides including galactose and glucose with a molar ratio of 6.55:38, and contents of 14.7 and 85.3%; the AlMZPS-1 was composed of mannose (4.82%), galactose (18.07%), glucose (77.11%) with the molar ratio of 1.0:3.75:16 (Fig. 7f), four monosaccharides (arabinose, mannose, galactose and

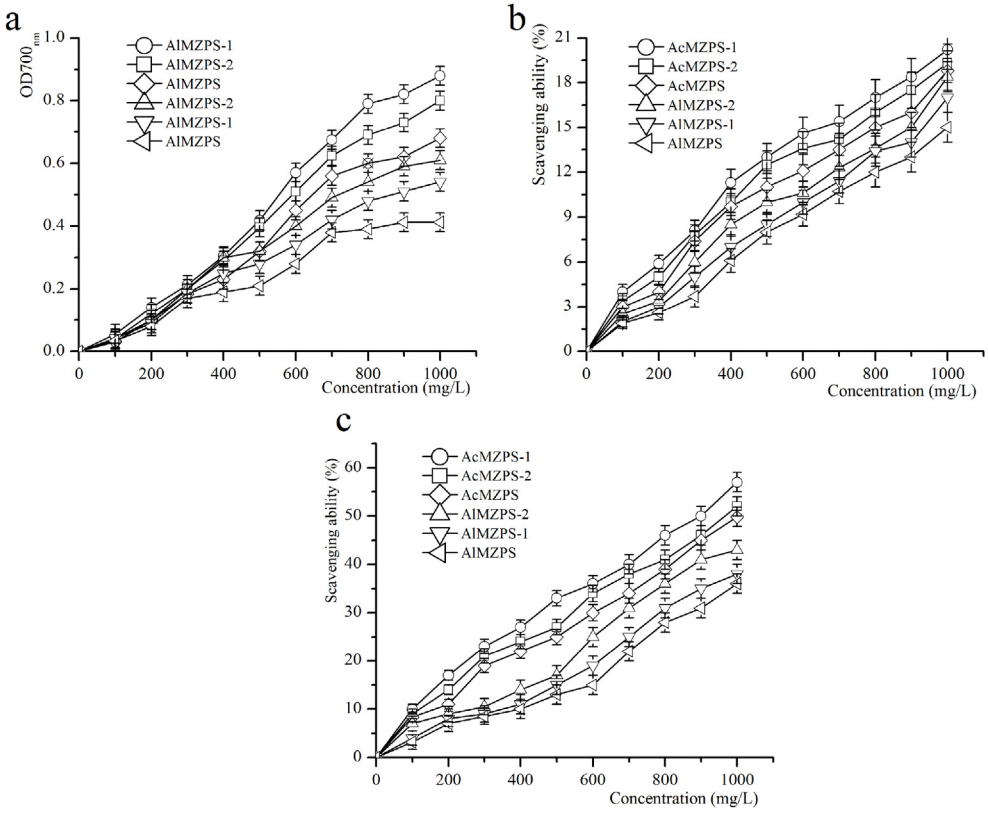

Fig. 6. Antioxidant activities of AcMZPS, AlMZPS, and their purified fractions *in vitro*. (a) Reducing power, (b) DPPH radicals, and (c) Hydroxyl radicals.

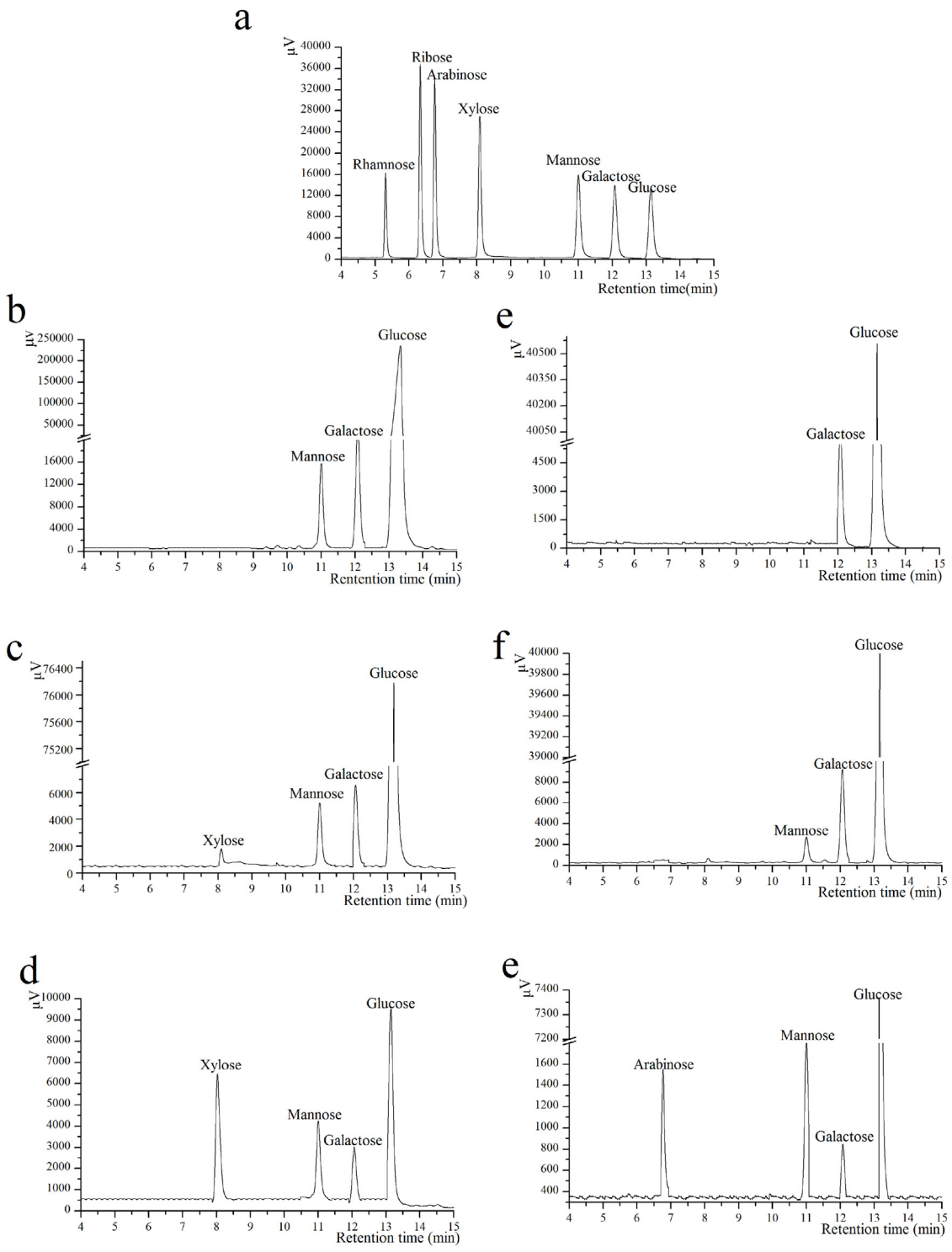

Fig. 7. GC chromatograms of (a) Standard samples, (b) AcMZPS-1, (c) AcMZPS-2, (d) AcMZPS, (e) AlMZPS-2, (f) AlMZPS-1, and (g) AlMZPS.

glucose) were identified in the AlMZPS-2, and monosaccharides contents were 10.1, 15.6, 7.80, and 66.5% with the molar ratio of 11.0:14.0:6.8:58, respectively (Fig. 7g). These data suggested that the four fractions were heteropolysaccharides, and glucose was the predominant monosaccharide in the four kings of polysaccharide.

4. Discussion

Recently, zinc-enriched polysaccharides, which were reported to possess a significantly higher antioxidant activity than regular polysaccharides, had been received more and more attentions [15]. However, to the best of our knowledge, there have been no comparative studies on the characterization of acidic-, alkali-extractable mycelium zinc polysaccharides present in *P. eryngii* var. *tuoliensis* (AcMZPS, AlMZPS). In this present work, AcMZPS and AlMZPS were considered as representative heteropolysaccharides due to the elution purified by DEAE-52 cellulose column.

The early hepatic damage could be determined by monitoring the serum enzymes activities of AST, ALT, and ALP, which could leach out of hepatocytes into blood circulation resulting the

enhancement of atherosclerosis [27] and blood viscosity [28,29] and. Clinically, the dysregulation of lipid metabolism was reconsidered as a fatal inducible factor of liver damage characterized by elevated levels of TG, TC, LDL-C, and VLDL-C, and decreased levels of HDL-C [6,12,17,28,30,31]. It had been demonstrated that the excess LDL-C and VLDL-C, which were the main carrier of TC and TG, could be aggregated at the blood vessel walls, could impose the increased risks for coronary heart diseases [27,32,33]. However, as an advantageous lipoprotein, physiological-high HDL-C levels could transport the TC from the peripheral tissues to the liver by the "reverse cholesterol transport" pathway for catabolism, reducing the lipid levels in serum [34]. In present study, the suppression of ALT, AST and ALP activities could be observed by treatment with polysaccharides, indicating that both AcMZPS and AlMZPS had potential effects in preserving the structural integrity of hepatic cells by increasing the stabilization of plasma membrane. Synchronously, both AcMZPS and AlMZPS significantly increased the HDL-C levels, and decreased LDL-C, VLDL-C, TC, and TG levels in serum, suggesting that the polysaccharides extracted from *P. eryngii* var. *tuoliensis* had potential effects in improving hyperglycemia and hyperlipemia in HFHCE-induced mice. This was also an important advantage in the prevention and treatment of hyperlipidemia with serious lipoprotein abnormality (Figs. 1 and 2).

In order to understand the antioxidant activities and hepatoprotective activities of AcMZPS and AlMZPS *in vivo*, the antioxidant enzyme activities and lipid contents of liver homogenate were also investigated. Hong et al. had demonstrated that the HFHCE could destroy the *in vivo* antioxidant enzyme defenses, leading cell and tissue damages [17]. During the enzymatic and non-enzymatic antioxidant system, the SOD catalyzed superoxide to hydrogen peroxide, and GSH-Px as well as CAT catalyzed hydrogen peroxide to water, alleviating the oxidative stress *in vivo* [35]. Furthermore, Young et al. had pointed out that the T-AOC activities, which reflected the non-enzymatic antioxidant capacity against various reactive oxygen radicals, could indicate the increased susceptibility to oxidative damage [33]. In this work, significant decreases of hepatic SOD, CAT, GSH-Px and T-AOC activities in HFHCM induced mice were observed, suggesting the serious oxidative stress occurred in the liver. In addition, the formation of LPO and MDA, which was the by-products of interaction between ROS and polyunsaturated fatty acids, were considered to be hallmarks of oxidative stress causing tissue damages [36]. Currently, remarkable increases of hepatic LPO and MDA contents could be observed in HFHCM-induced oxidative mice. However, the enhancement could be significantly reduced by the treatment with polysaccharides, especially Ac-MZPS at dose of 500 mg/kg bw, demonstrating that both Ac-MZPS and Al-MZPS had potential anti-hyperlipidemic and hepatoprotective activity in preventing atherosclerosis, liver fatty and other hyperlipidemia complications (Fig. 3).

Moreover, the HFHCE-induced mice showed the most severe injury as indicated by massive fatty changes, and ballooning degeneration (MC group) during the histopathological analysis. These histopathological changes were observably attenuated by the treatment with the tested polysaccharides, especially the AcMZPS at dosage of 500 mg/kg bw, suggesting that administration of AcMZPS and AlMZPS could decrease the histological alteration, and AcMZPS expressed the superior manners. The biochemical analysis was also consistent with the histopathological analysis of the livers (Fig. 4).

Previous literatures had been demonstrated that the DEAE-52 could combine with electriferous polysaccharides, and the acidic polysaccharide fractions were only being eluted by strong enough ionic force of saline solution (NaCl) [15]. Thus, AlMZPS-1 could be regarded as neutral-polysaccharides; the acidic fractions of AcMZPS-2, AlMZPS-1 and AlMZPS-2 were obtained from gradient elution (Fig. 5).

Documented literatures had reported that oxidative stress, usually caused by reactive oxygen species (ROS) including hydroxyl radicals, superoxide anion, hydrogen peroxide, and so on, could lead to damages or destructions in a variety of tissues and consequent development of hyperlipidemic and its complications [1–3,31]. And the hepatic oxidative injury induced by HFHCE was a well-characterized model system for screening hepatoprotective activity of antioxidants, since the exposure of HFHCE could result in hyperlipidemia, exacerbating the unnormal metabolisms in livers [6]. Hence, it would be great necessary to discover natural compounds with potential ROS scavenging activities for the treatment of oxidative-induced diseases [37]. There were also increasing proofs confirming that polysaccharides exerted their positive functions by scavenging free radicals [1,15,31]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity by breaking the free radical chain *via* the donation of a hydrogen atom [38]. Among the ROS, the hydroxyl radicals had the strongest chemical activities involving in the progress of biomolecules damage and lipid peroxidation [30]. And for DPPH radicals, stable free radicals played fatal role in the process of atherosclerosis and cardiovascular diseases, when DPPH encountered a proton-donating substance such as an antioxidant, the radical could be scavenged and the absorbance was reduced [39,40]. In this study, both AcMZPS and AlMZPS exhibited significant *in vitro* antioxidant activities directly by scavenging these free radicals, suggesting that the polysaccharides extracted from *P. eryngii* var. *tuoliensis* could probably be developed as potential natural antioxidants. In addition, it had been reported that the antioxidant properties of polysaccharides were mainly associated with their monosaccharide compositions [31]. Presently, the galactose contents in AcMZPS-2, AcMZPS-1 and AcMZPS were 32.37, 8.4 and 8.03%, which were agreed with the scavenging abilities, indicated that the galactose had potential effects in possessing the antioxidant activities. Compared with polysaccharides extracted from other *Pleurotus* species, the antioxidant activity of Ac-MZPS and Al-MZPS were higher than that isolated from *P. tuber-regium* [41] and *P. ostreatus* [42]. Interestingly, Ac-MZPS had been further testified by *in vivo* mice model subjected to high-fat emulsion induced hepatic damage (Figs. 6 and 7).

5. Conclusion

The present study showed that AlMZPS and AcMZPS displayed potential antioxidant actions and hepatoprotective activity in experimental hyperlipidemic mice induced by high-fat-high-cholesterol emulsion. Furthermore, four major fractions of AcMZPS-1, AcMZPS-2, AlMZPS-1, and AlMZPS-2 were purified, and their monosaccharide compositions as well as *in vitro* antioxidant activities were also investigated. The results demonstrated that the free radicals being released in the liver were effectively scavenged, providing a mechanistic basis for AcMZPS and AlMZPS using as potentially natural and functional foods in prevention and alleviation of hyperlipidemia and its complications.

Competing interests

The authors declare that they have no competing interests.

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