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Proteoglycan isolated from *Corbicula fluminea* exerts hepato-protective effects against alcohol-induced liver injury in mice

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Abstract

Corbicula fluminea (Asian clam), a freshwater bivalve mollusk, has been consumed in China for centuries as a health food and traditional Chinese medicine for treating liver diseases and alcoholism. This study aimed to evaluate the hepato-protective effects and potential mechanisms of a proteoglycan (PSP) from *C. fluminea* on alcohol-induced liver injury in mice. Results showed that PSP pretreatment significantly antagonized the increases in serum alanine aminotransferase, aspartate aminotransferase, triacylglycerides, and hepatic malondialdehyde levels; elevated the antioxidant enzyme activities and hepatic glutathione levels; and suppressed the levels of hepatic inflammatory cytokines in alcohol-induced liver injury in mice (P<0.05). Histopathological observation further revealed the potential hepato-protective effect of PSP against alcohol damage. Particularly, PSP pretreatment resulted in significantly decreased expression of cytochrome P450 2e1 (CYP2E1) while significantly upregulating the expression of hemeoxygenase-1 (HO-1) (P<0.05). These results suggested that PSP could protect the liver from hepatocyte injury induced by alcohol possibly by alleviating hepatic lipid metabolism, elevating antioxidant-enzyme activity, suppressing the immune inflammatory response, and reversing the expression levels of CYP2E1 and HO-1. Therefore, PSP may be developed as a food supplement that can be used to prevent liver diseases.

Keywords: *Corbicula fluminea*; Proteoglycan; Hepato-protective effect

1. Introduction

Alcohol abuse and its associated social consequences have become a major health problem worldwide. Alcohol consumption is related to approximately 3.3 million deaths every year (or 5.9% of all deaths) and to 5.1% of the global burden of disease [1]. In China, due to the change in diet and increased frequency of drinking, the incidence of alcoholic liver damage (ALD) has been gradually increasing per year. In fact, it is becoming the second largest liver disease after viral hepatitis, such as hepatitis A and hepatitis B [2]. ALD is believed to be a progressive disease that can be seriously harmful to people's health in the later stages and may eventually lead to liver cancer [3]. Based on histopathology, ALD is usually involved in a broad spectrum of liver disorders ranging from simple fatty liver to more severe forms of liver injury that present alcoholic hepatitis, cirrhosis, and superimposed hepatocellular carcinoma [4]. Generation of reactive oxygen species (ROS) and oxidative stress have been well recognized to play an important role in the development of ALD. In this context, over the years, many researchers have demonstrated that the hepato-protective effects of natural compounds are often associated with their potent antioxidant activities including reduction of ROS and/or increment of antioxidant enzymes [5, 6]. Moreover, accumulating evidence has revealed that ALD in the early stage was supposed to be reversible [7, 8]. Consequently, it is of meaningful significance to search for effective natural compounds with antioxidant potential for preventing or slowing down the development of ALD in the early stage, and thus becoming the focus of research in recent years.

Polysaccharides (PSs) represent a major class of bioactive molecules derived from natural resources, such as plants, animals, and microorganisms. To date, there is growing

attention to the use of PSs for various biomedical applications, functional foods, and cosmetics due to their prominent and multiple bioactivities, such as immunostimulatory, antitumor, and antioxidant activities, as well as other health benefits [9, 10]. A great number of studies have demonstrated that PSs possessed the excellent efficacy of hepato-protective function and positive therapeutic effect on experimental and chemical liver injury [6, 8, 11-14]. For example, Ozalp et al. reported that daily administration of 50 mg/kg of *Coprinus comatus* polysaccharide extract could greatly ameliorate the evaluated serum alanine transaminase (ALT) and aspartate transaminase (AST) activities in alcohol-treated mice, and considerably reduce the negative effects of alcohol on liver structure and function [15]. Wang et al. found that a homogeneous polysaccharide (DHP) purified from *Dendrobium huoshanense* effectively depressed the increased ratio of liver weight to body weight, reduced the elevated levels of serum AST, total cholesterol, total bilirubin, and low-density lipoprotein, and alleviated hepatic steatosis in mice with alcohol-induced subacute liver injury [8]. Lim and co-workers also demonstrated that supplementation of fucoidan from *Fucus vesiculosus* could effectively suppress the increased serum AST and ALT levels, and significantly reduce the increase in transforming growth factor beta 1 (TGF-β1) expression in alcohol-induced murine liver damage. Moreover, fucoidan significantly attenuated the production of inflammation-promoting cyclooygenase-2 and nitric oxide, but markedly upregulated the expression of the hemeoxygenase-1 (HO-1) on murine liver and HepG2 cells [16]. Taken together, these findings suggest that PSs exert potent protective effects against alcohol-induced liver injury through different underlying mechanisms, and their hepato-protective effects appear to be frequently associated with their antioxidant capacities

and their abilities to accelerate lipolysis and inhibit inflammatory responses. In this regard, it is of great significance to continue the search for bioactive PSs and their derivatives from foods or mushrooms to study their hepato-protective effects and underlying mechanisms against alcohol-induced liver injury.

Corbicula fluminea (*C. fluminea*), commonly known as Asian clam, is a freshwater bivalve mollusk that is mainly native to Asia, especially in China. The freshwater clam has been popularly consumed in East Asian countries and has received considerable attention all over the world due to its excellent edible values and health benefits. Modern pharmacological studies have shown its therapeutic effects on a wide range of diseases and conditions, such as improved appetite and eyesight, treatment of diuresis, liver disease, and measles, fever abatement, cough relief, reduced sputum, and as anti-alcoholism medicine [17, 18]. Moreover, *C. fluminea* has several potent bioactive properties including hepato-protective [17], anti-inflammatory [18], antitumor [19], antioxidant [20], and anti-hypertensive [21] activities. The multiple pharmacological effects of *C. fluminea* can be due to its chemical ingredients, such as PSs, proteins, amino acids, fatty acids, and trace elements. Among of them, PSs and their derivatives are supposed to be one of the major bioactive constituents that have been extracted and separated from *C. fluminea* [19, 20, 22]. In our research, we have recently prepared a proteoglycan (PSP) from *C. fluminea* by using three-phase partitioning (TPP) technique, and the partially purified PSP exhibit notable radical scavenging and antioxidant activities *in vitro* [22]. However, to the best of our knowledge, there are only a few literatures about the hepato-protective activity of PSP and its possible underlying mechanisms.

In this study, the protective effects of pretreatment with PSP from *C. fluminea* against

alcohol-induced hepatic damage in mice by measuring viscera indices, biochemical parameters, and inflammatory cytokines and by evaluating histopathological changes were investigated. Additionally, the possible mechanisms underlying the protective effect of *C. fluminea* PSP based on oxidative stress were also elucidated in this paper.

2. Materials and methods

2.1. Materials and chemicals

C. fluminea was supplied by Suqian Chengzihu Food Co., Ltd., Suqian, Jiangsu, China. The shell of *C. fluminea* was removed and the whole soft body was kept in ice and was transported to the laboratory, stored at -80°C for 48 h, freeze-dried, ground, sieved (60 mesh), and then sealed in airtight plastic bags at 4°C before use.

Monosaccharide standards [glucose (Glc), mannose (Man), galactose (Gal), arabinose (Ara), xylose (Xyl), rhamnose (Rha), glucosamine (GlcN), and galacturonic acid (GalA)], 1-phenyl-3-methyl-5-pyrazolone (PMP), trifluoroacetic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Silymarin (with a purity of ≥98%) was obtained from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). Alcohol in water (56%, v/v) was obtained from Beijing Red Star Co. Ltd. (Beijing, China). Commercial assay kits for alanine transaminase (ALT), aspartate transaminase (AST), triacylglycerides (TG), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), glutathione (GSH) and malondialdehyde (MDA) were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Murine interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Jianglai

Biotech Co. Ltd. (Shanghai, China). Bicinchoninic acid (BCA) protein assay kit, Trizol reagent and phenyl-methylsulfonyl fluoride (PMSF) were purchased from Auragene Biotech Co. Ltd. (Changsha, China). The antibodies to cytochrome P450 2e1 (CYP2E1) (ab151544), and HO-1 (ab68477) were purchased from Abcam (Abcam, Cambridge, UK). The secondary antibodies peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat anti-mouse IgG were obtained from Auragene Biotech Co. Ltd. (Changsha, China). All other chemicals and solvents were of laboratory grade and used without further purification.

2.2. Extraction and isolation of PSP

PSP was extracted and isolated from *C. fluminea* by using enzyme pretreatment and TPP technique as described previously [22]. In brief, the defatted *C. fluminea* powder (100 g) was immerged in 300 mL acetic acid-phosphate buffer (0.1 M, pH 6.0) and treated with 2% (w/w) papain at 60°C for 2 h under continuous stirring. After inactivation, the reaction mixture was centrifuged at 4000 rpm for 15 min, and the supernatant was collected and concentrated under reduced pressure. Subsequently, 20% (w/v) $(NH_4)_2SO_4$ was added to the supernatant and dissolved completely, and then *t*-butanol was added into the mixture at a *t*-butanol to supernatant ratio of 1.5:1.0 (v/v). The extraction was placed in the shaking incubator at 100 rpm and 35°C for 30 min, and centrifuged at 4000 rpm for 10 min for forming clear three phases. The lower aqueous phase was collected, dialyzed (MWCO: 8-12 kDa) against distilled water, concentrated and freeze dried to attain the partially purified PSP.

2.3. Physicochemical characteristics of PSP

The total carbohydrate content was determined via the phenol-sulfuric acid method with glucose as a standard [23]. The protein content was measured by the Bradford method with BSA as a standard [24]. The high-performance gel permeation chromatography was used to determine the molecular weight (MW) and MW distribution (MWD) of the PSP, and details of the instruments and experimental conditions as described previously [25]. Dextran MW standards ranging from 5.2 to 1482 kDa (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were utilized for calibration. HPLC-PMP derivation method was employed to analyze the constituent monosaccharides of the PSP, and details of experimental instruments and operated conditions were detailedly described in a previous study [26]. Monosaccharide standards were used for the identification and quantification of the corresponding peaks. An automated amino acid analyzer (HITACHI L-8800, Amino Acid Analyzer, Japan) was used to determine the amino acid composition of the PSP. Identification and quantification of amino acids were achieved by comparing the retention times of the peaks with amino acid standards [aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cysteine, valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, methionine, histidine, arginine, tryptophan, proline]. The Fourier-transform infrared (FT-IR) spectrum of PSP was obtained with a Nexus 670 FT-IR spectrometer (Thermo Nicolet Co., USA) in the wavenumber ranging from 500 to 4000 cm^{-1} with KBr pellets and then referenced against air, taken as a total of 16 scans with a 4 cm^{-1} resolution. β-elimination reaction was used to detect the linkage types between protein and polysaccharide in PSP as described previously [27]. In brief, PSP (5.0 μg/mL) was dissolved in 0.2 mol/L NaOH containing $NaBH_4$ and then incubated at 25°C. The resulting solution was measured with a Varian Cary 100 spectrophotometer (Varian Co., USA) in wavelength

range of 190-350 nm at 25°C. The PSP (5.0 μg/mL) without alkaline solution treatment was used as a control.

2.4. Animals and experimental design

SPF Kunming male mice (body weight, BW= 20 ± 2 g; 4 weeks old) were purchased from the Laboratory Animal Research Center of Jiangsu University (Zhenjiang, China). All the animals were housed for one week under standard conditions with a 12-h light/dark cycle at a temperature of 23 °C ± 0.5 °C and a humidity of 50% ± 5% before experiments. During the whole experimental period (including acclimation), all animals were allowed free access to commercial standard chow (Jiangsu Synergetic Biological Engineering Co. Ltd., Nanjing, China) and tap water. All animals used in this study were cared in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All experimental procedures involving the use of animals were approved by the Laboratory Animal Management Committee of Jiangsu University, China (Serial No. UJS-LAER-2017032201).

Before applied on alcohol-induced mice, acute oral toxicity was conducted to detect the PSP safety according to a previous study [28]. Single doses of PSP samples (600, 800, 1600, 2400 and 3200 mg/kg BW) were given to the groups of mice (10 animals per group) by gavage. The result showed that there were no significant difference (P>0.05) in BW in the treated mice as compared to the controls and also no any noticeable signs of toxicity and mortality during the whole experiment period (data not shown). Afterwards, the graded doses of PSP (100, 200, 400 and 600 mg/kg BW) was set and the treatment effect was detected to

confirm the doses of PSP applied on alcohol-induced mice. The result demonstrated that the PSP dose of 400 mg/kg BW exhibited better treatment effect than the doses of 100 and 200 mg/kg BW (P<0.05) (data not shown). By contrast, the dose beyond 400 mg/kg BW did not improve the treatment effect. Consequently, the PSP at a dose of 200 and 400 mg/kg BW was selected as a low-dose and a high-dose group for this study, respectively.

According to above preliminary experimental results, mice were randomly divided into five groups (n=10 in each group): (I) the normal control group, (II) the alcohol-treated model control group, (III) the silymarin-positive control group (100 mg/kg BW), (IV) the PSP low-dose group (200 mg/kg BW), and (V) the PSP high-dose group (400 mg/kg BW). Groups I and II were given 5 mL/kg BW physiological saline solution (0.9%, w/v) through gastric gavage. Group III was intragastrically treated with 5 mL/kg BW silymarin. Groups IV and V received with 5 mL/kg BW PSP through gastric gavage. After 4 h, all mice except those in the normal group were administered with 5 mL/kg BW alcohol in water (50%, v/v), whereas the normal group received 5 mL/kg BW physiological saline solution (0.9%, w/v) through gastric gavage. All administrations were conducted once daily for 20 consecutive days.

After 12 h fasting following the last drug administration, the mice were weighed and blood samples were immediately collected from each group in a centrifuge tube (Eppendorf, Germany) without anticoagulation. After 2 h, the serum was collected by centrifugation (3,000 rpm, 10 min) at 4 °C and then stored at −80 °C prior to further analysis. Then, all animals were sacrificed by cervical dislocation. The liver, spleen, and thymus were excised and weighed after washing with ice-cold PSS to calculate the organ indexes (each organ weight as a percentage of BW). A portion of the liver was fixed with 10% paraformaldehyde

for histopathological observation, and the other parts were homogenized in ice-cold PSS and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was then collected and stored at −80 °C for further analysis.

2.5. Biochemical analysis

Serum ALT, AST, and TG levels and the activities of SOD, CAT, and GSH-Px and the GSH and MDA levels in liver samples were measured by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. Cytokines (IL-1β and TNF-α) in the liver were determined with commercially available ELISA kits (Shanghai Jianglai Biotechnology Co. Ltd., Shanghai, China) according to the manufacturer's protocols. The corresponding protein concentration in liver was measured by using a Bradford assay using bovine serum albumin (BSA) as a standard [24].

2.6. Histopathological studies

A portion of fixed liver tissue was embedded in paraffin, cut into transverse sections of 5-μm thickness, and then stained with haematoxylin-eosin (H&E) by using standard techniques. A DM6000B light microscope (Leica, Germany) was used for general histopathology examinations.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the liver samples with Trizol reagent (Guangzhou Dongsheng Biotech Co. Ltd., Guangzhou, China) according to the manufacturer's protocol.

The purity and concentration of RNA were determined according to optical density measurements at 260 nm and 280 nm, respectively. Complementary DNA (cDNA) was synthesized from RNA (2.0 μg) in a 20-μL reaction mixture containing 2-μL DNTP mixture (10 mM), 1-μL RNase inhibitor (40 U/μL), 1-μL reverse transcriptase (200 U/μL), and 1-μL oligo-dT (0.5 μg/μL) at 42 °C for 60 min, and then heated at 70 °C for 10 min. Subsequently, the resultant cDNA was amplified through real-time PCR using a SYBR Green qPCR Mix (Guangzhou Dongsheng Biotechnology Co. Ltd., Guangzhou, China) and ABI 7300 real-time PCR instrument and software (ABI, USA). All quantifications were performed with murine β-actin as the internal reference gene. The primer sequences are listed in Table 1. Relative quantification was performed with the $2^{-\Delta\Delta Ct}$ method according to the previous report [29] and then the results were expressed as the extent of change with respect to control values.

2.8. Western blotting

The liver tissues were homogenized in radio immunoprecipitation assay (RIPA) buffer (Auragene Biotech Co. Ltd., Changsha, China) supplemented with protease inhibitors (Auragene Biotech Co. Ltd., Changsha, China) followed by incubation on ice for 20 min, and then centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatants were collected and protein concentrations were determined using BCA protein assay kit and then stored at −80℃ until further testing. The tissue proteins were subjected to electrophoresis on 10% SDS-PAGE gel and was subsequently transferred to a 0.22-μm nitrocellulose (NC) membrane. After incubation in blocking solution containing 3% BSA in Tris buffer with Tween 20 (TBST) at room temperature for 120 min, the NC membrane was incubated with primary rabbit

anti-CYP2E1 (1:4,000 dilution) and rabbit anti-HO-1 (1:3,000 dilution) overnight at 4 °C. Subsequently, the membranes were washed for five times with TBST and then incubated with goat anti-rabbit immunoglobulin G (IgG) (H&L)-horse radish peroxidase (HRP) (1:15,000 dilution) for 40 min at room temperature. Specific protein bands were visualized with the chemiluminescent reagent (ECL) (Auragene Biotech Co. Ltd., Changsha, China) and quantified using Gel-Pro Analyzer software (Media Cybernetics, Washington, MD, USA) using β-actin as the internal standard.

2.9. Statistical analysis

All experiments are conducted in three replicates and the mean ± standard deviation (SD) is used in the analysis. The statistical analysis was performed by analysis of variance (ANOVA) using OriginPro Software Version 8.0 (OriginLab Corp., MA, USA). $P<0.05$ indicated statistically significant differences.

3. Results and discussion

3.1. Preliminary physicochemical characterizations of PSP

According to the reported results [22], the extraction yield, carbohydrate and protein contents of PSP were determined to be 9.0%, 81.7% and 2.7%, respectively. PSP was an acidic proteoglycan with O-glycosylation mainly containing 14.2% aspartic acid, 16.3% glutamic acid, 14.4% cysteine and 9.0% valine. PSP was mainly composed of Glc, GlcN and Man with a molar ratio of 10.8:4.4:1.0 (Fig. 1b) with a high MW fraction (2113.4 kDa, 27.6%) and a low MW fraction (61.5 kDa, 72.4%) (Fig. 1c). FT-IR spectrum of PSP (Fig. 1d) further

demonstrated that the major characteristic IR peaks at around 3380, 2910, and 1000-1200 cm^{-1} were assigned to the O-H, C-H, C-O-H and C-O-C stretching vibrations, respectively, which is typical of polysaccharide structure. The signals at approximately 1641 cm^{-1} (amide I) and 1543 cm^{-1} (amide II) were attributed to the bending vibrations of amide or amino group ($-NH_2$) and the secondary –CONH- group of proteins, which indicated the protein is covalently present in PSP. In addition, a characteristic peak at about 816 cm^{-1}, which suggested the α-glycosidic bond existed in the PSP. UV spectrum (Fig. 1e) of the alkali-treated PSP showed an relatively obvious absorbance at around 240 nm as compared to the untreated PSP, which suggested that the β-elimination reaction took place and the α-amino acrylic or α-amino butenoic acid were formed [27], further implying the protein and polysaccharide were covalently linked by an O-glycosidic linkages in PSP. Collectively, these results suggested that the PSP was proven to be an acidic proteoglycan, and it was then further subjected to the subsequent *in vivo* hepato-protective potential through alcohol-induced liver injury in mice.

3.2. Effects of PSP on viscera indices

Table 2 shows the effects of PSP on liver, spleen, and thymus indices in alcohol-treated mice. A single application of alcohol led to a significant increase of up to 4.02% ± 0.12% in the liver index from 3.77% ± 0.18% of normal mice (P<0.01). Interestingly, in comparison with alcohol-induced group, PSP pretreatment at the tested concentrations caused a remarkable decrease in liver index of mice (P<0.05). Specifically, pretreatments with PSP at doses of 200 and 400 mg/kg BW showed dramatic decreases of up to 3.87% ± 0.15%, and

3.82% ± 0.13% in liver index (P<0.05), respectively, which were close to that of the silymarin at a dose of 100 mg/kg BW (3.80% ± 0.12%). Results indicated that pre-administration of PSP to mice resulted in the protective effects against alcohol-induced injury. Yang et al. reported that mice treated with water-soluble apple peel polysaccharides (APP) or apple flesh polysaccharides (AFP) from Pink Lady fruits at the high dosage of 500 mg/kg BW caused a significant decrease of up to 5.43% ± 0.88% and 5.35% ± 0.73% in the liver index as compared with that in carbon tetrachloride (CCl_4)-intoxicated mice (5.83% ± 0.35%, P<0.01) [30], which was similar to the results of our present work. As displayed in Table 2, we noted that the spleen and thymus indices showed a significant decrease after administration of alcohol as compared with the normal control group (P<0.05). However, compared with the alcohol model group, pretreatments with PSP and silymarin appeared to have remarkable effects by enhancing the spleen and thymus indices of alcohol-treated mice (P<0.05), which suggested that pretreatment with PSP effectively stimulated the immune functions in alcohol-induced liver injury and can have potentially protective effect against alcohol-caused toxicity.

3.2. Effects of PSP on ALT, AST, and TG in serum

The enzymatic activities of ALT and AST in serum have been commonly regarded as sensitive biomarkers of hepatic injury. Under healthy circumstances, these two enzymes are localized to the cytoplasm and would be released into the blood stream when there is hepatocellular plasma membrane damage, and is thus indicative of liver damage [31]. Thus, to elucidate the effect of PSP on liver function and hepatic lipid metabolism, the activities of

serum ALT and AST, as well as the level of serum TG in different groups, were investigated. As shown in Table 3, the administration of alcohol caused a remarkable elevation in serum ALT and AST levels in mice as compared with that of normal mice (P<0.001), suggesting that alcohol-induced liver injury model in mice was successfully established in the present study. The preventive administration of PSP exhibited a remarkable protective effect on the alcohol-caused liver injury by significantly preventing the elevation of serum levels of ALT and AST in a dose-dependent manner (P<0.05). Specifically, at dosages of 200 and 400 mg/kg BW, the serum levels of ALT and AST decreased by 4.97% and 13.02%, and 10.89% and 17.86%, respectively, when compared with those of alcohol model group, but were less than that of silymarin at a dose of 100 mg/kg BW (34.99% and 22.47%, respectively). Thus, the result indicates that PSP had potential hepato-protective effect against alcohol-induced liver injury in mice. Likewise, Wang et al. found that polysaccharides (HDPS) from the peduncles of *Hovenia duclis* at a dose of 600 mg/kg BW significantly prevented the elevation of ALT and AST levels in the serum of mice with alcohol-induced liver injury, and the serum levels of ALT and AST were reduced by about 36% and 42%, respectively [32]. As depicted in Table 3, the alcohol model group showed significant increase in the level of TG in comparison with the normal group. Compared with the alcohol group (1.68 ± 0.23 mmol/L), the PSP groups depressed the serum level of TG (1.59 ± 0.07 mmol/L for 200 mg/kg BW, and 1.29 ± 0.16 mmol/L for 400 mg/kg BW) and further limited lipid accumulation. However, the PSP group at a dose of 200 mg/kg BW was no significant different (P>0.05) (Table 3). The result indicated that PSP might play an important role in reversing the lipid dysfunction or repairing a damaged metabolism led by alcohol-induced liver injury. Taken together, PSP

pretreatments at the tested concentrations offered significant protection against alcohol intoxication in mice by attenuating ALT, AST, and TG elevation in serum. The results from our study also confirmed those from previous studies [12,16, 32].

3.3. Effects of PSP on antioxidant enzyme activities and lipid peroxidation in livers

Oxidative stress and lipid peroxidation are acknowledged as the two major mechanisms of alcohol-induced liver injury. Excessive free radicals that are potentially harmful to the body are produced during the metabolism process of ethanol, and thus trigger the chain reaction of lipid peroxidation [33]. In the current work, the effects of PSP and silymarin on the activities of antioxidant enzymes (SOD, CAT, and GSH-Px) in the livers of alcohol-treated mice are summarized in Table 4. The livers of mice challenged with alcohol alone were found to have dramatically lowered SOD, CAT, and GSH-Px activities compared with that in normal mice (P<0.001), which are indicative of damage to hepatic cells. PSP pretreatment significantly increased the activities of SOD (116.27 ± 3.54 U/mg prot for 200 mg/kg BW, and 122.60 ± 2.25 U/mg prot for 400 mg/kg BW), CAT (20.34 ± 0.95 U/mg prot for 200 mg/kg BW, and 26.20 ± 0.53 U/mg prot for 400 mg/kg BW), and GSH-Px (196.06 ± 11.22 U/mg prot for 200 mg/kg BW, and 222.23 ± 10.74 U/mg prot for 400 mg/kg BW) as compared with the alcohol administration group (108.97 ± 3.86, 19.30 ± 0.40 and 182.77 ± 5.93 U/mg prot, respectively) (P<0.05). Especially, PSP at a dose of 400 mg/kg BW showed comparable effects with silymarin at a dose of 100 mg/kg BW (130.38 ± 1.92, 27.29 ± 0.62 and 232.66 ± 4.22 U/mg prot, respectively) when compared with the alcohol model group. These findings suggest that PSP can effectively attenuate oxidative damage and exhibit strong

antioxidant activity in vivo by enhancing the activities of the evaluated antioxidant enzymes in the livers of alcohol-caused mice.

Usually, elevated alcohol toxicity has been associated with GSH decrease, which may reflect the consumption of GSH by the overproduction of ROS and subsequent oxidative stress caused by alcohol [34, 35]. Our present work (Table 4) showed that alcohol administration caused significant GSH depletion compared with the normal group (P<0.001). However, the pre-consumption with PSP (200 and 400 mg/kg BW) significantly retarded the subsequent alcohol-induced GSH depletion (3.33 ± 0.20 and 4.79 ± 0.27 mg/g prot, respectively) (P<0.05), which was close to that of the positive control group. This result indicated that the hepato-protective effects of PSP against alcohol are correlated to the increase in hepatic GSH content. As displayed in Table 4, compared with the normal control group, alcohol supplementation remarkably caused a twofold increase in the amount of MDA from 3.82 ± 0.26 mmol/mg prot to 8.38 ± 0.47 mmol/mg prot. However, pretreatment with PSP significantly reversed the effect of alcohol on MDA formation. At doses of 200 and 400 mg/kg BW, the MDA levels were remarkably decreased by up to 7.51 ± 0.51 and 6.04 ± 0.20 mmol/mg prot (P<0.01), respectively, in comparison with the alcohol model group, which indicated that PSP pretreatment significantly decreased the alcohol-induced lipid peroxidation. Consequently, the results from our present study agreed with those in previous studies [8, 32, 36, 37], and supported that oxidative damage is an important factor responsible for liver injury induced by alcohol. Moreover, our present study showed that pre-intakes with PSP at the tested concentrations markedly alleviated alcohol-caused oxidative stress and ameliorated liver functions either by suppressing alcohol-induced lipid peroxidation or by promoting

antioxidant enzyme activities. This finding further implied that PSP could provide anti-oxidative protection against hepatic oxidative injury.

3.4. Effects of PSP on pro-inflammatory cytokines in livers

In addition to oxidative stress and lipid accumulation, the crucial feature of ALD is also associated with cytokine metabolism. Many pathological events are closely related to the liver inflammatory processes after exposure to various hepatotoxins, and some pro-inflammatory mediators, such as TNF-α and IL-1β, were released by alcohol-induced reactive oxygen species [38]. In this study, compared with the normal control group, alcohol administration caused significant inflammatory responses (P<0.05), as strongly demonstrated by the increases in hepatic IL-1β and TNF-α levels (Fig. 2), which was consistent with previous studies [12, 39, 40]. However, pretreatments with PSP at the measured concentrations (200 and 400 mg/kg BW) remarkably retarded the increases of IL-1β (155.05 ± 19.33 and 143.64 ± 19.95 pg/mL, respectively) and TNF-α (1397.28 ± 95.03 and 1154.43 ± 42.66 pg/mL, respectively) as compared with the alcohol-treated mice (207.05 ± 9.57 and 1589.90 ± 47.50 pg/mL, respectively) (P<0.05). Interestingly, after pre-intake with PSP at a dose of 400 mg/kg BW, the levels of hepatic IL-1β and TNF-α significantly decreased by about 30.62% and 27.39%, respectively, in comparison with the alcohol-treated mice, which was slightly less than that of silymarin at a dose of 100 mg/kg BW (36.33% and 37.05%, respectively). Results suggested that the anti-inflammatory effects of PSP might play a key role in protecting the liver against alcohol-induced hepatotoxicity.

3.5. Histopathological studies

As illustrated in Fig. 3a, a section of liver tissue exhibited typical hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, and visible central veins in the normal mice. Furthermore, the hepatic sinus is normal with no swelling and cell inflammation. By contrast, alcohol treatment resulted in enlargements and irregular arrangements of the hepatocytes, higher steatosis (fat accumulation), inflammatory injury, telangiectasia, mild fibrosis of liver tissue, edema, and necrosis (Fig. 3b). In the positive control group, the hepatic lesions induced by alcohol were significantly improved by pretreatment with silymarin at a dose of 100 mg/kg BW (Fig. 3c), and the hepatocytes were neatly arranged without edema, which was similar to that of mice in normal group (Fig. 3a). Fig. 3d shows the photomicrograph of liver section from the PSP group at a dose of 200 mg/kg BW. It could be seen that the PSP pretreatment partially attenuated the inflammatory reactions and lipid change of hepatocytes in the liver tissues of mice as compared with the model control group (Fig. 3b). Specifically, in the PSP pretreated group at 400 mg/kg BW (Fig.3e), hepatic tissue showed the normal cellular architecture without evident inflammation zones and vacuolar degeneration, then numbers of the necrotic cells decreased and all structures were closed to that of normal liver. This result indicated that PSP might protect the liver from alcohol-induced hepatic damage.

3.6. Effects of PSP on the expression levels of CYP2E1 and HO-1

To further clarify the molecular hepato-protective mechanism of PSP, we explored the effects of PSPs on the mRNA and protein expression levels of CYP2E1 and HO-1, which are

two of the key enzymes in ethanol metabolism, and the results are shown in Figs 4 and 5. Many hepatotoxicants including alcohol are metabolically activated by CYP2E1, one of the isoforms of CYP450 system, to form reactive toxic metabolites, which further in turn induce liver injury [41]. As shown in Fig. 4a, compared with that in the normal control group, the mRNA expression of CYP2E1 was remarkably upregulated by alcohol administration in mice, whereas significantly downregulated by pre-consumption of PSP and silymarin (P<0.001). The relative mRNA expressions of CYP2E1 in the PSP groups (200 and 400 mg/kg BW) decreased by 35.78% and 51.25%, respectively, as compared with the model control group. Especially, pretreatment with PSP at a dose of 400 mg/kg BW showed comparable regulatory function on the mRNA expression of CYP2E1 with silymarin at a dose of 100 mg/kg BW, further indicating that PSP pre-intake can effectively suppress free radicals production and lipid peroxidation presumably by down-regulating the CYP2E1 level. Fig. 5a shows that alcohol treatment significantly upregulated the expression of CYP2E1 protein as compared with the normal mice. When pretreated at 200 and 400 mg/kg BW, the relative protein expressions of CYP2E1 in the PSP groups were reduced by 19.40% and 34.33%, respectively, as compared with the alcohol-treated mice (P<0.01). Results revealed that the hepatic mRNA changes were positively correlated with protein changes in the liver. Similarly, Hong et al. showed that fucoidan, a sulfated polysaccharide extracted from various brown seaweeds, significantly suppressed the protein expression of CYP2E1 in acetaminophen (AAP)-induced liver injury [42]. Therefore, suppressing the expression of CYP2E1 by administration and/or pretreatment of PSs plays an important role in protecting against alcohol-induced liver injury.

Recent studies have demonstrated that the induction of HO-1 prevents alcohol-induced

inflammation in the liver by increasing carbon dioxide (CO), which is its downstream mediator, because it is one of the important regulators of signal pathways that regulates hepatic inflammatory responses [43]. As shown in Fig. 4b, the mRNA expression of HO-1 after alcohol treatment was dramatically downregulated as compared with that of the normal mice (P<0.01). In contrast, pretreatment with PSP significantly upregulated the mRNA expression of HO-1 when compared with the alcohol-treated mice (P<0.05). For example, compare with the model control group, the relative mRNA expressions of HO-1 in the PSP groups (200 and 400 mg/kg BW) increased by 21.92% and 65.08%, respectively. Thus, the result indicated that PSP effectively ameliorates the mRNA expression of HO-1, and further plays an important role in protecting the liver against alcohol-induced injury. This may be attributed to the fact that PSP can activate the activity of HO-1 in the liver inhibited by alcohol intake and directly address HO-1 induction, and the induction of HO-1 may be related to the potent antioxidant activity of PSP. Fig. 5b shows the effects of PSP on the expression of HO-1 protein determined by using Western blot analysis. After alcohol exposure, the expression level of HO-1 protein remarkedly diminished when compared with the normal group (P<0.001). Interestingly, the expression level of HO-1 protein was significantly elevated by PSP and silymarin pre-supplementations in the presence of alcohol. Compared with the model group, the relative protein expression levels of HO-1 increased 53.86% and 161.54% by pretreatments with PSP at doses of 200 and 400 mg/kg BW, respectively, which was weaker than that of silymarin at a dose of 100 mg/kg BW (246.15%). Collectively, the present study revealed that PSP effectively enhanced the expression of hepato-protective HO-1 in alcohol-induced liver injury.

4. Conclusions

In summary, the present study demonstrated that PSP showed a potent hepato-protective effect on alcohol-induced liver injury in mice. PSP pretreatment significantly improved the liver, spleen, and thymus functions; attenuated the elevation of ALT, AST, and TG levels in serum and MDA level in liver tissue; and antagonized the decreases in hepatic antioxidant enzyme activities (SOD, CAT, and GSH-Px) and GSH level as compared with that of alcohol-treated mice. Histopathological examinations also confirmed that PSP could protect the liver from alcohol-induced histological changes. Moreover, pretreatment with PSP not only significantly prevented the alcohol-induced enhancement of hepatic IL-1β and TNF-α, but also reversed the expression levels of CYP2E1 and HO-1. Taken together, we suggested that antioxidative PSP could protect the liver from hepatocyte injury induced by alcohol possibly by alleviating hepatic lipid metabolism (AST, ALT, TG, and MDA), ameliorating the expression levels of CYP2E1 and HO-1, and thus leading to the elevation of antioxidant enzyme activity (SOD, CAT, and GSH-Px) and the suppression of immune inflammatory injury (viscera indices, IL-1β, and TNF-α). Therefore, PSP can be developed as an effective food supplement that may be potentially applied to alcohol-induced liver injuries. Further investigations on purification, structural elucidation and structure-activity relationship of the PSP are underway in our lab.

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References

[1] World Health Organization. Global status report on alcohol and health. Available from http://www.who.int/substance_abuse/publications/alcohol/en/, 2014.

[2] H. Zhuang, J.H. Zhang, Epidemiology of alcoholic liver disease. Chin. J. Gastroenterol. 8 (2003) 294-297.

[3] R. Bruha, K. Dvorak, J. Petrtyl, Alcoholic liver disease. World J. Hepatol. 4 (2012) 81-90.

[4] B. Gao, R. Bataller, Alcoholic liver disease: pathogenesis and new therapeutic targets. Gastroenterology 141 (2011) 1572-1585. https://doi.org/10.1053/j.gastro.2011.09.002

[5] T. Khanal, J.H. Choi, Y.P. Hwang, Y.C. Chung, H.G. Jeong, Saponins isolated from the root of *Platycodon grandiflorum* protect against acute ethanol-induced hepatotoxicity in mice. Food Chem. Toxicol. 47 (2009) 530-535. https://doi.org/10.1016/j.fct.2008.12.009

[6] X.Y. Wang, J.P. Luo, R. Chen X.Q. Zha, L.H. Pan, *Dendrobium huoshanense* polysaccharide prevents ethanol-induced liver injury in mice by metabolomic analysis. Int. J. Biol. Macromol. 78 (2015) 354-362. https://doi.org/10.1016/j.ijbiomac.2015.04.024

[7] S. Ramaiah, C. Rivera, G. Arteel, Early-phase alcoholic liver disease: an update on animal models, pathology, and pathogenesis. Int. J. Toxicol. 23 (2004) 217-231. https://doi.org/10.1080/10915810490502069

[8] X.Y. Wang, J.P. Luo, R. Chen, X.Q. Zha, H. Wang, The effects of daily supplementation of *Dendrobium huoshanense* polysaccharide on ethanol-induced substance liver injury in

mice by proteomic analysis. Food Funct. 5 (2014) 2020-2035. DOI:10.1039/C3FO60629E

[9] I. Giavasis, Bioactive fungal polysaccharides as potential functional ingredients in food and nutraceuticals. Curr. Opin. Biotech. 26 (2014) 162-173. https://doi.org/10.1016/j.copbio.2014.01.010

[10] A.M. Stephen, G.O. Phillips, P.A. Williams, *Food polysaccharides and their applications (2 edition)*. Boca Raton, FL: CRC Press, 2016.

[11] D. Cheng, H. Kong, The effects of *Lycium Barbarum* polysaccharide on alcohol-induced oxidative stress in rats. Molecules 16 (2011) 2542-2550. doi:10.3390/molecules16032542

[12] Y. Cui, Q. Ye, H. Wang, Y. Li, W. Yao, H. Qian, Hepatoprotective potential of *Aloe vera* polysaccharides against chronic alcohol-induced hepatotoxicity in mice. J. Sci. Food Agr. 94 (2014) 1764-1771. https://doi.org/10.1002/jsfa.6489

[13] D. Liang, Q. Zhou, W. Gong, Y. Wang, Z. Nie, H. He, J. Li, J. Wu, C. Wu, J. Zhang, Studies on the antioxidant and hepatoprotective activities of polysaccharides from *Talinum triangulare*. J. Ethnopharmacol. 136 (2011) 316-321. https://doi.org/10.1016/j.jep.2011.04.047

[14] Y.J. Liu, J.L. Du, L.P. Cao, R. Jia, Y.J. Shen, C.Y. Zhao, P. Xu, G.J. Yin, Anti-inflammatory and hepatoprotective effects of *Ganoderma lucidum* polysaccharides on carbon tetrachloride-induced hepatocyte damage in common carp (*Cyprinus carpio* L.). Int. Immunopharmacol. 25 (2015) 112-120. https://doi.org/10.1016/j.intimp.2015.01.023

[15] F.O. Ozalp, M. Canbek, M. Yamac, G. Kanbak, L.J.L.D. Van Griensven, M. Uyanoglu, H. Senturk, K. Kartkaya, A. Oglakci, Consumption of *Coprinus comatus* polysaccharide extract causes recovery of alcoholic liver damage in rats. Pharm. Biol. 52 (2014) 994-1002.

https://doi.org/10.3109/13880209.2013.874536

[16] J.D. Lim, S.R. Lee, T. Kim, S.A. Jang, S.C. Kang, H.J. Koo, E. Sohn, J.P. Bak, S. Namkoong, H.K. Kim, I.S. Song, N. Kim, E.H. Sohn, J. Han, Fucoidan from *Fucus vesiculosus* protects against alcohol-induced liver damage by modulating inflammatory mediators in mice and HepG2 cells. Mar. Drugs 13 (2015) 1051-1067. doi:10.3390/md13021051

[17] C.F. Chen, C.S. Lin, Hepatoprotective effects of clam extract supplementation on subacute hepatitis induced by carbon tetrachloride in rats. Taiwanese J. Agric. Chem. Food Sci. 41 (2003) 159-163.

[18] C.M. Lin, Y.L. Lin, N.M. Tsai, H.Y. Wu, S.Y. Ho, C.H. Chen, Y.K. Liu, Y.H. Chiu, L.P. Ho, R.P. Lee, K.W. Liao, Inhibition effects of chloroform extracts derived from *Corbicula fluminea* on the release of pro-inflammatory cytokines. J. Agric. Food Chem. 60 (2012) 4076-4082. DOI: 10.1021/jf2051202.

[19] N. Liao, S. Chen, X. Ye, J. Zhong, N. Wu, S. Dong, B. Yang, D. Liu, Antioxidant and anti-tumor activity of a polysaccharide from freshwater clam, *Corbicula fluminea*. Food Funct. 4 (2013) 539-548. DOI: 10.1039/c2fo30178d.

[20] N. Liao, J. Zhong, X. Ye, S. Lu, W. Wang, R. Zhang, J. Xu, S. Chen, D. Liu, Ultrasonic-assisted enzymatic extraction of polysaccharide from *Corbicula fluminea*: characterization and antioxidant activity. LWT-Food Sci. Technol. 60 (2015) 1113-1121. https://doi.org/10.1016/j.lwt.2014.10.009.

[21] J.S. Tsai, T.C. Lin, J.L. Chen, B.S. Pan, The inhibitory effects of freshwater clam (*Corbicula fluminea*, Muller) muscle protein hydrolysates on angiotensin I converting

enzyme. Process Biochem. 41 (2006) 2276-2281. https://doi.org/10.1016/j.procbio.2006.05.023.

[22] Y.Y. Wang, W.Y. Qiu, Z.B. Wang, H.L. Ma, J.K. Yan, Extraction and characterization of anti-oxidative polysaccharide-protein complexes from *Corbicula fluminea* through three-phase partitioning. RSC Adv. 7 (2017) 11067-11075. DOI: 10.1039/c7ra00117g.

[23] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (1956) 350-366. https://doi.org/10.1021/ac60111a017

[24] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248-254. https://doi.org/10.1016/0003-2697(76)90527-3.

[25] Z.B. Wang, J.J. Pei, H.L. Ma, P.F. Cai, J.K. Yan, Effect of extraction media on preliminary characterizations and antioxidant activities of *Phellinus linteus* polysaccharides. Carbohyd. Polym. 109 (2014) 49-55. http://dx.doi.org/10.1016/j.carbpol.2014.03.057

[26] J.K. Yan, Y.Y. Wang, W.Y. Qiu, L.X. Wu, Z.C. Ding, W.D. Cai, Purification, structural characterization and bioactivity evaluation of a novel proteoglycan produced by *Corbicula fluminea*. Carbohyd. Polym. 176 (2017) 11-18. http://dx.doi.org/10.1016/j.carbpol.2017.08.063

[27] Y. Chen, M.Y. Xie, S.P. Nie, C. Li, Y.X. Wang, Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. Food Chem. 107 (2008) 231-241. https://doi.org/10.1016/j.foodchem.2007.08.021

[28] S.S. Irudayaraj, C. Sunil, V. Duraipandiyan, S. Ignacimuthu, Antidiabetic and antioxidant activities of *Toddalia asiatica* (L.) Lam. leaves in streptozotocin induced diabetic rats. J. Ethnopharmacol. 143 (2012) 515-523. https://doi.org/10.1016/j.jep.2012.07.006

[29] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25 (2001) 402-408. https://doi.org/10.1006/meth.2001.1262.

[30] X. Yang, S. Yang, Y. Guo, Y. Jiao, Y. Zhao, Composition characterization of soluble apple polysaccharides, and their antioxidant and hepatoprotective effects on acute CCl_4-caused liver damage in mice. Food Chem. 138 (2013) 1256-1264. https://doi.org/10.1016/j.foodchem.2012.10.030.

[31] J. Ozer, M. Ratner, M. Shaw, W. Bailey, S. Schomaker, The current state of serum biomarkers of hepatotoxicity. Toxicology 245 (2008) 194-205. https://doi.org/10.1016/j.tox.2007.11.021.

[32] M. Wang, P. Zhu, C. Jiang, L. Ma, Z. Zhang, X. Zeng, Preliminary characterization, antioxidant activity in vitro and hepatoprotective effect on acute alcohol-induced liver injury in mice of polysaccharides from the peduncles of *Hovenia dulcis*. Food Chem. Toxicol. 50 (2012) 2964-2970. https://doi.org/10.1016/j.fct.2012.06.034.

[33] S.D. Das, D.M. Vasudevana, Alcohol-induced oxidative stress. Life Sci. 81 (2007) 177-187. https://doi.org/10.1016/j.lfs.2007.05.005.

[34] D. Cheng, H. Kong, The effects of *Lycium Barbarrum* polysaccharide on alcohol-induced oxidative stress in rats. Molecules 16 (2011) 2542-2550. DOI:

10.3390/molecules16032542.

[35] Y. Iimuro, B.U. Bradford, S. Yamashita, I. Rusyn, M. Nakagami, N. Enomoto, H. Kono, W. Frey, D. Forman, D. Brenner, R.G. Thurman, The glutathione precursor L-2-oxothiazolidine-4-carboxylic acid protects against liver injury due to chronic enteral ethanol exposure in the rat. Hepatology 31 (2000) 391-398. DOI: 10.1002/hep.510310219.

[36] D. Liang, Q. Zhou, W. Gong, Y. Wang, Z. Nie, H. He, J. Li, J. Wu, C. Wu, J. Zhang, Studies on the antioxidant and hepatoprotective activities of polysaccharides from *Talinum triangulare*. J. Ethnopharmacol. 136 (2011) 316-321. https://doi.org/10.1016/j.jep.2011.04.047.

[37] F.O. Ozalp, M. Canbek, M. Yamac, G. Kanbak, L.J.L.D. Van Griensven, M. Uyanoglu, H. Senturk, K. Kartkaya, A. Oglakci, Consumption of *Coprinus comatus* polysaccharide extract causes recovery of alcoholic liver damage in rats. Pharm. Biol. 52 (2014) 994-1002. https://doi.org/10.3109/13880209.2013.874536.

[38] H.Y. Park, H.D. Choi, H. Eom, I. Choi, Enzymatic modification enhances the protective activity of citrus flavonoids against alcohol-induced liver disease. Food Chem. 139 (2013) 231-240. https://doi.org/10.1016/j.foodchem.2013.01.044.

[39] Y. Han, Q. Xu, J. Hu, X. Han, W. Li, L. Zhao, Maltol, a food flavoring agent, attenuates acute alcohol-induced oxidative damage in mice. Nutrients 7 (2015) 682-696. Doi: 10.3390/nu7010682

[40] J. Zhao, H. Chen, Y. Li, Protective effect of bicyclol on acute alcohol-induced liver injury in mice. Eur. J. Pharmacol. 586 (2008) 322-331. https://doi.org/10.1016/j.ejphar.2008.02.059

[41] O. Tsedensodnom, A.M. Vacaru, D.L. Howarth, C. Yin, K.C. Sadler, Ethanol metabolism and oxidative stress are required for unfolded protein response activation and steatosis in zebrafish with alcoholic liver disease. Dis. Model. Mech. 6 (2013) 1213-1226. Doi:10.1242/dmm.012195.

[42] S.W. Hong, H.S. Lee, K.H. Jung, H. Lee, S.S. Hong, Protective effect of fucoidan against acetaminophen-induced liver injury. Arch. Pharm. Res. 35 (2012) 1099-1105. https://doi.org/10.1007/s12272-012-0618-5.

[43] A. Haschemi, O. Wagner, R. Marculescu, B. Wegiel, S.C. Robson, N. Gagliani, D. Gallo, J.F. Chen, F.H. Bach, L.E. Otterbein, Cross-regulation of carbon monoxide and the adenosine A2a receptor in macrophages. J. Immunol. 178 (2007) 5921-5929. doi: 10.4049/jimmunol.178.9.5921.

<Figure captions>:

Fig. 1. High-performance liquid chromatograms of (a) monosaccharide standards and (b) the proteoglycan (PSP). Peaks: 1, mannose (Man, R_t: 11.46 min); 2, glucosamine (GlcN, R_t: 13.97), 3, rhamnose (Rha, R_t: 14.60); 4, galacturonic acid (GalA, R_t: 18.19); 5, glucose (Glc, R_t: 20.49); 6, galactose (Gal, R_t: 22.40), 7, Xylose (Xyl, R_t: 22.89); 8, arabinose (Ara, R_t: 23.64). (c) Gel permeation chromatogram and (d) Fourier-transform infrared (FT-IR) spectrum of the PSP. (e) UV spectrum of PSP before and after β-elimination reaction.

Fig. 2. Effect of the proteoglycan (PSP) on the contents of (a) interleukin-1β (IL-1β) and (b) tumor necrosis factor-α (TNF-α) in the livers of alcohol-induced mice. Group I: normal control group; Group II: 50% (v/v) alcohol (5 mL/kg BW) model control group; Group III: silymarin (100 mg/kg BW) positive control group; Group IV: PSP low-dose (200 mg/kg BW) group; Group V: PSP high-dose (400 mg/kg BW) group. Values are expressed as mean ± standard deviation (SD, n=10). * P<0.05, ** P<0.01, and *** P<0.001, *vs*. model control group. $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, and $^{\#\#\#}$ P<0.001, *vs*. normal control group.

Fig. 3. Effect of the proteoglycan (PSP) on hepatic histological changes using H&E staining. (a) Liver section of normal group (I) showing normal hepatic lobular architecture and hepatic cell structure; (b) Liver section of alcohol model group (II); (c) Liver section of positive silymarin (100 mg/kg BW) group (III); (d) Liver section of PSP low-dose (200 mg/kg BW) group (V); (e) Liver section of PSP high-dose (400 mg/kg BW) group (V).

Fig. 4. Effect of the proteoglycan (PSP) on the expression levels of (a) cytochrome P450 2e1 (CYP2E1) mRNA and (b) hemeoxygenase-1 (HO-1) mRNA. Group I: normal control group; Group II: 50% (v/v) alcohol (5 mL/kg BW) model control group; Group III: silymarin (100 mg/kg BW) positive control group; Group IV: PSP low-dose (200 mg/kg BW) group; Group V: PSP high-dose (400 mg/kg BW) group. Values are expressed as mean ± standard deviation (SD, n=10). * P<0.05, ** P<0.01, and *** P<0.001, *vs.* model control group. $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, and $^{\#\#\#}$ P<0.001, *vs.* normal control group. The values normalized to β-actin.

Fig. 5. Effect of the proteoglycan (PSP) on the expression levels of (a) cytochrome P450 2e1 (CYP2E1) protein and (b) hemeoxygenase-1 (HO-1) protein. Group I: normal control group; Group II: 50% (v/v) alcohol (5 mL/kg BW) model control group; Group III: silymarin (100 mg/kg BW) positive control group; Group IV: PSP low-dose (200 mg/kg BW) group; Group V: PSP high-dose (400 mg/kg BW) group. Values are expressed as mean ± standard deviation (SD, n=10). * P<0.05, ** P<0.01, and *** P<0.001, *vs.* model control group. $^{\#}$ P<0.05, ## P<0.01, and ### P<0.001, vs. normal control group. β-actin was used as internal control, and the values normalized to β-actin.

Table 1. Primer sequences used for qRT-PCR.

Target gene	Direction	Primer sequence
CYP2E1	Forward	5’-TGAATGAAAATGGGAAGTT -3’
	Revese	5’-GAATAGCAGACAGGAGCAG -3’
HO-1	Forward	5’-GGTCCTGAAGAAGATTGCG -3’
	Revese	5’-GATGCTCGGGAAGGTGAA -3’
β-actin	Forward	5’-AGGCCCCTCTGAACCCTAAG -3’
	Revese	5’-CCAGAGGCATACAGGGACAAC -3’

Table 2. Effect of PSP on liver, spleen, and thymus indices in alcohol-induced mice.

Groups	Liver (%)	Spleen (%)	Thymus (%)
I	3.77 ± 0.18 **	0.22 ± 0.03 *	0.22 ± 0.02 *
II	4.02 ± 0.12 ##	0.20 ± 0.01 #	0.18 ± 0.01 #
III	3.80 ± 0.12 **	0.22 ± 0.02 *	0.22 ± 0.02 *
IV	3.87 ± 0.15 *	0.21 ± 0.04 #	0.21 ± 0.03*
V	3.82 ± 0.13 **	0.21 ± 0.02*	0.21 ± 0.01**

Group I: normal control group; Group II: 50% (v/v) alcohol (5 mL/kg BW) model control group; Group III: silimarin (100 mg/kg BW) positive control group; Group IV: PSP low dose (200 mg/kg BW) group; Group V: PSP high dose (400 mg/kg BW) group. Values are expressed as mean ± SD (n=10). * P<0.05, and ** P<0.01, *vs.* model control group. # P<0.05, and ## P<0.01, *vs.* normal control group.

Table 3. Effect of PSP on the levels of alanine transaminase (ALT), aspartate transaminase (AST) and triacylglycerides (TG) in serum in alcohol-induced mice.

Groups	ALT (U/L)	AST (U/L)	TG (mmol/L)
I	14.30 ± 4.17 ***	59.47 ± 4.56 ***	0.80 ± 0.43 ***
II	26.95 ± 3.65 ###	80.63 ± 7.48 ###	1.68 ± 0.23 ###
III	17.52 ± 2.52 ***	62.51 ± 3.81 ***	0.92 ± 0.06 ***
IV	25.61 ± 2.20 ###	71.85 ± 3.94 * ###	1.59 ± 0.07 ###
V	23.44 ± 1.86 * ###	66.23 ± 2.05 *** ###	1.29 ± 0.16 ** ##

Group I: normal control group; Group II: 50% (v/v) alcohol (5 mL/kg BW) model control group; Group III: silimarin (100 mg/kg BW) positive control group; Group IV: PSP low-dose (200 mg/kg BW) group; Group V: PSP high-dose (400 mg/kg BW) group. Values are expressed as mean ± SD (n=10). * P<0.05, ** P<0.01, and *** P<0.001, *vs.* model control group. # P<0.05, ## P<0.01, and ### P<0.001, *vs.* normal control group.

Table 4. Effect of PSP on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and the levels of glutathione (GSH) and malondialdehyde (MDA) in livers in alcohol-induced mice.

Groups	SOD (U/mg prot)	CAT(U/mg prot)	GSH-Px (U/mg prot)	GSH (mg/g prot)	MDA (mmol/mg prot)
I	135.00 ± 3.35 ***	27.20 ± 0.54 ***	241.06 ± 7.48 ***	6.55 ± 0.37 ***	3.82 ± 0.26 ***
II	108.97 ± 3.86 $^{\#\#\#}$	19.30 ± 0.40 $^{\#\#\#}$	182.77 ± 5.93 $^{\#\#\#}$	3.05 ± 0.29 $^{\#\#\#}$	8.38 ± 0.47 $^{\#\#\#}$
III	130.38 ± 1.92 $^{***\ \#}$	27.29 ± 0.62 ***	232.66 ± 4.22 $^{***\ \#}$	6.17 ± 0.23 ***	4.42 ± 0.19 $^{***\ \#\#\#}$
IV	116.27 ± 3.54 $^{**\ \#\#\#}$	20.34 ± 0.95 $^{*\ \#\#\#}$	196.06 ± 11.22 $^{*\ \#\#\#}$	3.33 ± 0.20 $^{*\ \#\#\#}$	7.51 ± 0.51 $^{**\ \#\#\#}$
V	122.60 ± 2.25 $^{***\ \#\#\#}$	26.20 ± 0.53 $^{***\ \#}$	222.23 ± 10.74 $^{***\ \#}$	4.79 ± 0.27 $^{***\ \#\#\#}$	6.04 ± 0.20 $^{***\ \#\#\#}$

Group I: normal control group; Group II: 50% (v/v) alcohol (5 mL/kg BW) model control group; Group III: silimarin (100 mg/kg BW) positive control group; Group IV: PSP low-dose (200 mg/kg BW) group; Group V: PSP high-dose (400 mg/kg BW) group. Values are expressed as mean ± SD (n=10). * P<0.05, ** P<0.01, and *** P<0.001, *vs.* model control group. $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, and $^{\#\#\#}$ P<0.001, *vs.* normal control group.

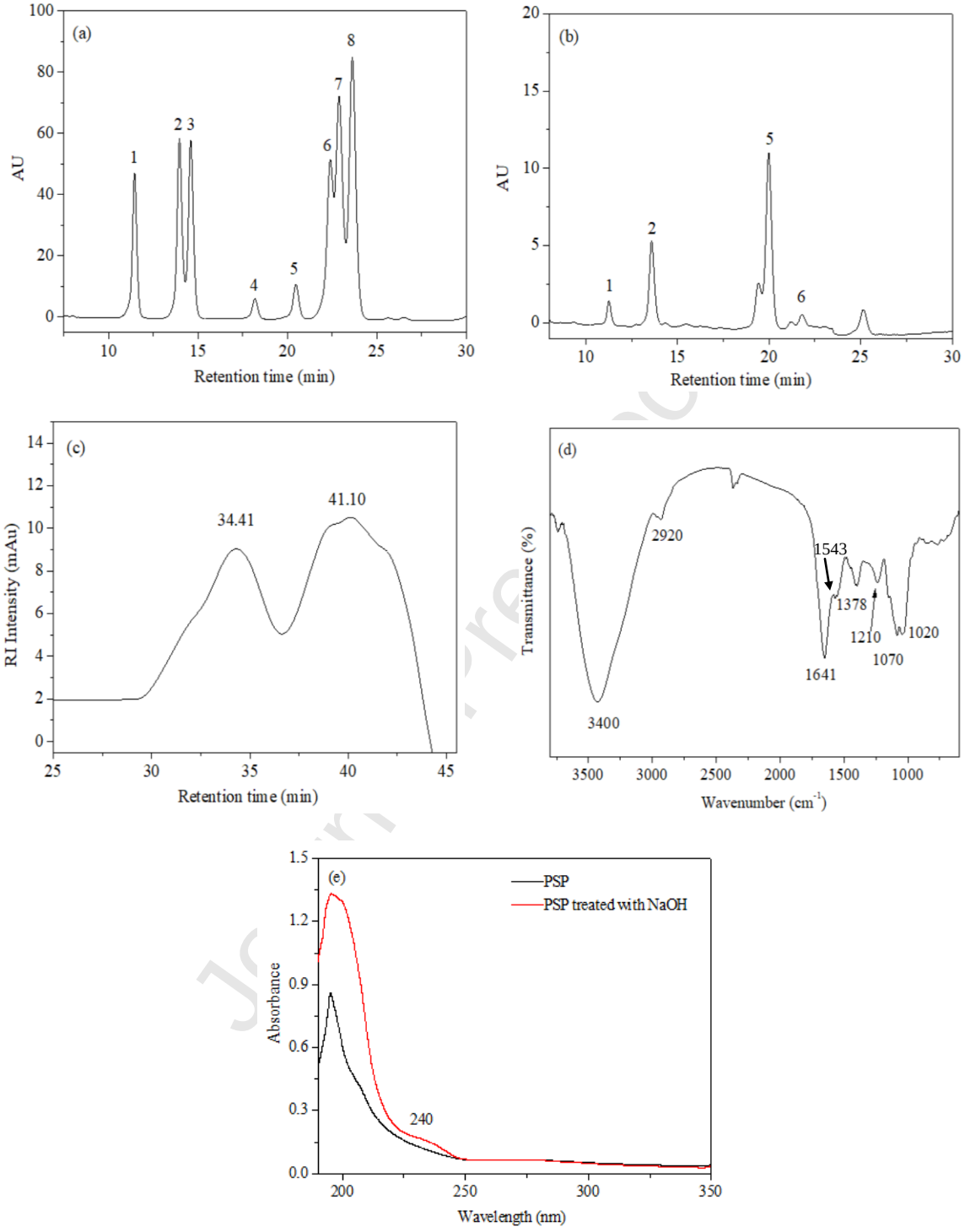
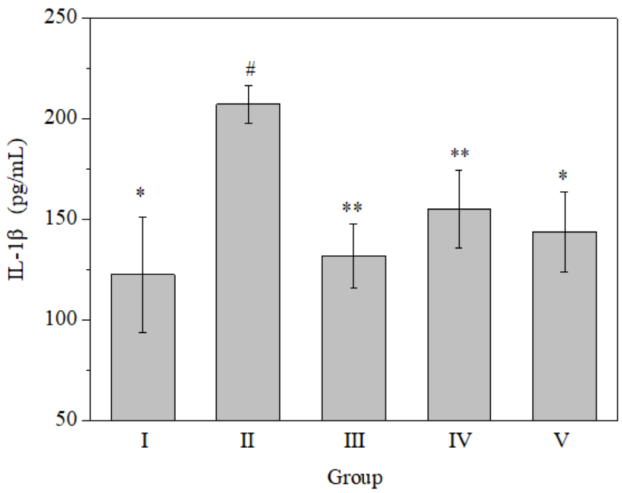


Fig. 1 Bai et al.

(a)



(b)

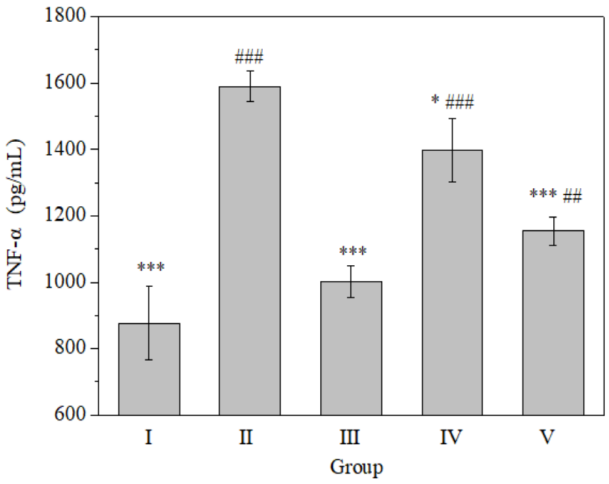


Fig. 2 Bai et al.

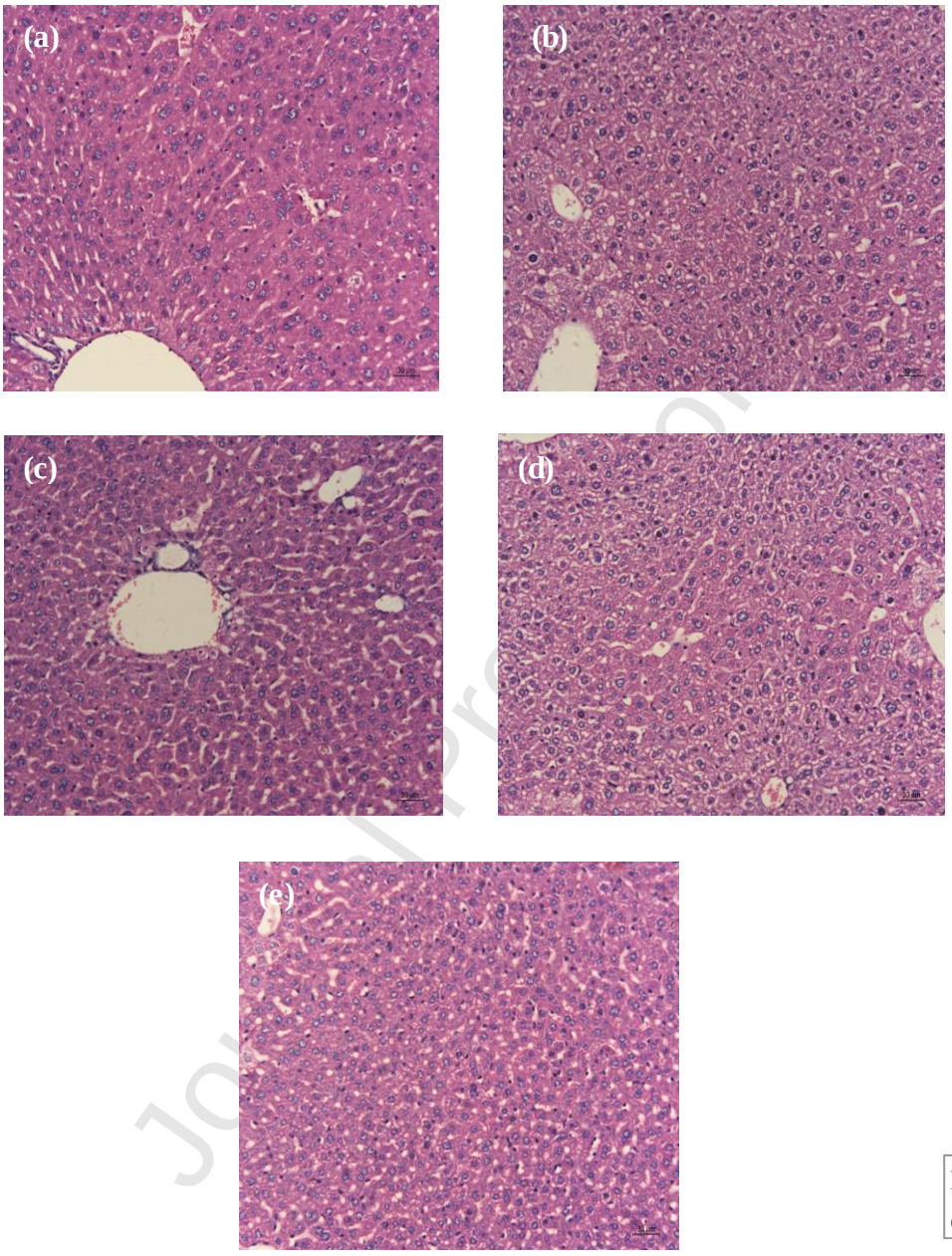


Fig. 3 Bai et al.

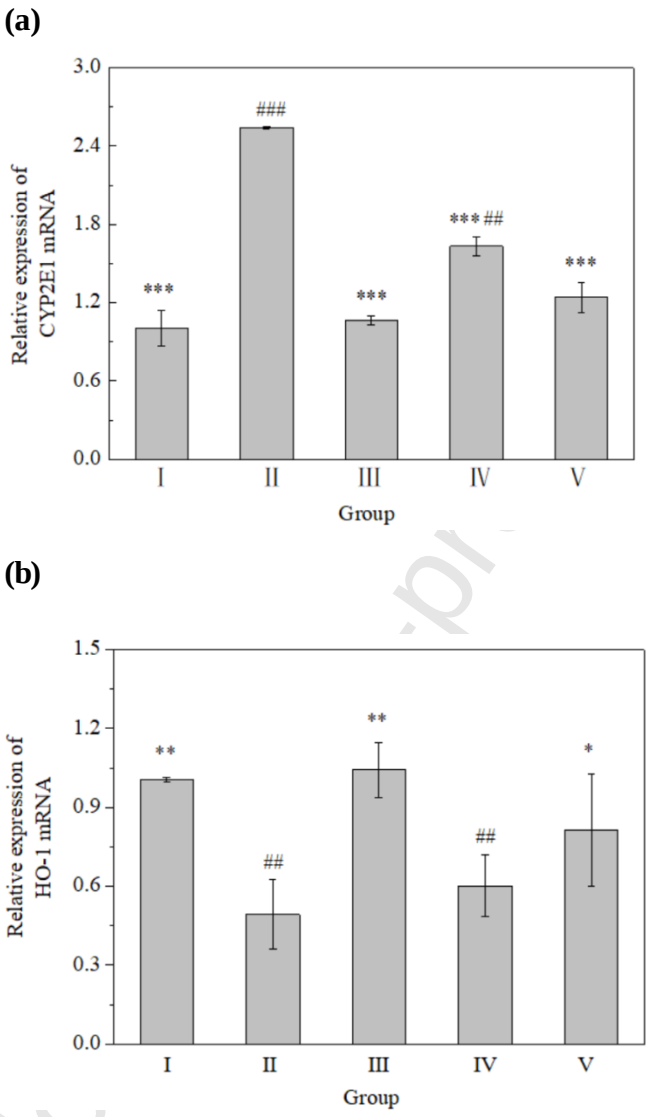
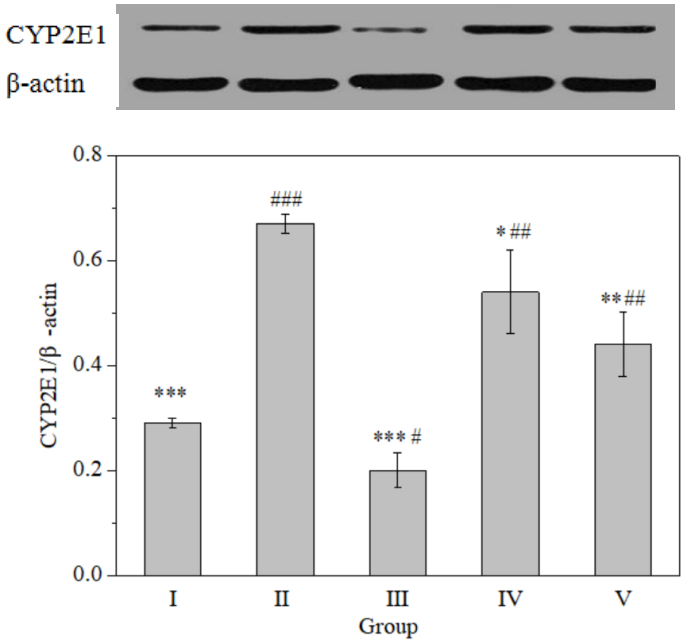


Fig. 4 Bai et al.

(a)



(b)

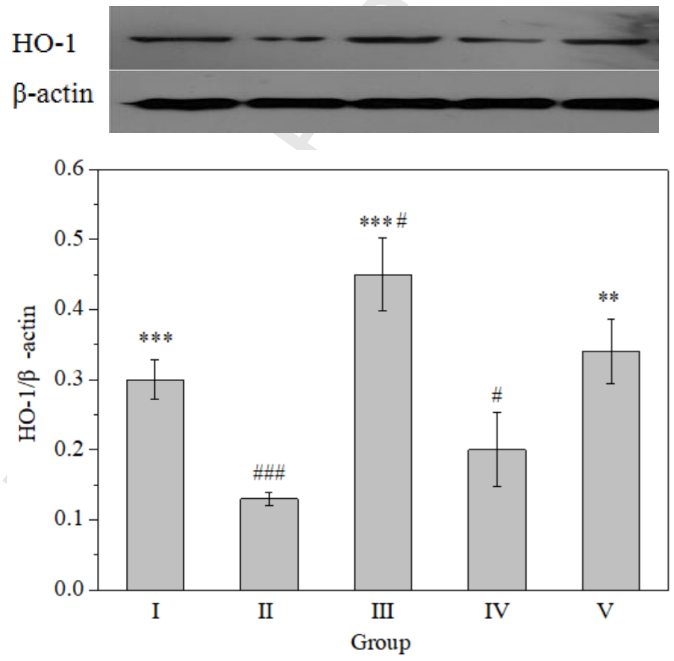


Fig. 5 Bai et al.

Author Statement

Jiafeng Bai: Conceptualization, Methodology, Software. **Yichang Chen**: Data curation, Writing-Original draft preparation. **Zhenxing Ning**: Visualization, Investigation. **Shaohua Liu**: Software, Validation. **Chunping Xu**: Supervision. **Jing-Kun Yan**: Writing- Reviewing and Editing. All authors read and approved the final manuscript. The authors have declared no conflicts of interest.

Highlights

- Hepato-protective effect of a *Corbicula fluminea* proteoglycan (PSP) was evaluated.
- PSP pretreatment ameliorated hepatic lipid metabolism of alcohol-induced mice.
- PSP pre-intake elevated antioxidant-enzyme activity and hepatic glutathione level.
- PSP pretreatment suppressed the immune inflammatory response.
- PSP pre-intake reversed the expressions of cytochrome P450 2e1 and hemeoxygenase-1.