Identification of triterpene acids in *Poria cocos* extract as bile acid uptake transporter inhibitors

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Running title page

Poria cocos triterpenes as bile acid transporter inhibitors

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Abbreviations:

ASBT, apical sodium-dependent bile acid transporter; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; IC\textsubscript{50}, half-maximum inhibition concentration; K\textsubscript{i}, inhibition constant; NTCP, sodium/taurocholate co-transporting polypeptide; *Poria cocos*, *P. cocos*; Tauro-nor-THCA-24-DBD, N-(24-[7-(4-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole)]amino-3α,7α,12α-trihydroxy-27-nor-5β-cholestan-26-oyl)-2′-aminoethanesulfonate; TCA, taurocholic acid sodium salt; \[^{3}\text{H}]\text{TCA}, \[^{3}\text{H}]\text{Taurocholic acid}; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry.
Abstract

Literature reports that *Poria cocos* (*P. cocos*) reduces blood lipid levels; however, the underlying mechanism remains unclear. Blood lipid levels are closely related to the enterohepatic circulation of bile acids, where uptake transporters playing a significant role. *P. cocos* extract is commonly used in traditional prescriptions and food supplements in China. We investigated the effects of *P. cocos* and its five triterpene acids on bile acid uptake transporters, including intestinal apical sodium-dependent bile acid transporter (ASBT) and hepatic sodium/taurocholate co-transporting polypeptide (NTCP). Triterpene acids were fingerprinted by high-performance liquid chromatography-TripleTOF and quantified by ultra performance liquid chromatography/tandem mass spectrometry. The inhibitory effect of *P. cocos* and its five major representative triterpene acids on ASBT and NTCP was investigated by *in vitro* assays using *Xenopus* oocytes expressing ASBT and NTCP. *P. cocos* extract exhibited significant inhibitory effects with half-maximum inhibition constants of 5.89 µg/mL and 14.6 µg/mL for NTCP and ASBT, respectively. Among five triterpene acids, poricoic acid A, poricoic acid B, and polyrenic acid C significantly inhibited NTCP function. Poricoic acid A, poricoic acid B, and dehydrotumulosic acid significantly inhibited ASBT function. The representative triterpene acid, poricoic acid A was identified as a competitive inhibitor of NTCP with inhibitory constant of 63.4 ±18.7 µM. In conclusion, our results indicate that both *P. cocos* extract and its major triterpenes are competitive inhibitors of ASBT and NTCP. Accordingly, it was suggested that competitive inhibition of these bile acid transporters is one of underlying mechanism for the hypolipidemic effect of *P. cocos*.

**Keywords:** *Poria cocos*; NTCP; ASBT; transporter; bile acid
Significance statement:

*Poria cocos*, a commonly used Chinese herbal medicine and food supplement, demonstrates significantly inhibitory effects on the function of ASBT and NTCP. It has potential that *Poria cocos* reduces the blood lipid through inhibition of these uptake transporters in enterohepatic circulation of bile acid.
1. Introduction

Several natural medicines and food supplements have been safely and effectively used to treat various metabolic syndromes. *Poria cocos* (*P. cocos*) is the dried sclerotium of the fungus *Poria cocos* (Schw.) Wolf (Polyporaceae), known as Fuling in Chinese, are widely used both as a food supplement and traditional Chinese medicine (Wang *et al.*, 2013). As a health-promoting food, *P. cocos* exerts beneficial effects on food absorption and metabolism. In the Chinese Pharmacopoeia, approximately 15% of traditional Chinese prescriptions contain *P. cocos* (Nie *et al.*, 2020). For example, *P. cocos* is present in many ancient Chinese prescriptions, including Si-jun-zi decoction and Lin-gui-zhu-gan decoction, used for treating hyperlipidemia (Wu *et al.*, 2009; Liu *et al.*, 2013). *P. cocos* has also been shown to reduce blood lipid levels, including triglycerides and cholesterol, both in rats and humans (Miao *et al.*, 2016; Mendes *et al.*, 2018). The major chemical constituents of *P. cocos* are triterpenes and polysaccharides (Ríos, 2011). Although several studies have investigated the effects of polysaccharides in alleviating hyperlipidemia (Wu *et al.*, 2019), the mechanism of triterpene acids in reducing blood lipids is not yet known.

Bile acids are physiological detergents that solubilize lipids in the intestinal tract, thereby promoting fat digestion and absorption in the small intestine. The synthesis of bile acids in the liver accounts for a major fraction of daily cholesterol turnover in humans (Chiang *et al.*, 2013). Hepatic cholesterol is converted to bile acids by oxidation and conjugation reactions in the liver and intestine by several enzymes. Nearly 95% of the bile acid secreted is reabsorbed at the terminal ileum and is recycled via the enterohepatic circulation. Approximately 50% of cholesterol is eliminated from the body by this pathway (Li *et al.*, 2014). Therefore, fecal excretion of bile acids and reduced return to the liver may result in the accumulation of cholesterol in the liver. Both ASBT (*SLC10A2*) and NTCP (*SLC10A1*) located on the luminal surface of ileal enterocytes and the basolateral membrane of the hepatocytes, respectively, are involved in the reuse of bile acids by contributing to the enterohepatic circulation of bile acids and cholesterol homeostasis. Moreover, inhibition of NTCP and ASBT has been reported to alleviate obesity and hypercholesterolemia (Xu *et al.*, n.d.; Claro da Silva *et al.*, 2013; Rao *et al.*, 2016; Donkers *et al.*, 2019). Therefore, *P. cocos* possibly exerts its lipid-lowering effect via inhibition of bile acid uptake transporters in the enterohepatic circulation to decrease
the return of bile acids to the liver, thereby increasing the conversion of cholesterol to bile acids. Because NTCP and ASBT regulate the blood lipid levels, it is hypothesized that *P. cocos* affects their functions in reducing blood lipid levels. Thus, we studied the effect of *P. cocos* extract and its major triterpene acids (Figure 1) on bile acid uptake transporters, ASBT and NTCP.
2. Materials and methods

2.1 Materials and reagents

Tauro-nor-THCA-24-DBD was purchased from Geno Membrane, Inc. (Kanagawa, Japan) and taurocholic acid sodium salt (TCA) are obtained from Nacalai Tesque, Inc. (Kyoto, Japan). [³H] Taurocholic acid ([³H] TCA) was purchased from American Radiolabeled Chemicals, Inc. (specific activity 20 Ci/mmol, St. Louis, MO). Five triterpene acids, including poricoic acid A, poricoic acid B, polyprenolic acid C, pachymic acid, and dehydrotumulosic acid, and P. cocos extract (alcohol extract) were purchased from Nanjing Spring & Autumn Biological Engineering Co., Ltd. (Nanjing, China). Plasmid pcDNA3.1(+)/NTCP and pGEMHE/ASBT were constructed in our laboratory. Chlorzoxazole (used as internal standard) was obtained from Dalian Meilun Biotechnology Co. Ltd., China. Methanol and acetonitrile of chromatographic grade were obtained from Fujifilm-Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ammonium acetate was procured from Sigma-Aldrich Chemie GmbH Fluka (Netherlands). Female Xenopus laevis frogs were purchased from Kato-S-Science (Chiba, Japan) and all the studies were approved by Ethics Committee for Animal Experimentation of Kanazawa University (Kanazawa, Japan).

2.2 Identification of triterpenes in P. cocos by HPLC-TripleTOF

Triterpene acids were identified by using a high-performance liquid chromatography (HPLC)-TripleTOF system. Five µL of the extract (100 µg/mL) was injected into the HPLC system (LC-20A, Shimadzu Corporation, Kyoto, Japan) and separated by a reverse phase C18 column (150 × 2.0 mm × 2.1 mm, Shimadzu, VP-ODS) at a flow rate of 0.4 mL/min for 30 min. Formic acid (0.1%) (A) and acetonitrile (B) were used as mobile phases. The gradient used was: 0 to 2 min, 20% B; 2 to 20 min, 90% B; 20 to 24 min, 90%–95% B; 27 to 30 min, 95%–5% B; 30 min, stop. The mass spectroscopy data were recorded by TripleTOF5600+ system (SCIEX, CA, USA). The mass spectrometer was equipped with an ESI source in negative ion mode with a declustering potential (DP) of −80.0 V and collision energy (CE) of −35.0 eV. The TripleTOF 5600+ system was operated in the information-dependent acquisition (IDA) mode consisting of a TOF/MS scan type with an accumulation time of 0.05 s. The TOF masses ranged from 50 to 1000 Da. A curtain gas of 35 psi, ion source gas 1
of 55 psi, and ion source gas 2 of 55 psi were optimized. The ion spray voltage floating (ISVF) was set to 4500 V, and the heater interface temperature was 550 °C.

2.3 Simultaneous determination of five triterpene acids in P. cocos extract by UPLC-MS/MS

The content of five triterpene acids in P. cocos extract was quantified by a UPLC-MS/MS system. The UPLC-MS/MS system consisted of a Shimadzu UPLC system (LC-30A, Shimadzu Corporation, Kyoto, Japan) and an AB SCIEX mass spectrometer 4000 QTRAP system (AB Sciex Pte. Ltd., Framingham, USA). Analytes were separated using a C18 column (150 × 2.0 mm, 2.1 µm, Shimadzu, VP-ODS) at 40°C with a flow rate of 0.4 mL/min. The mobile phase consisted of 0.1% formic acid and 0.5 mM ammonium acetate aqueous solution (A) and acetonitrile (B). The gradient elution condition was: 0 to 6 min, 40%–90% B; 6 to 9.2 min, 90% B; 9.2 to 9.5 min, 90%–40% B; 9.5 to 10 min, 40% B. The injection volume was 2 µL. The mode of the mass spectrum was MRM model for the detection and related mass parameters are shown in Table 1. Five triterpenes were identified and qualified according to the retention time and molecular ion peaks of standard substances.

During sample preparation, original stock solutions (5 mg/mL) of poricoic acid A, poricoic acid B, polyporenic acid C, pachymic acid, and dehydrotumulosic acid were dissolved in dimethyl sulfoxide (DMSO). The working standard solutions of five analytes at concentrations of 1000, 500, 200, 100, 50, 20 and 10 ng/mL were prepared by diluting the stock solutions with acetonitrile. The chlorzoxazone working solution, used as the international standard (IS), was prepared using acetonitrile at a final concentration of 5 µg/mL. Working standard solution (50 µL) and 50 µL of IS working solution were added to an EP tube and mixed to get calibration curve samples. The stock solution (5 mg/mL) of P. cocos extract was prepared and diluted to 5 µg/mL by acetonitrile. Next, 50 µL of extract samples were mixed with 50 µL IS working solution. After the mixtures were vortexed for 5 min, four parallel samples were withdrawn for UPLC-MS/MS analysis.

2.4 Uptake assays in Xenopus laevis oocytes

Human NTCP and ASBT plasmid DNAs cloned in pcDNA3.1(+) and pGEMHE vectors were linearized by SmaI (New England Biolabs Inc, Ipswich, UK) and NheI
Female *Xenopus laevis* frogs were anesthetized in a mixture of ice and water for 30 min. The oocytes were dissected from ovaries and extracted from *Xenopus laevis* (15-20 min in the collagenase solution, 1 mg/mL). After digestion by collagenase solution, oocytes were washed in the OR2 solution (Oocyte Ringer 2 Solution; 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$·6H$_2$O and 5 mM HEPES, pH 7.4) for ten times and MBS (Modified Barth’s Saline; 88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO$_3$)$_2$·6H$_2$O and 5 mM HEPES, pH 7.4) for 5 times, respectively. Oocytes were defolliculated one by one under the microscope. After 24 h culture in the MBS solution with 50 mg/L gentamicin at 18°C, defolliculated oocytes between stage IV and VI were micro-injected with 50 nL cRNA solution (500 ng/μL) or pure water as control. After 48 h incubation in the MBS solution, oocytes were used in the uptake assays.

N-(24-[7-(4-N,N-dimethylamino-sulfonyl-2,1,3-benzoxadiazole)]amino-3α,7α,12α-trihydroxy-27-nor-5β-cholestan-26-oyl)-2′-aminoethanesulphonate (tauro-nor-THCA-24-DBD, 10 µM), a fluorescent derivative of bile acid, was used as the probe substrate of NTCP and ASBT (Yamaguchi *et al.*, 2010; De Bruyn *et al.*, 2014; Zhu *et al.*, 2020) in the uptake studies. All oocytes were preincubated in the uptake buffer (MBS) for 5 min at 25 °C. Oocytes injected with pure water were used as a reference. Uptake assays were performed at 25°C for 45 min (ASBT) and 30 min (NTCP) in the presence and absence of test compounds, including *P. cocos* extract and five triterpenes. TCA (25 µM) was used as a positive control. All experiments were terminated by removing the uptake buffer. The oocytes were washed thrice with ice-cold uptake buffer. The whole process is shown in Figure 2. In the uptake assays of [³H] TCA (0.125 µCi/ml), the concentration of substrate was 5 µM and the uptake time was 10 min.

### 2.5 Detection of substrate Tauro-nor-THCA-24-DBD

For sample preparation, all oocytes were disrupted in ice-cold methanol (500 µL). A 450 µL-aliquot of the supernatant of each sample was withdrawn, vortexed for 5 min, and centrifuged at 15,000 rpm for 15 min. The resultant samples were volatilized for 3
h to dryness and each sample was dissolved in 100 µL of mobile phase for measurement. Samples in the uptake assays were quantified by an HPLC system (Jasco 2057 Plus, Japan) with a fluorescence detector (Jasco FP2020 Plus, Japan). Tauro-nor-THCA-24-DBD analysis was performed using a reverse-phase Mightysil RP-18 GP column (4.6 mm×250 mm, 5 µm). The flow rate was 1 mL/min and the eluent mobile phase consisted of 45% 10 mM ammonium acetate and 55% acetonitrile. The injection volume of samples was 50 µL. The excitation and emission wavelengths for detection were set at 454 nm and 570 nm, respectively.

2.6 Inhibition kinetics assays
Poricoic acid A was used as the representative inhibitor of NTCP in assays for assessing the inhibition kinetics. The experiments were conducted using a series of substrate concentrations (5, 10, 20, 40, and 80 µM) in the presence and absence of poricoic acid A (5, 25, and 75 µM). Samples were prepared by the method described above.

2.7 Data analysis
Inhibition kinetic parameters were estimated using non-linear least-squares analysis using GraphPad Prism 7.0 (GraphPad Software Inc.). The IC\textsubscript{50} and the inhibition ratio (the percent inhibition of the ASBT and NTCP uptake activity) was calculated using the following equations:

\[
\% \text{ of control} = \frac{100 \times I_{C50}}{I_{C50} + [I]} \\
\text{Inhibition ratio (\%) =} \frac{(Accumulation)_{\text{Transporter},I}-(Accumulation)_{\text{Control},I}}{(Accumulation)_{\text{Transporter},0}-(Accumulation)_{\text{Control},0}} \times 100
\]

where [I] is inhibitor concentration.

The type of inhibition and inhibition constant (K\textsubscript{i}) for NTCP-mediated tauro-nor-THCA-24-DBD transport was determined by fitting competitive,
noncompetitive, and uncompetitive models to the untransformed data by nonlinear regression analysis using GraphPad Prism 7.0. The best-fit model was assessed by R square of goodness-of-fit. Equations used for each inhibition model were as follows:

Competition model: 

\[
v = \frac{V_{\text{max}} \times S}{K_m \times \left(1 + \frac{1}{K_i}\right) + S}
\]

Noncompetition model:

\[
v = \frac{V_{\text{max}} \times S}{K_m \times \left(1 + \frac{1}{K_i}\right) + S \times (1 + 1/K_i)}
\]

Uncompetition model:

\[
v = \frac{V_{\text{max}} \times S}{K_m + S \times (1 + 1/K_i)}
\]

Where \( S \) represents the concentration of tauro-nor-THCA-24-DBD, \( I \) represents the concentration of poricoic acid A, \( v \) represents the rate of tauro-nor-THCA-24-DBD transport, \( V_{\text{max}} \) represents the maximal rate of tauro-nor-THCA-24-DBD transport, \( K_m \) represents the tauro-nor-THCA-24-DBD concentration that yields one-half maximal velocity of tauro-nor-THCA-24-DBD transport, and \( K_i \) represents the inhibition constant. All experiments were conducted at least 3 times, and results are expressed as the mean \( \pm \) SEM.
Results

3.1 Fingerprints of triterpene acids in *P. cocos* by HPLC-TripleTOF

According to the precise molecular mass and fragmentation information from previous reports (Zhao *et al.*, 2013; Wu *et al.*, 2016; Qian *et al.*, 2018; Zou *et al.*, 2019), eight triterpene acids in the *P. cocos* extract were identified (Figure 3). These were divided into lanostane and 3, 4-secolanostane skeletons (Ríos, 2011). The closed structure of lanostane imparts stability to the parent nucleus tetracyclic triterpenes in triterpene acids. Therefore, most of the quasi-molecular ion peaks were selected both as precursor ions and fragment ions. 3, 4-secolanostane skeletons can be identified more precisely because the opening ring in them produces fragment ions. The possible chemical structures of the identified triterpene acids are shown in Figure 3 and the chromatographic and mass spectral data of eight analyzed compounds are listed in Table 2. Five representative commercially available triterpenes included poricoic acid A, poricoic acid B, polyporenic acid C, pachymic acid, and dehydrotumulosic acid, with retention times of 15.93 min, 15.11 min, 16.71 min, 19.50 min, and 15.57 min, respectively.

3.2 Simultaneous determination of five triterpene acids in *P. cocos* extract by UPLC-MS/MS

Five triterpene acids in *P. cocos* extract were determined simultaneously. A series of standard mixture solutions of these five triterpene acids was used to determine the linearity between the standard mixture concentration and peak areas. The concentration ranges of five triterpene acids in the calibration curves are all from 10 to 1000 ng/mL. The regression equations of the standard calibration curve for five triterpene acids were $y = 0.00148x+0.000201$ (Poricoic acid A, $R^2=0.995$), $y = 0.00173x+0.00246$ (Poricoic acid B, $R^2=0.996$), $y = 0.00267x-0.00195$ (Dehydrotumulosic acid, $R^2=0.994$), $y = 0.00246x+0.00315$ (Polyporenic acid C, $R^2=0.995$), $y = 0.00361x+0.00522$ (Pachymic acid, $R^2=0.994$), respectively. In the content analysis, five triterpene acids occupied 26.08% of the *P. cocos* extract. Among the five triterpene acids in the *P. cocos* extract, poricoic acid A had the highest content, accounting for 18.03% of *P. cocos* extract (Table 3).

3.3 *In vitro* transporter inhibition study using *Xenopus laevis* oocytes
The inhibitory effect of five triterpene acids and *P. cocos* extract on ASBT- and NTCP-mediated tauro-nor-THCA-24-DBD uptake was assessed using *Xenopus laevis* oocytes expressing ASBT and NTCP, respectively. Tauro-nor-THCA-24-DBD is a substrate of ASBT and NTCP (Yamaguchi *et al.*, 2010; De Bruyn *et al.*, 2014; Zhu *et al.*, 2020). Oocytes expressing ASBT and NTCP exhibited a higher uptake of tauro-nor-THCA-24-DBD compared with those injected with water as a reference. Oocytes expressing ASBT were treated with five triterpene acids with different concentrations of 1, 5, 10, 50, and 100 µM and *P. cocos* extract with concentrations of 1, 5, 10, 50, and 100 µg/mL. For NTCP, 1, 5, 10, 50, and 100 µM of five triterpene acids and 0.1, 5, 10, 50, and 100 µg/mL *P. cocos* extract were used as inhibitors.

To verify the reliability of the uptake experimental model, 25 µM TCA was used as a positive control inhibitor. The inhibition ratio for TCA was 34.3% in the ASBT-mediated uptake assay and 49.3% in the NTCP-mediated uptake assay. The results of inhibition study by *P. cocos* are shown in Figures 4 and 5 and the IC$_{50}$ values are summarized in Table 4 (The non-corrected data are shown in the Supplemental Figure 1 and 2). *P. cocos* extract, poricoic acid A, poricoic acid B, and dehydrotumulosic acid inhibited ASBT with IC$_{50}$ value of 14.6 ± 1.42 µg/mL, 39.7 ± 1.63 µM, 67.3 ± 1.28 µM, and 38.7 ± 1.32 µM concentrations. Pachymic acid did not inhibit ASBT up to 100 µM. Polyporenic acid C inhibited ASBT; however, its IC$_{50}$ value was higher than 100 µM (Figure 4D). Regarding NTCP, IC$_{50}$ values of *P. cocos* extract, poricoic acid A, poricoic acid B, and polyporenic acid C were 5.89 ± 1.48 µg/mL, 14.3 ± 1.47 µM, 31.1 ± 1.16 µM, and 38.6 ± 1.47 µM, whereas IC$_{50}$ values of dehydrotumulosic acid and pachymic acid were higher than 100 µM (Figure 5). Poricoic acid A strongly inhibited both ASBT and NTCP.

3.4 Inhibition kinetics K$_i$ of poricoic acid A on NTCP

Poricoic acid A as a representative inhibitor, was used at varying concentrations of 0, 5, 25, and 75 µM to study inhibition kinetics because of its strongest inhibitory effect on NTCP-mediated tauro-nor-THCA-24-DBD. The uptake of tauro-nor-THCA-24-DBD in the presence of poricoic acid A was analyzed by competitive, noncompetitive and uncompetitive model in the nonlinear regression. The best-fit model was assessed by R square of goodness-of-fit. The R square in the competitive, noncompetitive and uncompetitive model is 94.6, 93.5 and 92.3,
respectively. Therefore, the best fitting is competitive model. The uptake of tauro-nor-THCA-24-DBD in the presence of poricoic acid A by fitting the competitive model is shown in Figure 6. The result demonstrates that poricoic acid A competitively inhibit NTCP. Estimated inhibition constant $K_i$ value of poricoic acid A on NTCP was 63.4 ±18.7 µM.
4 Discussion

Hyperlipidemia is an emerging health problem worldwide that increases the risk of more serious diseases such as atherosclerosis and coronary heart disease (Sorokin et al., 2007). Therefore, regulating the levels of cholesterol and triglycerides by controlling the synthesis and transport of bile acids is crucial. ASBT and NTCP are uptake transporters responsible for enterohepatic circulation of bile acid. Accordingly, we investigated the mechanism underlying the lipid-lowering effects of *P. cocos* extract and its five major triterpene acids on ASBT and NTCP.

The extract of *P. cocos* has complex components. The raw material and extraction methods could result in variations in their composition. Although our results are specific to one alcohol extract of *P. cocos* obtained commercially, five triterpene acids with the range of concentrations tested in the uptake assays encompass the possible variable concentration levels in the different ethanol-extractable *P. cocos*, including this specific extract and other similarly prepared extracts.

In the concentration-dependent uptake of the probe substrate, tauro-nor-THCA-24-DBD, the evaluated $K_m$ for NTCP was 22.1±2.4 μM (Supplemental Figure 3). It is close to the $K_m$ (10.5 ± 2.9 μM) of utilizing TCA as the substrate (Masuda et al., 2014). For NTCP, the affinity of Tauro-nor-THCA-24-DBD is considerably close to the affinity of taurocholate. $K_m$ of TCA and Tauro-nor-THCA-24-DBD for ASBT is 66.0 ± 10.6 μM and 40.6 ± 14.0 μM, respectively (Zhu et al., 2020). The Michaelis constants of two substrates for both NTCP and ASBT are close. The inhibitory effect on poricoic acid A was compared by using TCA and tauro-nor-THCA-24-DBD as the substrates in vitro. The IC$_{50}$ values are 10.85±1.04 μM and 14.3 ± 1.47 μM, respectively. It had low propensity to generate the different values (Supplemental Figure 4). Therefore, uptake assays using tauro-nor-THCA-24-DBD as an in vitro probe substrate to determine IC$_{50}$ values are feasible.

Poricoic acid A exhibited the strongest inhibitory effect on both NTCP- and ASBT-mediated uptake among five triterpene acids. The inhibitory effect could be attributed to the structural similarity between triterpene acids and bile acids. Results of kinetic assays confirmed poricoic acid A as a competitive inhibitor of bile acids during NTCP-mediated uptake. The observed IC$_{50}$ for *P. cocos* extract in this study converted to the concentration of single compound according to their contents in *P. cocos* extract.
are all lower than 10 μM. These concentrations are all lower than IC$_{50}$ values observed when assessing a single compound. Thus, other compounds in the P. cocos extract could contribute to the inhibition of ASBT and NTCP or only a mixture including different triterpene acids such as the P. cocos extract mentioned above inhibits ASBT and NTCP.

The IC$_{50}$ values of uptake transporters of ASBT and NTCP in the enterohepatic circulation indicated a possibility that increased bile acid excretion regulated in vivo bile acid synthesis. The recommended dose of P. cocos extract ranges from 10 to 30 mg as a food supplement (data provided by the manufacturer). The concentration of extract in the intestine can be up to 120 μg/mL (30 mg/250 mL water), which is eight times higher than the IC$_{50}$ in ASBT. However, concentrations of these components in the blood are still unknown because of the complexity of traditional Chinese medicine and lack of corresponding pharmacokinetic data. Therefore, more pharmacokinetic studies are required to obtain clinical relevance.

The currently available lipid-lowering drugs in the market, such as bile acid sequestrants as the combined medication of statins, reduce the reabsorption of bile salts. However, their therapeutic effects are associated with adverse reactions (McCrindle, 2003). Moreover, difficulties in their preparation and administration prevent their use as first-line drugs. Compared with these clinically available drugs, P. cocos, as a food supplement, can be used in daily life and has been proved to treat obesity (Mendes LC, 2018). Therefore, P. cocos extract and its triterpene acids represent potential therapeutics for hyperlipidemia. The mechanism should be further proved in the animal assessment level in the future.
Conclusion

In the present study, five representative triterpene acids in the alcohol extract of *P. cocos* were identified and quantified. Both *P. cocos* extract and its main triterpene acids displayed an inhibitory effect on the functions of ASBT and NTCP in the enterohepatic circulation of bile acids, thus increasing the conversion of blood cholesterol to bile acids. This could be one of the mechanisms responsible for lowered blood lipid levels observed *in vivo* after administration of *P. cocos*. 
Authorship Contribution

Participated in research design: Lu, Tamai, Chen
Conducted experiments: Cai, Zhu
Performed data analysis: Cai, Cheng
Wrote or contributed to the writing of the manuscript: Cai, Kong, Lu, and Tamai
References


UHPLC-QTOF-MS/MS method for the simultaneous determination of eight triterpene compounds from Poria cocos (Schw.) Wolf extract in rat plasma: Application to a comparative pharmacokinetic study. J Chromatogr B 1102–1103:34–44.


Wu LF, Wang KF, Mao X, Liang WY, Chen WJ, Li S, Qi Q, Cui YP, and Zhang LZ (2016) Screening and analysis of the potential bioactive components of Poria cocos (Schw.) Wolf by HPLC and HPLC-MSn with the aid of chemometrics. Molecules 21:227.


Zhao Y, Li SQ, Li HJ, and Lan WJ (2013) Lanostane triterpenoids from the fungus...


Conflicts of interest statement

The authors declare no conflicts of interest.

Footnotes

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Figure 1 Chemical structures of five representative triterpene acids in the *P. cocos* extract.

Figure 2 Expression of ASBT and NTCP in *Xenopus laevis* oocytes and uptake assay.

Figure 3 Chromatography and skeleton constructions of triterpene acids in *P. cocos* identified based on HPLC-TripleTOF.

Figure 4 Inhibitory effects of *P. cocos* extract and its five main triterpenes on oocytes expressing ASBT. Uptake of Tauro-nor-THCA-24-DBD (10 µM) was measured in the absence or presence of various concentrations of *P. cocos* extract (A), poricoic acid A (B), poricoic acid B (C), polyporenic acid C (D), dehydrotumulosic acid (E) and pachymic acid (F) for 45 min at 25 °C and pH 7.4. Data are expressed as mean ± S.E.M from 6-10 oocytes.

Figure 5 Inhibitory effects of *P. cocos* extract and its five main triterpenes in oocytes expressing NTCP. Uptake of Tauro-nor-THCA-24-DBD (10 µM) was measured in the absence or presence of various concentrations of *P. cocos* extract (A), poricoic acid A (B), poricoic acid B (C), polyporenic acid C (D), dehydrotumulosic acid (E) and pachymic acid (F) for 30 min at 25 °C and pH 7.4. Data are expressed as mean ± S.E.M from 6-10 oocytes.

Figure 6 Simulation of NTCP-mediated Tauro-nor-THCA-24-DBD uptake by competitive inhibition model in the presence or absence of different concentrations of poricoic acid A.
### Tables

Table 1 Mass spectrometry parameters of five analytes and internal standard

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Precursor ion (m/z)</th>
<th>Extracted fragment ion (m/z)</th>
<th>Retention time (min)</th>
<th>DP/CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poricoic acid A</td>
<td>C$<em>{31}$H$</em>{46}$O$_{5}$</td>
<td>497.5</td>
<td>N/A</td>
<td>5.55</td>
<td>-127/-25</td>
</tr>
<tr>
<td>Poricoic acid B</td>
<td>C$<em>{30}$H$</em>{44}$O$_{5}$</td>
<td>483.4</td>
<td>N/A</td>
<td>5.17</td>
<td>-127/-20</td>
</tr>
<tr>
<td>Dehydrotumulosic acid</td>
<td>C$<em>{33}$H$</em>{50}$O$_{5}$</td>
<td>483.4</td>
<td>N/A</td>
<td>5.44</td>
<td>-138/-20</td>
</tr>
<tr>
<td>Polyporenic acid C</td>
<td>C$<em>{31}$H$</em>{46}$O$_{4}$</td>
<td>481.5</td>
<td>N/A</td>
<td>5.98</td>
<td>-126/-20</td>
</tr>
<tr>
<td>Pachymic acid</td>
<td>C$<em>{33}$H$</em>{52}$O$_{5}$</td>
<td>527.4</td>
<td>N/A</td>
<td>7.37</td>
<td>-135/-20</td>
</tr>
<tr>
<td>Chlorzoxazone (IS)</td>
<td>C$<em>{7}$H$</em>{4}$ClNO$_{2}$</td>
<td>168</td>
<td>132</td>
<td>2.21</td>
<td>-35/-20</td>
</tr>
<tr>
<td>Peak</td>
<td>Rt(min)</td>
<td>Mass measured (Da)</td>
<td>Mass accuracy (ppm)</td>
<td>Fragment ion (m/z)</td>
<td>Formula</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>-------------------</td>
<td>---------------------</td>
<td>--------------------</td>
<td>---------</td>
</tr>
<tr>
<td>1</td>
<td>15.12</td>
<td>483.3108</td>
<td>-1.7</td>
<td>411, 409, 367, 237, 211, 197</td>
<td>C$<em>{30}$H$</em>{44}$O$_{5}$</td>
</tr>
<tr>
<td>2</td>
<td>15.59</td>
<td>483.3446</td>
<td>-7</td>
<td>435, 421, 337, 313</td>
<td>C$<em>{31}$H$</em>{48}$O$_{4}$</td>
</tr>
<tr>
<td>3</td>
<td>15.95</td>
<td>497.3257</td>
<td>-3.1</td>
<td>495, 425, 423, 424, 381</td>
<td>C$<em>{31}$H$</em>{46}$O$_{5}$</td>
</tr>
<tr>
<td>4</td>
<td>16.73</td>
<td>481.3292</td>
<td>-6.6</td>
<td>437,403,387,335,311,293,271,241</td>
<td>C$<em>{31}$H$</em>{46}$O$_{4}$</td>
</tr>
<tr>
<td>No.</td>
<td>Rt</td>
<td>M/Z</td>
<td>Retention Time</td>
<td>Mass (m/z)</td>
<td>Molecular Formula</td>
</tr>
<tr>
<td>-----</td>
<td>-----</td>
<td>-----------</td>
<td>----------------</td>
<td>---------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>5</td>
<td>18.14</td>
<td>511.3411</td>
<td>-3.4</td>
<td>435,417,379,351, 309,215,153</td>
<td>C_{32}H_{48}O_{5}</td>
</tr>
<tr>
<td>6</td>
<td>19.52</td>
<td>527.36974</td>
<td>-8.5</td>
<td>467</td>
<td>C_{33}H_{52}O_{5}</td>
</tr>
<tr>
<td>7</td>
<td>20.95</td>
<td>453.33449</td>
<td>-6.5</td>
<td>452,371,337</td>
<td>C_{30}H_{46}O_{3}</td>
</tr>
<tr>
<td>8</td>
<td>21.71</td>
<td>513.35349</td>
<td>-9.9</td>
<td>467</td>
<td>C_{32}H_{50}O_{5}</td>
</tr>
</tbody>
</table>

*Rt: Retention time*
Table 3 Quantitative data of five triterpene acids in *P. cocos* extract (*n* = 4)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content (ng µg⁻¹)</th>
<th>Content (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poricoic acid A</td>
<td>180.3</td>
<td>18.03</td>
<td>3.77</td>
</tr>
<tr>
<td>Poricoic acid B</td>
<td>45.5</td>
<td>4.55</td>
<td>3.54</td>
</tr>
<tr>
<td>Dehydrotumulosic acid</td>
<td>11.14</td>
<td>1.11</td>
<td>8.42</td>
</tr>
<tr>
<td>Polyporenic acid C</td>
<td>16.78</td>
<td>1.68</td>
<td>4.18</td>
</tr>
<tr>
<td>Pachymic acid</td>
<td>7.14</td>
<td>0.71</td>
<td>6.93</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>260.86</strong></td>
<td><strong>26.09</strong></td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 4 Summary of IC\textsubscript{50} values of inhibition of NTCP and ASBT by \textit{P. cocos} extract and five main triterpenes

<table>
<thead>
<tr>
<th>Components</th>
<th>ASBT IC\textsubscript{50}</th>
<th>NTCP IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{P. cocos} extract</td>
<td>14.64 ± 1.42 µg/mL</td>
<td>5.89 ± 1.48 µg/mL</td>
</tr>
<tr>
<td>Poricoic acid A</td>
<td>39.66 ± 1.63 µM</td>
<td>14.28 ± 1.47 µM</td>
</tr>
<tr>
<td>Poricoic acid B</td>
<td>67.33 ± 1.28 µM</td>
<td>31.08 ± 1.16 µM</td>
</tr>
<tr>
<td>Polyporenic acid C</td>
<td>&gt;100 µM</td>
<td>38.62 ± 1.47 µM</td>
</tr>
<tr>
<td>Dehydrotumulosic acid</td>
<td>38.68 ± 1.32 µM</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>Pachymic acid</td>
<td>&gt;100 µM</td>
<td>&gt;100 µM</td>
</tr>
</tbody>
</table>
Figure 1

Pachymic acid

Poricoic acid A

Poricoic acid B

Dehydrrotumulosic acid

Polyporenic acid C
Figure 2

Time line/day

0
- Harvest ovarian tissue by surgery from female frog
- Defolliculate the oocyte from tissue pieces

1
- Microinject 50 nL cRNA or pure water

2
- Culture for 3 days in 1*MBS (pH 7.4, gentamycin plus) at 18 °C

3

4
uptake
Figure 4

A. Uptake of Tauro-nor-THCA-24-DBD (% of control) vs. Poria cocos extract (µg/mL).

B. Uptake of Tauro-nor-THCA-24-DBD (% of control) vs. Poricoic acid A (µM).

C. Uptake of Tauro-nor-THCA-24-DBD (% of control) vs. Poricoic acid B (µM).

D. Uptake of Tauro-nor-THCA-24-DBD (% of control) vs. Polyporenic acid C (µM).

E. Uptake of Tauro-nor-THCA-24-DBD (% of control) vs. Dehydrotumulosic acid (µM).

F. Uptake of Tauro-nor-THCA-24-DBD (% of control) vs. Pachymic acid (µM).
Figure 5

A

Uptake of Tauro-nor-THCA-24-DBD (\% of control)

\( IC_{50} = 5.89 \pm 1.48 \mu \text{g/mL} \)

Porica cocos extract (\mu g/mL)

B

Uptake of Tauro-nor-THCA-24-DBD (\% of control)

\( IC_{50} = 14.28 \pm 1.47 \mu \text{M} \)

Poricoic acid A (\mu M)

C

Uptake of Tauro-nor-THCA-24-DBD (\% of control)

\( IC_{50} = 31.08 \pm 1.16 \mu \text{M} \)

Poricoic acid B (\mu M)

D

Uptake of Tauro-nor-THCA-24-DBD (\% of control)

\( IC_{50} = 38.62 \pm 1.47 \mu \text{M} \)

Polyporenic acid C (\mu M)

E

Uptake of Tauro-nor-THCA-24-DBD (\% of control)

\( IC_{50} > 100 \mu \text{M} \)

Dehydrotumulosic acid (\mu M)

F

Uptake of Tauro-nor-THCA-24-DBD (\% of control)

\( IC_{50} > 100 \mu \text{M} \)

Pachymic acid (\mu M)
Figure 6

The figure shows a graph plotting the rate of reaction (V, pmol/oocyte/30 min) against the concentration of [Tauro-nor-THCA-24-DBD] (μM). The data points are presented for different inhibitor concentrations: 0 μM (no inhibitor), 5 μM, 25 μM, and 75 μM.