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Isolation of antioxidants from *Psoralea corylifolia* fruits using high-speed counter-current chromatography guided by thin layer chromatography-antioxidant autographic assay

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ABSTRACT

A combinative method using high-speed counter-current chromatography (HSCCC) and thin layer chromatography (TLC) as an antioxidant autographic assay was developed to separate antioxidant components from the fruits of *Psoralea corylifolia*. Under the guidance of TLC bioautography, eight compounds including five flavonoids and three coumarins were successfully separated from the fruits of *P. corylifolia* by HSCCC with an optimized two-phase solvent system, *n*-hexane–ethyl acetate–methanol–water (1:1.1:1.3:1, v/v/v/v). The separation produced 5.91 mg psoralen, 6.26 mg isopsoralen, 3.19 mg psoralidin, 0.92 mg corylifol A, and 2.43 mg bavachinin with corresponding purities of 99.5, 99.8, 99.4, 96.4, and 99.0%, as well as three sub-fractions, in a single run from 250 mg ethyl acetate fraction of *P. corylifolia* extract. Following an additional clean-up step by preparative TLC, 0.4 mg 8-prenyldaidzein (purity 91.7%), 4.18 mg neobavaisoflavone (purity 97.4%) and 4.36 mg isobavachalcone (purity 96.8%) were separated from the three individual sub-fractions. The structures of the isolated compounds were identified by ^{1}H NMR and ^{13}C NMR. The results of antioxidant activity estimation by electron spin resonance (ESR) method showed that psoralidin was the most active antioxidant with an IC_{50} value of 44.7 μM. This is the first report on simultaneous separation of eight compounds from *P. corylifolia* by HSCCC.

1. Introduction

Psoralea corylifolia Linn is an annual plant of Fabaceae family widely distributed in China and Southeast Asia. Its fruits, known as Fructus Psoraleae (*Buguzhi* in Chinese), were traditionally used for the treatment of spermatorrhea, pollakiuria, asthma, and nephritis [1]. A number of studies have shown that coumarins and flavonoids are the major bioactive constituents in *P. corylifolia* fruits [2–10]. Some of these phytochemicals exhibited a wide range of biological activities such as antioxidant [6,8], antibacterial [7], anti-inflammatory [11], and antidepressant-like activities [12] as well as inhibitory activities of baculovirus-expressed BACE-1 [13], DNA polymerase and topoisomerase II [14], and nitric oxide synthase expression in lipopolysaccharide-activated macrophages [15]. Most of these studies were conducted within only one type of compounds, either coumarins or flavonoids. The comparative pharmacological actions between coumarins and flavonoids are still sparse due probably that the majority of these compounds are commercially unavailable. Generally, repeated column chromatography over silica gel has been frequently used for the purification of compounds from traditional Chinese medicines (TCM). However, this separation method always consumes large volumes of organic solvents and may cause irreversible adsorption of samples onto solid phase. Therefore, a green and preparative separation method is of great interest in recent years.

High-speed counter-current chromatography (HSCCC), a kind of liquid–liquid partition chromatography, has been widely used in preparative isolation of pure compounds from natural materials. The well-known advantages of HSCCC such as high recovery,

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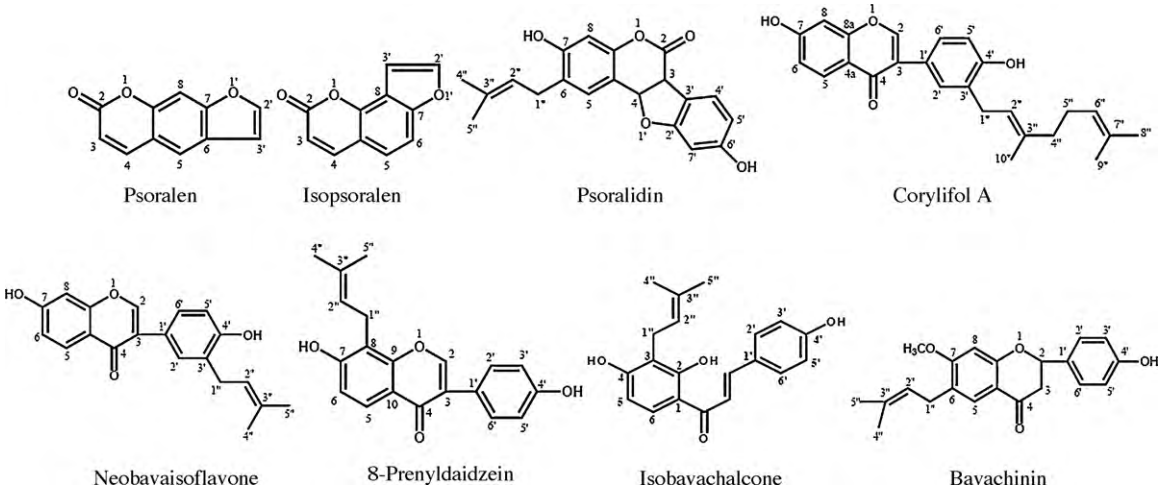

Fig. 1. Chemical structures of isolated compounds from *Psoralea corylifolia* fruits by HSCCC.

high efficiency and the ease to scale-up [16,17] were gradually confirmed by numerous reports on the isolation of various types of natural products [17]. HSCCC has also been used to effectively separate two coumarins (psoralen and isopsoralen) from *P. corylifolia* [18]. As part of our continuous efforts to screen antioxidants from TCM [19–21], the objective of the present study was to develop a HSCCC method for preparative isolation of natural antioxidants from *P. corylifolia*, guided by the TLC bioautographic assay against DPPH radicals. In addition, the antioxidant activity of the isolated compounds by electron spin resonance (ESR) method was described.

2. Materials and methods

2.1. Reagents

All reference standards of psoralen, isopsoralen, psoralidin, corylifol A, 8-prenyldaidzein, neobavaisoflavone, isobavachalcone and bavachinin were obtained from Shanghai Research & Development Center for Standardization of Chinese Medicines (Shanghai, China), the structures of which are shown in Fig. 1. All organic solvents used for TLC and HSCCC were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Acetonitrile and methanol used for HPLC analysis were of HPLC grade and obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were purchased from Sigma–Aldrich (Steinheim, Germany). Silica gel 60 F_{254} TLC plates used for TLC bioautography were purchased from Merck (Darmstadt, Germany). Silica gel used for preparative TLC was obtained from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). Sodium carboxymethyl cellulose for preparative TLC was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Water was bidistilled and all other chemicals were of analytical grade without further purification. The fruits of *P. corylifolia* were purchased from Kangqiao Decoction Pieces Factory (Shanghai, China) and authenticated by Dr. Lihong Wu, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, China.

2.2. Preparation of crude extracts

The fruits of *P. corylifolia* were ground to a fine powder to pass a 20 mess screen. The powder (1 kg) was then extracted three times with 10 l of 80% aqueous ethanol for 60 min. The extracts were combined and evaporated to dryness at 60 °C under reduced pressure. The resulting residue was re-suspended in distilled water and partitioned thrice in a separatory funnel with an equal volume of ethyl acetate each time. The ethyl acetate layers were combined and evaporated to dryness at 50 °C under reduced pressure. Then the dry residue (250 mg) of the ethyl acetate fraction was dissolved in 20 ml of the lower phase before HSCCC separation.

2.3. High-speed counter-current chromatography

HSCCC experiments were carried out using a model TBE-300A high-speed counter-current chromatograph (Tauto Biotech, Shanghai, China) with a set of three multilayer columns, connected in series (diameter of tube, 2.6 mm; total volume, 300 ml). The revolution radius was 5 cm, and the β values of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus was allowed to be regulated with a controller in the range of 0 and 1000 rpm. A HX-1050 constant temperature circulating implement (Beijing Boyikang Lab Implement Co., Ltd., Beijing, China) was used to control the separation temperature. The two-phase solvent system was pumped into the column with a Series S pump (Beijing Shengyitong Technology Development Co., Ltd., Beijing, China). A manual injection valve with 20 ml sample loop was used to introduce the sample into the column. The HSCCC chromatograms were recorded by a N2010 workstation (Intelligence Research Institute, Zhejiang University, Hangzhou, China).

2.4. Purification of compounds by preparative TLC

HSCCC peaks I, II, and VI were further purified by preparative TLC. The dry residues of peaks I, II, and VI were individually dissolved in methanol. These sample solutions were deposited (as bands) onto preparative TLC plates (20 cm × 10 cm). TLC plates were then developed in a presaturated solvent tank with petroleum ether–ethyl acetate (3:1) as developing reagents until the solvent front reached the top of plates. The developed TLC plates were removed from the solvents, and allowed to air-dry. The major bands observed under 254 nm were removed, collected, and extracted with methanol. The target compounds were obtained by evaporating the solvents under reduced pressure at 50 °C.