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Short communication

Purification of (+)-dihydromyricetin from leaves extract of *Ampelopsis grossedentata* using high-speed countercurrent chromatograph with scale-up triple columns

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Abstract

Purification of (+)-dihydromyricetin from an extract (16 g) of leaves of *Ampelopsis grossedentata* was performed using a preparative triple-column countercurrent chromatograph. With a solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:3:2:4, v/v) 11.3 g of (+)-dihydromyricetin was obtained at a high purity of over 99% by HPLC at 254 nm in 9 h.
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Keywords: Countercurrent chromatography; *Ampelopsis grossedentata*; Preparative chromatography; Dihydromyricetin

1. Introduction

The leaves of *Ampelopsis grossedentata* are used for beverages in South China. The main component in the leaves is (+)-dihydromyricetin (Fig. 2), also called (+)-ampelopsin, that occupies 15–20% (w/w) of dry tender leaves. (+)-Dihydromyricetin has minimum toxicity and can inhibit the contraction of rabbit aortic strips evoked by noradrenaline and high K^+ solution, which implies the effect of inhibiting hypertension [1]. It was also found to show an inhibitory activity on the ethanol-induced muscle relaxation in rats [2]. However, it is not easy to purify (+)-dihydromyricetin though it comprises about 70% of the direct methanol extract of the leaves [3].

This paper describes the preparative purification of (+)-dihydromyricetin from dried leaves of *Ampelopsis grossedentata* using a high-speed countercurrent chromatograph equipped with three scale-up columns [4,5].

2. Experimental

2.1. Apparatus

The present study employed a triplet coil planet centrifuge countercurrent chromatograph manufactured at the Institute of Food and Biological En-

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gineering, Hangzhou University of Commerce, Hangzhou, China. It holds a set of three multilayer coil separation columns each consisting of 42 m long×5 mm I.D. PTFE (polytetrafluoroethylene) tubing wound around a holder hub of 22 cm in length×7 cm in diameter. The three columns were connected in series to make a total capacity of 2460 ml.

The stationary phase was filled into the counter-current chromatography (CCC) column using nitrogen pressure through a special solvent container, and the mobile phase was pumped by a Waters 510 pump (Waters, Milford, MA, USA). A model 8823A-UV monitor (Beijing Institute of New Technology Application, Beijing, China) was used to continuously monitor the effluent at 254 nm. The chromatogram was recorded with an S-100 recorder (Shanghai Analysis Instrument Factory, Shanghai, China). The effluent was collected with a BS 100 fraction collector (Shanghai Puxi Instrument Factory, Shanghai, China).

2.2. Reagents

n-Hexane, methanol and ethyl acetate used for high-speed countercurrent chromatography (HSCCC) were analytical-grade solvents. Methanol used for HPLC analysis was HPLC grade reagent. All solvents were purchased from Shanghai Chemical Factory (Shanghai, China). The dried leaves of *Ampelopsis grossedentata* were purchased from the Tea Research Institute (Chinese Academy of Agricultural Sciences, Hangzhou, China).

2.2. Selection and preparation of two-phase solvent system

The two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water was tested by changing the volume ratio to obtain the optimum composition that gave suitable partition coefficient values for the target compound.

The preparative purification was performed with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:3:2:4, v/v) using a semipreparative HSCCC instrument [6] equipped with a 230-ml capacity column.

After thoroughly equilibrating the solvent mixture in a separatory funnel at room temperature, the two phases were separated shortly before use. The upper organic phase was used as the stationary phase, and the lower aqueous phase as the mobile phase.

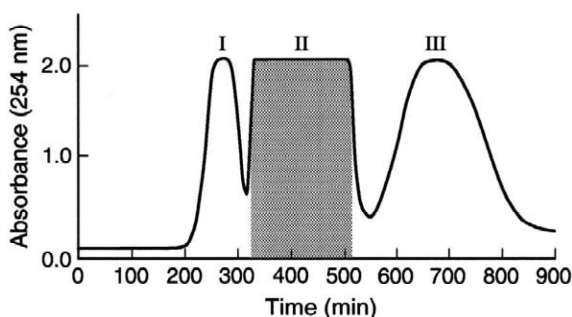

Fig. 1. HSCCC chromatogram of 16 g of the dried leaves extract of *Ampelopsis grossedentata*. Solvent system: *n*-hexane–ethyl acetate–methanol–water (1:3:2:4, v/v); stationary phase: upper phase; flow-rate of the mobile phase: 5.0 ml/min; revolution: 650 rpm; retention of stationary phase: 55%.

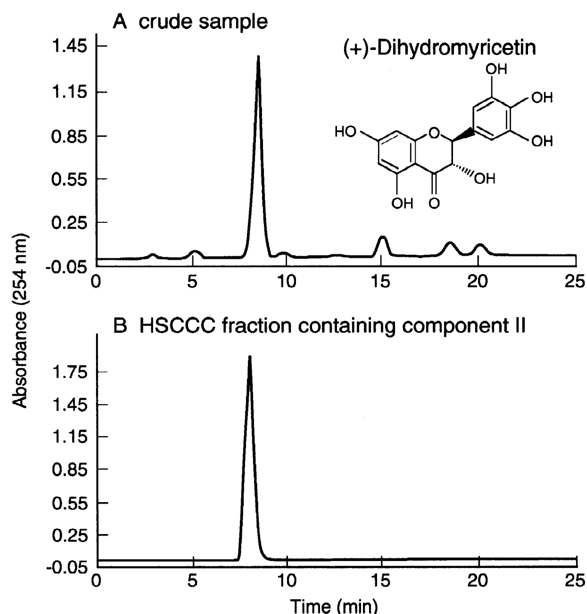

Fig. 2. HPLC analyses of the crude extract of *Ampelopsis grossedentata* and its HSCCC fraction. (A) Crude extract; (B) HSCCC fraction (component II) corresponding to peak II (shaded) in Fig. 1. Experimental conditions: ODS column: 150×4.6 mm, 5 μm, gradient elution: 30% methanol in water from 0 to 10 min and 30–70% methanol in water from 10 to 20 min.

2.3. Sample and preparation of sample solutions

Dried powder (500 g) of tender leaves of *Ampelopsis grossedentata* was extracted with 3 l of methanol three times. The extracts were combined and the solvent evaporated. The pigments were removed with chloroform after dispersing into 600 ml of water, and then extracted twice with 600 ml of ethyl acetate. The ethyl acetate was removed under vacuum and the residue lyophilized to yield 122 g of crude (+)-dihydromyricetin for purification by HSCCC. In each HSCCC separation the sample solution was prepared by dissolving 16 g of the crude (+)-dihydromyricetin in 250 ml of aqueous mobile phase of the solvent system used for separation.

2.4. Separation procedure

The multilayer coiled columns were first entirely filled with the upper organic stationary phase. Then the coils were rotated at a low speed (200 rpm). The sample solution was injected through the injection loop and the mobile phase was pumped into the column at 5 ml/min in the head-to-tail elution mode after the revolution speed was increased to 650 rpm. The effluent was monitored at 254 nm and collected using a fraction collector.

2.5. HPLC analysis

The HPLC system composed of two Waters HPLC 510 pumps, a manual injector, a Water 486 UV detector, an ODS column (150×4.6 mm, 5 μm, Beckmann, Germany), and a chromatogram processor (Elite, Dalian, China). The HPLC separation was performed with a gradient elution using 30% methanol in water from 0 to 10 min and 30–70% methanol in water from 10 to 20 min.

2.6. MS and NMR analyses

A MAT-711 mass spectrograph (electron impact at 70 eV) and a Brüker-AM300 NMR spectrometer were used for MS, ^{1}H-NMR and ^{13}C-NMR.

3. Results and discussion

3.1. HS-CCC separation

Fig. 1 shows the HSCCC separation of 16 g of the crude extract of (+)-dihydromyricetin. Three components, I, II and III, were obtained. Although HPLC analysis of the crude sample showed more than five peaks (Fig. 2A), component II (Fig. 1) showed a single peak (Fig. 2B). HPLC analyses also revealed that components I and III were found to be a mixture of two and three compounds, respectively. Except for myricetin present in component III, all other com-

Table 1
MS and NMR data of component II from HSCCC

MS (*m/z*)	Relative intensity (%)	δ values of ^{13}C-NMR in DMSO-d_6	
		At 125 MHz	At 300 MHz
320 (M^+)	48	197.4 (C-1)	4.42 (1H, C_3−H, dd, J=5.86, J=10.74 Hz)
302 (M^+-H_2O)	3	166.7 (C-4′)	4.90 (1H, C_2−H, d, J=10.74 Hz)
292 (M^+−CO)	7	163.2 (C-3′)	5.86 (1H, C_6–H, d, J=1.95 Hz)
291 (M^+−CHO)	65	162.4 (C-5′)	5.9 (1H, C_8−H, d, J=1.95 Hz)
166 ($M^+-C_7H_5O_4$−H)	33	145.6 (C-3)	6.40 (2H, s, H-2′, H-6′)
153 ($M^+-C_8H_6O_4$)	38	133.4 (C-5)	
		127.0 (C-7)	
		106.9 (C-9)	
		100.4 (C-4)	
		95.9 (C-8)	
		95.7 (C-6)	
		95.0 (C-6′)	
		94.8 (C-2′)	
		83.2 (C-2)	
		71.6 (C-1′)	

pounds were unknown. It suggested that component II could be the monomer of (+)-dihydromyricetin. Then, the fractions corresponding to component II (Fig. 1) were collected, the solvent evaporated, and the residue lyophilized to yield 11.3 g (over 99% pure by HPLC at 254 nm) of white powder which was subjected to MS and NMR analyses.

3.2. Structure confirmation

Table 1 lists the data from the MS, ^{13}C-NMR and ^{1}H-NMR in DMSO-d_6 at 300 MHz. The compound (slender white needles) showed a very high purity and possessed 15 carbons and six C-H [7]. All of the detailed information from the MS, ^{13}C-NMR and ^{1}H-NMR in DMSO-d_6 consisted with those of (+)-dihydromyricetin [8]. Therefore, component II (Fig. 1) was concluded to be (+)-dihydromyricetin.

The overall results of our studies indicate that HSCCC is a useful technique for preparative separation and purification of natural products such as (+)-dihydromyricetin.

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