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ScienceDirect
Chinese Journal of Natural Medicines 2017, **15**(4): 0265–0270
Chinese Journal of Natural Medicines

•Research Articles•

Accumulation of biomass and four triterpenoids in two-stage cultured *Poria cocos* mycelia and diuretic activity in rats

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Available online 20 Apr., 2017

[ABSTRACT] *Poria cocos* (Schw.) Wolf, an important medicinal and food fungus, is well known in East Asia. Due to growing market demand, long cultivation period, and consumption of pine trunk during cultivation, developing alternative methods for producing *P. cocos* and/or its active components is of interest. In the present study, the effects of different culture methods on biomass and accumulation of four triterpenoids were investigated. The ethanol extract of fermented mycelium (EFM) was orally administered to rats. Urine output and concentrations of electrolytes (Na^+, K^+, and Cl^-) were measured. Our results showed that mycelia grew better under continuous shaking culture condition (7.5 g DW·L^{-1}), and higher triterpenoid levels were accumulated in two-stage culture (112 mg·L^{-1}, 2.03%). The optimal starting time of static culture for triterpenoid yield was 4th d after shaking culture. Single administration of middle and high dose of EFM significantly increased urine output, Na^+ and Cl^- excretion, and Na^+/K^+ ratio. These results suggested that ethanol extract of cultured mycelia showed significant diuretic activity in rats and two-stage culture of *P. cocos* could be an alternative way to produce mycelia and triterpenoids.

KEY WORDS] *Poria cocos*; Two-stage culture; Diuretic activity; Triterpenoids; Content determination

CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2017)04-0265-06

Introduction

Higher fungi have received increasing attention in recent years as they produce numerous bioactive secondary metabolites [1]. For several thousand years, *Poria cocos* (Schw.) Wolf, a traditional Chinese medicinal and food fungus, has been used in China and other East Asian countries for its ability to clear damp, promote diuresis, and invigorate the spleen [2]. As reported previously, the chemical constituents of *P. cocos* include polysaccharides [3-6] and triterpenoids [7-10]. Chemical and pharmacological investigations have shown that polysaccharides separated from *P. cocos* have anti-tumor activity both *in vivo* [11] (sarcoma-180 solid tumors implanted in BALB/c mice) and *in vitro* (HL-60 tumor cells) [6], and antioxidant activities [12]. As for triterpenoids, the main constituents are dehydrotumulosic acid (DTA), polyporenic acid C (PAC), 3-epi-dehydrotumulosic acid (eDTA) and dehydropachymic acid (DPA) [13]. Triterpenoids are reported to be responsible for the diuretic [14], anti-tumor [15], and anti-inflammatory activities [8] of *P. cocos*.

Because of its nutritional and health-promoting values, *P. cocos* has gained wide popularity as a nutraceutical and functional food in China. Normally, *P. cocos* grows underground around the roots of living pine trees or dried trunks of pine trees, and needs a long cultivation period of about 8−9 months. In recent decades, fermentation culture of *P. cocos* has been developed because of the potential for increased production of mycelia and bioactive components in a compact space and shorter time. Studies have been conducted to examine the influence of different conditions on the accumulation of biomass, polysaccharides, and triterpenoids, including medium composition, temperature, and culture conditions [16-18] in continuous shaking condition. Two-stage culture, a method that combines shaking and static culture, dramatically improves the accumulation of ganoderic acids in *Ganoderma lucidum* [19]. *P. cocos* and *G. lucidum* belong to the same family of Ployporaceae and their effective components are both triter-

[Received on] 18-May-2016
[Research funding] This work was financially supported by the post-doctoral program of 54th China Postdoctoral Science Foundation (No. 2013M541255), Career Development Support Program for Young and Middle-aged Teachers of Shenyang Pharmaceutical University (No. ZQN2015034), and the funds from Shenyang Pharmaceutical University and Kanion Pharmaceutical Co. Ltd..
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These authors have no conflict of interest to declare.

penoids. Therefore, in the present study, two-stage culture was applied in *P. cocos*. The accumulation of biomass and four main triterpenoids was investigated and the ethanol extract of mycelia was used for diuretic activity determination in rats, in order to prove that the two-stage cultured mycelia of *P. cocos* can be an alternative resource for the production of effective triterpenoids with diuretic activity.

Materials and Methods

Chemicals and reagents

Dehydrotumulosic acid (DTA), polyporenic acid C (PAC), 3-epi-dehydrotumulosic acid (eDTA), and dehydropachymic acid (DPA) were provided by Kanion Pharmaceutical Co. Ltd. Research Center (Lianyungang, Jiangsu Province, China). Furosemide injection (20 mg/2 mL) was purchased from Henan Provincial Runhong Pharmaceutical Co. Ltd. (Xinzheng City, China). Normal saline injection (0.9%) was purchased from Chenxin Pharmaceutical Co. Ltd. (Jining, Shandong Province, China).

Fungus isolation and culture conditions

P. cocos sclerotium was collected freshly from a cultivation base located in Luotian country, Hubei Province, in October 2011, and was authorized by Associate Professor YUAN Jiu-Zhi in Shenyang Pharmaceutical University, Shenyng, China. The specimen was stored in the Herbarium of Shenyang Pharmaceutical University (voucher number SPU-F201109-10). Sterilized *P. cocos* sclerotium was cut into small pieces with about 0.5-cm diameter and inoculated onto potato dextrose agar (PDA) media for 3 d and growing mycelia were picked up using sterile toothpicks and inoculated onto new PDA plates. Purified *P. cocos* mycelia were maintained on PDA plates.

To create a seed culture, mycelia covering an area of 1 cm^2 were cut from the plates and inoculated into 200 mL of liquid media in Erlenmeyer flasks for 10 d. 5 mL of seed culture was then inoculated into Erlenmeyer flasks containing 45 mL of new media and cultured at 25 ± 1 °C in dark with shaking speed at 130 $r \cdot min^{-1}$. The liquid medium was consisted of following components ($g \cdot L^{-1}$): glucose 25, cone steep liquor 5, KH_2PO_4 6.0, $MgSO_4 \cdot 7H_2O$ 3.0, and thiamine hydrochloride 0.04. These flasks were divided into 4 groups: continuous shaking (Control), shaking for 4 d then static culture (SF4), shaking for 5 d then static culture (SF5), and shaking for 6 d then static culture (SF6). In static culture, the flasks were incubated statically at 25 ± 1°C in dark. Samples were collected at 7^{th}, 10^{th}, 13^{th}, 16^{th}, 19^{th}, 22^{ed}, and 25^{th} d after inoculation.

Determination of mycelia biomass, DTC, PAC, eDTC, and DPA

Mycelia were firstly collected by filtration through a stainless sieve (40 meshes), washed with distilled water, and then lyophilized. The weight of dried mycelia was recorded as biomass. The contents of DTA, PAC, eDTA, and DPA in mycelia were determined according to the previously reported methods [20]. 30 mg of each powder sample (60 meshes) of *P. cocos* was extracted using 1 mL of methanol under ultrasound (ultrasonic frequency: 40 KHZ) for 30 min, followed by centrifugation at 12 000 $r \cdot min^{-1}$ for 15 min. The supernatant was filtered through a 0.45-μm Millipore filter. The samples were analyzed using a Hitachi L-2000 HPLC with Dikma Platisil ODS columns (250 mm × 4.6 mm, 5 μm, Dikma Technology, Co. Ltd., Beijing, China) at 30 °C with ACN/0.5% phosphate solution (80 : 20) as the mobile phase at 1.0 $mL \cdot min^{-1}$. The detector was DAD with a scanning range from 200 to 400 nm and the detection wavelength was set at 242 nm.

To calculate the DTA, PAC, eDTA, and DPA contents of the *P. cocos* samples, standard curves were prepared using serial dilutions of each of the four compounds. Compound quantity (μg) (Y) and peak area (X) were used for the linear regression analysis. The standard formulas of four standard compounds are listed in Table 1.

Table 1 Retention time, standard formula, linear ranges, and coefficient of determination of four triterpenoids

Compound	t_R (min)	Standard formula	Linear range (μg)	R^2
DTA	9.82	$Y = 2 \times 10^{-6}X - 0.014\ 8$	0.021 25–1.7	0.999 2
PAC	13.79	$Y = 2 \times 10^{-6}X + 0.001\ 9$	0.005–0.1	0.999 7
eDTA	15.73	$Y = 2 \times 10^{-6}X + 0.001\ 7$	0.004 55–0.045 5	0.998 1
DPA	29.93	$Y = 2 \times 10^{-6}X + 0.015\ 8$	0.010 1–0.050 5	0.998 2

Preparation of the ethanol extracts of the fermented mycelia (EFM) and furosemide solution

The fermented mycelia (50 g) were extracted under reflux for 2 h with 500 mL of 95% ethanol and the solvent was recycled under reduced pressure at 45 °C and to yield a black syrup (6.5 g, yield rate: 13%). The contents of DTA, PAC, eDTA, and DPA in syrup were determined as 110.3, 1.7, 0.36, and 2.41 $mg \cdot g^{-1}$, respectively. The syrup was suspended in distilled water at certain concentrations before oral administration. Furosemide injection was diluted to final concentration of 2 $mg \cdot mL^{-1}$ with distilled water in 10-mL brown measuring flasks before experiment, sealed and kept at room temperature until use.

Animals and prescreening

Male SD rats (weighing 180−200 g, SPF, Batch No. 211002300007424) were bought from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, Liaoning Province, China), [License number for experimental animal production and utilization SCXK (Liao) 2010-0001]. All experiments and procedures were carried out according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China. The rats were maintained at 21 ± 2 ºC, relative humidity 40%−60%, with free access to food and water. The rats were housed in the metabolism cage for 3 d prior to the experiments. After deprived of food but not water for 18 h, the rats were admin-