Research Paper

Hepatoprotection of enzymatic-extractable mycelia zinc polysaccharides by *Pleurotus eryngii* var. *tuoliensis*

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ABSTRACT

The purpose of this work was designed to investigate the hepatoprotective and antioxidant effects of enzymatic-extractable mycelia zinc polysaccharides (En-MZPS) from *Pleurotus eryngii* var. *tuoliensis* on the hyperlipidemic mice induced by high-fat-high-cholesterol emulsion (HFHCM). The results showed that the supplementation of En-MZPS had potential anti-hyperlipidemia effects on reducing hepatic lipid levels by monitoring the serum enzyme activities (ALP, ALT and AST) and serum lipid levels (TC, TG, HDL-C, LDL-C and VLDL-C), enhancing the antioxidant enzymes (SOD, GSH-Px, CAT and T-AOC), and decreasing the lipid peroxidation (MDA and LPO). Furthermore, the *in vitro* scavenging results indicated that the inhibition effects of En-MZPS on hydroxyl radicals and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals reached $59.98 \pm 6.29\%$ and $37.01 \pm 2.15\%$, respectively. These conclusions demonstrated that the En-MZPS might be suitable for functional foods and natural drugs in preventing the HFHCM-induced hyperlipidemia and non-alcoholic fatty liver.

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1. Introduction

The hyperlipidemia, the most common lipid metabolic disturbance disease, has been incriminated as an important factor in inducing fatty liver, hypertension, atherosclerosis and cerebrovascular disease (Zheng et al., 2014). Clinical studies have demonstrated that taking too much high-fat or high-cholesterol food/diet is a well-known reason in metabolic dysfunctions, resulting hyperlipidemia, liver cirrhosis and even hepatocellular carcinoma (Yang, Yang, Guo, Jiao, & Zhao, 2013). The academic literatures have shown that these diseases are evidenced to be accompanied with the ultra-production of oxygen free-radicals, or generally known as reactive oxygen species (ROS) such as superoxide anion and hydroxyl radical (Bruck et al., 2004; Liang et al., 2011; Yang et al., 2013). It is believed that antioxidant agents are helpful for the treatment and prevention of these disorders because the antioxidants can scavenge the harmful active free radicals in body cells and reduce the potential mutations (Fu et al., 2010). Accumulating evidences had indicated that the hepatoprotective effects of substance may be linked to their known antioxidant and pre-oxidant properties. Hence, there is a strong need for safe and effective oral hepatoprotective agents that provide an alternative option for preventing and treating hyperlipidaemia and its complications.

In recent years, natural mushrooms polysaccharides, widely extracted from the fermentation broth, mycelia, and fruiting body during the mushroom cultivation, have received more and more academic attentions owing to their abundant sources of nutraceutical and pharmacological potentials such as antioxidant, anti-inflammatory (Im, Nguyen, Shin, Lee, & Lee, 2014), anti-aging (Zhang et al., 2014), antihypertensive (Miyazawa, Okazaki, & Ohga, 2008) and immunomodulatory activities (Cui et al., 2015). *Pleu-*
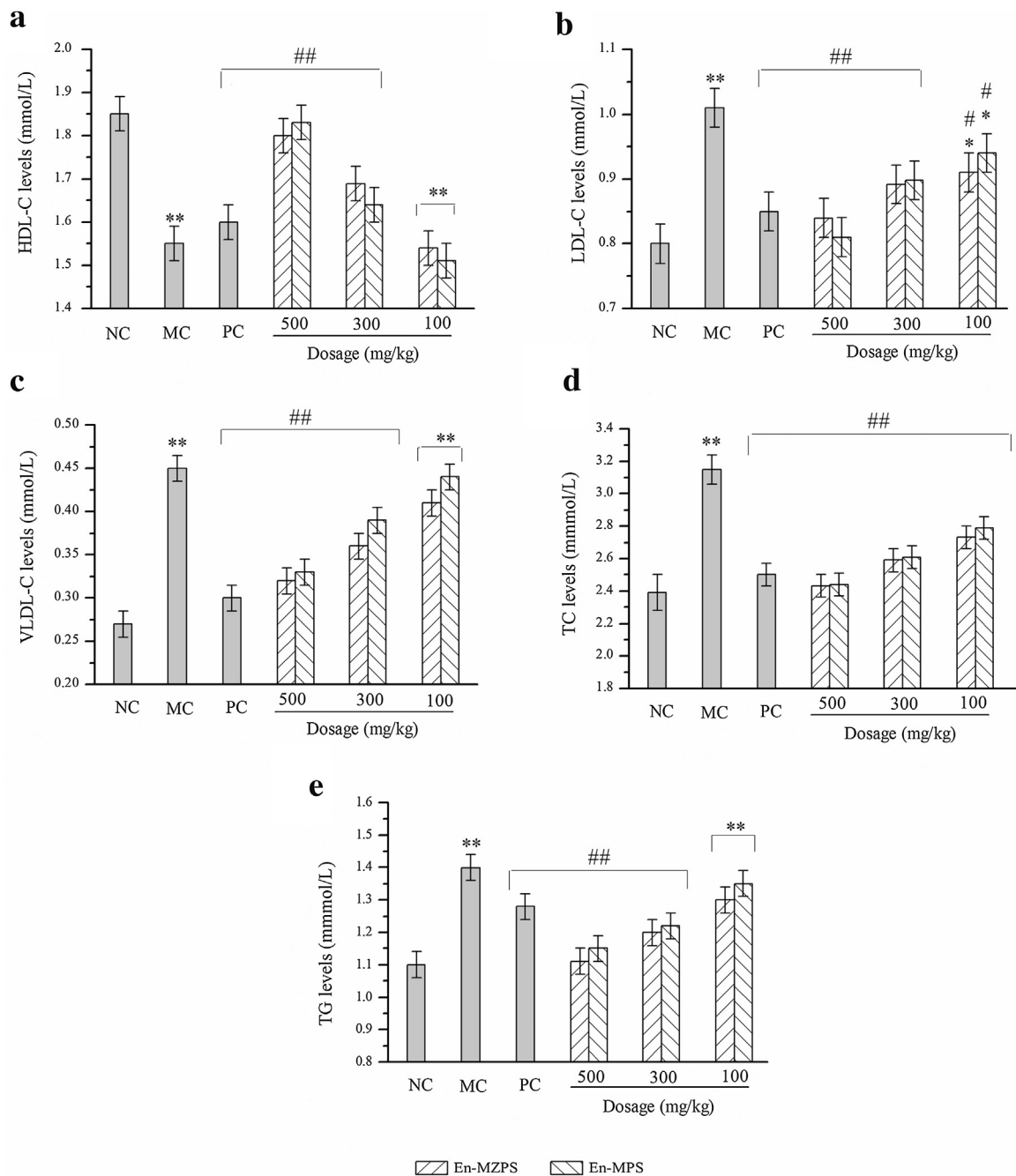
Fig. 1. Effects of En-MPS and En-MZPS on serum lipid in hyperlipidemic mice. (a) HDL-C levels, (b) LDL-C levels, (c) VLDL-C levels, (d) TC levels and (e) TG levels. (**) $P < 0.01$ and (*) $P < 0.05$ compared with NC groups; (##) $P < 0.01$ and (#) $P < 0.05$ compared with MC groups.

rotus eryngii var. tuoliensis, an edible and medicinal mushroom belongs to the family *Pleurotaceae* in the phylum *Basidiomycota*, has been commonly used as a functional food to promote health and longevity (Zheng et al., 2014; Zhang et al., 2014; Miyazawa et al., 2008; Cui et al., 2015; Lv et al., 2009; Zhang et al., 2015; Irudayaraj, Sunil, Duraipandiyan, & Ignacimuthu 2013). As the best known and most potent mushroom-derived substances, polysaccharides extracted from the fruiting bodies of *P. eryngii* var. *tuoliensis* showed beneficial immunomodulatory, antioxidant activities, as well as protective effects on hepatocytes (Miyazawa et al., 2008; Lv et al., 2009). Furthermore, as for the extraction of polysaccharides, the enzymatic-extractable extraction is undoubtedly an emerging technology with many advantages such as high extraction yield and reproducibility, lower investment costs and energy requirements, and simplified manipulations (Jin, Yang, Hu, Shen, & Zhao, 2012). Taken together, it is quite necessary and significative to explore the hepatoprotective effects and antioxidant activity of the enzymatic-extractable polysaccharides extracted from the zinc-enriched mycelia of *P. eryngii* var. *tuoliensis* in preventing the HFHCE induced hyperlipidemia.

The aim of present work was conducted to investigate the hepatoprotective effects and antioxidant activities of enzymatic-extractable mycelia zinc polysaccharides (En-MZPS) from *P. eryngii* var. *tuoliensis* on hyperlipidemic mice induced by HFHCE for seeking possible hypoglycemic mechanisms and their health benefits in food and pharmaceutical industry. Furthermore, primary monosaccharide compositions of En-MZPS were also processed.
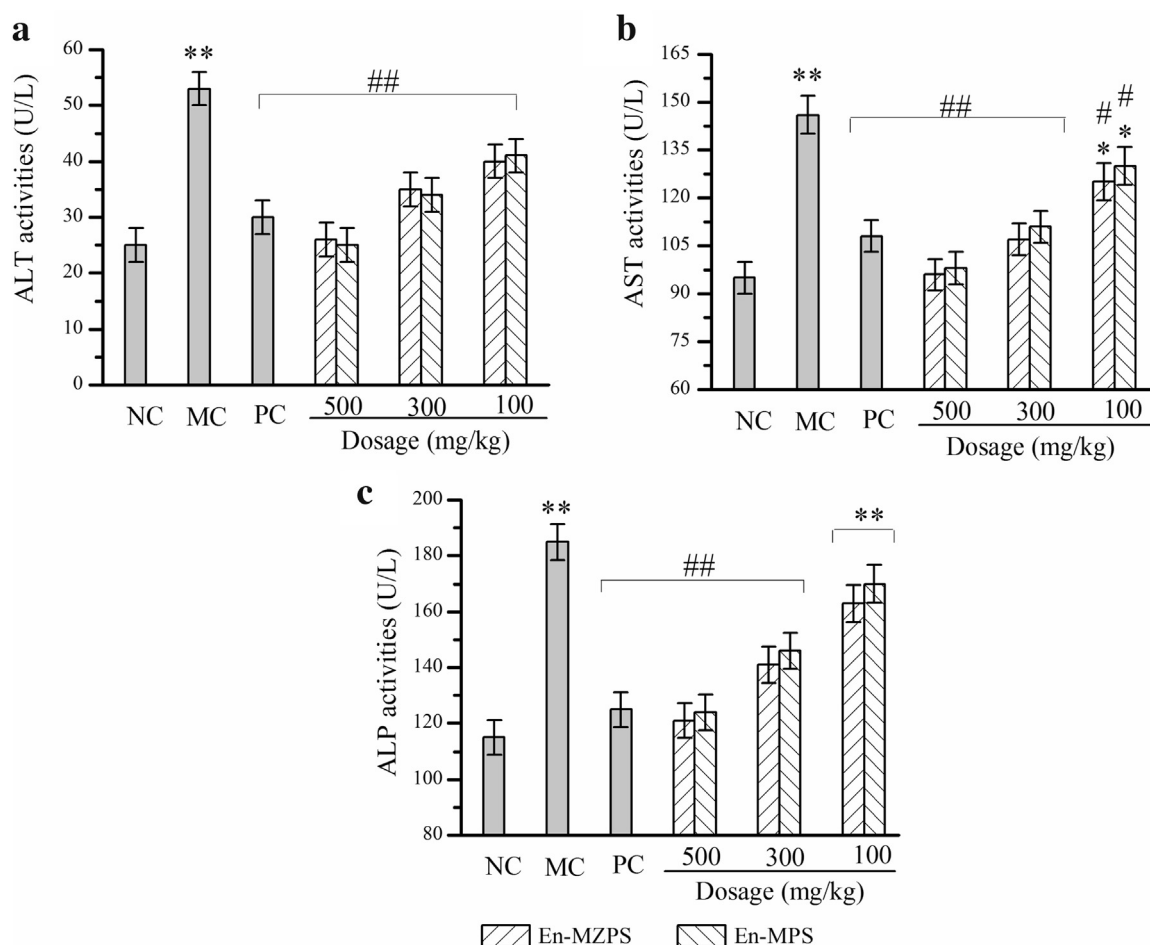
Fig. 2. Effects of En-MZPS and En-MPS on serum enzyme activities in hyperlipidemic mice. (a) ALT activities, (b) AST activities, and (c) ALP activities. (**) $P < 0.01$ and (*) $P < 0.05$ compared with NC groups; (##) $P < 0.01$ and (#) $P < 0.05$ compared with MC groups.

2. Materials and methods

2.1. Fermentation and chemicals

The strain of *P. eryngii* var. *tuoliensis* used in this experiment was provided and identified by Fungi Institute of Academy of Agricultural Sciences (Taian, China), and the fermentation was applied on a fermentation cylinder (Luoyang, China) with the liquid medium of glucose (20 g/L), potato (200 g/L), KH_2PO_4 (1.5 g/L), $MgSO_4$ (1 g/L), and zinc acetate (0.5 g/L). Standard monosaccharide samples were purchased from Sigma Chemicals Company (St. Louis, USA). The reagents for assaying superoxide dismutase (SOD), glutathione peroxide (GSH-Px), catalase (CAT), total antioxidant activity (T-AOC), malondialdehyde (MDA) and lipid peroxidation (LPO), and the diagnostic kit for the total antioxidant activity (T-AOC) were purchased from Nanjing Jiancheng Bioengineering Insititute (Nanjing, China). All other chemicals used in present work were analytically grade and purchased from local chemical suppliers in China.

2.2. Preparation of En-MZPS

The En-MZPS was prepared according to our published literature (Zhang et al., 2015). After 7 days of fermentation, the dried mycelia were mixed with three-fold volumes of snailase solution (4%) for 4 h at 37 °C (w/v). The supernatant homogenate (pH 7) was precipitated with three volumes of ethanol (95%, v/v) overnight (4 °C). The precipitate was collected by centrifugation (3000 r/min, 10 min), and deproteinated by employing the Sevage

method (Miao et al., 2013). Finally, the En-MZPS and enzymatic-extractable mycelia polysaccharides (En-MPS) were obtained by lyophilization and used for further experiments.

2.3. Animal experiments

The Kunming strain male mice weighing 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 experiments were performed as approved by the institutional animal care and use committee of Shandong Agricultural University, and in accordance with the Animals (Scientific Procedures) Act. 1986 (amended 2013).

After a 7-day acclimatization period, all animals were randomly distributed into six dosage groups (10 in each group) including En-MZPS groups (500, 300, 100 mg/kg) and En-MPS groups (500, 300, 100 mg/kg), as well as three control groups including 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) (Wang et al., 2015). During the experiment procedure, the gavage of HFHCE and polysaccharides in dosage groups were processed every other day, using distilled water in NC and MC groups, and simvastatin (200 mg/kg) in PC
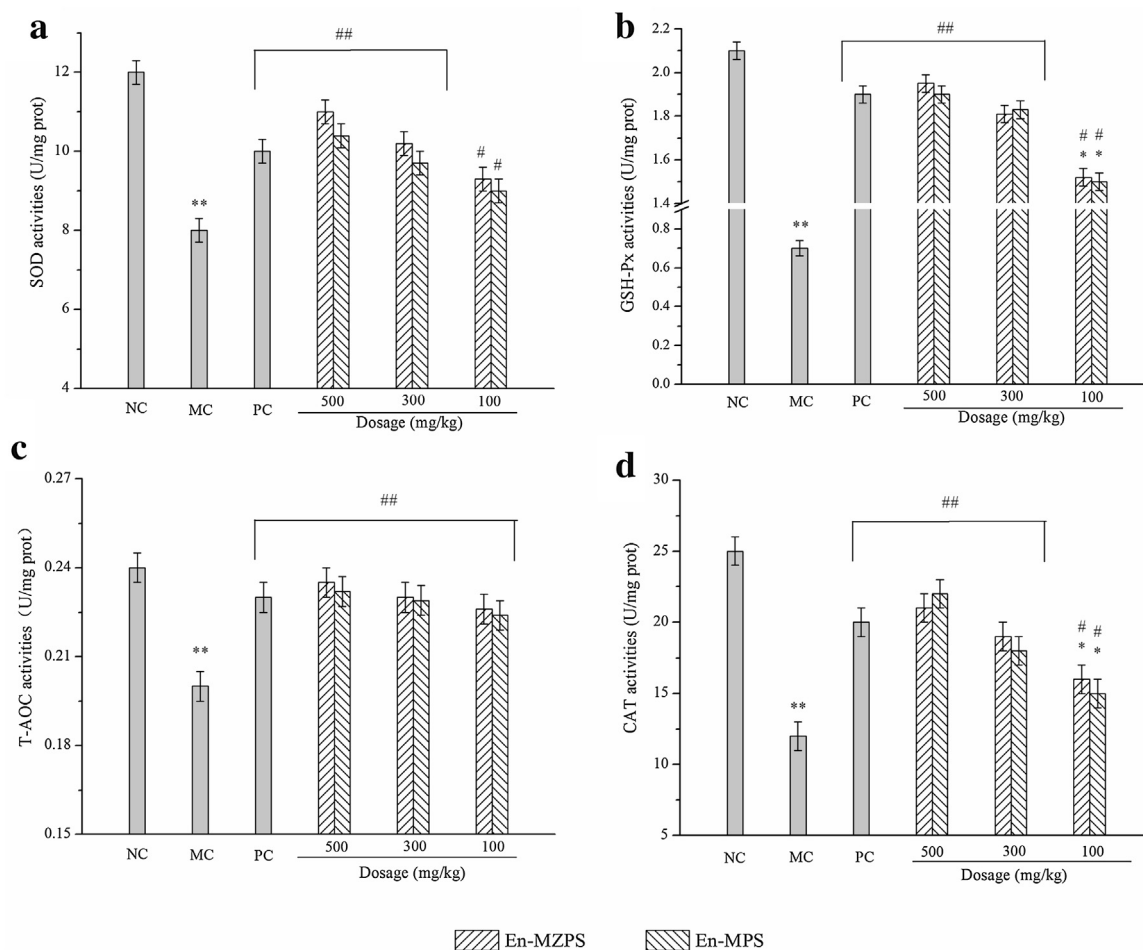
Fig. 3. Effects of En-MPS and En-MZPS on hepatic SOD (a), GSH-Px (b), T-AOC (c) and CAT (d) activities in hyperlipidemic mice. (**) $P < 0.01$ and (*) $P < 0.05$ compared with NC groups; (##) $P < 0.01$ and (#) $P < 0.05$ compared with MC groups.

groups as control. The whole treatment was lasted for 40 consecutive days.

At the end of the experiment, all mice were overnight fasting, and sacrificed by exsanguinations under diethyl ether anesthesia. The serum was separated from blood by centrifugation (12,000 r/min, 10 min). The tissue of liver was immediately excised, weighed and homogenized (1:9, g/mL) in phosphate buffer solutions (PBS, 0.2 M, pH 7.4). The homogenates were centrifuged (3000 r/min, 4 °C) for 20 min, and the supernatants were collected for further analysis.

2.4. Determination of serum lipid

The alkaline phosphatase (ALP) activities, alanine aminotransferase (ALT) activities, aspartate transaminase (AST) activities, total cholesterol (TC) levels, triacylglycerols (TG) levels, high-density lipoprotein cholesterol (HDL-C) levels, low-density lipoprotein cholesterol (LDL-C) levels and very low-density lipoprotein cholesterol (VLDL-C) levels in serum were measured using automatic biochemical analyzer (ACE, USA).

2.5. Antioxidant activities in vivo

The SOD activity was measured according to the method of Hong, Wu, Ma, Liu, & He (2009). The SOD activity was calculated by Formula (1) and 1 U/mg prot was expressed as 50% inhibition of

photochemical reduction of the substance (Nitroblue tetrazolium, NBT) in relative units per milligram protein.

$$SOD\ activity\,(U/mgprot) = \frac{A_0 - A_s}{A_0} \times \frac{1}{2} \times N \qquad (1)$$

Where A_0 was the absorbance of PBS, A_s was the absorbance of samples, and N was the constant dilution ratio (20), respectively.

The GSH-Px activity was analyzed by the reported method of Flohé and Günzler (1984) and 1 U/mg prot of GSH-Px was expressed as mM of NADPH oxidized per minute per milligram of protein.

The CAT activity was measured by the method of ammonium molybdate colorimetry (Kong, 2011). And the CAT activity was calculated in terms of μM of H_2O_2 consumed/(min mg) protein by the following Formula (2).

$$CAT\ activity\,(U/mgprot) = \frac{A_1 - A_2}{A_0} \times \frac{60 \times 2}{0.5} \qquad (2)$$

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 hepatic T-AOC activities were analyzed using commercial kits.

The LPO contents were measured according to the methods reported by Zuo (2009), and the contents of LPO were calculated using the Formula (3).

$$LPO\ contents\,(mmol/g\ prot) = \frac{A_i}{A_j} \times 40 \qquad (3)$$

510 nm. The hydroxyl radical scavenging ability was calculated by Formula (5).

$$Scavenging\ ability(\%) = (1 - \frac{A}{A_0}) \times 100\% \qquad (5)$$

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

The DPPH scavenging activities were measured according to our method of Ma et al. (2015) established by Brand-Williams, Cuvelier, & Berset (1995) and Kong, Mat-Junit, Aminudin, Ismail, & Abdul-Aziz (2012). The reaction mixture contained 2.0 mL polysaccharide sample and 2.0 mL DPPH solution (0.2 mM) or ethanol (95%, w/v). The mixtures were well-mixed and left still for 30 min in the dark. The absorbance of the mixture was determined at 517 nm. The scavenging ability was calculated by Formula (6).

$$Scavenging\ ability(\%) = (1 - \frac{A}{A_0}) \times 100\% \qquad (6)$$

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

2.8. Monosaccharide composition analysis

The determination of monosaccharide compositions was analyzed by gas chromatography (GC, GC-2010, Shimadzu, Japan) equipped with a capillary column of Rtx-1 ($30 \times 0.25 \times 250$ mm) using the reported method (Sheng et al., 2007). The relative molar ratios of monosaccharide were investigated by the area normalization method according to the standard chromatograms of xylose, arabinose, glucose, rhamnose, galactose, ribose, inositol, and mannose.

2.9. 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 while $P < 0.05$.

3. Results

3.1. Effects of En-MZPS on body weight and liver index

Table 1 showed the changes in body weight and liver index of different groups of experimental mice. As shown in Table 1, the difference between the initial body weight and the weight at the end of the experiment was remarkably. There were no significant differences for initial body weight among nine groups. 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, $P = 0.0021$). The mice treated by En-MZPS were less weight gain than that treated by En-MPS, compared to the MC group the high dose group of En-MZPS (35.44 ± 0.70 g, $P = 0.0073$) and En-MPS (34.40 ± 0.67 g, $P = 0.0013$) the body weight were declined obviously. The results indicated that both En-MZPS and En-MPS had potential contribution in suppressing the weight gain induced by HFHCE and the effect of En-MZPS was superior to that of En-MPS.

It could be seen in Table 1 that the liver index of the mice in MC groups significantly increased after the HFHCE treatment when compared with that in the NC group ($P = 0.0044$). However, this increase could be mitigated by pretreatment with En-MZPS and En-MPS at their dose concentrations (100, 300, 500 mg/kg), similar to the results of pretreatment with the simvastatin (PC groups) at a dosage of 200 mg/kg.
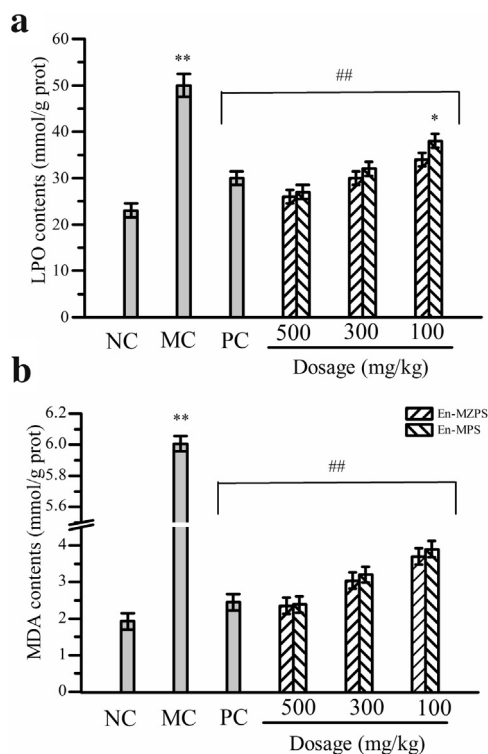

Fig. 4. Effects of En-MPS and En-MZPS on hepatic LPO (a) and MDA (b) contents in hyperlipidemic mice. (**) $P < 0.01$ and (*) $P < 0.05$ compared with NC groups; (##) $P < 0.01$ and (#) $P < 0.05$ compared with MC groups.

Where A_i was the absorbance of samples, and A_j was the absorbance of 1,1,3,3-tetraethoxypropane (TEP).

The content of MDA was measured by the method of Zhao, Shi, and Dong (2002), and the content of MDA was calculated by the Formula (4).

$$MDA\ contents(mmol/gprot) = 6.45 \times (OD_{532nm} - OD_{600nm}) \times OD_{450nm} \qquad (4)$$

2.6. Morphologic observation

The fresh liver tissue masses (4–5 μm thickness) were fixed in 4% formaldehyde solution (pH 7.4) overnight, embedded in paraffin, and stained with hematoxylin-eosin (HE). Each section was photo-graphed under microscope showing the histopathological changes ($\times 400$ magnifications).

2.7. Antioxidant activity in vitro

Reducing power was determined according to the method with slight modifications established by Oyaizu (1986). Each one milliliter polysaccharide samples were mixed with 2.5 mL PBS (pH 6.6, 0.2 M) and 1.0 mL potassium ferricyanide solution (1%, w/v). The mixture was water-bath heated for 20 min at 50 °C. After the reaction was cooled in flowing water, the reaction was terminated via adding 2.0 mL of trichloroacetic acid (10%, w/v), and 1.2 mL ferric chloride (0.1%, w/v) were added subsequently and the mixture was kept warm for 20 min at room temperature. The absorbance of the reaction mixture was measured at 700 nm using spectrophotometer.

The hydroxyl radical scavenging was measured according to the method of Smironff and Cumbes (1989) with little modifications. The reaction mixture contained 1 mL ferrous sulfate (9 mM), 1 mL salicylic acid (9 mM) and 1 mL hydrogen peroxide (8.8 mM, v/v). After addition of 1 mL polysaccharide samples, the mixture was incubated at 37 °C for 30 min, and the absorbance was measured at

3.2. Effects of En-MZPS on serum lipids levels

As shown in Fig. 1, mice treated with HFHCE (MC groups) showed early liver damage as evidenced by significant decrease of HDL-C levels ($P = 0.0080$), 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 after 40 days of gavage with En-MZPS ($P = 0.0036$) and En-MPS ($P = 0.0131$) at three dosages. The VLDL-C, TC, and TG levels in groups of En-MZPS at 500 mg/kg reached 0.32 ± 0.03 (Fig. 1c), 2.43 ± 0.17 (Fig. 1d), and 1.11 ± 0.13 mmol/L (Fig. 1e), which were 28.33, 22.75, and 20.71% than that in MC groups (with all $P < 0.01$), and 2.32, 0.41, and 4.14% lower than that in En-MPS groups at 500 mg/kg (with all $P > 0.05$), while the LDL-C levels reached 0.69 mmol/L (Fig. 1b), which were 8.13% lower than that in MC groups ($P = 0.0034$), but 32% higher than that in En-MPS groups at 500 mg/kg ($P > 0.05$). Meanwhile, the HDL-C levels was 1.83 mmol/L at the same dosage treatment, with 18.38% significant higher than that in MC groups, but 1.94% non-significant lower than that treated with En-MPS at the same dosage (Fig. 1a). These results indicated that En-MZPS was superior to En-MPS in antihyperlipidemic. In addition, simvastatin (PC group) at a dosage of 200 mg/kg also demonstrated significant hepatoprotective effects.

3.3. Effects of En-MZPS on ALT, AST, and ALP activities

Enzyme activities in serum were commonly used for the investigation of early liver damage. As displayed in Fig. 2, significant increase of ALT, AST, and ALP activities were observed in mice after treating with HFHCE (MC groups), indicating that liver damage had been occurred. As shown in Fig. 2a, the ALT activities reached 24.62 ± 3.15 U/L in En-MZPS groups at the dosage of 500 mg/kg, with 53.58% lower than that in MC groups ($P = 0.0052$), but 5.38% higher than that in En-MPS groups (500 mg/kg, $P > 0.05$). As for AST and ALP, it showed that the AST and ALP activities were 96.01 ± 9.85 and 120.25 ± 3.15 U/L in dosage groups of En-MZPS at 500 mg/kg, which were 33.79% and 35.04% significant lower than that in MC groups, and 2.03% and 3.41% non-significant lower than that in dosage groups at 500 mg/kg (Fig. 2b and c). These results testified that the alleviated effects in serum enzyme activities of En-MZPS surpassed that of En-MPS.

3.4. Effects of En-MZPS on SOD, GSH-Px, CAT, and T-AOC activities

The decline of several enzyme activities (SOD, GSH-Px, and CAT) and non-enzyme activity (T-AOC) were commonly used as biochemical marker for monitoring early oxidative stress *in vivo*. As shown in Fig. 3, significant decreases in the hepatic activities of SOD, GSH-Px, CAT and T-AOC were observed in HFHCE-induced hyperlipidemia mice as compared to the NC group ($P = 0.0026$), respectively, indicating that early damage had been occurred in liver.

As shown in Fig. 3a, in the dosage groups of 500 mg/kg treated with En-MZPS ($P = 0.0070$), the SOD activities reached 11.02 ± 0.15 U/mg prot ($P = 0.0013$), with 8.98% higher than that of groups treated with En-MPS at the dosage of 500 mg/kg and 37.51% higher than that of the MC group (8.03 ± 0.12 U/mg prot, $P = 0.0028$). Furthermore, the hepatic SOD activity in the mice treated with En-MZPS at the dosage of 500 mg/kg was approximate to the NC groups (11.41 ± 0.24 U/mg prot).

In present study, the hepatic GSH-Px activities of dosage groups treated with En-MZPS and En-MPS at dosage of 500 mg/kg reached 1.95 ± 0.15 and 1.91 ± 0.14 U/mg prot, respectively, which was 78.57% and 72.86% higher than that of the MC group (0.7 ± 0.11 U/mg prot, $P < 0.0001$) (Fig. 3b). Furthermore, the hepatic GSH-Px activity in the mice treated with En-MZPS at the dosage of 500 mg/kg was more 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, $P = 0.0314$).

Fig. 3c showed that the CAT activities in the mice treated with En-MZPS and En-MPS at the dosage of 500 mg/kg reached 22.12 ± 0.15 and 21.03 ± 0.14 U/mg prot, higher than that of the MC groups (12.07 ± 0.44 U/mg prot, $P = 0.0006$), respectively, and were almost approximate to the NC groups (25.23 ± 0.24 U/mg prot).

For analysis of the T-AOC activities in dosage groups treated with En-MZPS and En-MZPS, as illustrated in Fig. 3d, it reached the maximum of 0.235 ± 0.07 U/mg prot and 0.232 ± 0.05 U/mg prot at the highest doses, 17.51% and 16.04% higher than that of the MC group (0.70 ± 0.11 U/mg prot, $P = 0.0023$). The activity of En-MZPS at dose of 500 mg/kg was better than the PC group (0.20 ± 0.07 U/mg prot, $P = 0.0343$).

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

3.5. Effects of En-MZPS on LPO and MDA contents

The products of lipid peroxidation (LPO and MDA) were also investigated. As illustrated in Fig. 4, HFHCE significantly improve LPO and MDA contents in MC groups (50.03 ± 7.15, 6.007 ± 1.32 mmol/g prot) compared with that in NC group (23.10 ± 3.21, 1.93 ± 0.42 mmol/g prot, all $P < 0.01$). The hepatic LPO contents of mice treated with En-MZPS and En-MPS at 500 mg/kg reached 26.38 ± 4.15, and 27.18 ± 4.33 mmol/g prot, with about 48.06%, and 46.25% lower than that of MC groups (Fig. 4a, $P = 0.0005$), while the hepatic MDA contents reached 2.35 ± 0.02 and 2.39 ± 0.01 mmol/g prot, which were 60.88% and 60.21% lower than that of MC groups (Fig. 4b, $P = 0.0044$), respectively. Moreover, simvastatin-treated mice also demonstrated significant protective against HFHCE ($P = 0.0007$).

These data indicated that both En-MZPS and En-MPS had potential anti-hyperlipidemia effects by improving SOD, GSH-Px, CAT, and T-AOC activities, and reducing MDA and LPO contents in the liver of mice.

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

Groups	Body weight (g)		Liver index (%)
	Initial body weight	Final body weight	
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
En-MZPS			
100 mg/kg	31.04 ± 0.46	$37.08 \pm 0.65^{*}$	$7.11 \pm 0.32^{**}$
300 mg/kg	30.58 ± 0.51	$35.84 \pm 0.69^{†}$	$5.44 \pm 0.28^{†}$
500 mg/kg	31.90 ± 0.49	$35.44 \pm 0.70^{***}$	$4.97 \pm 0.30^{***}$
En-MPS			
100 mg/kg	31.18 ± 0.48	$38.35 \pm 0.71^{*}$	$7.17 \pm 0.31^{**}$
300 mg/kg	30.72 ± 0.49	$36.32 \pm 0.68^{†}$	$5.32 \pm 0.28^{†}$
500 mg/kg	30.68 ± 0.53	$34.40 \pm 0.67^{***}$	$4.87 \pm 0.29^{***}$

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.
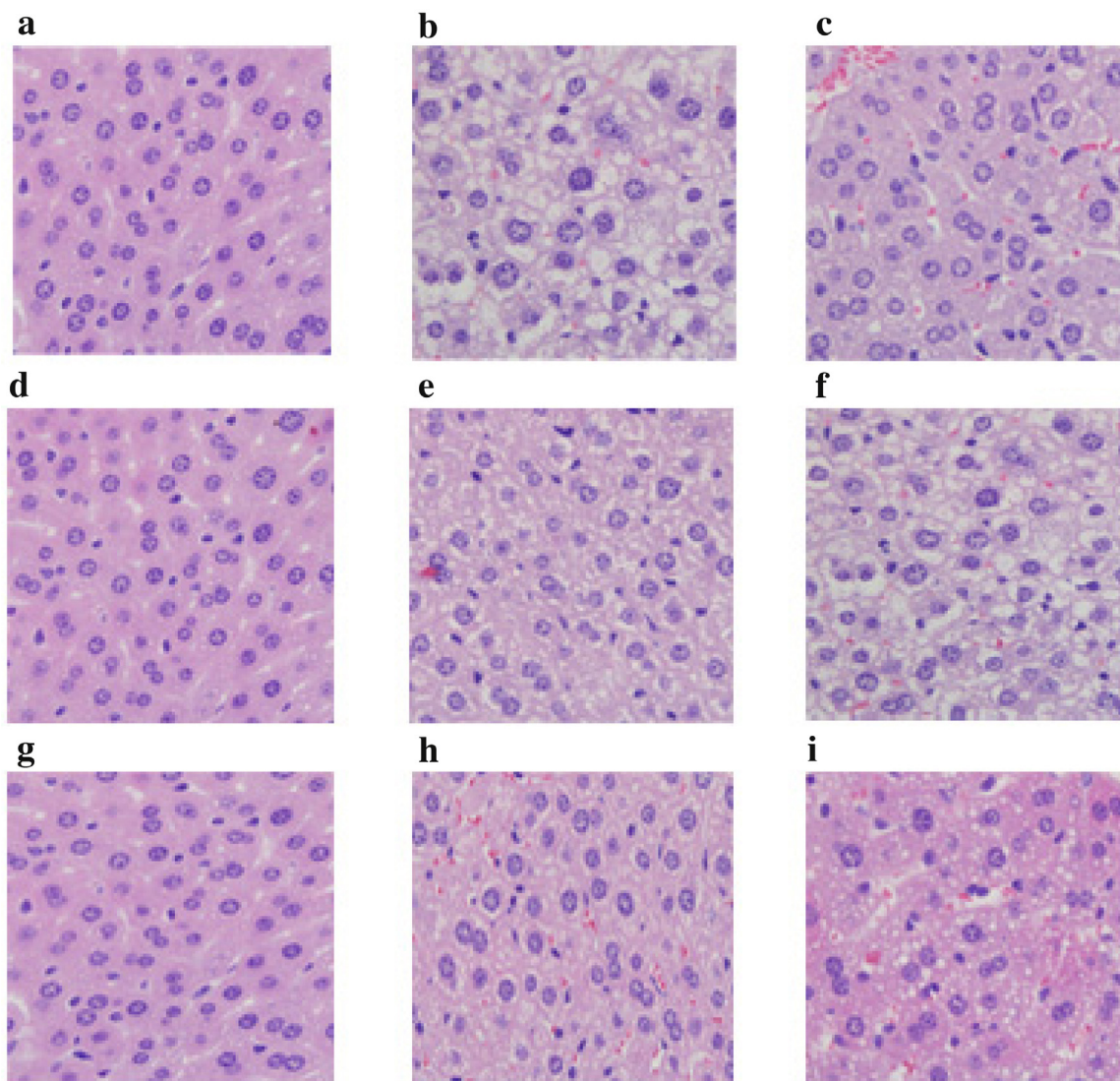[†] $P < 0.05$ compared with MC groups.

Fig. 5. 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-f) liver of mice fed with En-MPS at dosage of 500, 300 and 100 mg/kg, and (g-h) liver of mice fed with En-MZPS at dosage of 500, 300 and 100 mg/kg, respectively.

3.6. Histopathological analysis

In the current study, the histopathological observations, corroborating the evidence from biochemical analyses, were observed by optical microscope (×400) and shown in Fig. 5. Obviously, the control mice had a normal hepatic architecture with normal hepatocyte morphology and orderly arranged hepatic cell cords, showing no symptoms of fat degeneration (Fig. 5a). While the HFHCE-induced hyperlipemia liver samples showed pathological changes including the swelling liver cells, large fat vacuoles accumulation, and the disappear of nuclei, demonstrating that the obvious hepatic steatosis, necrosis, inflammatory, and vesicular degeneration were occurred in the liver (Fig. 5b). Changes on the structures of the hepatocyte in the dosage groups could be seen in Fig. 5d–i. Significantly, after the intervention of En-MZPS, the fat vacuoles and hepatocyte degeneration were reduced and decreased, respectively. Especially in the high-dose group of En-MZPS, the hepatic architecture was even similar to the normal hepatic architecture (Fig. 5g). Meanwhile, the simvastatin treatment had the similar restoring effects on the HFHCE-induced pathological changes (Fig. 5c). These results indicated that En-MZPS had obviously inhibition on HFHCM induced morphologic changes.

3.7. Antioxidant capacities in vitro

In this experiment, to analyze the antioxidant of En-MZPS and En-MPS *in vitro*, three parameters including reducing power, DPPH radicals scavenging ability and hydroxyl radicals scavenging ability were investigated and the results were shown in Fig. 6.

As shown in Fig. 6a, during the concentration of 0–1000 mg/L, a concentration-dependent manner of reducing power was observed. Apparently, at the concentration of 1000 mg/L the reducing power of En-MZPS reached 0.911 ± 0.05, which was 54.12% higher than that of En-MPS (0.418 ± 0.01, $P = 0.0002$). The results indicated that the En-MZPS had stronger reducing power than En-MPS *in vitro*.

It could be seen from Fig. 6b that the DPPH radicals scavenging abilities dose-dependently increased with the increasing concentration of the components. Both of the components had strong hydroxyl radical scavenging abilities, and the scavenging ability of En-MZPS reached $37.01 \pm 2.15\%$ at the concentration of 1000 mg/L, with 16.04% higher than that of En-MPS ($20.97 \pm 1.89\%$, $P = 0.0018$). The DPPH scavenging results revealed that En-MZPS and En-MPS probably contained substances that were hydrogen donors and
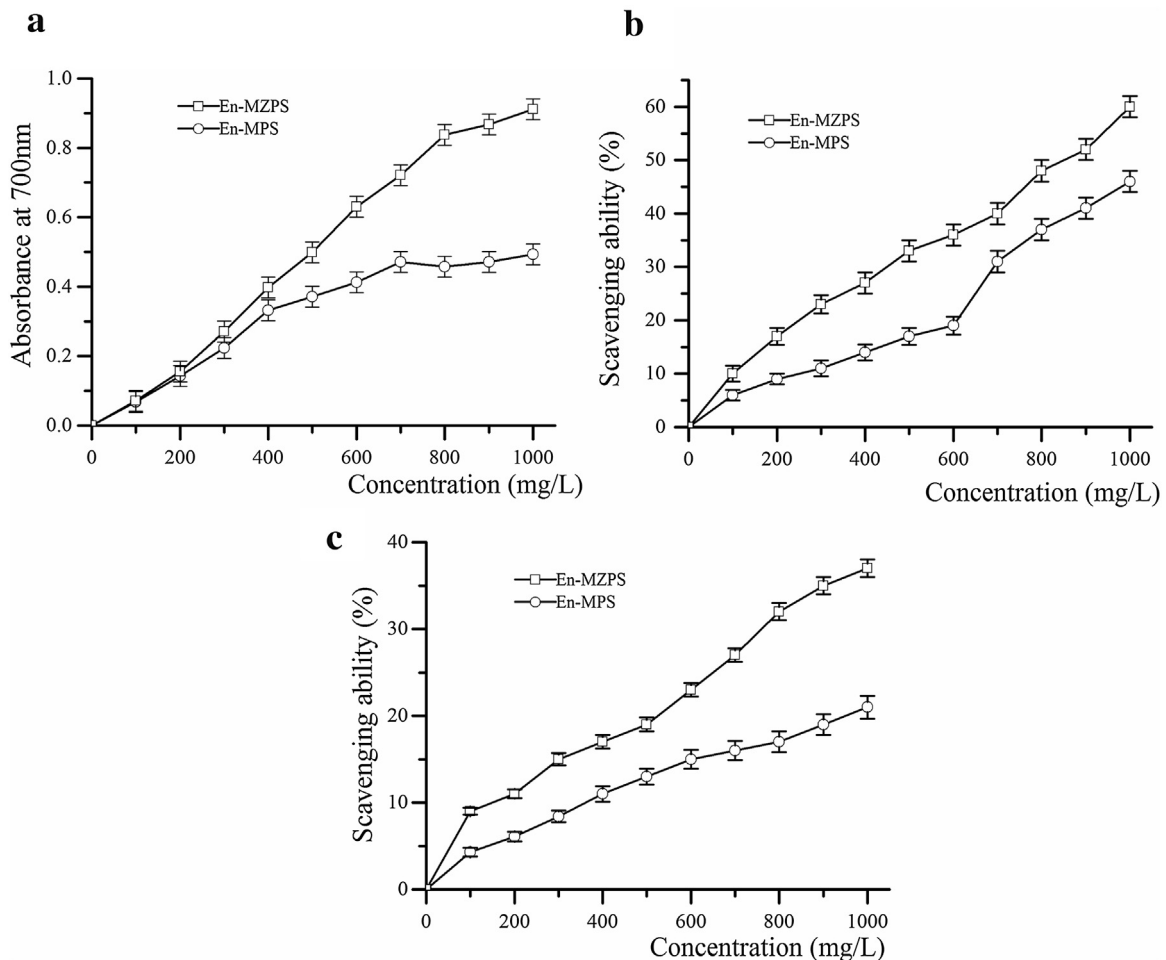
Fig. 6. Antioxidant activities of En-MPS and En-MZPS *in vitro*. (a) reducing power, (b) scavenging abilities on DPPH, and (c) scavenging abilities on hydroxyl radical.

could react with free radicals to convert them to stable diamagnetic molecules.

As displayed in Fig. 6c, both En-MZPS and En-MPS expressed dose-dependent hydroxyl radical scavenging abilities. When the polysaccharides concentrations were raised from 0 to 1000 mg/L, the hydroxyl radicals scavenging abilities increased from 0 to 59.98 ± 6.29% for En-MZPS and from 0 to 46.02 ± 5.43% for En-MPS, indicating that En-MZPS expressed stronger hydroxyl radicals scavenging abilities than that of En-MPS (P = 0.0006). Our data indicated that En-MZPS had stronger antioxidant and oxidative defense mechanism capacities *in vitro*.

3.8. Monosaccharide composition analysis

As identified by comparing the retention time of standards (Fig. 7a), the gas chromatography analysis showed that En-MZPS was composed of arabinose, mannose, galactose and glucose with a mass percentage of 15.62, 13.20, 14.11, and 57.07% with a molar ratio of 1.3:1.1:1.0:4.7 (Fig. 7b), while En-MPS contained three monosaccharides of glucose, galactose, and mannose in a mass percentage of 60.82, 20.08, and 19.10% with a molar ratio of 1.3:1.0:3.8 (Fig. 7c).

4. Discussion

In recent years, enzyme-assisted extraction had been gained more and more academic attention in food industry owing to many advantages of high extraction yield and reproducibility,

lower energy requirements and investment costs, environment-friendly technology, and simplified manipulation as compared with conventional extraction methods of maceration, heat extraction, ultrasound assisted and acidic hydrolysis (Zhang et al., 2015). Meanwhile, it had been reported that the zinc-enriched polysaccharides were superior to regular polysaccharides in possessing antioxidant activities (Zhang et al., 2014). However, little was known about the enzymatic-extractable polysaccharides from *P. eryngii* var. *tuoliensis* submerged with zinc acetate (En-MZPS).

Hyperlipidemia was known to be the leading risk factor for atherosclerosis and cardiovascular diseases. And the HFHCE, acted as chemical agents that caused the disordered circulatory of lipoproteins, had been widely used to induce hyperlipidemia in several animals (Irudayaraj et al., 2013). Clinically speaking, the high serum LDL-C, VLDL-C, TC, and TG levels, as well as low serum HDL-C levels, were fatal in increasing the blood viscosity, which was the premonition of atherosclerosis (Liu et al., 2012). In addition, as the main carrier of TC, the excess LDL-C could be aggregated at the blood vessel walls, leading the formation of atherosclerotic plaque lesion (Liu et al., 2012; Zhu, Nie, Liang, & Wang, 2013). However, as an advantageous lipoprotein, physiological-high HDL-C levels could transport TC from the peripheral tissues to the liver by the "reverse cholesterol transport" pathway for catabolism (Wang et al., 2013). In present study, obviously, both En-MZPS and En-MPS significantly increased HDL-C levels, and decreased LDL-C, VLDL-C, TC, and TG levels. This was an important advantage in the prevention and treatment of hyperlipidemia most prevalently presents as lipoprotein abnormality (Hu, Yang, & Tong, 2005). Ren, Noda,
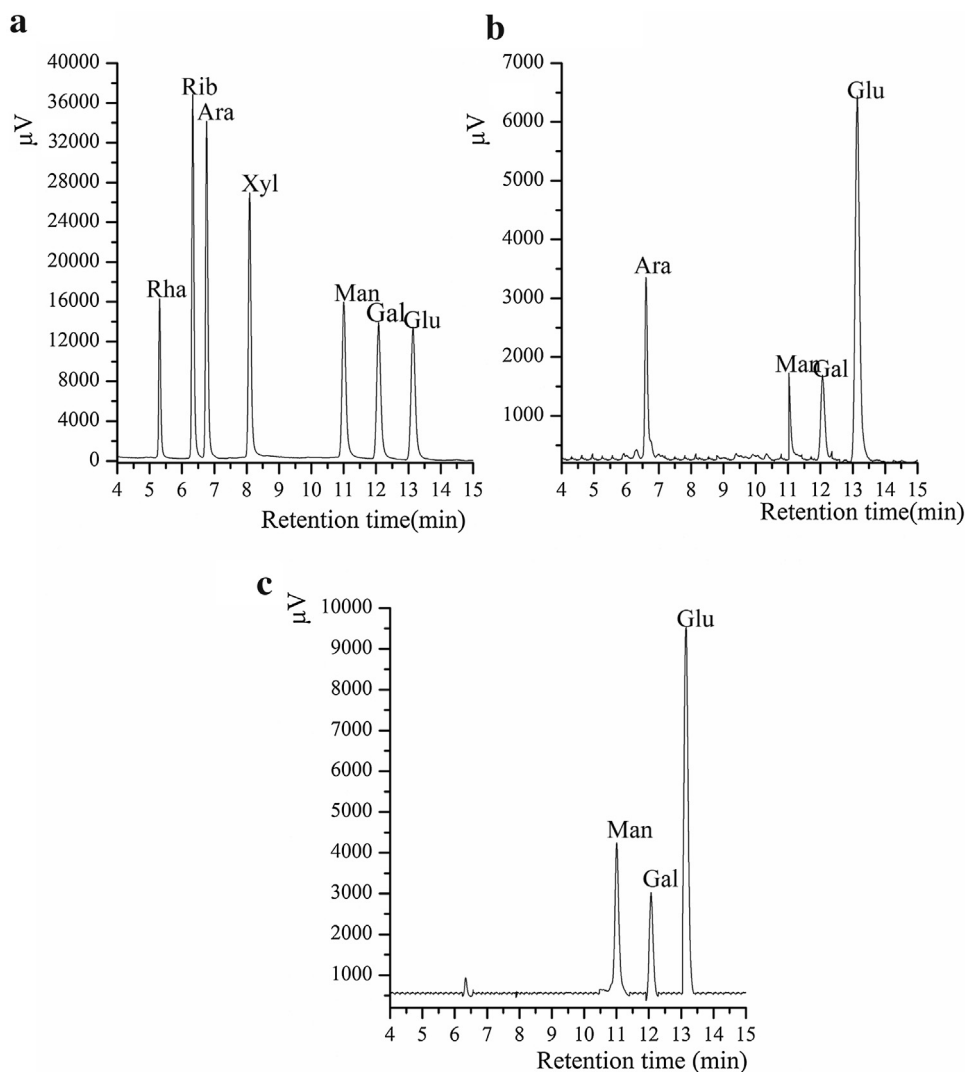
Fig. 7. GC chromatograms of monosaccharides. (a) Standard sugars, (b) En-MPS, and (c) En-MZPS.

Amano, Nishino, & Nisizawa (1994) had proved that the elevated TC and LDL-C were considered to be related with biosynthesis of cholesterol, suggesting that polysaccharides extracted from *P. eryngii* var. *tuoliensis* may combine with lipids and act as a carrier to participate in cholesterol metabolism, accelerating transport and excretion of serum lipids. Furthermore, the serum ALT, AST, and ALP activities, commonly used as biomarkers for liver damage, were markedly heightened when liver damaged occurred. Huang et al. (2012) had reported that the leakage of large quantities of enzymes into the bloodstream was associated with massive centrilobular necrosis, ballooning degeneration, and cellular infiltration of the liver. Currently, the suppression of ALT, AST and ALP activities after treatment with En-MZPS and En-MPS indicated the stabilization of plasma membrane and the repair of hepatic tissue damages caused by HFHCE.

Documented literatures had reported that "oxidative stress" was responsible for many human diseases including liver diseases (Kosecik, Erel, Sevinc, & Selek, 2005). In order to analyze the relationship between antioxidant activities and hepatoprotective effects *in vivo*, the antioxidant enzymes and lipid contents of liver homogenate were investigated. Hong et al. (2009) had demonstrated that the HFHCE could destroy the antioxidant enzyme defenses (Wang et al., 2015; Zheng et al., 2014), thereby allowing reactive oxygen species (ROS) to damage cells and tissues. Antiox-

idant enzymes, such as SOD, GSH-Px, T-AOC and CAT, formed the defense system against ROS in the organism, converting active oxygen molecules into nontoxic compounds (Zhang et al., 2014; Zhang et al., 2015). The mechanism might be that the superoxide radicals could be metabolized to H_2O_2 by SOD, which later could be decomposed to H_2O and O_2 by GSH-Px and CAT, thereby the formation of ROS was prevented (Yao et al., 2005). Besides, Young (2001) had pointed out that the T-AOC activities, which reflected the non-enzymatic antioxidant capacity against various reactive oxygen radicals, could indicate oxidative stress or increased susceptibility to oxidative damage. Therefore, these enzymes acted cooperatively in the scavenging free radicals in the liver. Meanwhile, lipid peroxidation could be very sensitive biomarkers for investigating antioxidant effects of En-MZPS and En-MPS under oxidative stress since lipid peroxidation could lead to hydroperoxide generation to yield products like LPO and MDA (Zhang et al., 2016). In this work, significantly decreases of hepatic SOD, CAT, GSH-Px and T-AOC activities (Fig. 3), and increases of hepatic MDA and LPO contents (Fig. 4) in HFHCE-induced model groups were observed when compared with the control group. Interestingly, Yan, Jing, & Wang (2015) also indicated that the PNPA, a polysaccharides extracted from *P. nebrodensis,* had antioxidant-increasing effects on oxidative rats. Thus, administration of En-MZPS significantly changed the decrease/increase in dosage groups mice

compared to the model control mice, suggested that En-MZPS had antioxidant effects, at least in part, from activation of enzyme activities and suppression of lipid contents. When compared with other polysaccharides, Wu et al. (2010), had proved that the polysaccharides extracted from *Lycium barbarum* had potential inhibition against live oxidative injury of high-fat mice.

On the other hand, the oxidative stress, usually caused by reactive oxygen species (ROS) of hydroxyl radicals, superoxide anion radicals, DPPH radicals and hydrogen peroxide (H_2O_2), played fatal roles in the process of atherosclerosis and cardiovascular diseases (Ma et al., 2015; Zhang et al., 2016). These radicals could cause oxidative damage by oxidizing biomolecules leading to cells death and tissues damage. Thus, the scavenging abilities on the radicals seemed to be important in the evaluation of a natural substance. In present experiment, in order to determine the *in vitro* radical scavenging abilities of En-MZPS and En-MPS, antioxidant evaluations including hydroxyl and DPPH radical scavenging assay and reducing power analysis were processed. As reported by Meir, Kanner, Akiri, & Philosoph-Hadas (1995) 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. The hydroxyl and DPPH radicals had the strongest chemical activity among ROS causing tissue damages. There were also increasing proofs confirming that polysaccharides exerted their positive functions by scavenging free radicals (Chen Zhang, Jiang, Mu, & Miao, 2012; Cui et al., 2008). In this study, En-MZPS exhibited significant antioxidant activity by directly scavenging free radicals, suggesting that En-MZPS extracted from *P. eryngii* var. *tuoliensis* could probably be developed as a potential natural antioxidant in hepatic-protection. Compared with polysaccharides extracted from other *Pleurotus* species, the antioxidant activities of En-MZPS was higher than that isolated from *P. tuberregium* (Wu, Hu, Li, Huang, & Jiang, 2014) and *P. ostreatus* (Vamanu, 2013).

However, it was well-known that the antioxidant properties of polysaccharides were mainly associated with their characterizations such as monosaccharide compositions (Han et al., 2011). In the present study, the monosaccharide compositions of En-MZPS and En-MPS were analyzed. The results demonstrated that the major component of En-MZPS and En-MPS were glucose (Fig. 5b and c). Compared with other literature, Yan, Jing, & Wang also found that the glucose was the major monosaccharide in mycelia polysaccharide (MPS) of *P. nebrodensis*, but Capek, Machová, & Turjan (2009) had reported that galactose had superior abilities of enhancing antioxidant activities. The differences of monosaccharide compositions possibly resulted from different species of *Pleurotus*, the enzyme-assisted extraction technologies and the analysis methods for the polysaccharides.

5. Conclusions

In the present work, a novel enzymatic-extractable zinc polysaccharide (En-MZPS) with major component of glucose was isolated from the mycelia of *P. eryngii* var. *tuoliensis*. The results showed that En-MZPS exhibited impressive prevention effects on the HFHCE-induced hyperlipidemia in mice paralleled with simvastatin as a prophylactic agent, suggesting that En-MZPS from *P. eryngii* var. *tuoliensis* had a potential in the prevention and treatment with hyperlipidemic and liver damage induced by HFHCE.

Competing interests

The authors declare that they have no competing interests.

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