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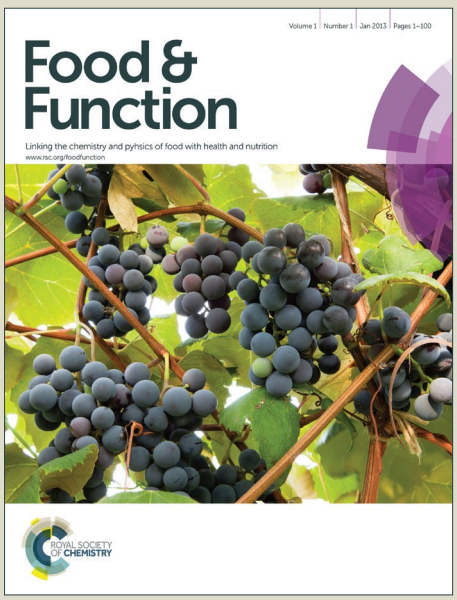

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Hepatoprotective effect of aqueous extracts of *Penthorum chinense* Pursh against acute alcohol-induced liver injury is associated with ameliorating hepatic steatosis and reducing oxidative stress

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Abstract

The aim of present study was to evaluate the effects of *Penthorum chinense* Pursh (PCP), a health food and folk medicine, against acute alcohol-induced liver injury and further to elucidate its probable mechanisms. Male C57BL/6 mice were treated with aqueous extract of PCP (5.2 and 10.3 g/kg BW) once daily for 7 consecutive days priors to ethanol gavage (4.7 g/kg) every 12 h for a total of three doses. Pretreatment with PCP significantly decreased the elevations of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and hepatic triglyceride after the last ethanol administration. PCP suppressed the elevation of malondialdehyde (MDA) level, restored glutathione (GSH) level and enhanced the activities of superoxide dismutase (SOD) and catalase (CAT) in both serum and liver, which were associated with the inhibition of hepatic cytochrome P450 2E1 (CYP2E1). Additionally, alcohol exposure markedly induced the lipolysis of white adipose tissue (WAT) through up-regulating protein expression of adipose triglyceride lipase (ATGL) and phosphorylation of hormone-sensitive lipase (p-HSL), and enhancing fatty acid uptake capacity in liver by elevated hepatic CD36 expression, which were attenuated by PCP treatment. These data demonstrated that pre-treatment of PCP protected against acute ethanol-induced liver injury, possibly through reducing CYP2E1-dependent oxidative stress and ameliorating dysfunctional WAT derived-fatty acid influx to liver. Our findings suggested that PCP might be a promising agent for the prevention of acute alcohol-induced liver injury.

Key words: *Penthorum chinense* Pursh; Alcoholic liver disease; Hepatic steatosis; Oxidative stress; Lipolysis.

1. Introduction

Alcoholic liver disease (ALD) is the most prevalent cause of advanced liver disease and contributes substantially to 4% of global mortality.[1] ALD encompasses a histological spectrum of liver injury that ranges from early-stage steatosis to cirrhosis, and ultimately hepatocellular carcinoma.[2] Hepatic steatosis, defined as excess lipid accumulation in the cytoplasm of hepatocyte, has been widely recognized to be the early consequence of alcohol consumption. The multiple mechanisms contribute to alcoholic hepatic steatosis, involving the increased hepatic *de novo* lipogenesis, the impaired mitochondrial fatty acid β-oxidation and the decreased very low-density lipoprotein (VLDL) secretion.[2] In recent years, the pathogenesis of "increased mobilization of fatty acids from adipose tissue influx toward the liver" in alcoholic hepatic steatosis has attracted more attention.[3, 4] Chronic alcohol exposure stimulates wgite adipose tissue (WAT) lipolysis through up-regulating key enzymes involved in intracellular degradation of triacylglycerol, such as adipose triglyceride lipase (ATGL) and phosphorylated hormone-sensitive lipase (HSL), and elevates hepatic fatty acid uptake capability by up-regulating the expression of CD36, fatty acid transport protein-5 (FATP-5), leading to the increased WAT-derived fatty acid influx to the liver.[3-5] Furthermore, lipid droplets in hepatocytes occupy cytoplasmic space, which may impair cellular functions and make the hepatocytes more susceptible to toxic or stress factors, especially oxidative stress. It is well-known that oxidative stress plays a critical role in the pathogenesis of ALD.[6] The enzymes cytochrome P450 2E1 (CYP2E1), alcohol dehydrogenase (ADH), and catalase all contribute to oxidative metabolism of ingested alcohol. The CYP2E1-dependent ethanol oxidation assumes the most important role in metabolizing ethanol to acetaldehyde during alcohol intake, and it requires oxygen activation and produces reaction oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2), superoxide anion radical (O_2-·) and hydroxyl radical (·OH). [6-8] Free radicals have a great potential to react with polyunsaturated fatty acids, leading to lipid peroxidation (LPO) which results in cell membrane damage. Malonyldialdehyde (MDA), an end-product of LPO, has been widely used as indicator of LPO and a marker for the status of oxidative stress. The elevated ROS generation, along with the depletion or inhibition of endogenous non-enzymatic (e.g. glutathione, (GSH)) and enzymatic antioxidants (e.g. superoxide dismutase (SOD) and catalase (CAT)) by ethanol, can cause the oxidative stress in liver, subsequently lead to liver injury through various mechanisms. [6-8]

Despite the profound detrimental impact of ALD, little progress has been made in the management of ALD, in particular, through medication.[9] Herbal medicines have attracted much attention as potential

therapeutic agents in the prevention and treatment of ALD, due to their multi-target actions and less adverse effects.[10] *Penthorum chinense* Pursh (PCP), belonging to the family of Saxifragaceae, is a well-known Miao ethnomedicine and has been used for a long time as health food and folk medicine for liver protection in China.[11-13] The tea made from the whole plant of PCP is becoming popular among the bartenders in local wineries and local residents who often drink wine. Recently, several *in vitro* studies have demonstrated that PCP and its ingredients possess potent antioxidant [11, 12] and anti-complement properties,[12] as well as anti-hepatocarcinoma.[11] Our previous study also indicated that PCP could protect against chronic ethanol-induced liver injury through suppressing CYP2E1-mediated oxidative stress and enhancing the oxidant defense systems via the activation of Nrf2/HO-1 pathway [14]. However, the impact of PCP on acute alcohol-induced liver injury, particularly in aspect of hepatic steatosis, has been not addressed yet. Alcohol binge drinking is the most common form of alcohol intake for human.[15] Animal model of binge drinking was well-established to mimic acute alcohol consumption.[16] Therefore, in the present study, the possible protective effects of the aqueous extract of PCP against acute ethanol-induced liver injury and its underlying mechanisms on were investigated in a mouse model of binge drinking.

2. Materials and methods

2.1 Herbal materials and preparation

P. chinense Pursh was collected from Gulin County, Luzhou City, Sichuan Province, China, and provided by Sichuan New Lotus Traditional Chinese Herb Limited Company (Chengdu, China). The voucher specimen (No. GHC201401) was deposited at 4 °C in Institute of Chinese Medical Sciences, University of Macau, Macao, China. Dried aerial parts of PCP (100 g) was cut into pieces and successively boiled in 1000 ml of distilled water three times for 2 h each, the decoctions were combined and filtered by gauze, subsequently concentrated in a rotary vacuum evaporator at 65 ℃ followed by lyophilization. The freeze-dried extract was dissolved in distilled water for *in vivo* study. The chemical components of aqueous extract of *P. chinense* was profiled using the established HPLC-UV method [14]. The chemical marker, pinocembrin-7-O-β-D-glucoside, in *P. chinense* was also quantified to control the quality of the tested sample. Upon examining, its content in raw *P. chinense* was 3.49 mg/g.

2.2 Animals and treatments

All of the mice were treated according to the animal procedure approved by institutional animal ethics committee. Eight-week-old male C57BL/6 mice were purchased from Laboratory Animal Services Center, The Chinese University of Hong Kong (Hong Kong, China). Animals were housed three to four per cage, and maintained on individually ventilated cage (IVC) system and allowed free access to water and standard lab chow. All mice were randomly divided into four groups i.e. control group, ethanol group, and two PCP treatment groups (5.2 and 10.3 g/kg of body weight) [14]. A mouse model of binge drinking was used to induce acute alcohol-induced liver injury. Animal in treatment groups were orally administered PCP at doses of 5.2 and 10.3 g/kg BW, respectively, for 7 days prior to ethanol challenge. Meanwhile, mice in control and ethanol groups received an equal volume of Milli-Q water. The high dose of PCP (10.3 g/kg) was roughly calculated and converted according to usage description of the China approved drug, Gansukeli (WS3-B-2526-97), which made from aqueous extract of *P. Chinense* for the treatment of viral hepatitis. On day 8, mice were treated with ethanol (4.7 g/kg BW), diluted in water (60%, v/v) by oral gavage every 12 h for a total of three doses. Control group were pair-fed an isocaloric maltose solution to eliminate the difference in energy between the alcohol and control groups. This alcohol dose could cause significant liver injury and hepatic steatosis, as described in our previous study. [17] At 4 h after the last dosing, mice were sacrificed and blood sample was collected for biochemical analysis. Whole liver and epididymal fat were immediately collected. The portion of tissues from the same lobe of liver in each mouse was embedded in OCT (frozen tissue matrix) for histological analysis. The remaining liver tissue and epididymal fat were stored at -80 °C until analyzed.

2.3 Serum biochemical assays

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and serum triglyceride (TG) were determined by automatic biochemical analyzer in clinical laboratory at the fifth affiliated hospital of Zunyi Medical University, Zhuhai, China.

2.4 Histological assay

Liver tissues were embedded with frozen tissue matrix and cut into thin sections (8μm). Liver cryostat sections were fixed in 4% phosphate-buffered paraformaldehyde (pH=7.4) and stained with Oil Red O (Sigma-Aldrich) and counterstained with hematoxylin (Sigma-Aldrich) using a standard protocol. The

stained sections were examined and recorded by an Olympus CX-31 light microscopy with CCD camera (Olympus Crop, Tokyo, Japan).

2.5 Hepatic triglyceride determination

Hepatic triglyceride level was quantitatively determined by a Triglyceride Quantification Kit (Beijing BHKT Clinical Reagent Co., Ltd, Beijing, China) according to the manufacturer's instruction. Briefly, 50 mg of liver tissue was homogenized in 450 μL of chloroform/methanol solution (2:1, *v/v*). After extraction for 16 h at 4 ℃, samples was added 500 μL saline, then centrifuged at 2000 rpm for 15 min. The chloroform layer (lower) was transferred to a new sterile tube and dried under nitrogen gas. The residue was dissolved in 100 μL PBS containing 1 % Triton X-100 and the triglyceride content was examined by using the commercial kit. The value of hepatic triglyceride level was normalized to tissue wet weight and expressed as mg/g of liver.

2.6 Determination of MDA, GSH, SOD and CAT levels in serum and liver

Liver homogenates (5%-10%) were prepared with cold phosphate buffer (8 mM KH_2PO_4, 12 mM K_2HPO_4, 1.5% KCl, pH 7.4), then centrifuged at 12,000 g for 15 min at 4 ℃. This resulting supernatant of liver homogenate or serum plasma samples were used to determine the levels of MDA and reduced glutathione, as well as the activities of SOD and CAT in liver or serum by using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. All values of liver were normalized to hepatic total protein.

2.7 Measurement of hepatic cytokines

Liver TNF-α and IL-6 were measured using commercial TNF-α and IL-6 ELISA kits, respectively. Briefly, 50 mg of liver tissue was homogenized in 450 μL ice-cold RIPA lysis buffer (50 mM Tris , 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS sodium orthovanadate, sodium fluoride, EDTA, leupeptin, pH 7.4) with 1% protease inhibitor cocktail. After incubation on ice for 30 min, the homogenate was centrifuged at 12,000 g for 15 min at 4 °C. The supernatants were then used for ELISA assay by Mouse TNF-α and Il-6 ELISA MAX™ Standard kits (BioLegend Inc., San Diego, CA, USA) according to the manufacturer's instructions. The results were corrected by protein quantification and expressed as pg/mg of protein. The levels of TNF-α and IL-6 were examined by using Mouse TNF-α

and IL-6 ELISA MAX™ Standard kits (BioLegend Inc., San Diego, CA, USA), respectively. The values of cytokines were normalized to hepatic total protein and expressed as pg/mg of liver protein.

2.8 Measurement of adipose tissue lipolysis

Lipolysis of adipose tissue was measured as the release of free fatty acid (FFA) into the culture medium *ex vivo*. Briefly, the fresh epididymal adipose tissue explants were washed in culture plates with pre-warmed Dulbecco's PBS containing 100 U/mL penicillin and 100 mg/mL streptomycin. After removing possible blood vessels and connective tissues, approximate 30 mg of adipose tissue was transferred to 12-well plates, cut into small pieces and then cultured in DMEM containing 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin and 2% fatty acid-free bovine serum albumin for 3 h. Free fatty acid (FFA) in culture medium and serum samples were determined by using a Fatty Acid Quantification Kit (Biovision, Milpitas, CA) according to its manufacturer's protocol.

2.9 Immunoblotting analysis

Total proteins were extracted from liver and epididymal adipose tissue in a cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) with 1% phosphatase inhibitor cocktail and 1% phenylmethanesulfonyl fluoride (PMSF). The protein concentrations of the tissue extracts were determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). Equal amounts of protein samples (60 ug) were separated by 10% SDS- PAGE gel and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. After blocking, the membranes were immunoblotted with the primary antibodies, including CD36 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) , CYP2E1 (Abcam, Cambridge, UK), HSL, phospho-HSL, ATGL and β-actin (Cell Signaling Technology, Beverly, MA, USA), at 4 ℃ overnight. Then, the blotted membrane was incubated with the secondary antibody (anti-rabbit peroxidase conjugate, 1:5000 dilutions in TBST; Cell Signaling Technology) for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Bio-Sciences., Piscataway, NJ, USA) under Bio-Rad ChemiDoc™ XRS System (Bio-Rad Laboratories, Inc., Hercules, USA). Protein quantity was determined by densitometry analysis using ImageJ software (version 1.47).

2.10 Statistical analysis

All other values were expressed as means ± SD. Statistical comparisons between groups were done using GraphPad Prism 5.0 software by student's t-test. The level of significance was considered at $P<0.05$ for all the statistical tests.

3 Results

3.1 Effect of *P.chinense* on body weight, liver index and fat mass

The body weight (BW), liver index (liver weight/BW, %) and the proportion of abdominal fat to BW were compared across four groups and listed in **Table 1**. Three doses of ethanol significantly reduced the average body weight of mice, and PCP did not affect animal body weight. Compared to the control group, liver index in ethanol group was remarkably increased by 14.4%, the most plausible explanation was that alcohol exposure led to the severe lipid accumulation in liver. This increase was prevented by PCP treatments, either low-dose (5.2 g/kg BW) or high-dose (10.3 g/kg BW). In addition, alcohol exposure significantly reduced the masses of abdominal fat depots, including epididymal and perirenal WAT, and the weight loss was 20.1% and 36.7%, respectively. This decrease was reversed by the pre-treatment of PCP, especially by high-dose. Low-dose PCP showed a decreasing tendency compared to ethanol group, but there was no significant difference.

3.2 PCP attenuates acute alcohol-induced hepatoxicity

The serum activities of ALT and AST were most commonly used as reliable primary indicators for clinical monitoring of liver injury.[18] As shown in **Fig. 1A and 1B**, three doses of ethanol led to hepatotoxicity in mice, as indicated that serum levels of ALT and AST in ethanol group were greatly increased by 58.7% (39.5 ± 10.7 *vs*. 24.9 ± 3.6 U/L) and 78.4% (157.0 ± 12.9 vs. 88.0 ± 6.9 U/L), respectively when compared with control group. The pretreatment with PCP, either low-dose or high-dose, was effective in protecting the acute alcohol-induced liver injury by decreasing the serum levels of ALT and AST. But this decrease did not appear to be dose-dependent.

3.3 PCP reduces inflammatory cytokines in liver

To examine the effect of PCP treatment on inflammatory cytokines induced by acute ethanol exposure, two important cytokines, i.e. TNF-α and IL-6, in liver were determined by using commercial ELISA kits. As shown in **Fig. 1C and 1D**, the hepatic levels of both TNF-α and IL-6 were significantly increased in ethanol group when compared to control group. These increases were attenuated by PCP treatments in a dose-dependent manner.

3.4 PCP relieves ethanol-induced oxidative stress

The term of oxidative stress is characterized by an imbalance between endogenous pro-oxidants and antioxidants. To evaluate the effect of PCP pretreatment on acute ethanol-induced oxidative stress, lipid peroxidation, non-enzymatic antioxidant level and enzymatic antioxidant activity were examined in both serum (**Table 2**) and liver tissue (**Fig. 2**). MDA, an principal product of lipid peroxidation, has been widely used as an indicator for the status of oxidative damage [19]. Three doses of alcohol induced the dramatic increase of MDA level in serum and liver by 50.2 % and 336.1 % (p<0.01), respectively, which were significantly attenuated by either low-dose or high-dose PCP treatment. Conversely, acute ethanol exposure might impair endogenous non-enzymatic (e.g. GSH) and enzymatic antioxidant (e.g. SOD and CAT) systems that protect hepatocyte against oxidative damage [10], which was consistent with our results that three doses of ethanol intake greatly reduced GSH level, SOD and CAT activities in both serum and liver when compared to control mice. This depletion of these antioxidans were remarkably ameliorated by pretreatment of PCP, especially high-dose of PCP (10.3 mg/kg BW). Low-dose of PCP showed an increasing tendency in hepatic CAT, hepatic GSH and serum CAT, but no significant difference.

To understand the mechanism involving in the attenuation of ethanol-induced oxidative stress by PCP, the protein expression of CYP2E1, a major contributor to ROS production, was examined by immunoblot analysis. As shown in **Fig. 3**, three doses of ethanol gavage notably up-regulated the protein expression of CYP2E1 by 63.2 % when compared to control group. This increase of CYP2E1 expression was significantly inhibited by PCP (10.3 g/kg). Our results indicated that the protective effect of PCP on ethanol-induced oxidative stress was associated with down-regulation of CYP2E1 in liver.

3.5 PCP alleviates acute ethanol-induced hepatic steatosis

To assess the impact of PCP on hepatic steatosis induced by acute ethanol exposure, lipid accumulation in liver was qualitatively examined by Oil Red O staining and quantitatively determined by a

triglyceride quantification kit. As illustrated by Oil Red O staining (**Fig. 4A-4D**), the mice in the ethanol group and ethanol + PCP groups exhibited obvious microvesicular steatosis in their livers when compared to control group. The hepatic lipid droplets in PCP-treated groups were much smaller and fewer than those in the ethanol group. Quantitative TG determination was consistent with histopathological assay. Acute ethanol gavage dramatically increased the hepatic TG level in mice by 186 % (p <0.001), and this elevation was significantly blunted by pre-treatment of PCP at dose of 5.2 and 10.3 g/kg in a dose-dependent manner (**Fig.4E**). In addition, serum triglyceride level in PCP-treated mice (10.3 g/kg) was significantly lower when compared to that of ethanol group (**Fig. 4F**). These data clearly demonstrated that PCP could effectively protect against acute ethanol-induced hepatic steatosis.

3.6 Effect of PCP on WAT lipolysis and hepatic fatty acid uptake

To understand how acute alcohol exposure reduces adipose tissue mass, we examined the lipolysis capacity of WAT and the expressions of the related genes involving in fatty acid transportation from adipose tissue to liver. Lipolysis capacity was determined by incubating freshly isolated epididymal WAT. As shown in **Fig. 5A**, three doses of alcohol apparently stimulated WAT lipolysis as indicated by 1.6 fold increase in FFA release during 3 hrs incubation when compared to control group, which partly explained the lower ratio of WAT to BW after acute ethanol exposure. Pre-treatment with PCP (10.3 g/kg) abolished the increase of FFA release from epididymal WAT explants induced by acute ethanol ingestion. Low-dose of PCP showed a decreasing tendency, but no significant difference. This result was consistent with the alteration in serum FFA level, PCP could substantially decrease the serum FFA levels elevated by acute ethanol exposure in dose-dependent manner (**Fig. 5B**). Adipose ATGL and HSL are key enzymes involved in intracellular degradation of TG in adipose tissue, and HSL activity is regulated by post-translational phosphorylation.[20, 21] Therefore, the protein expression levels of ATGL, total HSL, and phosphorylated HSL (p-HSL) were examined and compared by western blotting analysis. As shown in **Fig. 5C and 5D**, acute ethanol exposure obviously up-regulated the protein expressions of ATGL and p-HSL in epididymal WAT, which could be partially attenuated by PCP treatment (10.3 g/kg), whereas, the total HSL protein level was not affected. Our data demonstrated that PCP treatment ameliorated the acute ethanol-induced WAT dysfunction, which is associated with the down-regulation of ATGL and p-HSL expressions.

To understand the molecular mechanism underlying protective effect of PCP on hepatic lipid accumulation induced by acute ethanol exposure, the protein expression of CD36, a major fatty acid translocases that contributes to fatty acid uptake in liver,[22] was also examined (**Fig. 6**). Acute ethanol exposure significantly increased the hepatic protein level of CD36, which was normalized by PCP treatment (10.3 g/kg). These data suggested that PCP might decrease the elevation of hepatic fatty acid uptake stimulated by alcohol exposure.

4. Discussion

Oxidative stress has been suggested to play a central role in ethanol-induced liver injury and the pathogenesis of ALD.[8] Either acute or chronic alcohol exposure increases the production of reactive oxygen species (ROS), leading to oxidative stress in the liver. ROS generation is normally counterbalanced by non-enzymatic antioxidants (e.g. GSH), enzymatic antioxidants (e.g. SOD and CAT) and other redox molecules (Yu, 1994). Excess ROS depletes endogenous anti-oxidative defenses and causes the deleterious effects on hepatocytes by damaging DNA and denaturing proteins.[7] Alcohol-induced ROS also reacts with polyunsaturated fatty acids, leading to cell membrane damage. MDA, an end-product of lipid peroxidation, is often used as indicator of oxidative damage.[19] Our data showed that the pre-treatment with PCP (10.3 g/kg) could prevent the elevation of MDA levels, completely reverse depletion of GSH and decrease of SOD and CAT activities in both liver and serum induced by acute ethanol exposure. However, whether this dramatic enhancement of antioxidants was a direct response to PCP treatment or the secondary effect of the reduced oxidative stress was still unknown. In order to gain more insight into the mechanisms underlying the anti-oxidative stress effect of PCP, the protein expression of cytochrome CYP2E1 in liver was examined. CYP2E1 is a central functional enzyme in alcohol metabolism and could produce ROS during its catalytic circle. The level of CYP2E1 can be elevated by ethanol administrate which is considered as a major contributor to ethanol-induced hepatic oxidative stress.[23] The results of the present study indicated that pre-treatment of PCP down-regulated the ethanol-induced elevation of hepatic CYP2E1 expression, which contributed to its anti-oxidative stress effect. Acute ethanol exposure of small intestine appears to increase the permeability and integrity of its epithelium which is critical for the barrier of intestine,[24] resulting in endotoxins to enter the systemic circulation and contribute to an inflammatory response in the liver. Our data presented that PCP treatment significantly decreased acute alcohol-induced elevation of TNF-α and IL-6 level in liver. These results also indicated the anti-inflammatory effect of PCP against alcohol-induced liver injury.

Hepatic steatosis is the earliest stage in the progression of ALD and most common response of liver to either acute or chronic alcohol exposure. The excessive lipids occupy the cytoplasmic space of the hepatocytes, which severely affect cellular function and render the hepatocytes more susceptible to hepatotoxins.[3] Our data demonstrated that pre-treatment with PCP dramatically decreased hepatic steatosis induced by three doses of alcohol exposure, as indicated by Oil Red O staining and hepatic TG quantification. Increasing number of studies have also indicated that chronic alcohol exposure stimulates lipolysis of WAT and elevates hepatic fatty acid uptake, leading to the increased fatty acid release and influx to the liver, which also involved in pathogenesis of alcoholic steatosis.[3, 4] This pathogenesis has been well-documented in chronic alcohol exposure model. However, its role in acute alcohol exposure has not been addressed yet. Although hepatic *de novo* lipogenesis has been considered to be main mechanism of hepatic steatosis induced by acute ethanol exposure, it appears that there is not enough time to express a series of lipogenesis-related genes and biosynthesize the large amount of fatty acids from glucose in very short time.

Adipose tissue derived-fatty acids influx to the liver might be a more important and economical contributor to acute ethanol-induced hepatic steatosis. Our results indicated that binge drinking by giving three doses of alcohol (4.7 g/kg BW) every 12 h significantly reduced the WAT index, including epididymal and perinephric WAT (**Table 1**). Lipid homeostasis in WAT is generally dependent on fatty acids uptake and its lipolysis which mainly cause the change in fat mass.[25] Recent studies have shown that ethanol exposure inhibited the ability of fatty acids uptake in WAT by down-regulating the expression of CD36, fatty acid transport protein-1 (FATP-1) and FATP-4.[26] Whereas ethanol exposure also stimulated the WAT lipolysis, leading to the increased circulating FFA level,[3] which is consistent with our results. Our finding also demonstrated that pre-treatment with PCP could ameliorate the WAT mass lose induced by acute alcohol exposure via inhibition of WAT lipolysis. ATGL is the rate-limiting enzyme for the initial step in TG hydrolysis in adipose tissue. The phosphorylated HSL is a principal enzyme responsible for hydrolyzing both TG and diacylglycerols (DAG) with the release of a free fatty acid.[27] Our data indicated that PCP treatment decreased the protein levels of ATGL and phosphorylated HSL, but not total HSL, which mainly contributes to its inhibitory effect on WAT lipolysis.

The increased hepatic fatty acids uptake ability contributes to the pathogenesis of hepatic steatosis. [28, 29]Actually, the total amount of fatty acids influx to liver mainly depends on hepatic fatty acid uptake capacity and the circulating FFA concentration.[30] The increased circulating FFA concentration provides sufficient sources to allow for increased uptake into hepatocytes after alcohol exposure, subsequently increased TG content in liver. The circulating FFA is mainly derived from dietary and excess WAT lipolysis.[30] However, the factor of dietary may be ignored due to pair-feeding in our study. Our data indicated that pre-treatment with PCP could not only reduce the elevated serum FFA level released from dysfunctional WAT, but also inhibit the up-regulated the protein expression of CD36, a key transport protein involved in regulating the uptake of fatty acids in hepatocytes. These effects of PCP might contribute to its protective effect against hepatic injury induced by acute alcohol exposure.

In summary, the pre-treatment with aqueous extract of *P. chinense* (10.3 g/kg) reduces acute ethanol-induced liver injury. These protective effects might be associated with reducing CYP2E1-dependent oxidative stress and ameliorating hepatic steatosis. Our findings also suggested that PCP might be a promising agent for the prevention of acute alcohol-induced liver injury.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Tables

Table 1. Effects of *P. chinense* (PCP) on acute alcohol-induced changes in body weight, liver index and fat mass

Group	Body weight (g)	Liver index (%)	eWAT / BW (%)	pWAT / BW (%)
Control	25.2±1.7	3.88±0.39	1.99±0.20	0.60±0.12
Ethanol	24.4±1.4*	4.44±0.20**	1.59±0.42*	0.38±0.21*
Ethanol + PCP (5.2 g/kg)	23.2±1.2	4.13±0.19$^{\#}$	1.63±0.50	0.45±0.17
Ethanol + PCP (10.3 g/kg)	25.0±1.5	4.15±0.14$^{\#\#}$	2.07±0.49$^{\#}$	0.62±0.16$^{\#}$

Data are expressed as means ± SD (n=7~10); eWAT, epididymal white adipose tissue; pWAT, perinephric white adipose tissue, $^{*}p<0.05$, $^{**}p<0.01$, *vs.* control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, *vs.* ethanol group.

404 **Table 2.** Effects of *P. chinense* (PCP) on the productions of malondialdehyde (MDA), glutathione (GSH)
405 level, and activities of superoxide dismutase (SOD) and catalase (CAT) in serum.

Group	MDA (nmol/ml)	SOD (U/ml)	GSH (umol/L)	CAT (U/ml)
Control	9.87±2.72	73.2±3.31	155.5±5.62	5880.5±304.6
Ethanol	14.83±1.66**	58.3±5.72***	97.1±10.21***	5337.1±425.8*
Ethanol + PCP (5.2 g/kg)	12.30±1.52$^{\#}$	66.4±5.47$^{\#}$	111.5±6.89$^{\#}$	5461.3±349.0
Ethanol + PCP (10.3 g/kg)	9.53±2.67$^{\#\#}$	72.1±6.62$^{\#\#}$	130.2±21.58$^{\#\#}$	5842.5±271.8$^{\#}$

406 Values represent means ± SD (n=4~8); $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, *vs.* ethanol group.
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Figure Captions

Fig. 1. Effects of *P. chinense* (PCP) on serum enzyme activities of (**A**) ALT and (**B**) AST, and hepatic levels of (C) TNF-α and (D) IL-6. Values represent means ± SD (n=7~8); **p<0.01, ***p<0.001, *vs*. control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}p$<0.001 *vs*. ethanol group.

Fig. 2. Effects of *P. chinense* (PCP) on the levels of MDA and GSH, and the activities of SOD and CAT in liver. Values represent means ± SD and are normalized to mg or g protein content, n=7~8; *p<0.01, **p<0.01, ***p<0.001, *vs*. control group; $^{\#}$ p<0.05, $p^{\#\#}$<0.01 *vs*. ethanol group.

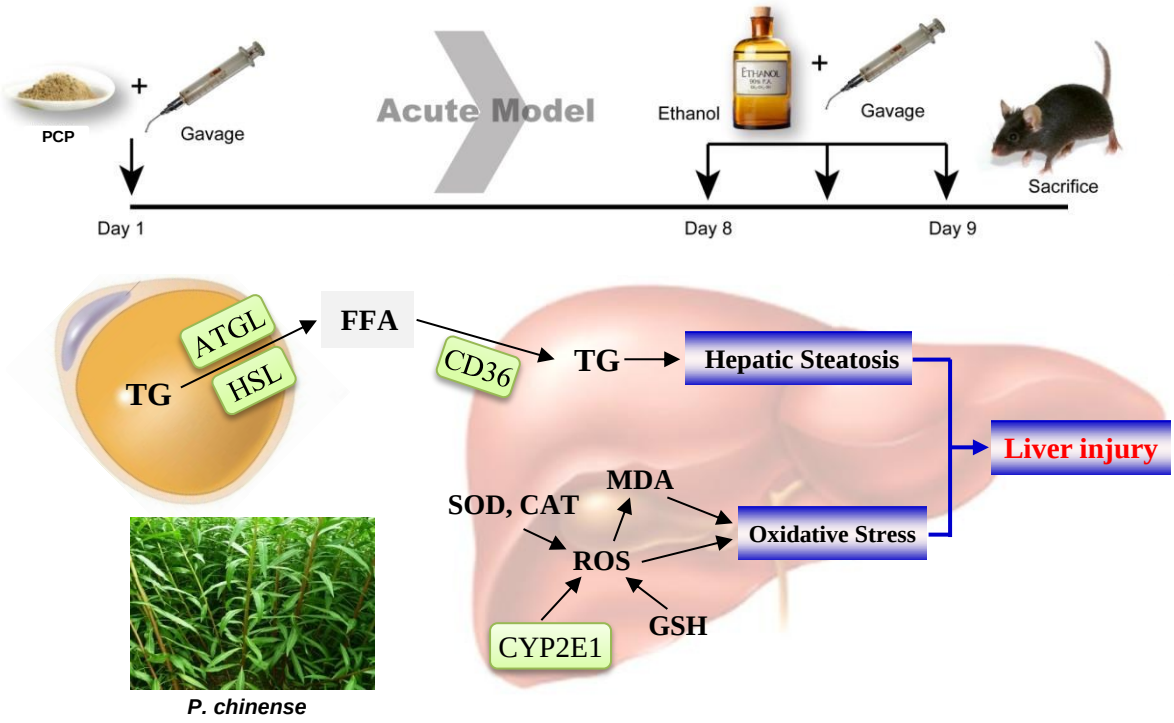
Fig. 3. Effect of PCP on protein expression of cytochrome P450 2E1 (CYP2E1) in the livers. (A) Immunoblot analysis; (B) quantification of the data from A by densitometry analysis. Data are means ± SD (n=3). ***p<0.001, *vs*. control group; $^{\#}$ p<0.05, *vs*. ethanol group.

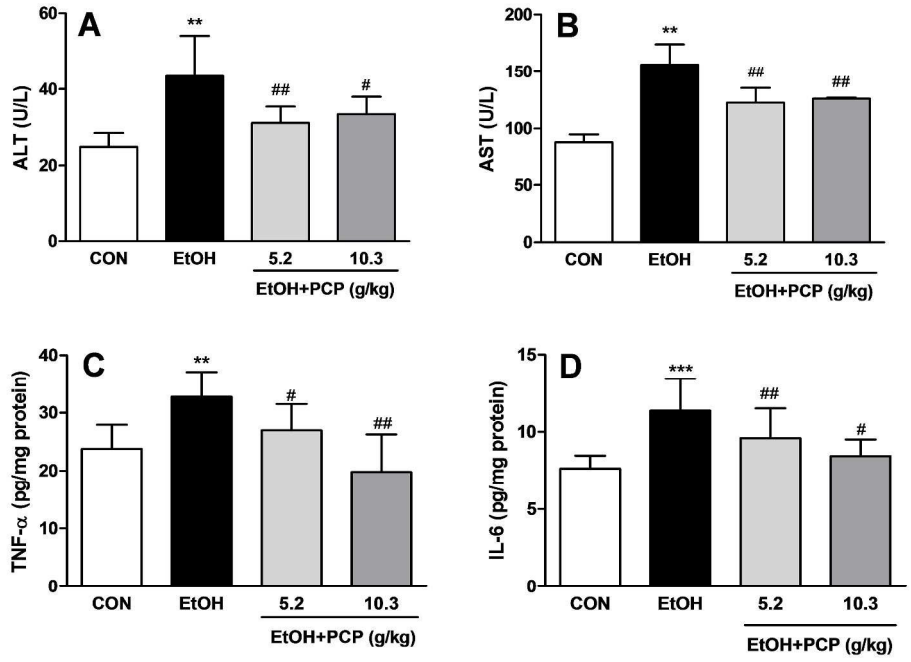
Fig. 4. *P. chinense* (PCP) attenuates acute alcohol-induced fat accumulation in liver. Representative Oil Red O-stained sections of livers (200 × magnification) from (A) control, (B) ethanol, (C) ethanol + PCP (5.2 g/kg) and (D) ethanol + PCP (10.3 g/kg); (E) hepatic triglyceride levels; (F) serum triglyceride levels. Values represent means ± SD, n=7~8; **p<0.05, ***p<0.001, *vs*. control group; $^{\#}$ p<0.05, $^{\#\#}$ p<0.01, *vs*. ethanol group.

Fig. 5. *P. chinense* (PCP) ameliorated acute ethanol-induced dysfunctional lipid metabolism of white adipose tissue. (**A**) Fatty acid released from epididymal WAT explant *ex vivo*; (**B**) Serum FFA levels; (**C**) Immunoblot analysis of p-HSL, HSL and ATGL proteins related to lipolysis in epididymal WAT; (**D**) The immunoblot bands were quantified by densitometry analysis. Value represents means ± SD (n=7~8 in A and B; n=3 in C and D). *p<0.01, **p<0.01, ***p<0.001, *vs*. control group; $^{\#}$ p<0.05, *vs*. ethanol group.

Fig. 6. Effect of PCP on protein expression of CD36 in the livers. (A) Immunoblot analysis; (B) quantification of the data by densitometry analysis. Data are means ± SD (n=3). *p<0.05, *vs*. control group; $^{\#}$ p<0.05, *vs*. ethanol group.

Graphic Abstract





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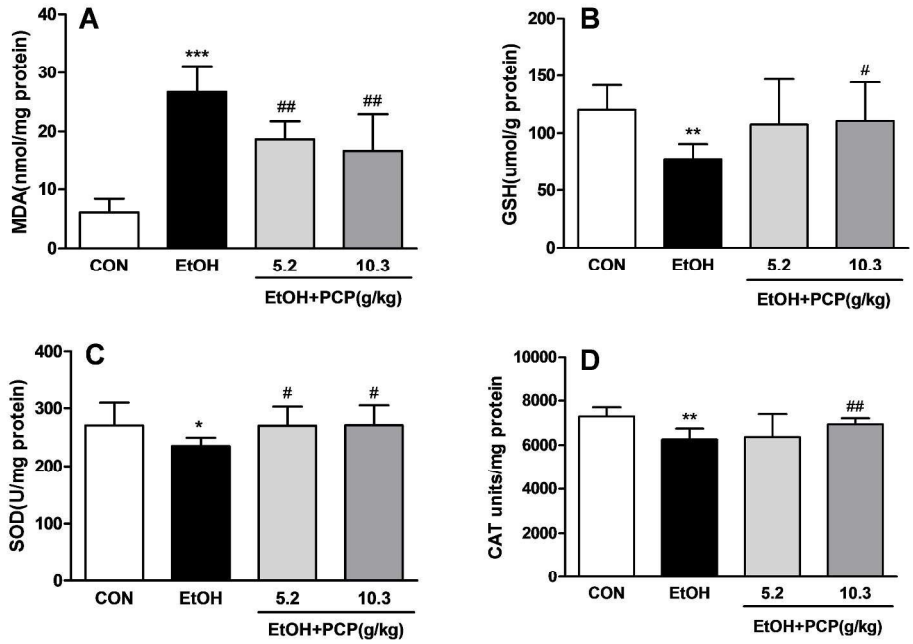

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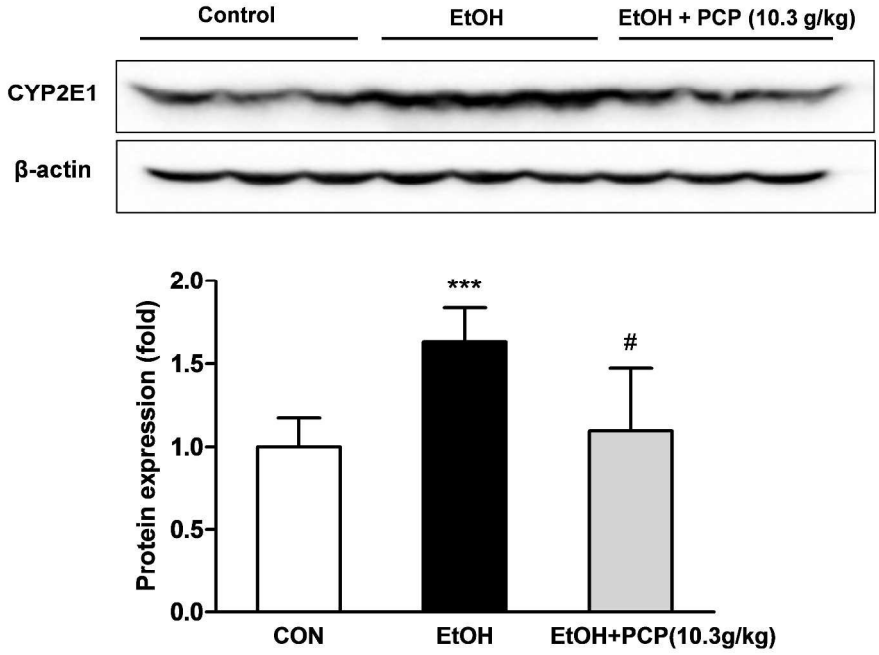

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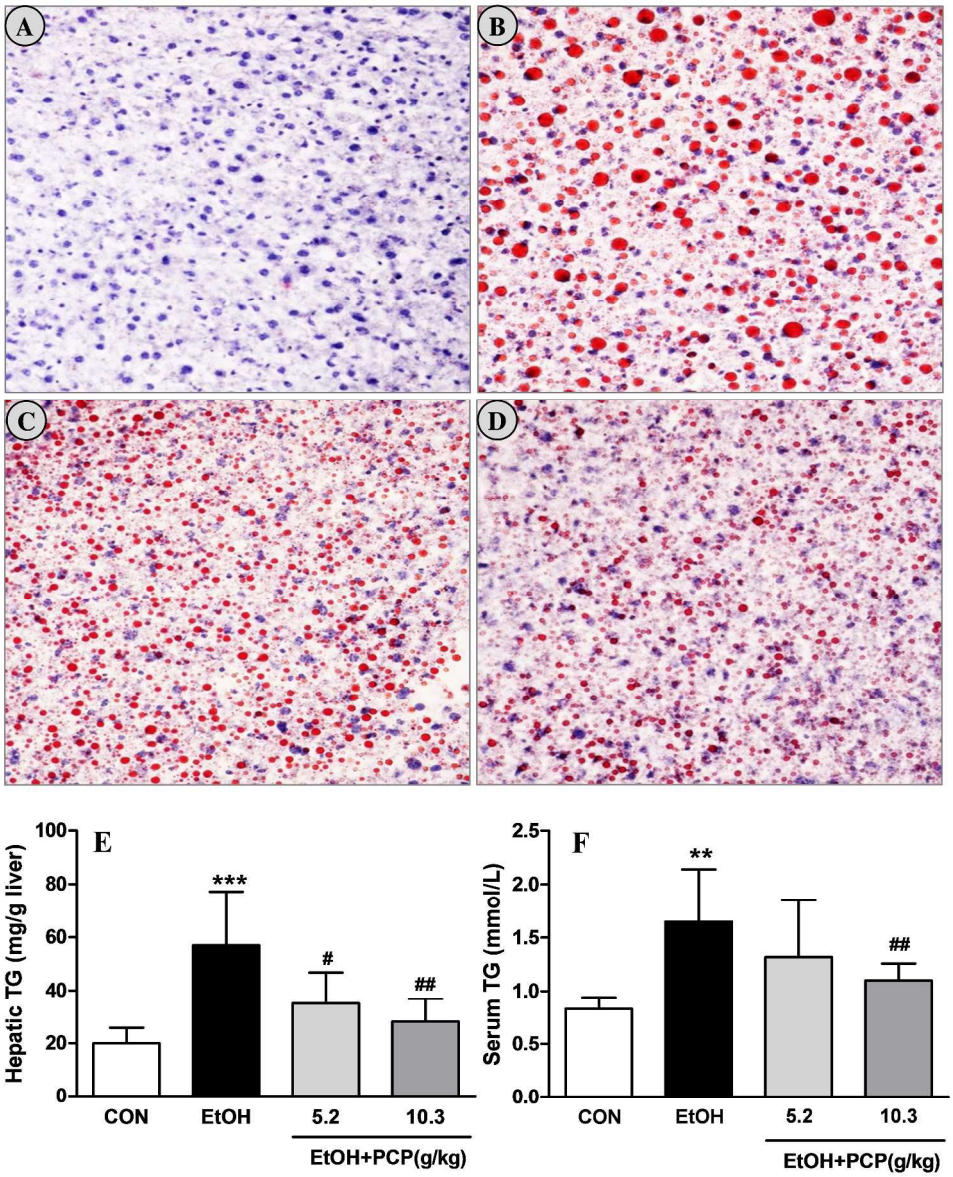

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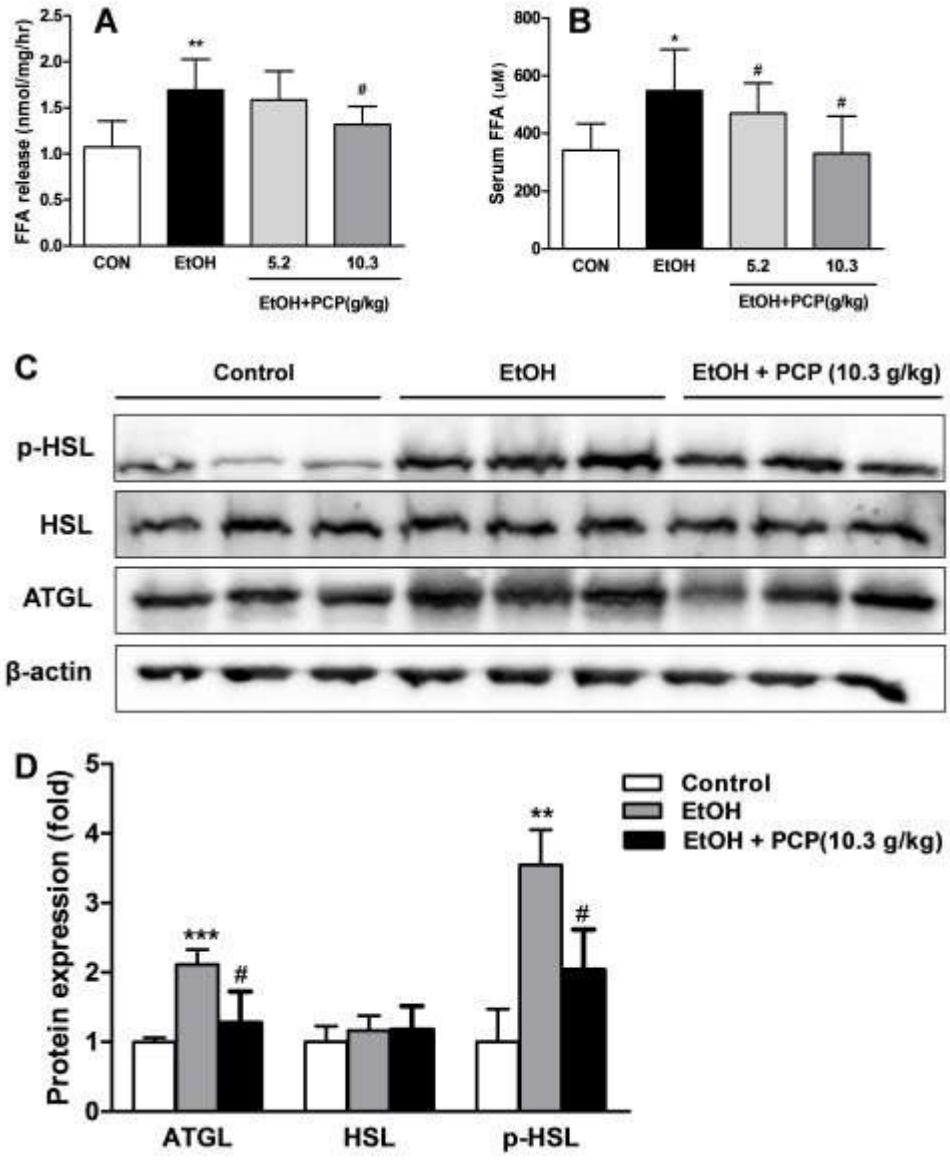

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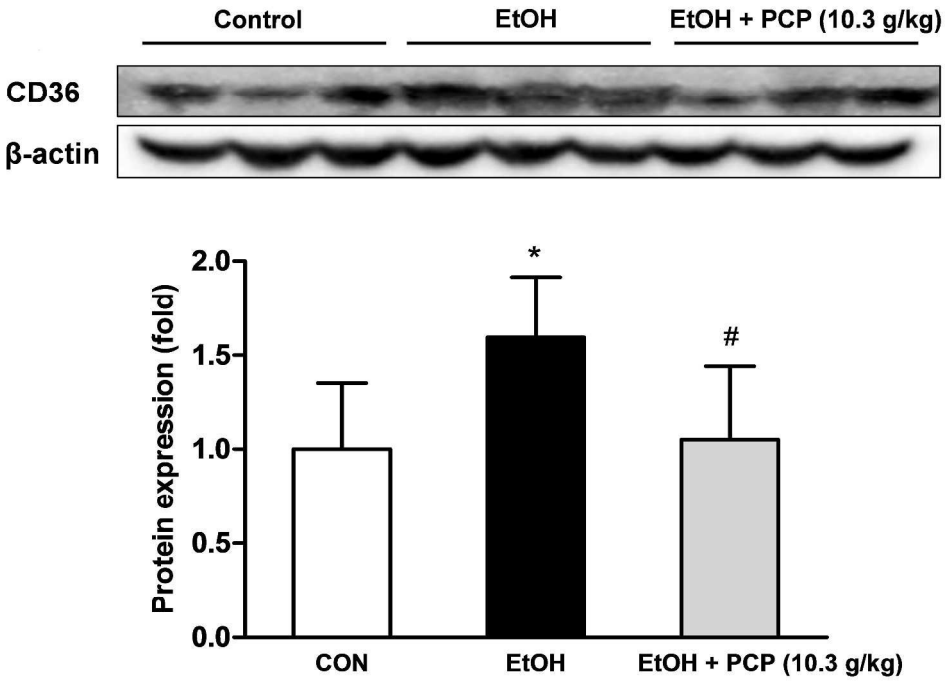

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