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Short communication

Isolation of a novel flavanone 6-glucoside from the flowers of *Carthamus tinctorium* (Honghua) by high-speed counter-current chromatography

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Abstract

A novel flavanone glycoside, (2*S*)-4′,5,6,7-tetrahydroxyflavavone 6-*O*-β-D-glucopyranoside was isolated from the ethyl acetate extract of the flowers of *Carthamus tinctorium* by high-speed counter-current chromatography (HSCCC). Using an optimized two-phase solvent system composed of ethyl acetate–methanol–water (5:1:5, v/v), target compound (52 mg) with purity of 98.0% was obtained from 2.0 g of sample by HSCCC in seven times run. The structure of the target compound was elucidated by means of spectroscopic methods including IR, MS, 1D and 2D NMR techniques.

Keywords: *Carthamus tinctorium*; High-speed counter-current chromatography; (2*S*)-4′,5,6,7-Tetrahydroxyflavavone 6-*O*-β-D-glucopyranoside

1. Introduction

Carthamus tinctorium belongs to the family Compositae. In the Chinese Pharmacopoeia [1], the flowers of *C. tinctorium* are used as the traditional Chinese medicine "Honghua" for the treatment of cardiovascular diseases. Previous phytochemical studies on this plant revealed the presence of various compounds such as flavonoids [2,3], polyacetylenes [4], serotonin derivatives [5], steroids [6], lignans [7,8], and alkane diols [9,10].

High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatographic technique, eliminates the risk of irreversible adsorption of phenolic components that is often the case with the classical multiple chromatographic steps on silica gel.

This paper describes the isolation of a novel flavanone glycoside from an ethyl acetate extract of the flowers of *C. tinctorium* by HSCCC. The chemical structure is elucidated by means of several spectroscopic methods including IR, MS, 1D and 2D NMR techniques.

2. Experimental

2.1. Apparatus

Preparative HSCCC was performed using a Model GS10A2 multilayer coil of 110 m long, 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing with a total capacity of 230 ml. The β values of this preparative column range from 0.5 to 0.8. The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application) at 280 nm. A manual sample injection valve with a 20 ml loop (Tianjin High New Science technology Company,

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Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The high-performance liquid chromatography (HPLC) equipment used was a Waters Millennium^{32} system consisting of a Waters 2996 Photodiode Array Detector, a Waters 600E Multi-solvent Delivery System, a Waters 600 system controller, a Waters Delta 600 pump, and a Millennium^{32} workstation (Waters, Milford, MA, USA).

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Methanol used for HPLC analysis was of chromatographic grade. Flowers of *C. tinctorium* were purchased from the Anguo herbal drug market in Hebei Province, China. Plant identification was verified by Professor Pengfei Tu (School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing, China).

2.3. Preparation of sample solution

Dry flowers (15 kg) of *C. tinctorium* were extracted three times with 95% ethanol. Rotatory evaporation of the extract gave 360.0 g residue. The residue was suspended in water (8 l) and extracted successively with petroleum ether (bp, 60–90 °C)(24 l) and ethyl acetate (24 l) which yielded 120.5 g of petroleum ether extract and 100.0 g of ethyl acetate extract after rotatory evaporation under reduced pressure. The ethyl acetate extract was chromatographed on a silica column (650 mm × 100 mm, 2 kg of silica gel G 100, Qingdao Haiyang Chemica, Qingdao, China) by eluting stepwise with chloroform–methanol (15:1, 12:1, 10:1, 6:1, 2:1 and 1:1, v/v), each collected fraction was 500 ml. All collected fractions were analyzed by silica gel TLC and 10 fractions were obtained. Fraction 8 yielded 6.3 g, 2.0 g of which were subjected to HSCCC. Sample solution for HSCCC was prepared by dissolving the residue in the lower phase solvent at a concentration of 20 mg/ml.

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

The solvent systems utilized in the present study were prepared by mixing ethyl acetate–methanol–water (5:1:5, v/v), and thoroughly equilibrating in a separatory funnel at room temperature. The two phases were separated shortly before use.

2.5. HSCCC separation procedure

HSCCC was performed as follows. The multilayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the column at a flow-rate of 2.0 ml/min, while the apparatus was run at 800 rpm. After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet, 15 ml of sample solution (20 mg/ml) was injected using an injection valve in seven times run. The effluent from the tail end of the column was continuously monitored with a UV detector at 280 nm. Peak fractions were manually collected according to the chromatogram.

2.6. HPLC analysis and identification of (2S)-4′,5,6,7-tetrahydroxyflavavone 6-O-β-D-glucopyranoside

The sample obtained after the silica clean-up step and peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a 250 mm × 46 mm I.D. Alltech C_{18} 5 μm column at room temperature. The mobile phase composed of methanol–0.05% TFA solution (55:45, v/v) was eluted at a flow-rate of 1.0 ml/min, and the effluent was continuously monitored at 280 nm. The concentration of the target compound was estimated by the peak area percent at 280 nm.

The identification of the target compound (2*S*)-4′,5,6,7-tetrahydroxyflavavone 6-*O*-β-D-glucopyranoside was carried out by HRFAB-MS, IR, 1D and 2D NMR spectra.

3. Results and discussion

3.1. Measurement of partition coefficients

To achieve successful separation, the two-phase solvent system should satisfy the following requirements: (1) the settling time of the solvent system should be shorter than 30 s; (2) the partition coefficient (*K*) value of target compounds should be close to 1, and the separation factor α should be greater than 1.5. In the present study, *K* values of compounds were determined in the following two solvent systems: ethyl acetate–methanol(ethanol)–water and chloroform–methanol–water each at various volume ratios.

Table 1 shows that chloroform–methanol–water (8:6:4) and ethyl acetate–methanol–water (10:2:10), (10:3:10) could be used to separate the target compound. After trying all of the above solvent system, the ethyl acetate–methanol–water (10:2:10) was the best system.

Fig. 1 showed the separation of HSCCC using this solvent. The target compound (Fraction B) was eluted at the retention time of 190 min. Fig. 2 shows HPLC analyses of the sample from the silica clean-up step (A) and HSCCC peak fractions (B and C). Fraction 8 of the ethyl acetate extract of *C. tinctorium* from the silica clean-up step contained the target compound in a purity of 7.6% as estimated by the peak area percent. After HSCCC, peak fraction 1 was collected and evaporated to dryness, yielding 52.1 mg of the target compound with 98.0% purity.

Table 1
Experiment procedure: each solvent mixture was thoroughly equilibrated in a test tube and the two phases separated

Solvent systems	Peak l	Peak 2	Peak 3
Target compound			
Chloroform–methanol–water (8:6:4)	3.02	0.46	0.43
Chloroform–methanol–water (3:5:4)	3.72	0.57	0.50
Chloroform–methanol–water (3:3:4)	21.30	1.73	1.53
Ethyl acetate–ethanol–water (10:2:10)	3.51	31.56	33.24
Ethyl acetate–methanol–water (10:2:10)	2.70	26.52	23.37
Ethyl acetate–methanol–water (10:3:10)	2.13	21.73	31.26

The *K* values were determined as follows: 2 ml of each pre-equilibrated phase was delivered into a test tube to which about 2 mg of the sample was added. The test tube was shaken vigorously to equilibrate the sample with two phases. Then 1 ml of each phase was evaporated to dryness. The residues were diluted with methanol to 1 ml and analyzed by HPLC. The *K* value was defined as the peak areas in the upper phase divided by that in the lower phase.

3.2. Identification of (2S)-4′,5,6,7-tetrahydroxyflavavone 6-O-β-D-glucopyranoside

Compound **1** was obtained as white needles, mp 216–218 °C. Its IR spectrum (KBr) showed absorption bands for hydroxyl (3388 cm^{-1}), conjugated carbonyl (1658 cm^{-1}) groups and aromatic rings (1614 and 1514 cm^{-1}). HRFABMS showed an $[M+1]^+$ peak at *m/z* 451.1208 corresponding to the molecular formula $C_{21}H_{22}O_{11}$. The ^1H NMR spectrum of **1** in DMSO showed three sets of double doublets at δ 5.43 (1H, dd, J = 12.5, 3.0 Hz), 3.28 (1H, dd, J = 17.0, 12.5 Hz), 2.68 (1H, dd, J = 17.0, 3.0 Hz) typical of H-2, H-3_{ax}, H-3_{eq} of ring-C of a flavanone moiety. It also showed signals assigned to a 4-substituted phenyl moiety at δ 7.31 (2H, d, J = 8.5 Hz, H-2′,6′), 6.79 (2H, d, J = 8.5 Hz, H-3′,5′), an isolated aromatic proton at δ 5.96 (1H, s), and two exchangeable phenolic hydroxyl protons at δ 12.24 (1H, s, 5-OH), 9.60 (1H, s, 4′-OH), which revealed that **1** was a 4′,5,6,7 or a 4′,5,7,8-tetraoxygenated flavanone. The ^1H NMR data also showed signals attributed to an anomeric protons at δ 4.63 (1H, d, J = 7.5 Hz, 1″-H), together with five glycosyl protons in the range δ 3.9–3.1. The ^{13}C NMR and DEPT spectra of **1** displayed 21 carbon signals consisted of six carbons of glycosyl moiety. All of the above data revealed that **1** is a flavanone monoglycoside with a β sugar unit. The signals in the ^1H and ^{13}C NMR spectra were unambiguously assigned by HMQC and HMBC experiments. In the HMBC spectrum (see Fig. 3) long-range correlations from 5-OH (δ 12.24) to C-6 (δ 126.2) and H-1″ (δ 4.63) to C-6 (δ 126.2) unequivocally established that the aglycon was not 4′,5,7,8- but 4′,5,6,7-tetrahydroxyflavanone and a β-D-glucopyranosyl moiety was located at C-6 of the aglycon. The absolute configuration at C-2 was found to be *S* as it showed positive and negative Cotton effects at 334 and 290 nm, respectively, in the CD spectrum [11]. Accordingly, the structure of **1** was determined as (2*S*)-4′,5,6,7-tetrahydroxyflavavone 6-*O*-β-D-glucopyranoside.

The HRFAB-MS, IR, 1D NMR and CD spectra data was as follows:

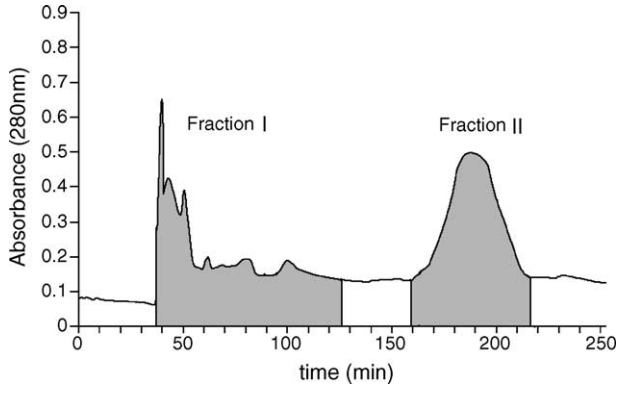

Fig. 1. HSCCC chromatogram of Fraction 8 from the silica clean-up step of the ethyl acetate extract of *Carthamus tinctorium*. Fraction I: Unknown components; Fraction II: (2*S*)-4′,5,6,7-tetrahydroxyflavavone 6-*O*-β-D-glucopyranoside; solvent system: ethyl acetate–methanol–water (10:2:10, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml min^{-1}; revolution speed: 800 rpm; sample: 300 mg dissolved in 20 ml lower phase; retention of the stationary phase: 43.5%.

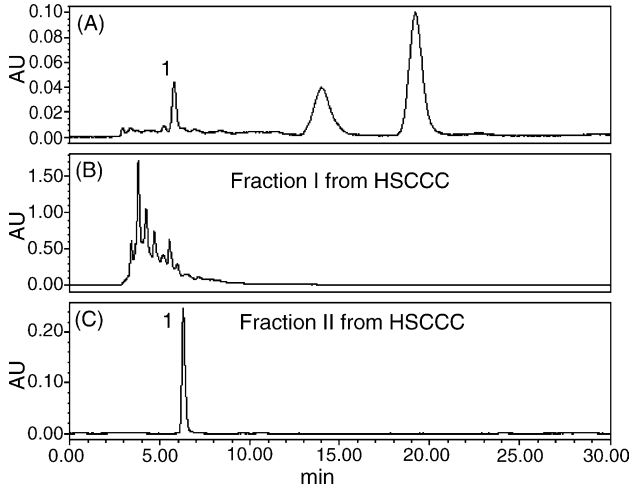

Fig. 2. (A): HPLC analysis of Fraction 8 from silica gel step with chloroform–methanol; (B) and (C): HPLC analysis of HSCCC Fraction I and II in Fig. 1. Peak 1 was the target compound; column: Alltech C_{18} (5 um, 250 mm × 4.6 mm I.D.); mobile phase: methanol–0.05% TFA solution (55:45, v/v); flow-rate: 1.0 ml min^{-1}.

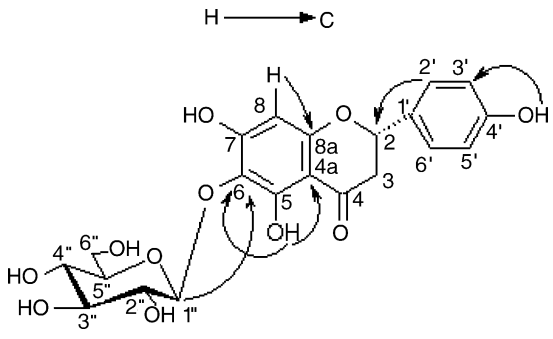

Fig. 3. Key HMBC correlations of (2*S*)-4′,5,6,7-tetrahydroxyflavavone 6-*O*-β-D-glucopyranoside.

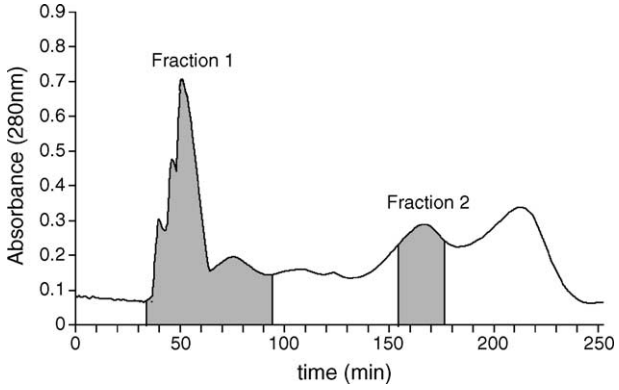

Fig. 4. HSCCC chromatogram of the fraction containing the target compound from silica gel step with ethyl acetate–methanol. Fraction 1 were unknown components, Fraction 2 contained the target compound: (2*S*)-4′,5,6,7-tetrahydroxyflavavone 6-*O*-β-D-glucopyranoside; solvent system: ethyl acetate–methanol–water (10:2:10, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml min^{-1}; revolution speed: 800 rpm; sample: 300 mg dissolved in 20 ml lower phase; retention of the stationary phase: 43.5%.

White needles (MeOH), mp 216–218 °C; CD; $\Delta\varepsilon_{214}$ + 10.97, $\Delta\varepsilon_{290}$ −11.94, $\Delta\varepsilon_{334}$ + 3.88 (MeOH, c 0.30); IRυ_{max}^{KBr} cm^{-1}: 3388 (hydroxyl), 1658 (conjugated carbonyl rings), 1614 and 1514 (aromatic groups). ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.24 (1H, s, 5-OH), 9.60 (1H, s, 4′-OH), 7.31 (2H, d, *J* = 8.5Hz, H-2′,6′ 6.79 (2H, d, *J* = 8.5 Hz, H-3′,5′), 5.96 (1H, s, H-8), 5.43 (1H, dd, *J* = 12.5, 3.0 Hz), 4.63 (1H, d, *J* = 7.5 Hz, 1″-H), 3.28 (1H, dd, *J* = 17.0, 12.5 Hz, H-3_{ax}), 2.68 (1H, dd, *J* = 17.0, 3.0 Hz, H-3_{eq}); ^{13}C NMR (500 MHz, DMSO-d_6): δ 197.1 (C-4), 159.4 (C-7), 158.6 (C-8a), 157.7 (C-4′), 155.0 (C-5), 128.8 (C-1′), 128.4 (C-2′,6′), 126.2 (C-6), 115.2 (C-3′,5′), 104.7 (C-1″), 101.8 (C-4a), 95.0 (C-8), 78.5 (C-2), 77.2 (C-5″), 76.1 (C-3″), 73.9 (C-2″), 69.5 (C-4″), 60.7 (C-6″), 42.0 (C-3); HRFABMS (*m*/*z*): 451.1208 $[M + 1]^+$ (calcd for $C_{21}H_{23}O_{11}$ 451.1240).

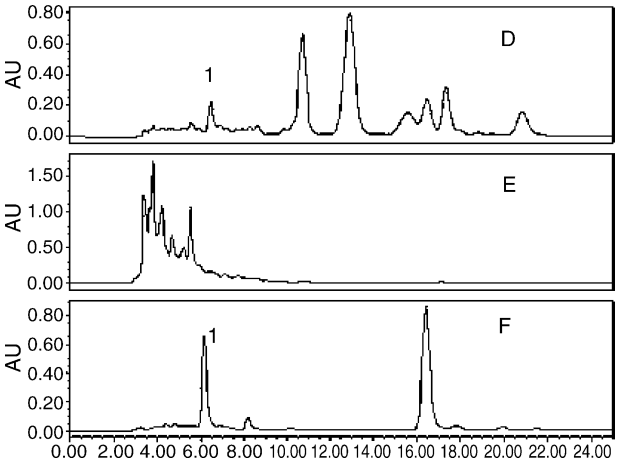

Fig. 5. (D): HPLC analysis of the fraction containing the target compound from silica gel step with ethyl acetate–methanol; (E) and (F): HPLC analysis of HSCCC Fraction 1 and 2 in Fig. 4. Peak 1 was the target compound; column: Alltech C_{18} (5 um, 250 mm × 4.6 mm I.D.); mobile phase: methanol–0.05% TFA solution (55:45, v/v); flow-rate: 1.0 ml min^{-1}.

3.3. Solvents in silica gel chromatography

Because $CHCl_3$ is a toxic and expensive solvent, we tried to replace it with other solvents for the silica column. We have tried several systems in silica step including petroleum ether–acetone, ethyl acetate–methanol, ethyl acetate–ethanol. Among these systems, ethyl acetate–methanol (10:1) is the best. However, the target compound cannot be separated with ethyl acetate–methanol (10:1) as effectively as chloroform–methanol system in the silica step, and an impurity compound mixed with the target compound in HSCCC step as showed in Fig. 4. So we found chloroform was the best solvent for the separation of the target compound (Fig. 5).

4. Conclusions

The result of our studies showed HSCCC is a valuable method not only in separating major components but also finding new minor components which is easily disappeared due to the irreversible adsorption in silica gel chromatography. Being a support-free liquid–liquid partition, HSCCC made a minor new compound prepared with high purity of 98.0% from the ethyl acetate extract of the flowers of *C. tinctorium*.

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