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Hepatoprotective and Antioxidant Effects of Total Triterpenoids from *Poria cocos*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors XH, YW, GW, XY and JZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author GW contributed equally to this work and should be considered co-first authors. Authors JZ and XY managed the analyses of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To prepare the *Poria cocos* total triterpenoids (PCTT) from the surface layer of *Poria cocos* and evaluate its pharmacological effect on alcohol induced-liver injury.
Study Design: PCTT was prepared from the surface layer of *Poria cocos* and characterized. Its effects on alcohol induced-liver injury models were investigated in vitro and in vivo.
Place and Duration of Study: School of Pharmacy, Guangdong Pharmaceutical University, between January 2014 and March 2014.
Methodology: PCTT was prepared via D101 macroporous resin chromatography and characterized by high performance liquid chromatography. The hepatoprotective and antioxidant effects of PCTT against alcohol induced-liver injury were investigated in L-02 cell line and mice.
Results: PCTT containing 63.95% triterpenoids showed potent radical-scavenging activities in vitro. PCTT (10 *μ*g/mL and 20 *μ*g/mL) treatments increased the viability of cells significantly in alcohol-treated L-02 cells. In vivo, pretreated with PCTT suppressed the acute ethanol gavage induced increase of the serum aminotransferase (AST), aminotransferase (ALT) levels and liver triacylglycerol (TG) level in mice. Simultaneously, PCTT also enhanced the glutathione peroxidase

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(GSH-Px) activity and restored glutathione (GSH) level in liver.
Conclusion: This study suggests that PCTT, containing 63.95% triterpenoids, could significantly improve the impairments of liver induced by alcohol and suitable for alcohol induced-liver injury patients as medicine or functional food, which would be a new candidate for the treatment of alcohol liver disease (ALD).

Keywords: Poria cocos; total triterpenoids; hepatoprotective; antioxidant; alcohol induced-liver injury.

ABBREVIATIONS

ALD : Alcohol liver disease;
ALT : Aminotransferase;
AST : Aminotransferase;
DPPH : 2, 2-Diphenyl-1-picrylhdrazyl;
FBS : Foetal bovine serum;
GSH-Px: Glutathione peroxidase;
GSH : Glutathione peroxidase;
HPLC : High-performance liquid chromatography;
L-02 : Human normal liver cell line;
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
NBT : Nitroblue tetrazolium chloride;
PMS : Phenazine methosulfate;
TG : Triacylglycerol

1. INTRODUCTION

Alcohol liver disease (ALD) is a group of diseases associated with a spectrum of liver injury ranging from steatosis and steatohepatitis to fibrosis or cirrhosis. Due to the increased frequency of drinking and change of diet formulation, the incidence of ALD increased quickly and has become an important risk factor for morbidity and mortality in addition to viral hepatitis [1]. It is estimated that almost 5.9% of all deaths worldwide are attributable to alcohol, and 5.1% of the global burden of disease is attributable to alcohol consumption [2]. ALD has been considered as a major health and economic problem worldwide. During the past few decades, numerous attempts have been made to investigate and develop effective therapy and hepatoprotective substances as preventive agents for ALD [3]. However, except alcohol abstinence, satisfactory treatment strategy for ALD is still undefined. Alcohol-induced liver injury, the common consequence of long-term and over-consumption of alcohol, is one of the most common causes of ALD. It has been recognized that oxidative stress and generation of free radicals play crucial roles in the development of ALD, although a comprehensive understanding of ALD mechanisms is not complete. Natural products with antioxidant activity have attracted great attention as potential functional dietary supplements to alleviate alcohol-induced liver injury, due to their multiple targets and less toxic side effects [4].

Poria cocos Wolf (Polyporaceae) a saprophytic fungus that grows around the old roots of pine trees is a well-known edible medicinal fungus in Asia. Its dried sclerotia was frequently prescribed as one of the chief ingredients in compound prescriptions in Traditional Chinese Medicine to promote urination, to invigorate the spleen function, and to calm the mind [5]. *Poria cocos* is rich of biological components related to both nutritional and nutraceutical values including all essential amino acids, vitamins, minerals polysaccharides, and fibres which is commonly served as food and food supplement. *Poria cocos* has also been found to have various secondary metabolites, such as triterpenoids, and steroids, in which triterpenoids have been reported to possess many bioactivities including anti-tumor activity [6,7], anti-inflammatory activity [8,9], inhibition of DNA polymerases and DNA topoisomerases [10], anti-hyperlipidemic activity [11], as well as anti-diabetic activity [12]. As reported previously, the surface layer of *Poria cocos* (“Fu-ling-pi” in Chinese) has the higher triterpenoids contents than the inner part. The ethanol extract of Fu-ling-pi has been reported to have diuretic effect in rat [13] and protective activity of chronic kidney disease [14,15]. To the best of our knowledge, the potential of *Poria cocos* total triterpenoids (PCTT) administration in the treatment of alcohol-induced liver injury have not been previously reported.

The main objective of this research was to concentrate PCTT using D101 resin, to evaluate its antioxidant and hepatoprotective effects *in vivo* and *in vitro*.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

D101 macroporous resin was purchased from Xi’an Lanxiao Resin Corporation Ltd. (Xi’an,

China). HPLC-grade methanol was provided by Oceanpak Chemical Co. (Gothenburg, Sweden). PMS was purchased from J&K Chemical Ltd. (Shanghai, China). FBS was obtained from Gibco Life Technologies (Grand Island, NY, USA). DPPH was obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China). All other analytical grade reagents were purchased Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2 Instrumentation

HPLC analysis was performed on a Waters chromatographic system (Waters Corp., Milford, MA, USA) using an analytical HPLC column (Amethyst C18-H, 4.6×250 mm, 5 μm, Sepax Technologies Inc., Newark, NJ, USA). A Waters 600 pump model equipped with a Waters 996 PDA detector connected to Empower software for data acquisition. A microplate reader (ST-360) was product of Kehua Technologies, Inc. (Shanghai, China).

2.3 Extraction and Concentration of the Total *Poria cocos* Triterpenoids (PCTT)

The surface layer of *Poria cocos* was collected from Anhui Province, China. A voucher specimen (No. GDPU-NPR-2013-PC) was deposited in the School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, China.

The dried surface layer of *Poria cocos* (6.0 kg) was crushed into small pieces and then extracted twice using 5 volumes methanol for 2 h at reflux [16,17]. The filtrate was evaporated under vacuum at 55℃ to afford methanol extract (670 g). 200 g of the methanol extract was then suspended in water and subjected to a D101 macroporous resin column (100 $\times$ 1100 mm). The resin column was successively eluted with water, 50% methanol, 70% methanol and methanol respectively. The methanol elution was concentrated in vacuum, yielding a white powder (99.8 g).

2.4 Quantitation of Total Triterpenoids

The total triterpenoid content in PCTT was determined by colorimetric methods using the reported protocol with modifications [18]. Briefly, ursolic acid (UA) standards (20 ~ 100 μg/mL) were prepared fresh in methanol before use. Added 1 mL sample or UA standard solution into each test tube (15×150 mm), and then taken to dryness in a water bath at 60℃. Then, 0.3 mL 5% vanillin-glacial acetic acid solution and 1.0 mL perchloric acid was added to each tube, which was reacted in a 60◦C water bath for 10 min. Then, 5 mL glacial acetic acid was added and mixed well. The absorbance was measured at 548 nm. Data were reported as mean ± SD for at least three replicates.

2.5 HPLC Analysis of PCTT

The analysis of PCTT was performed on a Sepax Amethyst C18-H analytical column (4.6 × 250 mm, 5 μm) using a Waters 600 chromatographic system equipped with a 996 PDA detector. The mobile phase composed of phase A (H_2O: H_3PO_4 = 100: 0.5) and phase B (MeOH). The linear gradient (0–65 min) was performed as follows: 76% phase B was held constant for 30 min, then phase B increased to 80% in 4 min, to 90% in 6 min, to 100% in 15 min and held constant for another 5 min. The flow rate was 1.0 mL/min and the column oven was 30℃.

2.6 Antioxidant and Hepatoprotective Activities Assay *in vitro*

DPPH and superoxide radicals scavenging activities were determined according to the reported procedures [19]. Potent protective effects of PCTT against ethanol-induced injury on L-02 cells were determined according the reported protocol [20].

2.7 Animal Experiments

All protocols involving animal experiments were confirmed by the Animal Ethics Committee of Guangdong Pharmaceutical University, China. At the end of the experiments, the mice were sacrificed under diethyl ether anesthesia to minimize suffering. The sacrificed mice were handled by the animal testing center after the experiment.

Male Kun-Ming SPF mice weighing 21–30 g were bought from the Laboratory Animal Center of Guangdong Pharmaceutical University. Mice were randomly allocated to 5 groups namely, Control group, Model group, PCTT groups (50, 200 mg/kg/d) and Biphenyldicarboxylate group (150 mg/kg/d). Each group had 10 mice. PCTT or Biphenyldicarboxylate was suspended in distilled water containing 0.3% CMC-Na. The control and normal groups received distilled

water containing 0.3% CMC-Na. The PCTT groups were gavaged with PCTT at 50 and 200 mg/kg, respectively. The mice of all groups were treated with the corresponding samples for 27 days. From the day 23^{td}, the mice of the model and PCTT groups were gavaged with 50% ethanol (12 mL/kg/d) 3 hours after the treatment of PCTT or vehicle, the control group was treated with the same volume of distilled water. On the day 28th, overnight-fasted mice were sacrificed under diethyl ether anesthesia. Blood was collected from the ophthalmic vein and allowed to clot at room temperature for 30 min. The serum was separated by centrifuging at 4000 rpm for 10 min with a refrigerated centrifuge. The liver was excised and rinsed with ice-cold saline. Samples were stored at −20℃ until analysis.

2.8 Measurement of Serum and Liver Variables

10% solution of liver tissue homogenate was prepared with a previously reported method. The supernatants were separated and stored at 4℃ for analysis. The protein content of tissue homogenate was determined according to the Bradford method using bovine serum albumin as standard [21]. The activities of serum ALT and AST, the TG, GSH-Px and GSH contents in liver homogenate were measured by colorimetric methods using the respective commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.9 Statistical Analysis

Experimental values were presented as mean ± SD. Comparison of mean values between groups was performed by one-way-analysis of variance followed by Tukey's test using the SPSS software (Version 20 for windows, IBM, Chicago, USA).

3. RESULTS

3.1 Quantitation of Total Triterpenoids in PCTT

Expressed as milligram of UA equivalents per gram of sample on a dry weight basis, the total triterpenoid content in PCTT was 63.95 ± 4.2% as measured by the colorimetric method, after concentration with a D101 macroporous resin column. The *Poria cocos* triterpenoids (PCTT) was used for the further study.

3.2 HPLC-PDA Analysis and Structure Identification of Triterpenoids in PCTT

The main active chemical constituents of *Poria cocos* are polysaccharides and triterpenoids, There are numerous reports on the triterpenoids composition in *Poria cocos*. In our study, The HPLC chromatography was obtained as shown in Fig. 1, and fifteen characteristic compounds (Fig. 2.) among them were identified

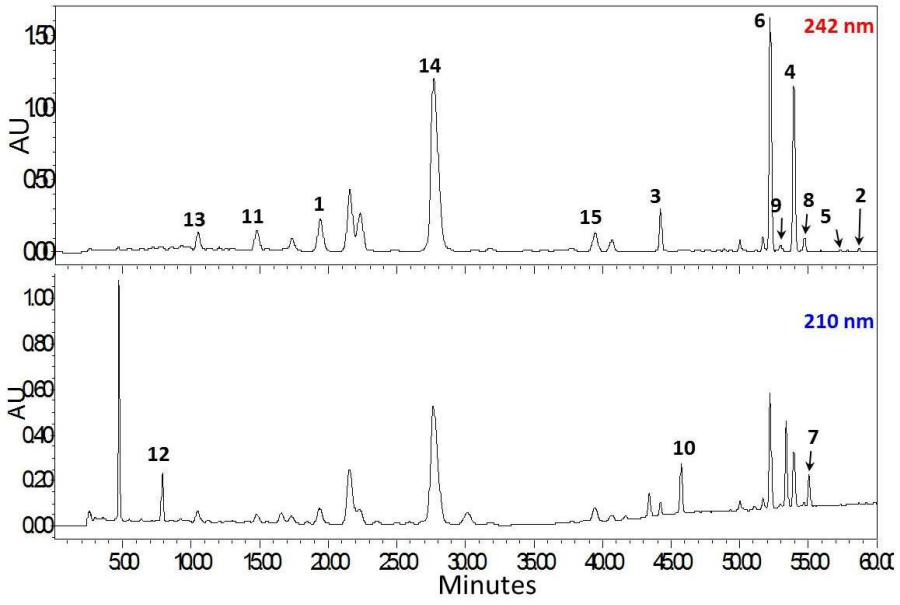

Fig. 1. HPLC-PDA chromatogram of PCTT
Flow rate, 1.0 mL/min

as dehydrotumulosic acid (**1**), dehydroeburicoic acid monoacetate (**2**) [22], dehydropachymic acid (**3**), dehydroeburicoic acid (**4**), 3β-acetoxylanosta-7,9(11),24-trien-21-oic acid (**5**), dehydrotrametenolic acid (**6**), 3β,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid (**7**), dehydroeburiconic acid (**8**), dehydrotrametenonic acid (**9**), pachymic acid (**10**), eburicoic acid (**11**), trametenolic acid (**12**), poricoic acid D (**13**), poricoic acid A (**14**), poricoic acid AM (**15**) by comparing their retention times. Compounds **7**, **8** and **10** were found to be the three major peaks. These ascribed compounds in the fingerprints could be considered as the characteristic profile of the PCTT.

3.3 *In vitro* Antioxidant and Hepatoprotective Activity of PCTT

In the present study, the antioxidant activities of PCTT were evaluated using DPPH free radical scavenging and superoxide radical scavenging assays. As indicated in Fig. 2A and Fig. 2B, PCTT exhibited scavenging activities on both DPPH and superoxide radicals with EC_{50} values of 0.79 ± 0.03 mg/mL and 0.22 ± 0.02 mg/mL respectively. In particular, it should be noted that the superoxide anion radical scavenging activity of PCTT was above 70% at a concentration of 0.5 mg/mL. Fig. 2C depicted the protective effect of bifendate and PCTT on alcohol-induced cell death in the L-02 cells. A significant decrease in cell viability was observed in L-02 cells exposed to alcohol as compared with the normal control group. Both PCTT (10 *μ*g/mL and 20 *μ*g/mL) and bifendate (1.0 *μ*M) treatments increased the viability of cells significantly.

3.4 *In vivo* Hepatoprotective Effect

Fig. 3 (panels A and B) shows the effects of PCTT and bifendate on alcohol administration induced alteration of AST and ALT levels in serum. Significant increases in serum AST and ALT levels were observed in mice after 5 days administration of alcohol when compared with the normal control group, indicating the alcohol-induced hepatotoxicity in mice was well-established. The elevated serum AST and ALT levers in mice those pretreated with PCTT were significantly reduced. Compared with the alcohol control group, the levels of AST and ALT decreased 23% and 24% respectively, pretreated with PCTT at a dose of 200 mg/kg/d.

Alcohol-induced lipid accumulation in liver was also observed in mice as showed in Fig. 3 (panels C, D, and E), after 5 days consumption of alcohol the liver TG levels of alcohol control group increased 66% when compared with the normal control group. The elevated liver TG level decreased significantly in a concentration-dependent manner in mice pretreated with PCTT. At a high dose of 200 mg/kg/d, the TG level reduced 45% when compared with the alcohol control group. These data indicated that PCTT could attenuate acute alcohol intake induced liver injury. Furthermore, the administration of PCTT significantly enhanced GSH and GSH-Px activities in a concentration-dependent manner when compared with the alcohol control group. At a dose of 200 mg/kg/d of PCT, the activities of GSH and GSH-Px increased 20% and 13% respectively when compared with the alcohol control group as shown in Fig. 3. These results were consistent with the changes in serum levels of AST and ALT, indicated that pretreated with PCTT could alleviate the ethanol induced oxidative stress.

4. DISCUSSION

Poria cocos is used in Traditional Chinese Medicine and prescribed as sedative, diuretic and tonic [23]. The aqueous extract of *Poria cocos* displayed inhibitory effect on $FeCl_2$-ascorbic induced lipid peroxidation in rat liver homogenate *in vitro* and scavenging activity on superoxide anion [24]. The ethanol extract of *Poria cocos* showed potent scavenging activity on hydroxyl radical at a concentration of 100 *μ*g/mL [25]. Previous studies indicate that the main active chemical constituents of *Poria cocos* are polysaccharides and triterpenoids [26-29].

The DPPH and superoxide radicals have been widely used for evaluating the preliminary radical scavenging capacity of plant extracts or antioxidant compounds. Superoxide anion radical ($O_2^{\bullet-}$), arising from the addition of one electron to dioxygen either through metabolic processes or following oxygen activation by physical irradiation, is considered as the primary reactive oxygen species (ROS). It can further interact with other molecules to generate secondary ROS either directly or prevalently through enzyme or metal catalyzed processes [30]. In this study, the total triterpenoids was extracted and concentrated from the surface layer of *Poria cocos* (PCTT). In the *in vitro* antioxidant assay, PCTT showed potent DPPH and superoxide radical scavenging capacity. Particularly, the superoxide anion radical scavenging activity of PCTT was above 70% at a concentration of 0.5

mg/mL. When treated with PCTT, the alcohol-induced cell death on L-02 cells was significantly decreased. These results of the present work may be used to explain the bioactivities of *Poria cocos*.

Excessive triacylglycerol accumulation in the liver is the common character of hepatic steatosis in the early stage of ALD. Abnormal retention of lipids within hepatocyte leads to liver damage [31]. ALT and AST are released from the cytoplasm of hepatocytes after cellular damage occurred, their serum activities were commonly used as reliable primary indicators for clinical monitoring of liver injury [3]. As we know, oxidative stress plays an important role in the development of alcohol-induced liver injury and ALD. Numerous experimental studies have shown that both acute and chronic alcohol increases the production of ROS, such as hydroxyl radical, superoxide radical and hydrogen peroxide leading to oxidative stress in liver [3,32]. GSH is the most prevalent low-molecular-weight thiol in mammalian cells, which plays a crucial role in the antioxidant defense [33]. The *in vivo* hepatoprotective experiment showed that pretreated with PCTT could effectively suppress the acute ethanol gavage induced increase of the AST and ALT levels in serum and the TG level in liver. Pretreated with PCTT also enhanced the GSH-Px activity and restored GSH level in liver. All these data indicated that PCTT might have a protective effect against alcohol-induced liver injury.

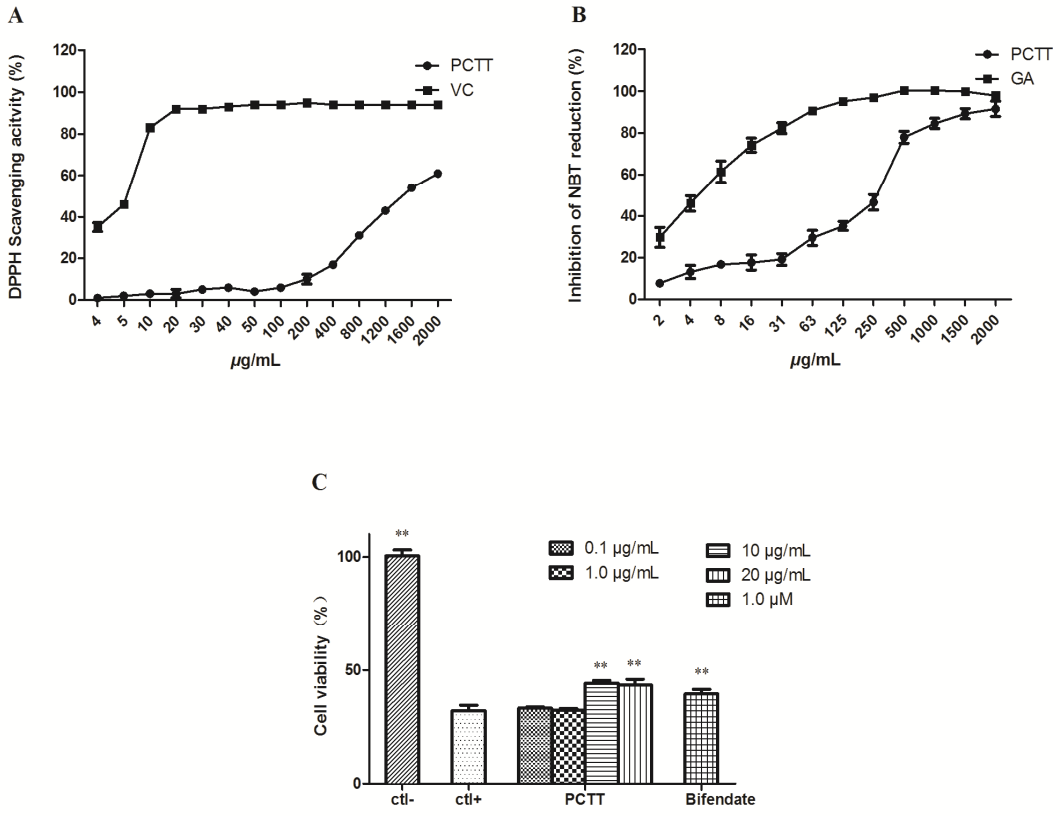

Fig. 2. *In vitro* antioxidant and hepatoprotective activity of PCTT

*Antioxidant Activities of PCTT were evaluated by DPPH free radicals (panel A, Vitamin C (VC) was positive control) and superoxide anion radicals (panel B, Gallic acid (GA) was positive control) scavenging assays. Panel C: Hepatoprotective activity of PCTT was determined by MTT assay. Control wells consisted of cells incubated with medium only, and the cells pretreated with 6% ethanol for 4 hours acted as the negative control. Values are the mean ± SD, n = 3. **p < 0.01 vs the negative control*

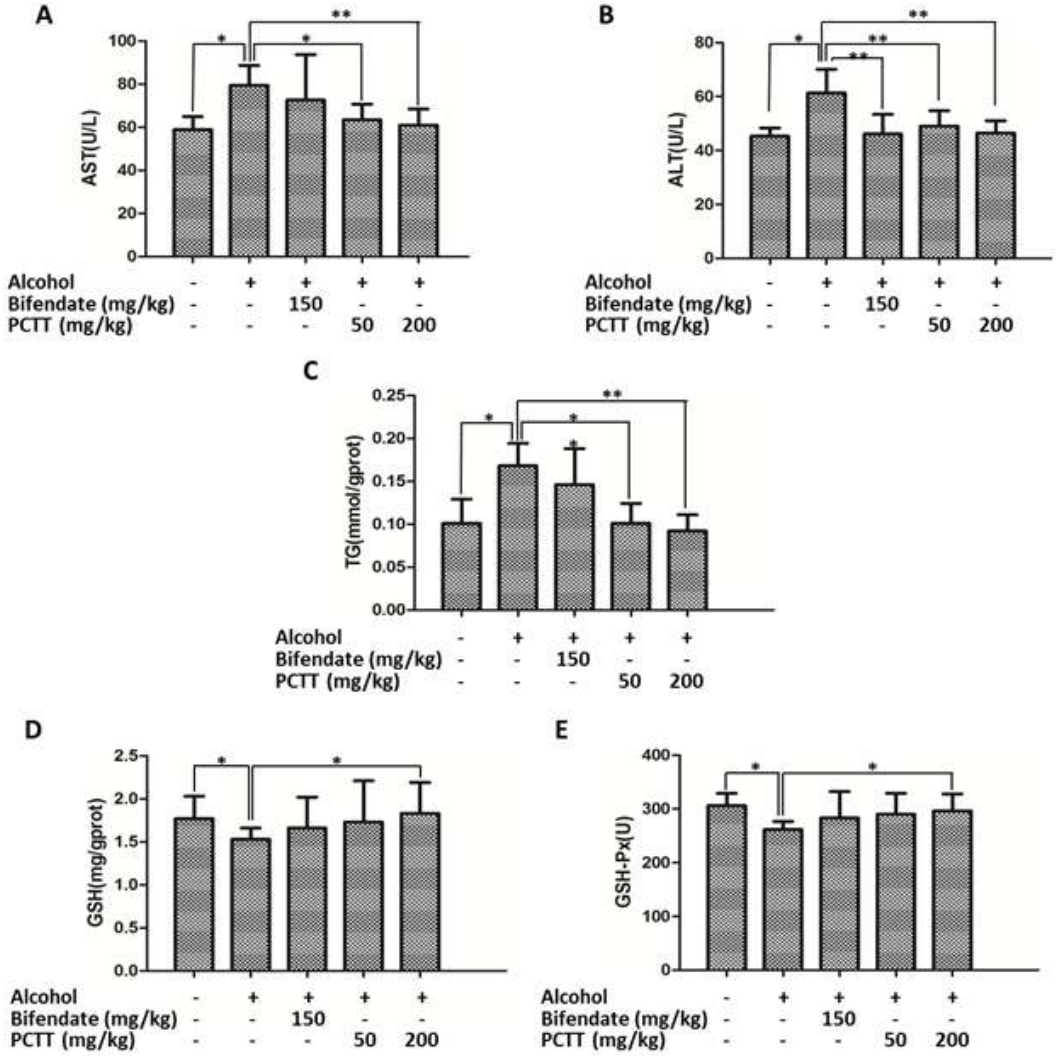

Fig. 3. Effects of PCTT pretreatment on alcohol-induced alteration in serum enzyme activities of AST (panel A) and ALT (panel B), and the TG content (panel C) and enzyme activities of GSH (panel D) and GSH-Px (panel E) in liver

*Values are the mean ± SD, n = 8. *P < 0.05, **p < 0.01*

5. CONCLUSION

In this study PCTT with high contents of triterpenoids was prepared from the surface layer of *Poria cocos* by extraction with 70% ethanol and concentration with D101 resin. The main components of PCTT was identified. *In vitro*, PCTT showed potential radical-scavenging activities against DPPH and superoxide anion radicals, and protective activity against alcohol-induced cell death on L-02 cells. One the basis of the *In vivo* study, it demonstrated that PCTT could effectively attenuat ethanol induced liver injury and remarkably restored the liver activity of GSH-Px. Finding of this work suggested that PCTT had the potential to be developed as functional ingredients to protect against ALD. Furthermore, the mechanism of the hepatoprotective activity of PCTT should be investigated in a future study.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experiments have been examined and approved by the appropriate ethics committee of

Guangdong Pharmaceutical University, China. (201402016)

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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