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Short communication

Separation of flavonoids from the seeds of *Vernonia anthelmintica* Willd by high-speed counter-current chromatography

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Abstract

Several flavonoids including 2′,3,4,4′-tetrahydroxychalcone, 5,6,7,4′-tetrahydroxyflavone and butin, were separated from the seeds of *Vernonia anthelmintica* Willd by high-speed counter-current chromatography using a two-step operation. Two different types of solvent systems were used: chloroform–dichloromethane–methanol–water (2:2:3:2, v/v) and 1,2 dichloroethane–methanol–acetonitrile–water (4:1.1:0.25:2, v/v). From 1 kg of seeds of *Vernonia anthelmintica* Willd the method yielded about 45 mg of 2′,3,4,4′-tetrahydroxychalcone, 40 mg of 5,6,7,4′-tetrahydroxyflavone, and 55 mg of butin. Each isolated component showed 95–97% purity as determined by high-performance liquid chromatography analysis. These purified compounds were characterized by MS and NMR.

Keywords: *Vernonia anthelmintica* Willd; 2′,3,4,4′-Tetrahydroxychalcone; 5,6,7,4′-Tetrahydroxyflavone; Butin

1. Introduction

The crude extract of the seeds of *Vernonia anthelmintica* Willd is a traditional Chinese medicine as vermicide [1], also used by Uygurs for the treatment of vitiligo for several centuries [2], and most recently found as an effective medicine for treating breast cancer [3]. Some components of the seed such as vernodlin, vernodalol, and vernolic acid, have been isolated and identified [4,5], but the effective components for vitiligo are unknown. Our research is to separate and identify the effective components from the seeds of *Vernonia anthelmintica* Willd to develop a new medicine for vitiligo. In order not to lose any possible effective component, we cannot use the conventional separative methods such as column chromatography because of its irreversible adsorption of samples onto the solid support. High-speed counter-current chromatography (HSCCC) is a very suitable alternative, because it is a support-free liquid–liquid partition chromatography, which eliminates irreversible adsorption in the conventional chromatography [6], and has been successfully applied to analysis and separation of various natural products [7,8]. Three flavonoids were isolated from the seeds and their structures were identified by MS and NMR. The chemical structures of these flavonoids are given in Fig. 1.

2. Experimental

HSCCC separations were performed with the following two models of counter-current chromatography (CCC):

1. Model GS10A2 preparative CCC (Beijing Institute of New Technology Application, Beijing, China): The multilayer coil was prepared from 1.6 mm i.d. PTFE tubing. The total capacity is 240 ml. The value varied from 0.5 at the internal terminal to 0.75 at the external terminal. The revolution speed is adjustable from 0 to 1000 rpm, but 800 rpm was used in the present studies.

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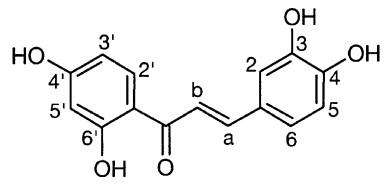
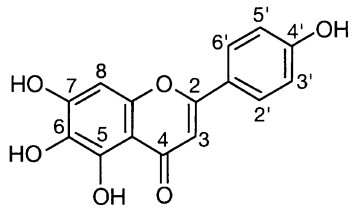
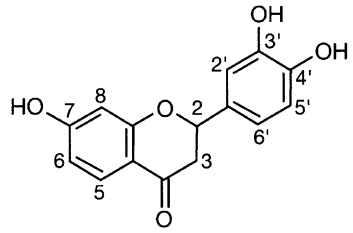


2',3,4,4'-tetrahydroxychalcone



5,6,7,4'-tetrahydroxyflavone



7,3',4'-trihydroxydihydroflavone (butin)

Fig. 1. The chemical structures of flavonoids.

2. Model CCC-1000 (Pharma-Tech Research, MD, USA) equipped with three PTFE multilayer coils of 2.6 mm i.d. that were serially connected to make up a total capacity of 860 ml [9].

These two HSCCC systems are equipped with Model MS-1007 constant-flow pump, a Model 8823A-UV monitor operating at 254 nm, a Yokogawa Model 3057 recorder, and a sample injection valve with a 20 or 40 ml sample loop.

2.1. Reagents

All organic solvents used for HSCCC and HPLC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. All standardized samples were purchased from National Institute for the Control of Pharmaceutical & Biological Products, Ministry of Health, Beijing, China. The seeds of *Vernonia anthelmintica* Willd were purchased from a local store in China.

2.2. Preparation and sample solution of two-phase solvent system

About 1.0 kg amount of the seeds of *Vernonia anthelmintica* Willd was soaked in a glass bottle (2.5 l capacity) with 1.5 l of light petroleum (b.p. 30–60) for 48 h, and then the seeds were extracted with 1.5 l ethanol solution (60% in water) for three times (a total volume of 4.5 l) at room temperature. The extracts were combined, and evaporated to dryness under reduced pressure, and then redissolved in 500 ml of water. The aqueous solution was again extracted with ethyl acetate (500 ml × 6 times), and these extracts was combined and evaporated to yield about 150 g of the crude sample. Then, 1 g of the crude extract was dissolved in 10 ml of each phase for preparative Model GS10A2 preparative separation and 3.5 g crude extract was dissolved in 20 ml of each phase for Model CCC-1000 preparative HSCCC separation. The sample solutions were sonicated for several minutes before loading into the column.

For the present study, the following two solvent systems was prepared: chloroform–dichloromethane–methanol–water (2:2:3:2, v/v); 1,2-dichloroethane–methanol–acetonitrile–water (4:1.1:0.25:2, v/v). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

2.3. HSCCC separation produces

For each separation, the column was first entirely filled with the upper aqueous stationary phase. Then the sample solution was injected through the sample port and the organic lower mobile phase was pumped through the column at a flow-rate of 2 ml/min while the column was rotated at 800 rpm. The effluent from the outlet of the column was monitored with a UV detector at 254 nm. Peak fractions were collected according to the chromatogram. After the desired peaks were eluted, the rotation and elution were stopped and the column contents were collected in a graduated cylinder by N_2 at approximately 0.5 MPa. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

2.4. HPLC analysis

HPLC analyses were performed with a Shimadzu LC-10AVP system including LC-10AT liquid chromatograph, SPD-M10AVP photodiode array detector, SCL-OAVP system controller and CTP-10SVP column oven using an Intersil ODS-3 column (150 mm × 4.6 mm i.d.) at a column temperature of 30 °C. The mobile phase, composed of methanol–0.5% H_3PO_4 (gradient flow, 40/60–80/20 in 20 min, v/v) was eluted at a flow-rate of 1.0 ml/min and effluent monitored by PDA detector.

3. Results and discussion

In the present study, the seeds of *Vernonia anthelmintica* Willd were extracted with light petroleum and 60% ethanol successively, and then 350 g of the 60% ethanol extract was further extracted with ethyl acetate. Pharmacodynamic test

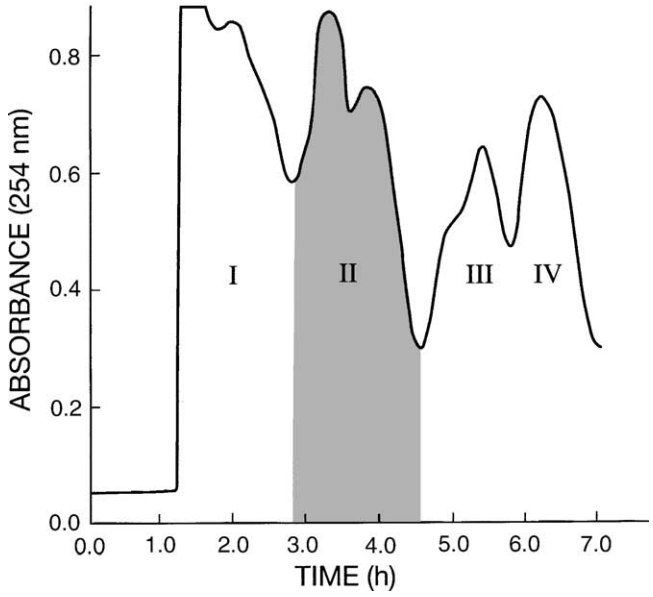


Fig. 2. HSCCC separation of the ethyl acetate extract of *Vernonia anthelmintica* Willd by Model GS10A2. Experiment conditions: rotation speed: 800 rpm; solvent system: chloroform–dichoromethane–methanol–water (2:2:3:2, v/v); mobile phase: lower organic phase; flow-rate: 2 ml/min; sample size: 1 g; retention of the stationary phase: 65%.

[10] on 100 mg each of light petroleum, 60% ethanol, ethyl acetate and water extracts revealed a high anti-vitiligo activity in the ethyl acetate extract. Consequently, the ethyl acetate extract was separated by HSCCC using the first solvent system composed of chloroform–dichloromethane–methanol–water at a volume ratio of 2:2:3:2 yielding five fractions (I–IV, and the residual in the column) (Fig. 2) among which fraction II showed the highest anti-vitiligo activity. The HPLC analysis of this fraction indicated that it mainly contained five components. This partially purified fraction was further subjected to HSCCC using the second solvent system composed of 1,2-dichloroethane–methanol–acetonitrile–water (4:1.1:0.25:2, v/v). In the second separation, three flavonoid components, 2′,3,4,4′-tetrahydroxychalcone, 5,6,7,4′-tetrahydroxyflavone and butin, were obtained at a purity of over 95–97% by HPLC analysis, and all of them showed anti-vitiligo activity, especially butin (Fig. 3).

In order to increase the quantity of samples for the Pharmacodynamic tests, Model CCC-1000 preparative HSCCC was used to separate the ethyl acetate extract (Fig. 4) instead of Model GS10A2 preparative HSCCC which increased the sample loading capacity from 1 to 4 g and the fraction II from 150 to 650 mg. From 1 kg of seeds of *Vernonia anthelmintica* Willd the method produced about 45 mg of 2′,3,4,4′-tetrahydroxychalcone, 40 mg of 5,6,7,4′-tetrahydroxyflavone, and 55 mg of butin.

Each purified component was analyzed by MS and NMR, the data of which are as follows.

Peak A [2′,3,4,4′-tetrahydroxychalcone] yellow needles, $C_{15}H_{12}O_5$. EI-MS *m/z* (%): 272 (77.95), 271 (37.46), 163 (32.75), 150 (26.71), 127 (100). 1H-NMR (500 MHz in

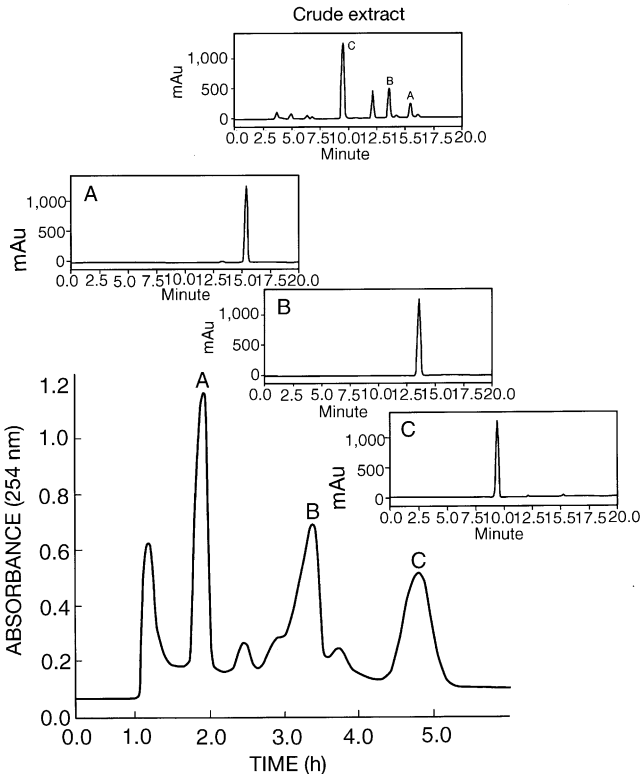


Fig. 3. HSCCC separation of fraction II (Fig. 2) and HPLC analyses of purified fractions. Experiment conditions: rotation speed: 800 rpm; solvent system: 1,2-dichloroethane–methanol–acetonitrile–water (4:1.1:0.25:2, v/v); mobile phase: lower organic phase; flow-rate: 2 ml/min; detection: 254 nm; sample size: 100 mg; retention of the stationary phase: 60%. Peak A: 2′,3,4,4′-tetrahydroxychalcone; Peak B: 5,6,7,4′-tetrahydroxyflavone; Peak C: butin.

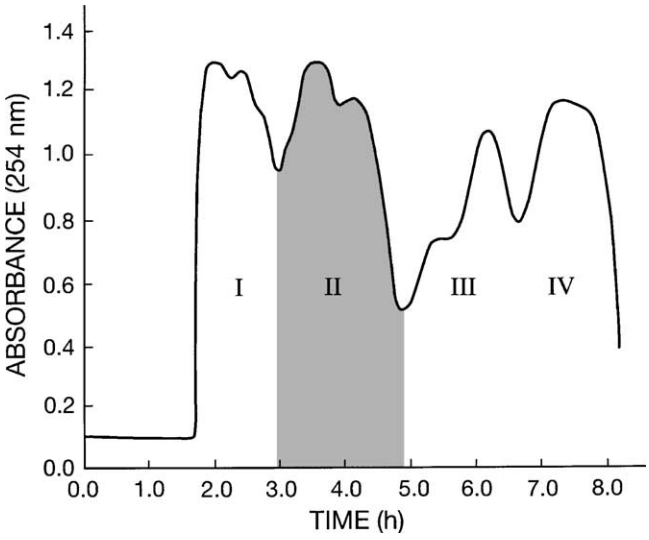


Fig. 4. HSCCC separation of the ethyl acetate extract of *Vernonia anthelmintica* Willd by Model CCC-1000. Experiment conditions: rotation speed: 1000 rpm; solvent system: chloroform–dichloromethane–methanol–water (2:2:3:2, v/v); mobile phase: lower organic phase; flow-rate: 5 ml/min; sample size: 4 g; retention of the stationary phase: 62%.

DMSO): 6.23 (1H, H-3), 6.35 (1H, H-5), 6.76 (1H, H-5), 7.06 (1H, H-6), 7.12 (1H, H-2), 7.47 (1H, H-a), 7.67 (1H, H-b), 7.89 (1H, H-6). 13C-NMR (500 MHz in DMSO): 128.9 (C-1), 117.1 (C-2), 147.3 (C-3), 150.4 (C-4), 116.3 (C-5), 124.1 (C-6), 115.2 (C-1′), 166.8 (C-2′), 104.3 (C-3′), 168.0 (C-4′), 109.6 (C-5′), 133.8 (C-6′), 118.7 (C-a), 146.6 (C-b), 193.9(C=O).

Peak B [5,6,7,4′-tetrahydroxyflavone] yellow needles, $C_{15}H_{10}O_6$. EI-MS *m*/*z* (%): 287 (19.24), 286 (100.00), 168 (38.20), 121 (9.68). 1H-NMR (500 MHz in D_2O): 6.57 (1H, H-3), 6.73 (1H, H-8), 6.91 (2H, H-3/H-5), 7.90 (2H, H-2/6). 13C-NMR (500 MHz in D_2O): 164.2 (C-2), 102.9 (C-3), 182.7 (C-4), 154.0 (C-5), 129.9 (C-6), 147.7 (C-7), 94.6 (C-8), 150.4 (C-9), 104.7 (C-10), 122.2 (C-1'), 129.1 (C-2′/C-6′), 116.6 (C-3′/C-5′), 161.7 (C-4′).

Peak C [butin] colorless needles, $C_{15}H_{12}O_5$. EI-MS *m*/*z* (%): 272 (73.86), 271 (34.58), 163 (25.42), 137 (100). 1H-NMR (500 MHz in CD_3OD): 2.62 (1H, H-3), 2.94 (1H, H-3), 5.25 (1H, H-2), 6.29 (1H, H-8), 6.42 (1H, H-6), 6.72 (2H, H-5/H-6), 6.67 (1H, H-2), 7.65 (1H, H-5). 13C-NMR (500 MHz in CD_3OD): 81.1 (C-2), 45.0 (C-3), 193.5 (C-4), 129.6 (C-5), 111.7 (C-6), 166.7 (C-7), 103.6 (C-8), 165.5 (C-9), 115.0 (C-10), 132.1 (C-1′), 114.7 (C-2′), 146.5 (C-3′), 146.8 (C-4′). 116.3 (C-5′), 119.2 (C-6′).

The results of our studies indicate that HSCCC is useful tool to purify the bioactive components from the crude seed extract of *Vernonia anthelmintica* Willd. The quantity of the sample can be greatly increased using a large multilayer coil separation column suggesting that the method is also ideal for preparation of the standard of effective components.

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