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Preparative separation of minor bioactive compounds from flower of *P. cuspidatum* by high-speed counter-current chromatography and comparison of their antioxidant activity

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ABSTRACT

Two runs of preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:7:1:7, *v/v*) were employed to separate the compounds from the flower of *Polygonum cuspidatum*. Three minor compounds, 8.6 mg of (+)-catechin (1), 12.3 mg of (−)-epicatechin (2) and 7.4 mg of 1-(S)-phenylethyl β-D-glucopyranoside (3) were obtained from 240 mg of the crude extract of the flower of *P. cuspidatum* with purities of 96.8%, 98.5% and 98.3%, respectively, as determined by HPLC. The structures of these compounds were identified by ESI-MS, ^{1}H NMR and ^{13}C NMR spectroscopy. Among them, compounds 2 and 3 were obtained from *P. cuspidatum* for the first time. In addition, antioxidant activities of (+)-catechin and (−)-epicatechin were evaluated and compared by the methods of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Both (+)-catechin and (−)-epicatechin showed high radical scavenging activities with their EC_{50} values being 15.08 ± 0.46 and 9.44 ± 0.13 μg/ml in DPPH radical method.

1. Introduction

Polygonum cuspidatum Sieb. et Zucc (Huzhang in Chinese), a very popular Chinese traditional medicinal herb belongs to the *Polygonaceae* family, is widely distributed in southern areas of the yellow river in China. Traditionally, its root and rhizome have been used for the treatment of inflammation, cough, abscess and gonorrhea [1]. Also, it has been used for menoxenia, skin burn, gallstone and hepatitis. In the recent years, *P. cuspidatum* was found to show potent estrogenic activity due to anthraquinones it contains [2,3]. Recently, plenty of literatures have been reported that the contents of stilbenes in *P. cuspidatum* are the highest [4–6].

So far, quite a few literatures have been developed for the analysis of the anthraquinones or stilbenes from *P. cuspidatum*, but they only focus on the roots of *P. cuspidatum* [7–10]. And very few data on chemical components of flower of the *P. cuspidatum* plant is available.

Traditional separation methods such as silica gel column chromatography or normal-phase thin-layer, were widely used for the separation and purification of bioactive compounds from herbal medicines. The disadvantages of these methods are often high costing, long time-consuming, and not available for isolation and purification in bulk [11–13]. So exploring an efficient method to separate and purify chemical compositions from the flower of *P. cuspidatum* is very important.

High-speed counter-current chromatography (HSCCC) invented by Ito [14] is a support-free liquid–liquid partition chromatography technique. HSCCC is distinguished from all the other separation methods because it has a unique separation mode. Firstly, there is no sample loss in theory. Working like a continued solvent partition, it ensures all the chemicals of the injected sample be screened infractions as finally obtained, which is significantly different between HSCCC and other separation methods [15,16]. Secondly, the solvent ratio of the solvent system is tolerant and flexible by increasing the percentage of the non-polar solvent relative to the polar solvent, which is available for separation of both water-soluble and alcohol-soluble components from various natural products. Another advantage of HSCCC is less solvent consumption since water is the majority of the mobile phase in most cases [17,18].

Herein, we report an efficient method of separation and purification of (+)-catechin (1), (−)-epicatechin (2) and 1-(S)-phenylethyl β-D-glucopyranoside (3) from the flower of *P. cuspidatum*.

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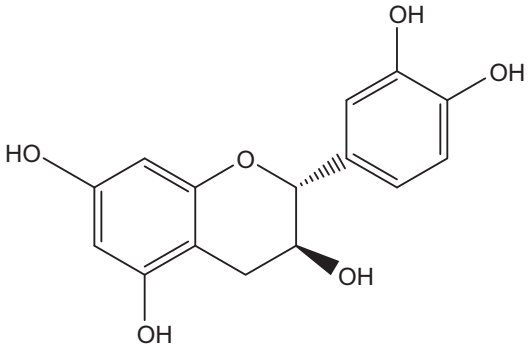
This research is necessary for quality control, pharmacological research and exploring new products from the flower of *P. cuspidatum*. The chemical structures were shown in Fig. 1.

2. Experimental

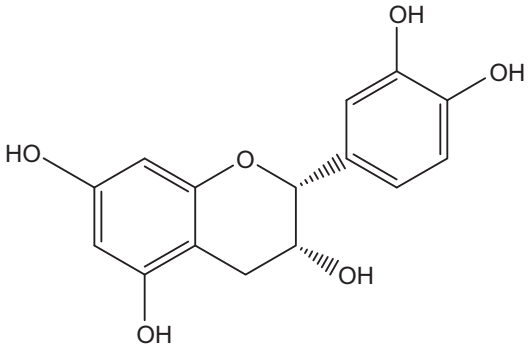
2.1. Chemicals and materials

N-hexane, ethyl acetate, methanol used for fractions preparation and HSCCC separation were of analytical grade and obtained from the Tianjin Chemical Factory, Tianjin, China. Acetonitrile used for HPLC was of chromatographic grade (Yongda Chemical Factory, Tianjin, China), and water used was redistilled water. Dimethyl sulfoxide (DMSO-d_6) and CD_3OD were used as the solvent for NMR determination. Column chromatography was performed on silica gel (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd., Shandong, China) and Sephadex LH-20 (GE Healthcare Biosciences AB, Uppsala, Sweden).

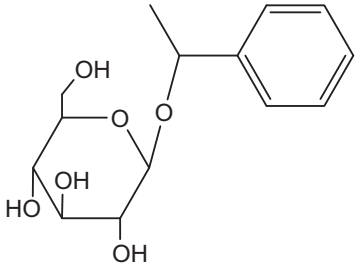
The flower of *P. cuspidatum* was collected from the Medicinal Plant Farm of Shandong Agricultural University in September 2012, Taian, China, and was identified by Doctor Jianhua Wang. The sample was pulverized into powder form by a disintegrator, and then sieved with stainless steel sieves to classify the particle size. The powdered samples were oven-dried at 60 °C for 6 h, and then kept in a dry and dark place until use.



(+)-catechin (1)



(–)-epicatechin (2)



1-(S)-phenylethyl β-D-glucopyranoside (3)

Fig. 1. The Chemical structures of compounds 1–3.

2.2. Apparatus

HSCCC instrument employed in this study is a Model GS-10A high-speed counter-current chromatography (Beijing Institute of New Technology Application, Beijing, China). The apparatus was equipped with a polytetrafluoroethylene multilayer coil (i.d. of the tubing = 1.6 mm, total volume = 230 mL) and a manual sample injection valve with a 10 mL loop. The distance between the holder axis and the central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and the central axis of the centrifuge). The revolution speed of the apparatus could be regulated with a speed controller in the range between 0 and 1000 rpm. The HSCCC system used in the present study was equipped with a Model NS-1007A constant-flow pump, a Model 8823B-UV Monitor at 280 and 254 nm and BF-2002 CT11 Signal collection cell (Chromatography Center of Beifenruili Group Company, Beijing, China). The data were collected with HW-2000 chromatography workstation (Qianpu Software Co. Ltd., Shanghai, China).

The high performance liquid chromatography (HPLC) equipment was Waters 600E (USA) HPLC system, including a 4-Solvent delivery system 600E start-up kit, a 600 pump (0–20 ml/min), a 2996 photodiode array detector, an Empower Add-on Single System, a Degasser in-line 4 chamber and a 600E controller.

Nuclear magnetic resonance (NMR) spectrometer was Avance 500 (Bruker, Switzerland). The mass spectrometer employed was an Agilent 1100 LC/MSD ESI System (Agilent Technologies, USA).

2.3. Preparation of crude extract

8 kg Dried and powdered flower (40 mesh) of *P. cuspidatum* was macerated and extracted with 30 L ether three times (12 h for each time). The residue was reflux extracted with 40 L methanol three times (3 h for each time). The methanol extracts combined and evaporated in vacuo to yield a crude extract (360 g).

Sephadex LH-20 (150 g) was macerated with 10% methanol (800 ml) for 12 h, then suspended in 10% methanol and transferred to the column (120 cm length × 4 cm i.d.). The column was rinsed with 1000 ml of 10% methanol. The methanol extract (8.8 g) was loaded on a Sephadex LH-20 column, eluted with EtOH-H_2O (v/v) gradient solvent system 10%, 20%, 30%, 50% and 70%, respectively. The volume of each gradient elution was 1000 ml, and the flow rate was 5 ml/min. B fraction (550 mg) obtained by elution of 20% methanol was combined and concentrated under reduced pressure. It was stored in a refrigerator for subsequent HSCCC separation.

2.4. Selection of two-phase solvent system

In the present study, *n*-hexane–ethyl acetate–methanol–water was used as the two-phase solvent system of HSCCC. The composition of the solvent system was selected according to the partition coefficients (K) of the target compounds of the crude sample. The K values of the target components were determined according to the literature [19] by HPLC analysis as follows: about 3 ml of each phase of the equilibrated two-phase solvent system was added to approximately 8 mg of a test sample. The test tube was caped and shaken vigorously for several minutes to equilibrate the sample thoroughly, then, the peak areas of the upper phase analyzed