



## **Triterpenoids from *Poria cocos* and Their Potential *In vitro* Hepatoprotective Effects**

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### **Authors' contributions**

*This work was carried out in collaboration between all authors. Authors XH, YW, GW and XY 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 GW and XY managed the analyses of the study. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aims:** To isolate triterpenoids from the surface layer of *Poria cocos* and evaluate their hepatoprotective activities *in vitro*.

**Study Design:** Triterpenoids were isolated and identified from the surface layer of *Poria cocos*. Their effects on alcohol induced-liver injury models were investigated *in vitro*.

**Place and Duration of Study:** School of Pharmacy, Guangdong Pharmaceutical University, between September 2013 and December 2013.

**Methodology:** Triterpenoids were purified by Silica gel, ODS as well as HPLC chromatography and characterized by MS, 1D and 2D NMR. The hepatoprotective effects of the isolated compounds against alcohol induced-liver injury were investigated in L-02 cell line.

**Results:** Eight triterpenoids were isolated and identified from the surface layer of *Poria cocos* (1–8), Compounds 2, 3, 6 showed potent protective effects against ethanol-induced injury on L-02 cells.

**Conclusion:** This study suggests that triterpenoids from the surface of *Poria cocos* could protect

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L-02 cells from death 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 alcoholic liver disease.

**Keywords:** *Poria cocos*; triterpenoids; hepatoprotective; L-02.

## ABBREVIATIONS

**ALD** : Alcohol liver disease;

**FBS** : Foetal bovine serum;

**HPLC**: High-performance liquid chromatography;

**L-02** : Human normal liver cell line;

**MTT** : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

## 1. INTRODUCTION

*Poria cocos* Wolf (Polyporaceae) (Fuling in Chinese) is a saprophytic fungus that grows around the old roots of pine trees. It's a well-known edible fungus which commonly served as food and food supplement for its high content of biological components related to both nutritional and nutraceutical values, including all essential amino acids, vitamins, minerals, and fibres. The content of dietary fibre prepared from the sclerotia of *P. cocos* was more than 70% of the dry weight [1]. In China, Fuling jiating also known as Fuling bing, made from *P. cocos*, flour and sugar is a traditional pancake-like snack food of Beijing. *P. cocos* has also been found to have various secondary metabolites, such as triterpenoids, polysaccharides and steroids, in which triterpenoids have been reported to possess many bioactivities including anti-tumor activity, anti-inflammatory activity, inhibition of DNA polymerases and DNA topoisomerases [2-4]. Recently, potential anti-hyperlipidemic, anti-diabetic, hepatoprotective and antiviral activities of *P. cocos* were also reported [5-11]. It's 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. While the surface layer of *P. cocos* (Fuling pi in Chinese) was only used as diuretic agents and usually imposed of as a solid waste during the industrial processing. However, the surface layer of *P. cocos* have the higher triterpenoids contents than the inner part as previously reported. The ethyl acetate and n-butanol fractions of the ethanol extract from Fuling pi have been demonstrated to have diuretic effect in rat [12-15] and can ameliorate chronic kidney disease by intervening in some metabolic pathways [16]. Thus, it is evident that the surface layer of *P. cocos* represents a

suitable potential source of health-promoting triterpenoids in the production of food supplement and nutraceuticals.

The main objective of this research was to extract and concentrate bioactive triterpenoids from the surface layer of *P. cocos*, to evaluate their antioxidant and hepatoprotective activities.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

D101 macroporous resin was purchased from Xi'an Lanxiao Resin Corporation Ltd. (Xi'an, China). Silica gel (200–300 mesh, Anhui Liangchen Silicon Material Co. Ltd. Anhui, China) and ODS (40–60  $\mu\text{m}$ , Merck KGaA, Darastadt, Germany) were used for column chromatography. HPLC-grade methanol was provided by Oceanpak Chemical Co. (Gothenburg, Sweden). FBS was obtained from Gibco Life Technologies (Grand Island, NY, USA). All other analytical grade reagents were purchased Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

### 2.2 Instrumentation

IR spectra were obtained from a Perkin Elmer Spectrum 100 FT-IR spectrometer (Perkin Elmer Inc., Waltham, MA, USA). NMR spectra were acquired with a Bruker Avance III 500 MHz digital NMR spectrometer using Bruker BioSpin GmbH pulse programs (Bruker, Switzerland). HR-ESI-MS data were obtained on an Acquity UPLC-Q-TOF Micro MS mass spectrometer (Waters Corp., Milford, MA, USA). Semi-preparative HPLC was carried out on a HPXL solvent delivery system equipped with a UV-D II detector model (Rainin Instrument Co. Inc., Woburn, MA, USA) and an ODS column (Amethyst C18-H, 10x250 mm, 5  $\mu\text{m}$ , Sepax Technologies Inc., Newark, NJ, USA). Microplate reader (ST-360) was the product of Kehua Technologies, Inc. (Shanghai, China).

### 2.3 Fungal Material

The surface layer of *P. cocos* was collected from Anhui Province of China and identified by

Prof. X.J. He of Guangdong Pharmaceutical University. A voucher specimen was deposited in the School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, China.

## 2.4 Extraction and Purification

The dried surface layer of *P. cocos* (6.0 kg) was crushed into small pieces and then extracted twice using 5 volumes methanol for 2 h at reflux. The filtrate was evaporated under vacuum at 55°C 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 × 1100 mm). The resin column was successively eluted with water, 50% methanol, 70% methanol and methanol respectively. The methanol elution was concentrated under vacuum, yielding a white powder (99.8 g). Part of the obtained powder (37.8 g) was dissolved in methanol and then filtered with filter paper. The filtrate was subjected to a silica gel column using cyclohexane/EtOAc (100:0 to 0:100) gradient elution. The cyclohexane/EtOAc (5:1, v/v) elution (218.6 mg) was further purified using a semi-preparative HPLC column (Amethyst C18-H, 10.0×250 mm 5 μm) eluting with a MeOH/H<sub>2</sub>O mixture to afford Compound **8** (10.0 mg). The cyclohexane/EtOAc (2:1-1:1, v/v) elution (19.66 g) was chromatographed on an ODS column (300 × 30 mm) eluted gradient with MeOH/H<sub>2</sub>O ((the ratios of MeOH/H<sub>2</sub>O were from 50:50 to 0:100) to yield two sub-fractions which were further purified by RP-HPLC to obtain compounds **2** (62.1 mg), **3** (50.4 mg) and **4** (23.1 mg). The methanol insoluble residue (3.0 g) of the white powder was dissolved in chloroform and then purified by silica gel chromatography and eluted with a CHCl<sub>3</sub>/Acetone gradient elution (100:0 to 100:50, v/v). The CHCl<sub>3</sub>/Acetone (100:8, v/v) elution was subjected to RP-HPLC using a mixture of methanol and water as mobile phase, to yield compounds **1** (45.5 mg), **5** (17.6 mg) and **7** (19.6 mg). Compound **6** (23.2 mg) were purified from the CHCl<sub>3</sub>/Acetone (100:20, v/v) elution by RP-HPLC.

## 2.5 Hepatoprotective Activities Assay *In vitro*

Potent protective effects of PCTT against ethanol-induced injury on L-02 cells were determined according the reported protocol. The human normal liver cells (L-02) obtained from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in the DMEM

medium (HyClone, Thermo Scientific, Logan, UT) supplemented with 10% FBS, 4.00 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and were maintained at 37°C in a humidified atmosphere of 95% air to 5% CO<sub>2</sub>. A total of 1.5 × 10<sup>4</sup> cells in 100 μL growth media were placed in each well of a 96-well flat-bottom plate. After 24 hrs of incubation, the growth medium was replaced by fresh prepared media containing 6% alcohol. After 4 hrs of incubation the medium was replaced by fresh prepared media containing different concentrations of the test samples. After 24 hrs of incubation, cell viability was determined by colorimetric MTT assay reading at 546 nm for each concentration compared to the control. At least three replications for each sample were performed. Bifendate was used as positive control. Control wells consisted of cells incubated with medium only, and the cells with 6% ethanol acted as the negative control [17].

All protocols involving cell experiments were approved by the cell Ethics Committee of Guangdong Pharmaceutical University, China.

## 2.6 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 (IBM, Chicago, USA). Difference with *P* value < .05 was perceived to be statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1 Structure Identification of the Purified Triterpenoids

The dried surface layer of *P. cocos* was extracted with methanol and then successively chromatographed on D101 macroporous resin C.C, silica gel CC, ODS CC, and finally purified using semi-preparative HPLC to afford 8 triterpenoids (1-8). Their chemical structures were shown in Fig. 1.

Compound **1** was obtained as white amorphous powder. UV (MeOH) λ<sub>max</sub> (log ε) 242 nm. The molecular formula of C<sub>31</sub>H<sub>46</sub>O<sub>3</sub> was determined by the negative-ion HR-ESI-MS peak at *m/z* [M-H]<sup>-</sup> C<sub>31</sub>H<sub>45</sub>O<sub>3</sub> 465.3389 (calcd for C<sub>31</sub>H<sub>45</sub>O<sub>3</sub> 465.3369). In the <sup>1</sup>H NMR spectrum, compound **1** showed signals due to five tertiary methyl groups at δ<sub>H</sub> 0.98 (3H, s), 1.04 (3H, s), 1.06 (3H, s), 1.13 (3H, s), 1.13 (3H, s), two secondary

methyl groups at  $\delta_{\text{H}}$  1.02 (3H, d,  $J = 5.1$  Hz) and 1.03 (3H, d,  $J = 4.7$  Hz), an exocyclic methylene group at  $\delta_{\text{H}}$  4.90 (1H, s) and 4.94 (1H, d,  $J = 1.1$  Hz), two olefinic methines at  $\delta_{\text{H}}$  5.57 (1H, d,  $J = 6.5$  Hz), 5.33 (1H, d,  $J = 5.8$  Hz). There were 30 carbons in the  $^{13}\text{C}$  NMR spectrum, which included two carbonyl carbon and six olefinic carbons. Carbon signal at 215.6 and 178.8 ppm due to carboxyl groups of C-3 and C-21 respectively. The olefinic methines at 121.1 ppm (C-7) and 118.0 ppm (C-11), and olefinic quaternary carbons at 143.2 ppm (C-8) and 145.3 ppm (C-9) revealed the presence of the  $\Delta^{7,9(11)}$  conjugated diene system [18].

Carbon resonances at 107.4 ppm (C-31) and 156.2 ppm (C-24) were assigned to the exomethylene group of  $\Delta^{24(31)}$ . These data consistent with the literature, therefore compound 1 was established to be dehydroeburonic acid [19,20].

Compounds 2–8 were identified as, dehydrotrametenonic acid (2) [21], pachymic acid (3) [22], eburicoic acid (4) [23], trametenolic acid (5) [23], poricoic acid D (6) [23], poricoic acid A (7) [19], and poricoic acid AM (8) [23] by comparing the UV, NMR and MS data with those reported in the literature.

**Table 1.**  $^{13}\text{C}$  NMR (125 MHz) data of compounds 1-8 in Pyridine- $d_5$

Position	$\delta_{\text{C}}$ (ppm)							
	1	2	3	4	5	6	7	8
1	37.1	37.1	35.7	36.4	36.4	36.7	36.7	36.2
2	35.3	35.3	24.8	29.0	29.0	30.5	30.5	29.9
3	215.6	215.6	81.0	78.3	78.3	177.0	177.0	174.9
4	47.8	47.8	38.3	39.8	39.9	149.6	149.6	149.5
5	51.3	51.3	51.0	51.2	51.2	51.0	51.1	51.1
6	24.2	24.2	18.7	19.0	19.0	28.9	28.9	28.8
7	121.1	121.1	27.1	27.1	27.1	118.3	118.3	118.3
8	143.2	143.2	135.3	135.5	135.5	142.2	142.2	142.1
9	145.3	145.3	134.7	134.6	134.6	137.8	137.8	137.5
10	37.8	37.8	37.4	37.7	37.7	39.1	39.1	39.1
11	118.0	118.0	21.2	21.6	21.6	120.6	120.6	120.8
12	36.3	36.3	30.0	29.7	29.7	37.3	37.3	37.3
13	44.5	44.5	49.1	45.2	45.2	45.9	45.9	45.9
14	50.7	50.7	46.6	50.2	50.2	49.6	49.6	49.6
15	31.9	31.9	44.0	31.2	31.2	44.1	44.1	44.1
16	27.6	27.6	77.0	27.8	27.8	76.7	76.7	76.7
17	48.4	48.4	57.6	48.0	48.1	57.9	57.9	57.9
18	16.6	16.6	18.1	16.7	16.7	18.6	18.7	18.7
19	22.3	22.3	19.5	19.7	19.7	22.5	22.5	22.5
20	49.3	49.3	49.0	49.5	49.4	48.9	48.7	48.8
21	178.8	178.9	179.1	179.0	179.1	179.1	179.0	179.0
22	32.1	33.6	31.9	32.1	33.6	32.5	31.7	31.6
23	33.1	27.1	33.6	33.0	27.1	30.3	33.5	33.5
24	156.2	125.2	156.4	156.2	125.2	158.2	156.3	156.4
25	34.5	132.1	34.4	34.5	132.1	72.9	34.4	34.4
26	22.4	25.9	22.4	22.3	26.1	30.3	22.3	22.4
27	22.2	18.1	22.2	22.2	18.1	30.3	22.2	22.2
28	26.0	26.1	28.3	28.9	29.0	112.5	112.5	112.6
29	22.7	22.7	17.1	16.7	16.7	22.6	22.6	22.4
30	25.9	25.9	25.7	24.8	24.8	25.2	25.2	25.1
31	107.4		107.4	107.4		107.3	107.3	107.4
<u>MeCO</u>			21.5					
<u>MeCO</u>			170.9					
<u>MeO</u>								51.7

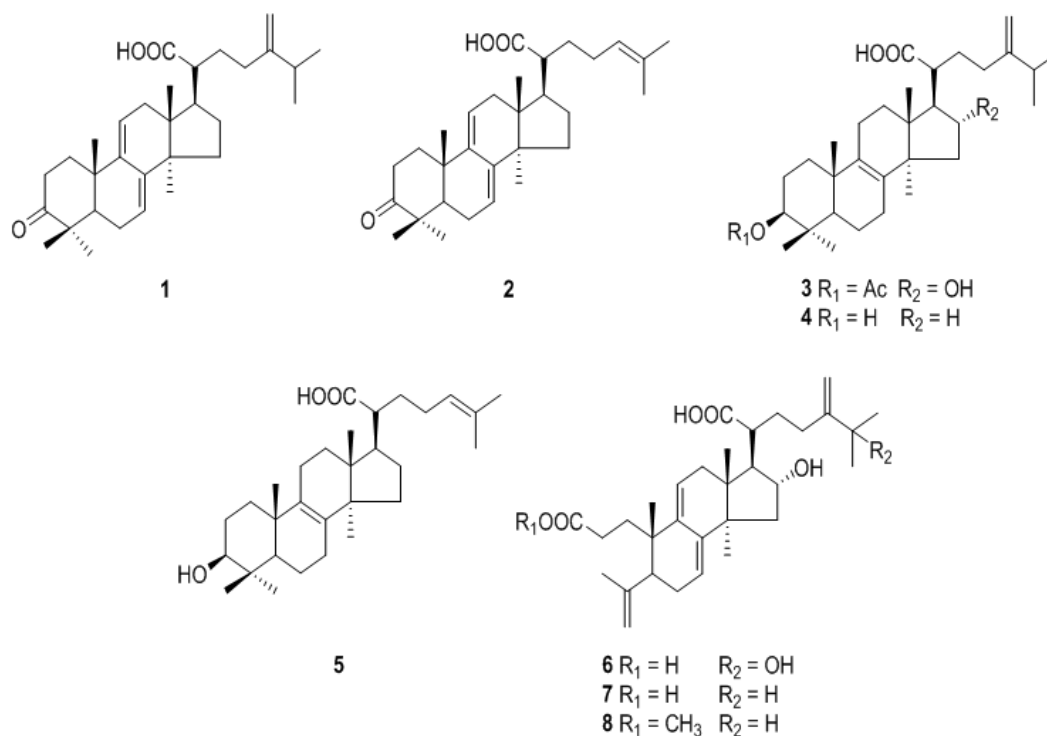


Fig. 1. Chemical structures of triterpenoids 1–8 isolated from *Poria cocos*

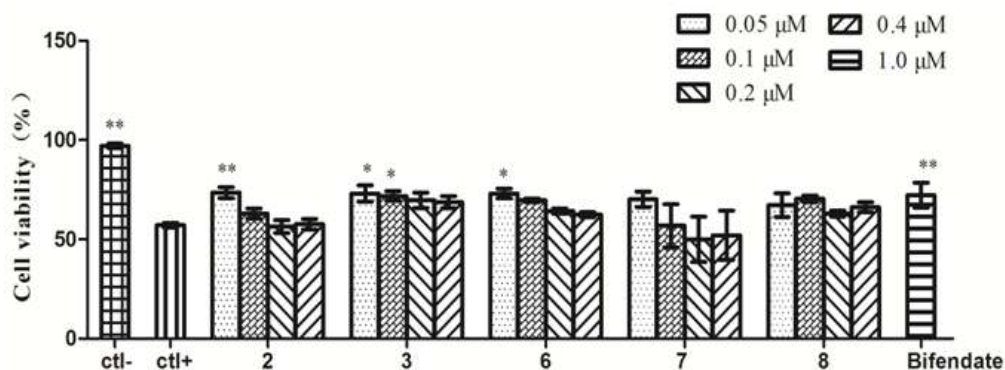


Fig. 2. Effects of the triterpenoids on L-02 cells against alcohol-induced cell death  
Hepatoprotective activities were determined by MTT assay. Control wells consisted of cells incubated with medium only, and the cells pretreated with 6% ethanol for 4 hours acted as the negative control.  
Test samples: significant from negative control. \* $P < .05$ , \*\* $P < .01$   
Values are the mean  $\pm$  SD,  $n = 3$

### 3.2 *In vitro* Hepatoprotective Activity

In the present study, compounds 2, 3, 6, 7, 8 were evaluated for their protective effects on the alcohol-induced cell death in L-02 cells. The results were summarized in above Fig. 2. Compounds 1, 10, 11, 14 showed protective effects at the dose of 0.05  $\mu$ M.

### 4. CONCLUSION

In this study, investigation of the surface layer of *P. cocos* led to the isolation of eight triterpenoids (1-8). Their structures were identified on the basis of MS and NMR data. Compounds 2, 3, 6 showed potent protective effects against ethanol-induced injury on L-02 cells. These results

suggested the potential utilization of the surface layer of *P. cocos* in functional foods and dietary supplement products.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

All protocols involving cell experiments were approved by the cell Ethics Committee of Guangdong Pharmaceutical University, China.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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