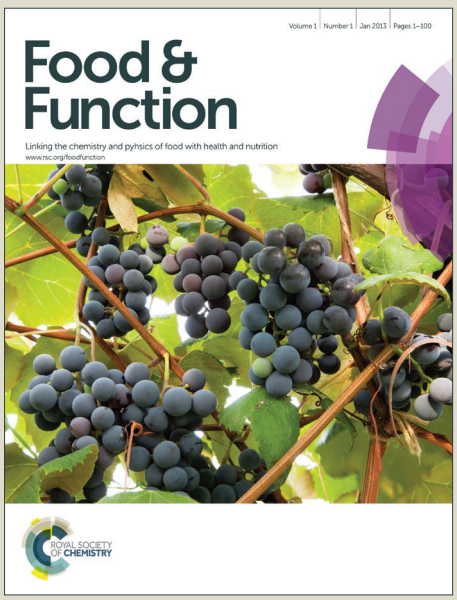
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Platycodin D isolated from the aerial parts of *Platycodon grandiflorum* protects alcohol-induced liver injury in mice

Wei Li[a, b], Ying Liu[a], Zi Wang[a], Ye Han [b], Yu-Hong Tian[a], Gui-Shan Zhang[c], Yin-shi Sun[b] and Ying-Ping Wang[b*]

[a] College of Chinese Medicinal Materials, Jilin Agricultural University, Changchun 130118, China.

[b] Institute of Special Wild Economic Animals and Plant, Chinese Academy of Agricultural Sciences, Changchun 132109, China.

[c] College of Animal Science and Technology, Jilin Agricultural University, Changchun 130118, China.

Abbreviation:

APPG	aerial parts of *P. grandiflorum*;	**TG**	triglyceride
PD	Platycodin D	**MDA**	malondialdehyde
AST	aspartate transaminase	**SOD**	superoxide dismutase
ALT	alanine aminotransferase	**GSH**	glutathione
CAT	catalase	**TNF-α**	Tumor necrosis factor-α
TC	total cholesterol	**IL-1β**	interleukin-1β
L-DLC	Low density lipoprotein cholesterol	**IL-6**	interleukin-6

Correspondence

Professor Ying-Ping Wang, Institute of Special Wild Economic Animals and Plant, Chinese Academy of Agricultural Sciences, Changchun 132109, China.

E-Mail: yingpingw@126.com;

Tel. /Fax: +86-431-81919856.

ABSTRACT

Platycodin D (PD) is the main active saponin of *Platycodon grandiflorum* (PG), and reported to exhibit multiple biological effects including anti-tumor, anti-inflammation, and anti-obesity. Recently though there were many research reports on chemical constituents on roots parts, few works have ever been reported on the aerial parts of PG. In the present paper, we reported the first isolation of PD from the aerial parts of PG and its protective effect on acute alcohol-induced liver oxidative injury and inflammatory response in mice. In brief, the protective effect was evaluated by biochemical markers, enzymatic antioxidants and proinflammatory cytokines in serum and liver tissue. The results indicated that PD pretreatment significantly decreased the levels of triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (L-DLC) in serum and malondialdehyde (MDA) in liver. PD was also found to increase the catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities in liver ($p < 0.05$). Additionally, PD markedly decreased the levels of proinflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 caused by alcohol exposure ($p < 0.05$). In contrast, histopathological examination revealed that PD pretreatment noticeably prevented alcohol-induced hepatocyte apoptosis and steatosis. Collectively, the present study clearly suggested that the protective effect exhibited by PD on alcohol-induced liver oxidative injury may be via alleviation of oxidative stress and inflammatory response.

Keywords: Platycodin D; the aerial parts of *Platycodon grandiflorum*; alcohol liver injury; oxidative stress; inflammatory response

1. Introduction

Increasing evidences showed that long-term alcohol abuse and alcohol dependence result in many diseases including malnutrition and alcoholic liver diseases (ALD) [1]. Generally, ALD encompasses the morphological features of liver steatosis, steatohepatitis, fibrosis and ultimately cirrhosis [2]. Among the mechanisms implicated in pathogenesis of ALD, oxidative stress and inflammatory response are known to play a pivotal role in the pathogenesis of alcohol induced liver injury [3, 4]. Since oxidative stress is involved in the development of ALD, using antioxidants would potentially blunt ethanol-induced oxidative stress and prevent their pathogenesis[5, 6]. Therefore, the predominant source of antioxidants and their role in preventing ethanol-induced liver injury is an important target.

The roots of *Platycodon grandiflorum* (Campanulaceae), Platycodi Radix, are often used as food material or a herbal medicine in China, Korea, and Japan. As a traditional medicine, *P. grandiflorum* can effectively treat cough, abscess, excessive phlegm, and sore throat [7]. The principal bioactive constituents of this herb are triterpenoid saponins, which exhibit a variety of pharmacological activities, such as anti-inflammatory [8], anti-obesity [9, 10], anti-cancer [11, 12], anti-diabetes [13, 14], and hepatoprotective effects [15, 16]. In the past decades, saponins from the roots have received a great deal of attention, especially platycodin D (Figure 1) [17, 18]. Interestingly, though there were many research reports on chemical constituents on roots parts, few works have ever been reported on the aerial parts of *P. grandiflorum* (APPG). Recently, flavonoids and phenolic acids were isolated from the aerial parts in *P. grandiflorum* with potent antioxidant activity *in vitro* [19]. Additionally, the roots were harvested 2 ~ 3 years after seeding, whereas the aerial parts of *P. grandiflorum* can be harvested annually. Thus, the aerial parts of *P. grandiflorum* would be worthy enough for exploration and development. According to our previous report, the kind and number of saponins in the aerial parts were similar to the roots of *P. grandiflorum* via HPLC analysis. Therefore, it was of great interest to find active saponin from the aerial parts of *P. grandiflorum* for further investigation.

Prior to this study, although the protective effect of total extract and crude saponins of Platycodi Radix on alcohol-induced liver damage were investigated [20, 21], fewer investigations of single saponin (platycodin) on oxidative stress injury were investigated and the active principle(s)

responsible for this action is still poorly understood. Recently, it is reported that platycodin D has contributed to attenuating bile duct ligation-induced hepatic fibrosis and cisplatin-induced nephrotoxicity in mice [22, 23]. Since the saponin isolation of the aerial parts of *P. grandiflorum* has not previously been reported, the aims of the present study were to isolate the active saponins and to evaluate the protective effect of main platycodin against alcohol-induced hepatotoxicity.

2. Materials and methods

2.1 Plant materials

The dried aerial parts of *P. grandiflorum* were collected in the medicinal plant garden of Jilin Agricultural University and identified by Professor Ying-ping Wang. Its voucher specimen (No. 06081103) was deposited in College of Chinese Medicinal Material, Jilin Agricultural University. Prior to the extraction experiment, the cut pieces were ground to obtain a relatively homogenous drug powder and then sieved through 40-mesh screen. The powder was dried at 60℃ until constant weight and well blended before use.

2.2 Reagents and kits

Commercial assay kits for alanine aminotransferase (ALT), aspartate transaminase (AST), triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (L-DLC), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 were purchased from R&D system (Minneapolis, MN, USA). Other chemicals such as alcohol were all of analytical grade from Beijing Chemical Factory (Beijing, China).

2.3 Sample preparation

2.3.1 Extraction of crude saponins

About 500 g of powdered aerial parts of *P. grandiflorum* was extracted with 70% ethanol by ultrasonic-assisted extraction for 60 min for 3 times. The combined extract was concentrated with an evaporator, lyophilized and dissolved in H_2O, then partitioned with ethyl acetate to obtain an ethyl acetate soluble fraction. The H_2O-soluble fraction was then partitioned with butanol to give

n-butanol layer. The *n*-butanol layer was confirmed to contain crude saponins by HPLC method, and it was used for further isolation. The flow chart of extraction and isolation was showed in Figure 1.

Insert Figure 1

2.3.2 Isolation and identification of platycodin D

The *n*-butanol layer (35.5 g) was chromatographed over a macroporous resin D101 column and eluted with H_2O, 30% ethanol, and 70% ethanol. The 70% ethanol elution was evaporated under a vacuum to obtain a residue (9.8 g). The above residue was subject to repeated silica gel column (solvent, $CHCl_3$: MeOH=15:1 to 10:1) to yield eight fractions (Fr. 1-8). All fractions were determined by HPLC to analyze the saponins fraction. Fraction 2 and 3 were found to mainly contain platycodin D, and were evaporated, lyophilized and stored at -20℃ until required. The 1.5 g of the above fractions was separated with semi-preparative HPLC (solvent, MeOH: H_2O=40:60) to yield platycodin D.

The chemical structure of platycodin D was identified on the basis of UV, IR, ^{1}H NMR, ^{13}C NMR, ESI-MS, retention times of HPLC by comparison of spectral and elemental analyses of standard reference, and our previous reported data [24].

2.3.3 HPLC analysis

The assay of saponins in aerial parts of *P. grandiflorum* was performed with SHIMADZU LC-20AT system with UV detector. Liquid chromatographic separations were achieved using a Hypersil ODS2 column (4.6×250 mm, 5μm). The column temperature was set at 25℃ and detection wavelength was set at 210 nm. The mobile phase consisted of a mixture of acetonitrile (A) and water (B) with flow rate of 1.0 mL/min. The gradient elution was programmed as follows: 0–30 min, 18–22% A; 30–60 min, 22–25% A. The 20 μL of sample solution was directly injected into the chromatographic column manually.

Pneumatic assisted electrospray positive ionization (ESI+) detection and cracking voltage is 160 V, atomizing air pressure is 276 kPa (40 psi), drying temperature is 350 ℃, and the drying gas flow rate is 12 L/min. HPLC chromatogram and MS spectra are shown in Figure 2.

Insert Figure 2

2.4 Animals and treatments

Male ICR mice (22-25g) were obtained from the Experimental Animal Holding of Jilin University, Changchun, China (Quality Certificate No. SCXK (JI) 2011-0004). The mice were housed under standard conditions with a 12/12 h light/dark cycle at a temperature of 23 ± 2℃ and a humidity of 60 ± 5% during the whole experiments. The experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). All experimental procedures were approved by the Ethical Committee for Laboratory Animals of Jilin Agricultural University.

After acclimatization for one week, the animals were randomly divided into four groups of 8 mice each: (1) control group; (2) alcohol group; (3) alcohol + PD low dose group (10 mg/kg, PD-L); (4) alcohol + PD high dose group (20 mg/kg, PD-H). The dosages of PD were based on preliminary range-finding studies. PD was mixed in a solution of 0.5% carboxymethylcellulose sodium (CMC-Na) in distilled water. Mice were administered intragastrically with PD at 10 and 20 mg/kg once daily for 7 consecutive days, respectively. The control and alcohol groups were administered appropriate vehicles. Three hours after PD administration on the seventh day, mice were administered intragastrically a one-time grant of alcohol in water (50%, v/v) with dose of 12 mL/kg. Then all the mice were kept fasting for 12 h, subsequently killed and the blood were collected. The serum was separated by centrifugation (1500 rpm, 10min, and 4℃) and stored at -20℃ for biochemical analysis. Livers and spleens were dissected quickly, washed twice with saline, blotted dry on a filter paper, and weights were measured. At the same time, the size, appearance, and texture cut surface were recorded as well. Liver sections of formalin-fixed liver were stained with hematoxylin and eosin (H&E), and frozen sections were stained with Sudan III. The remaining liver tissues were stored at -80℃ for hepatic homogenate preparation. Figure 3 shows the experiment design.

Insert Figure 3

2.5 Serum biochemical markers assay

The levels of ALT, AST, TC, TG, and L-DLC in serum were measured by colorimetric enzymatic methods using commercial kits according to the manufacturer's protocols provided by Nanjing

Jiancheng Institute of Biotechnology (Nanjing, China). The enzyme activities of ALT and AST were calculated as U/L. The contents of TC, TG, and L-DLC were calculated as mmol/L.

2.6 Hepatic antioxidant enzymes assay

For the determination of hepatic lipid peroxidation and antioxidant capacity, the weighted liver tissues were homogenized in a cold buffer (50 mM phosphate, pH 7.0). The homogenates were centrifuged at 13, 000 g for 15 min at 4℃, and then the supernatant was used for CAT, GSH-Px, SOD, and MDA assay according to the manufacturer's instructions (Nanjing Jiancheng Institute of Biotechnology). The amount of protein was measured using the Bradford assay.

2.7 Proinflammatory cytokines measurements

Levels of TNF-α, IL-1β, and IL-6 in liver were determined by using the ELISA kits obtained from R&D system (Minneapolis, MN, USA) according to the protocols provided by the manufacturer. Briefly, adding prepared reagent, samples and standards, antibodies labeled with enzyme, reacting 60 min at 37℃. After adding stopping solution, measuring and calculating the OD value within 10 min.

2.8 Histopathological examination and assessment

For histopathological analysis, the liver tissues (n=8 per group) were fixed in 10% neutral formalin buffer [formalin: phosphate buffer (0.01 M, pH 7.4) =1:1] for over 24h, subsequently processed by routine paraffin embedding and sectioned for 5 μm thickness. After hematoxylin-eosin (H&E) staining, slides were observed for histopathological changes using Nikon TE 2000 fluorescence microscope (Nikon, Japan). Representative images were presented. The histopathological characters were used for assessment of histological changes of the liver, including hepatocyte degeneration or necrosis, fatty degeneration, inflammatory cell infiltration and congestion.

2.9 Statistical analysis

All experiments were performed three times independently. Data were presented as means ± S.D. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by least significance difference (LSD) multiple comparison tests using SPSS 16.0 software (SPSS Inc., IL, USA). The Nonparametric Test (Ridit analysis) was used for the histological examination comparison. $p < 0.05$ was considered to be significant.

3. Results

3.1 Isolation and identification of platycodin D

Recently, interest in the chemical constituents and pharmacological activity of the roots of *P. grandiflorum* has been growing gradually. However, little investigation on the aerial parts has ever been reported. In the present study, PD was isolated from the aerial parts of *P. grandiflorum* by the combination of silica column chromatography and preparative HPLC separation for the first time (Figure 1). The chromatographic peak of PD was confirmed by comparing its retention time with that of the reference standard. As shown in Figure 2, an intense peak *m*/*z* 1223.6 corresponding to the deprotonated ion $[M-H]^-$ of PD was observed, and the content of PD was 0.081% by HPLC method.

3.2 Effects of PD on body weight and organ coefficients

Body weight and organ coefficients of the liver and spleen were evaluated in mice. As shown in the Table 1, the body weight of mice treated with alcohol did not change in comparison with control and PD groups. Similar to previous studies [15], liver and spleen coefficients were significantly increased in mice exposed to alcohol ($p < 0.05$). However, the alcohol-induced increase in the liver coefficient was reduced by PD at the dose of 10 and 20 mg/kg, respectively ($p < 0.05$). Interestingly, groups treated with PD showed a dose-dependent attenuation of alcohol-induced changes in the liver and spleen coefficients.

Insert table 1

3.3 Effect of PD on ALT and AST in serum

Release of the ALT and AST into serum is frequently used as a measure of alcohol-induced liver injury [25]. Table 2 and Figure 3 shows that the levels of ALT and AST in serum were significantly increased after exposure to alcohol compared with those in normal control ($p < 0.05$), indicating liver cell damage and the model of alcohol-induced liver injury had been established successfully. However, compared with the alcohol group, pretreatment with PD (10 and 20 mg/kg) for consecutive 7 days has significantly reduced the elevation of ALT and AST ($p < 0.05$).

3.4 Effect of PD on TG, TC, and L-DLC in serum

To investigate the effect of PD pretreatment on lipid metabolism in alcohol-induced mice, the serum TG, TC, and L-DLC were determined. As shown in Table 2, the levels of serum TG, TC and L-DLC in the alcohol group were significantly elevated compared with the control ($p < 0.05$), indicating that

acute alcohol consumption could result in dyslipidemia of mice. However, mice treated with 10 and 20 mg/kg of PD showed a significant reduction in TG, TC, and L-DLC levels, which is consistent with a previous report that PD exerted cholesterol-lowering effect in hypercholesterolemic ICR mice [26].

Insert table 2

3.5 Effect of PD on CAT, GSH-Px, SOD and MDA levels in liver

Previous studies indicated that alcohol exposure might impair enzymatic antioxidant (*e.g.* CAT, SOD and GSH-Px) systems that protect hepatocyte against oxidative damage [20, 21]. As shown in Figure 4, alcohol exposure greatly reduced the hepatic antioxidant CAT, GSH-Px and SOD activities by 48.2%, 45.1%, and 21.3%, respectively, compared to control mice ($p < 0.05$). However, pretreatment with 10 and 20 mg/kg of PD for 7 days remarkably reversed the decrease ($p < 0.05$). MDA is an end product of the breakdown of polyunsaturated fatty acids and related esters, and its formation is an indicator of lipid peroxidation in alcohol-induced mice. The results indicated that alcohol exposure induced the dramatic increase of MDA by 53.2% in the alcohol group ($p < 0.05$) comparing with the normal group, which was significantly attenuated by PD pretreatment ($p < 0.05$). The above results were consistent with the alteration in serum ALT and AST.

Insert Figure 4

3.6 Effect of PD on hepatic inflammatory markers

Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, play key roles in the inflammatory response caused by alcohol exposure [25]. In the present study, the expression of TNF-α, IL-1β, and IL-6 in liver were determined by enzyme-linked immunosorbent assay (ELISA). As indicated in Figure 5, one-time ethanol consumption (alcohol group) caused significantly higher hepatic TNF-α, IL-1β, and IL-6 concentrations than those in control group ($p < 0.05$). In contrast, hepatic TNF-α, IL-1β, and IL-6 concentrations in PD pretreatment groups were significantly lower than those in alcohol group ($p < 0.05$).

Insert Figure 5

3.7 Pathological observations

The representative photomicrographs of liver tissue sections were shown in Figure 6. The results indicated that the liver of normal group showed slight steatosis, normal lobular architecture with central veins, but no obvious inflammation or necrosis was observed (Figure 6A). However, in the alcohol group, typical pathological characteristics including necrosis, inflammatory infiltration and extensive vacuolar degeneration confirmed the successful establishment of alcoholic induced liver injury (Figure 6B). Pretreatment with PD for consecutive 7 days before alcohol exposure noticeably attenuated the apoptotic cells and inflammation, while almost similar to the normal group (Figure. 6C-D).

Insert Figure 6

3.8 Histopathological examination and classification

Systems for grading and staging incorporate the view that necroinflammation is not only a measure of severity but also of ongoing disease activity and the parameter most potentially responsive to therapy [27]. As shown in Table 3, pathological changes in liver is mainly hepatic steatosis and mainly in the central veins around. Alcohol group prior to normal group presented a significant liver injury. PD treatment groups alleviated the steatosis state in different degrees.

Insert Table 3

4. Discussion

Alcoholic liver disease (ALD), one of the most popular public health problems, has resulted in critical personal health hazards and serious public health burdens in the world [2]. Most ingested alcohol is metabolized in the liver, and excessive alcohol intake results in ALD including hepatic steatosis, inflammation, hepatitis, fibrosis and even cirrhosis [28]. Accumulated evidences indicated that alcohol can produce reactive oxygen species (ROS) by a plurality of pathways [4]. ROS over-production induced by alcohol metabolism can result in GSH depletion, decreasing antioxidant activities and elevating lipid peroxidation [29]. After alcohol exposure, there is significant inflammation reaction in the liver and it is believed that the inflammatory cytokines play an important role in the pathogenesis of ALD [3]. Despite years of ongoing research, the underlying mechanisms contributing to ALD remain obscure. The therapeutic strategy of reducing

ethanol-induced liver injury has been focused on the protective function of natural medicines in corresponding with the pathogenesis of alcoholic liver injury.

The root of *P. grandiflorum*, Platycodi Radix, is used as a food and traditional medicine in China, Korea and Japan [7]. Platycodin D (PD), a potent and major saponin isolated from the root of *P. grandiflorum*, exerts various pharmacological activities including anti-inflammatory, anti-tumor and anti-obesity effects [9, 12]. *P. grandiflorum* is a perennial grass and its roots were harvested 2 ~ 3 years after seeding, whereas the aerial parts can be harvested annually. Due to the aerial parts of PG is not recorded in China Pharmacopoeia, it is normally discarded during collecting roots [19]. The findings in our preliminary experiment indicated that the aerial parts of *P. grandiflorum* showed the chemical components sharing partly with them on the roots, especially saponins. Thus, the aerial parts of *P. grandiflorum* exerts potential application in form of natural extract. To date, despite many research reports on chemical constituents on roots parts, few works have ever been reported on the aerial parts of *P. grandiflorum* (APPG). In the present investigation, platycodin D was isolated from the aerial parts of *P. grandiflorum* for the first time. In the present study, the platycodin D in APPG were quantified and analyzed by HPLC method with content of 0.081%. According to our previous investigation, the content of PD in roots ranged from 0.201 to 0.278% [24]. Though the content of PD in roots was three times more than that in aerial parts, the aerial parts would be worthy enough for being employed in the form of natural extract, especially for extracting saponins.

Previous studies have shown that Platycodi Radix supplementation can protect alcohol-induced damage in rats via oxidative stress defense [30]. In addition, researchers reported that the water extract of *P. grandiflorum* exerts a significant protective activity on carbon tetrachloride/thioacetamide-induced liver damage in mice [31, 32]. However, there is no report involving hepatoprotective effect of PD so far. Here, we observed that PD exhibited remarkable hepatoprotective effect on alcohol induced liver injury *in vivo*.

The leakage of ALT and AST in the blood indirectly reflects liver failure caused by alcohol-induced hepatotoxicity [6]. However, pretreatment with PD for 7 consecutive days significantly reversed the elevation of serum ALT and AST to near normal control mice in a dose-dependent manner (p <0.05). In addition, the present study showed the elevation of serum TG

level and hepatic lipid accumulation after acute alcohol exposure. However, PD effectively reversed alcohol-induced lipid accumulation in the liver and increase of serum TG and L-DLC (p <0.05).

Previous studies have demonstrated that oxidative stress is one of the mechanisms of alcohol-induced liver injury [4]. Generally, oxidative stress is originally defined as the disequilibrium between prooxidants and antioxidants in biological systems. This imbalance will lead to lipid peroxidation [5]. The hepatic MDA level was widely used as an index of lipid peroxidation and a major parameter for the status of oxidative stress [29]. It was reported that the level of MDA in liver was increased under the enhancement of oxidative stress in mice [33]. Noh, *et al*. [20, 21] reported that the crude saponins of *P. grandiflorum* may play an important role in protection against alcohol-induced oxidative damage, possibly by inhibition of lipid accumulation and peroxidation through the enhancement of the antioxidant defense system. Among these saponins, PD has the most potent effect on peroxyl radical scavenging activity and pharmacological effects [34]. Similar to the previous studies, PD pretreatment was found to dramatically inhibit the elevation of MDA levels in alcohol-induced liver damage in terms of preventing lipid peroxide formation and blocking oxidative chain reaction, indicating that the hepatoprotective effect of PD is associated with antioxidant activities [22]. As we all know, CAT helps to remove hydrogen peroxide and avoid the production of greater toxicity of hydroxyl radicals. GSH-Px plays a protective role in the low levels of oxidative stress by removing lipids and other organic peroxides effectively. While SOD gets through continuous oxidation and reduction of metal ions of transition state, and then catalyzes the removal of superoxide radicals [35]. The decrease of these antioxidant enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide [36]. In the present study, the activities of hepatic CAT, GSH-Px, and SOD were significantly decreased in the alcohol group compared to the control group (p <0.05). However, PD pretreatment preserved the activities of these enzymes, which consequently prevented alcohol-induced oxidative stress.

In addition to lipid accumulation and oxidative stress, inflammatory response is also a major feature in the development of ALD. Accumulating evidences suggest that many illnesses of individuals with alcoholic liver diseases can be explained readily by a high level of circulating proinflammatory cytokines [37]. TNF-α, IL-1β and IL-6, secreted by Kupffer cells and peripheral blood

monocytes, are three important proinflammatory cytokines involved in ALD [25]. Numerous studies have demonstrated that TNF-α plays a pivotal role in the ethanol-induced liver pathology and it mediates acute and chronic inflammation and infection [38]. In addition, clinical investigations indicated that the levels of TNF-α and IL-1β in serum of ALD patients were closely related to acute phase response markers, liver function, and clinical outcome [39]. In this study, hepatic TNF-α, IL-1β and IL-6 levels significantly increased in mice after alcohol exposure, which indicates that acute alcohol exposure triggered the release of proinflammatory cytokines during the development of ALD. On the contrary, PD pretreatment lowered hepatic proinflammatory cytokine concentrations than those in control group. These findings were consistent with those obtained in previous studies, which demonstrated an anti-inflammation effects of saponins of *P. grandiflorum* on lipopolysaccharide-stimulated BV2 microglial cells and ethanol-induced hepatotoxicity in mice [8, 20].

The imbalance of fatty acid anabolism and catabolism will lead to liver steatosis, which is the most common consequence of acute ALD [20]. Liver steatosis might contribute to the progression of hepatic injury characterized by increased concentration of TG. Through the results of H&E staining (Figure 6), treatment with 20 mg/kg of PD can remarkably improve liver histopathological changes including steatosis, hepatocellular necrosis, inflammatory infiltration, and disorganized liver structure induced by alcohol. In addition, the application of Ridit analysis may be an appropriate method for evaluating pathological changes in liver though there is no widely accepted system for grading and staging ALD [6]. As shown in Table 3, pretreatment of PD can alleviate the steatosis state in different degrees, which is consistent with results of TG.

Conclusions

In this study, platycodin D was isolated from the aerial parts of *Platycodon grandiflorum* for the first time, and exerted protective role on acute alcohol-induced liver injury in mice possibly via alleviation of oxidative stress and inflammatory response. To the best of our knowledge, this is the first observation of the hepatoprotective effect of platycodin D against alcohol-induced mouse model. Findings from the present studies permit us to conclude that platycodin D can be a good candidate for illness of individuals with liver injury caused by alcohol, although further studies should be needed prior to its clinical application.

Conflicts of Interest

The authors declare no conflict of interest

Acknowledgments

This work was supported by the grants of National Natural Science Foundation of China (NO. 31201331), and of Jilin Science & Technology Development Plan (NO. 201201102, 20130303096YY, and 20150204050YY).

References

1. A. Gramenzi, F. Caputo, M. Biselli, F. Kuria, E. Loggi, P. Andreone and M. Bernardi, *Alimentary pharmacology & therapeutics*, 2006, **24**, 1151-1161.
2. M. G. Neuman, S. W. French, C. A. Casey, K. K. Kharbanda, R. M. Nanau, K. Rasineni, B. L. McVicker, V. Kong and T. M. Donohue, Jr., *Experimental and molecular pathology*, 2013, **95**, 376-384.
3. C. C. Tang, H. P. Huang, Y. J. Lee, Y. H. Tang and C. J. Wang, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2013, **62**, 786-796.
4. T. Xu, L. Zheng, L. Xu, L. Yin, Y. Qi, Y. Xu, X. Han and J. Peng, *Archives of toxicology*, 2014, **88**, 739-753.
5. H. Liu, X. Qi, S. Cao and P. Li, *Journal of natural medicines*, 2014, **68**, 521-529.
6. T. Zeng, F. F. Guo, C. L. Zhang, S. Zhao, D. D. Dou, X. C. Gao and K. Q. Xie, *Chemico-biological interactions*, 2008, **176**, 234-242.
7. E. Nyakudya, J. H. Jeong, N. K. Lee and Y. S. Jeong, *Preventive nutrition and food science*, 2014, **19**, 59-68.
8. K. J. Jang, H. K. Kim, M. H. Han, Y. N. Oh, H. M. Yoon, Y. H. Chung, G. Y. Kim, H. J. Hwang, B. W. Kim and Y. H. Choi, *International journal of molecular medicine*, 2013, **31**, 1357-1366.
9. E. J. Lee, M. Kang and Y. S. Kim, *Planta medica*, 2012, **78**, 1536-1542.
10. B. J. Xu, L. K. Han, Y. N. Zheng, J. H. Lee and C. K. Sung, *Archives of pharmacal research*, 2005, **28**, 180-185.
11. M. O. Kim, D. O. Moon, Y. H. Choi, D. Y. Shin, H. S. Kang, B. T. Choi, J. D. Lee, W. Li and G. Y. Kim, *Cancer letters*, 2008, **261**, 98-107.
12. D. Y. Shin, G. Y. Kim, W. Li, B. T. Choi, N. D. Kim, H. S. Kang and Y. H. Choi, *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 2009, **63**, 86-94.
13. Y. M. Ahn, S. K. Kim, J. S. Kang and B. C. Lee, *The Journal of pharmacy and pharmacology*, 2012, **64**, 697-704.
14. J. Zheng, J. He, B. Ji, Y. Li and X. Zhang, *Plant foods for human nutrition*, 2007, **62**, 7-11.
15. H. K. Kim, D. S. Kim and H. Y. Cho, *Bioscience, biotechnology, and biochemistry*, 2007, **71**, 1550-1552.
16. K. J. Lee, H. J. You, S. J. Park, Y. S. Kim, Y. C. Chung, T. C. Jeong and H. G. Jeong, *Cancer letters*, 2001, **174**, 73-81.

17. C. Y. Shin, W. J. Lee, E. B. Lee, E. Y. Choi and K. H. Ko, *Planta medica*, 2002, **68**, 221-225.
18. C. Wang, G. B. Schuller Levis, E. B. Lee, W. R. Levis, D. W. Lee, B. S. Kim, S. Y. Park and E. Park, *International immunopharmacology*, 2004, **4**, 1039-1049.
19. C.-H. Jeong, G. N. Choi, J. H. Kim, J. H. Kwak, D. O. Kim, Y. J. Kim and H. J. Heo, *Food Chemistry*, 2010, **118**, 278-282.
20. T. Khanal, J. H. Choi, Y. P. Hwang, Y. C. Chung and H. G. Jeong, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2009, **47**, 530-535.
21. J. R. Noh, Y. H. Kim, G. T. Gang, J. H. Hwang, S. K. Kim, S. Y. Ryu, Y. S. Kim, H. S. Lee and C. H. Lee, *Annals of nutrition & metabolism*, 2011, **58**, 224-231.
22. T. W. Kim, H. K. Lee, I. B. Song, J. H. Lim, E. S. Cho, H. Y. Son, J. Y. Jung and H. I. Yun, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2013, **51**, 364-369.
23. T. W. Kim, I. B. Song, H. K. Lee, J. H. Lim, E. S. Cho, H. Y. Son, S. J. Park, J. W. Kim and H. I. Yun, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2012, **50**, 4254-4259.
24. W. Li, Y.-s. Sun, Z. Wang and Y.-n. Zheng, *Journal of Liquid Chromatography & Related Technologies*, 2012, **35**, 547-557.
25. Y. L. Chen, H. C. Peng, Y. C. Hsieh and S. C. Yang, *Alcohol*, 2014, **48**, 701-706.
26. H. L. Zhao, K. H. Cho, Y. W. Ha, T. S. Jeong, W. S. Lee and Y. S. Kim, *European journal of pharmacology*, 2006, **537**, 166-173.
27. E. M. Brunt, *Hepatology*, 2000, **31**, 241-246.
28. J. R. Noh, Y. H. Kim, G. T. Gang, J. H. Hwang, H. S. Lee, S. Y. Ly, W. K. Oh, K. S. Song and C. H. Lee, *Food Chem Toxicol*, 2011, **49**, 1537-1543.
29. M. Galicia-Moreno and G. Gutierrez-Reyes, *Rev Gastroenterol Mex*, 2014, **79**, 135-144.
30. J. H. An, D. S. Kim, Y. H. Lee, J. N. Ho, H. K. Kim, O. J. Kang, I. S. Shin and H. Y. Cho, *Journal of medicinal food*, 2009, **12**, 1190-1198.
31. T. W. Kim, J. H. Lim, I. B. Song, S. J. Park, J. W. Yang, J. C. Shin, J. W. Suh, H. Y. Son, E. S. Cho, M. S. Kim, S. W. Lee, J. W. Kim and H. I. Yun, *Journal of nutritional science and vitaminology*, 2012, **58**, 187-194.
32. J. H. Lim, T. W. Kim, S. J. Park, I. B. Song, M. S. Kim, H. J. Kwon, E. S. Cho, H. Y. Son, S. W. Lee, J. W. Suh, J. W. Kim and H. I. Yun, *Journal of toxicologic pathology*, 2011, **24**, 223-228.
33. V. Balasubramaniyan, J. Kalaivani Sailaja and N. Nalini, *Pharmacol Res*, 2003, **47**, 211-216.
34. C. S. Ryu, C. H. Kim, S. Y. Lee, K. S. Lee, K. J. Choung, G. Y. Song, B.-H. Kim, S. Y. Ryu, H. S. Lee and S. K. Kim, *Food Chemistry*, 2012, **132**, 333-337.
35. M. R. Venukumar and M. S. Latha, *Indian J Physiol Pharmacol*, 2002, **46**, 223-228.
36. R. Rukkumani, K. Aruna, P. S. Varma and V. P. Menon, *J Physiol Pharmacol*, 2004, **55**, 551-561.
37. H. J. Wang, S. Zakhari and M. K. Jung, *World journal of gastroenterology : WJG*, 2010, **16**, 1304-1313.

38. S. Candel, S. de Oliveira, A. Lopez-Munoz, D. Garcia-Moreno, R. Espin-Palazon, S. D. Tyrkalska, M. L. Cayuela, S. A. Renshaw, R. Corbalan-Velez, I. Vidal-Abarca, H. J. Tsai, J. Meseguer, M. P. Sepulcre and V. Mulero, *PLoS Biol*, 2014, **12**, e1001855.
39. Y. L. Chen, H. C. Peng, Y. C. Hsieh and S. C. Yang, *Alcohol*, 2014.

Figure legends

Figure 1. The flow chart of extraction and isolation of the aerial parts of *P grandiflorum* (A) and chemical structure of platycodin D (B).

Figure 2. HPLC chromatogram of extract from the aerial parts of *P grandiflorum* (A) and ESI/MS (-) spectra (B) of PD. HPLC separations were achieved using a Hypersil ODS2 column (250×4.6 mm, 5μm). The column temperature was set at 25℃ and detection wavelength was set at 210 nm. The mobile phase consisted of a mixture of acetonitrile (A) and water (B) with flow rate of 1.0 mL/min. The gradient elution was programmed as follows: 0–30 min, 18–22% A; 30–60 min, 22–25% A.

Figure 3. Experimental design on alcohol-induced liver injury (A) and the effect of PD on serum ALT and AST in mice (B). After acclimatization for 7 days, mice were administered intragastrically with PD at 10 and 20 mg/kg once daily for 7 consecutive days, respectively. Three hours after PD administration on the seventh day, mice were administered intragastrically a one-time grant of alcohol in water (50% v/v) with dose of 12 mL/kg. Then all the mice were kept fasting for 12 h, subsequently killed and the blood were collected.

Figure 4. Effects of PD on hepatic CAT (A), GSH-Px (B), SOD (C), and MDA (D) activities in alcohol-induced mice. Data represent the mean ± S.D. Significant differences were indicated by $^{a}p < 0.05$ *vs*. control group. $^{b}p < 0.05$ *vs*. alcohol group.

Figure 5. Effects of PD on hepatic TNF-α (A), IL-1β (B), and IL-6 (C) in alcohol-induced mice. Data represent the mean ± S.D. Significant differences were indicated by $^{a}p < 0.05$ *vs*. control group. $^{b}p < 0.05$ *vs*. alcohol group.

Figure 6. Photomicrographs of liver sections obtained from control group (A), alcohol group (B), PD with 10 mg/kg (C), PD with 20 mg/kg (D). (magnification, all 100×, Bar: 100 μm)

Table 1. Effects of PD on body weight and organ index in mice

Group	Dosage (mg/kg)	Body weights (g)		Organ coefficients (%)	
		Initial	Final	Liver	Spleen
Control	—	21.23 ± 2.78	26.15 ± 3.43	4.21 ± 0.28	0.32 ± 0.03
Alcohol	—	21.45 ± 2.67	26.16 ± 3.56	6.32 ± 0.36 [a]	0.46 ± 0.02 [a]
PD-L	10	21.62 ± 3.15	25.32 ± 4.24	5.55 ± 0.34 [b]	0.42 ± 0.03 [b]
PD-H	20	21.75 ± 3.31	25.11 ± 3.71	4.98 ± 0.26 [b]	0.39 ± 0.02 [b]

Values represent the mean ± S.D., n=8. [a] $p < 0.05$ vs. control group; [b] $p < 0.05$ vs. alcohol group.

Table 2. Effects of PD on serum ALT, AST, TG, TC, and L-DLC levels in mice

Group	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	TG (mmol/L)	TC (mmol/L)	L-DLC (mmol/L)
Control	—	22.5±5.8	16.5±3.8	0.89±0.13	2.75±0.24	0.87±0.12
Alcohol	—	54.2±6.7 [a]	37.8±4.3 [a]	1.34±0.24 [a]	3.82±0.25 [a]	1.44±0.34 [a]
PD-L	10	37.5±4.9 [b]	27.9±2.4 [b]	1.11±0.18 [b]	3.25±0.18 [b]	1.12±0.15 [b]
PD-H	20	32.1±3.8 [b]	22.6±2.8 [b]	1.05±0.12 [b]	3.10±0.22 [b]	0.93±0.17 [b]

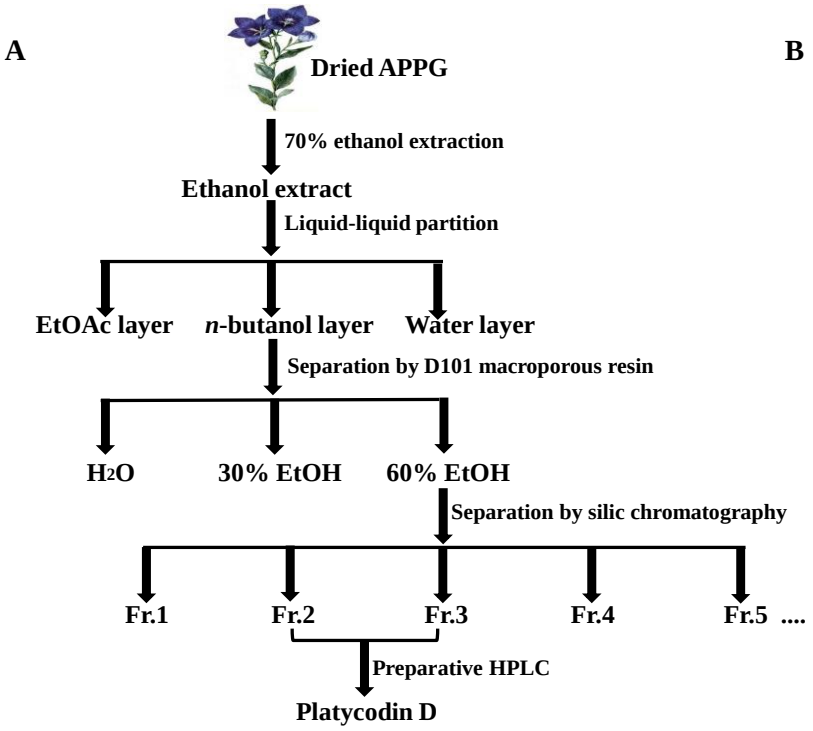
Values represent the mean ± S.D. (n=8); [a] $p < 0.05$ *vs*. control group, [b] $p < 0.05$ *vs*. alcohol group.

Table 3. Pathological changes in the liver and Ridit analysis

Groups	Dosage (mg/(kg))	*n*	Steatosis grade					Ridit analysis
			0	1	2	3	4	
Control	----	8	8	0	0	0	0	0.25
Alcohol	----	8	0	2	4	2	0	0.78 [a]
PD-L	10	8	3	1	2	2	0	0.61 [b]
PD-H	20	8	5	1	1	1	0	0.45 [b]

Note: The steatosis stages were classed on the basis of the H&E staining of liver sections. The data were analyzed by Ridit analysis. Values represent the mean ± S.D. (n=8); [a] $p < 0.05$ *vs*. control group, [b] $p < 0.05$ *vs*. alcohol group. Grading standard: Level 0 means lipid droplets scattered, rare and normal in the cell of liver; Level 1 means liver cells containing lipid droplets of no more than 1/4; Level 2 means liver cells containing lipid droplets of no more than 1/2; Level 3 means liver cells containing lipid droplets of no more than 3/4; Level 4 means liver tissue was almost instead of lipid drops.

1



2

3 **Figure 1**

4

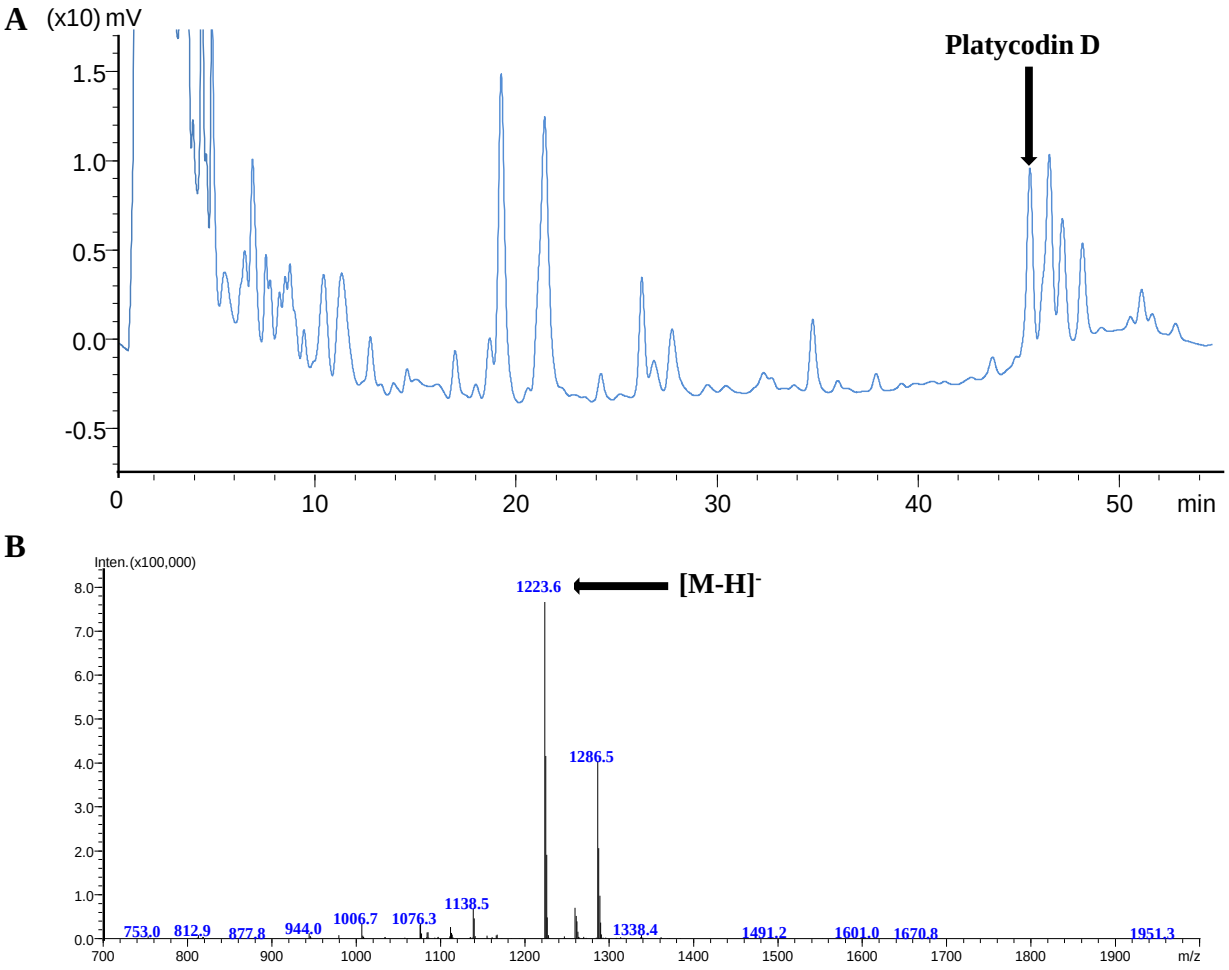


Figure 2

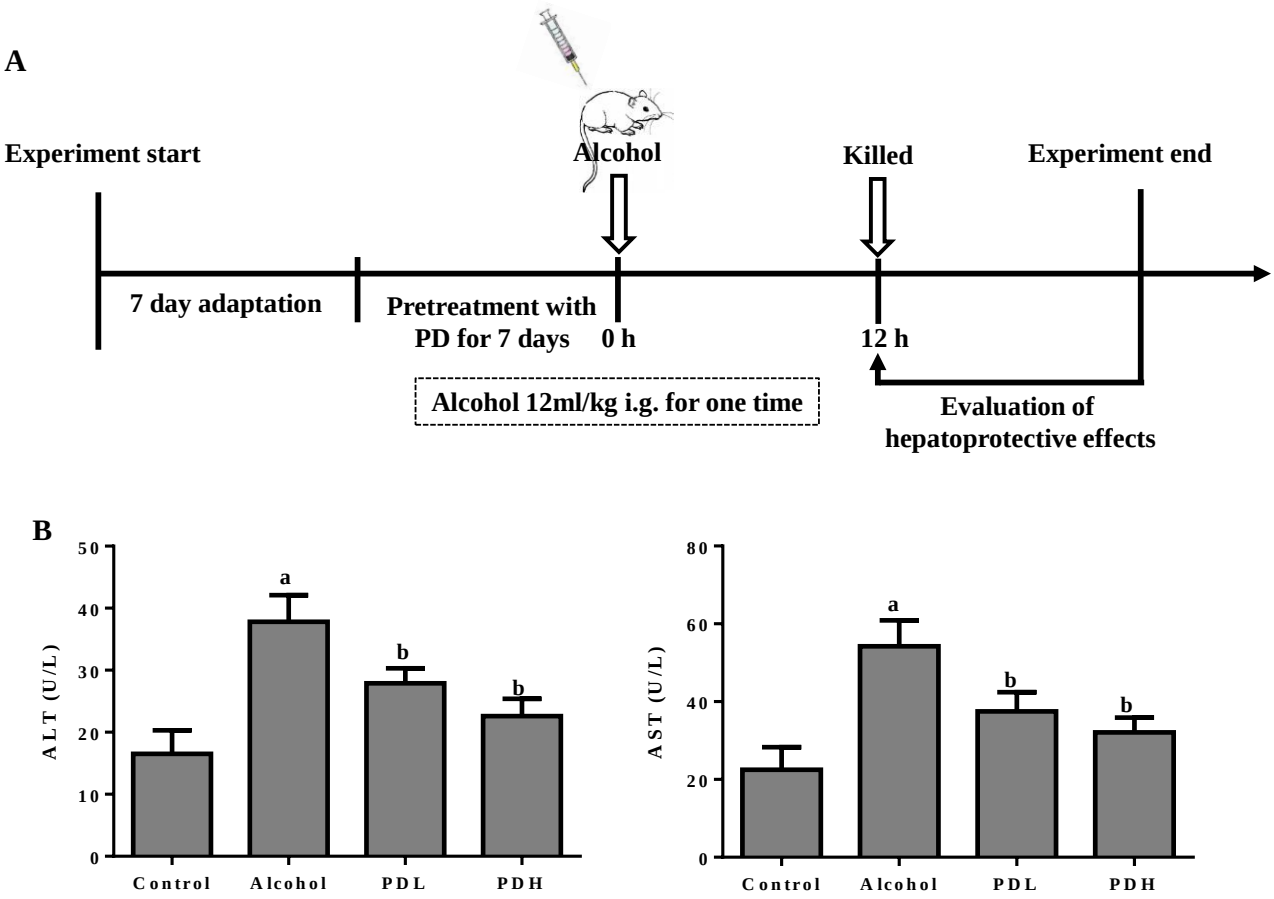


Figure 3

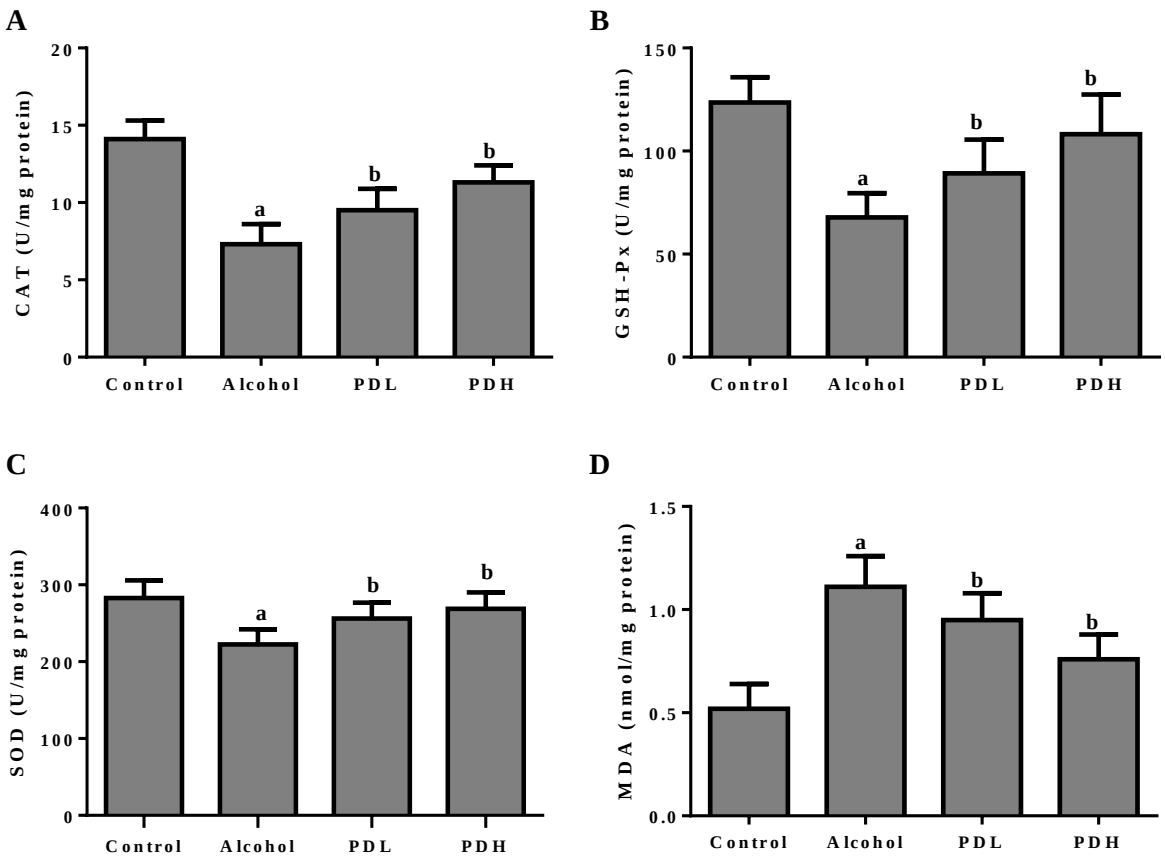


Figure 4

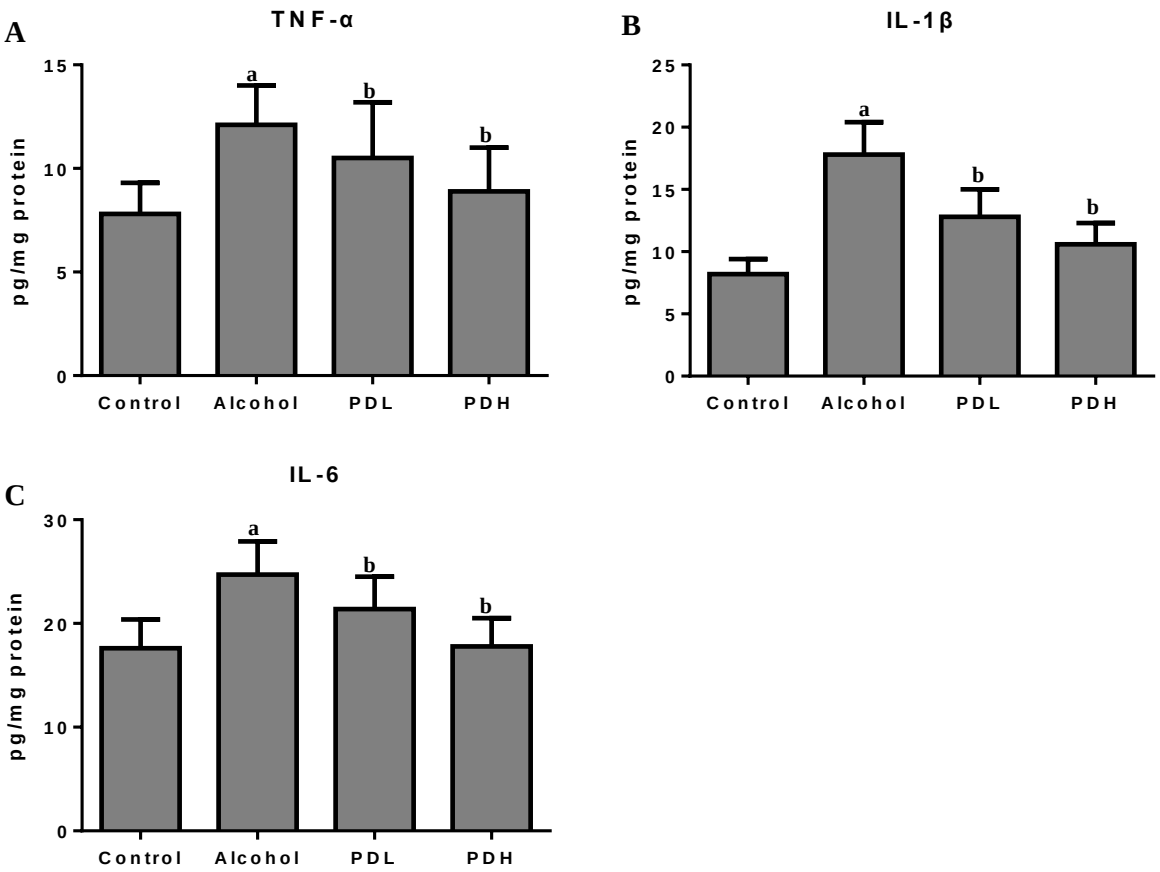


Figure 5

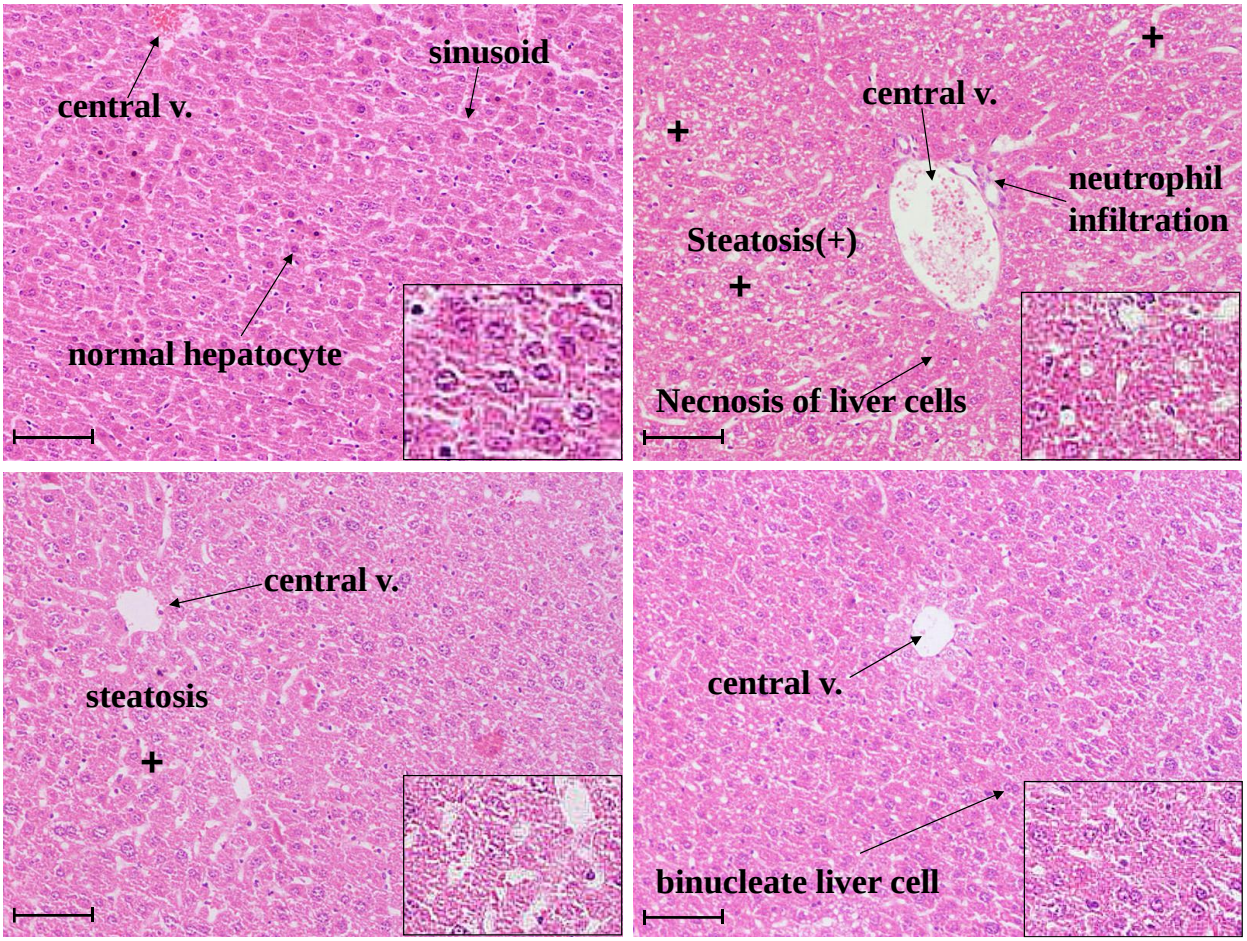


Figure 6