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Short communication

Purification of icariin from the extract of *Epimedium segittatum* using high-speed counter-current chromatography

Qizhen Du[a], Ming Xia[a], Yoichiro Ito[b,*]

[a]*Institute of Food and Biological Engineering, Hangzhou University of Commerce, Hangzhou 310035, China*

[b]*Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 50, Room 3334, 50 South Drive MSC 8014, Bethesda, MD 20892, USA*

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Abstract

Icariin was purified from the extract of *Epimedium segittatum* by high-speed counter-current chromatography with a two-phase solvent system composed of *n*-hexane–*n*-butanol–methanol–water (1:4:2:6, v/v). We used two multilayer coil planet centrifuges with different capacities. A 300 mg amount of the extract was separated using a semipreparative instrument equipped with a 230-ml capacity column to yield 103 mg of icariin at 86.2% purity. An 8 g amount of the extract was separated with a large preparative instrument equipped with a 2460-ml capacity column to produce 2.45 g of icariin at 85.7% purity. From these fractions over 98% pure icariin was obtained by recrystallization with water.

Keywords: *Epimedium segittatum*; Counter-current chromatography; Icariin

1. Introduction

Icariin is a flavonoid isolated from *Epimedii herba*. Previous studies indicated that icariin gave various pharmaceutical effects such as anti-fatigue, immunoregulation, improvement of liver function and relaxation on corpus cavernosum smooth muscle [1–4]. Conventional silica column chromatography has been used for purification of icariin yielding a low recovery due to irreversible adsorption of the compound onto the silica support [5–7].

The present paper describes the separation of icariin from an ethanol extract of *Epimedium segittatum* by high-speed counter-current chromatography (HSCCC).

2. Experimental

2.1. Apparatus

Two HSCCC instruments with different column capacities were used. One is a Model GS10A multilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a 230-ml capacity multilayer coil consisting of 110 m×1.6 mm I.D. PTFE tubing. The other is a triple-column planet centrifuge counter-current chromatograph (Hangzhou Tea Research Institute, Hangzhou, China) which holds a set of

*Corresponding author. Tel.: +1-301-496-1210; fax: +1-301-402-3404.

E-mail address: itoy@nhlbi.nih.gov (Y. Ito).

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three multilayer coils each prepared from a 42 m×5 mm I.D.×6 mm O.D. PTFE tubing by winding it onto a column holder hub of 22 cm×7 cm O.D. The total capacity measured 2460 ml.

The stationary phase was introduced into the CCC column by N_2 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) was used for continuous monitoring of the effluent at 254 nm and the chromatogram was recorded with a strip-chart recorder while the effluent was collected with a fraction collector: BS160 collector for small-scale separations and BS100 (Shanghai Puxi Instrument Factory, Shanghai, China) for large-scale separations.

2.2. Reagents

n-Hexane, *n*-butanol and ethyl acetate used for HSCCC separations were of an analytical grade. Methanol used for high-performance liquid chromatography (HPLC) analysis was an HPLC grade reagent. Icariin standard was a gift from Professor Liu Zhonghai of Hunan Agricultural University, Changsha, China.

2.3. Preparation of two-phase solvent system

The HSCCC experiments were performed with a two-phase solvent system composed of *n*-hexane–*n*-butanol–methanol–water (1:4:2:6, v/v). After thoroughly equilibrating the solvent mixture in a separatory funnel at room temperature, two phases were separated shortly before use. The upper organic phase was used as stationary phase, and the lower aqueous phase as mobile phase.

2.4. Preparation of sample solutions

A 1.0 kg amount of dried power of *E. segittatum* (areal parts) was extracted with 5 l of 95% ethanol three times. The extracts were combined and evaporated into extractum in vacuum. Then, hydrophobic components of the extractum were removed by successive extraction with *n*-hexane, dichloromethane and ethyl acetate. Finally, the residue was extracted with *n*-butanol three times. The extracts were evaporated in vacuum and freeze–dried to yield 62 g of crude sample for HSCCC separation. The HSCCC sample solvents were prepared by dissolving 5% of the crude sample in the mobile phase of the solvent system.

2.5. Separation procedure

The multilayer coiled column was first entirely filled with the upper organic phase. After the apparatus was rotated, the sample solution was introduced into the separation column through an injection loop. Then, the mobile phase was pumped into the column at a desired flow-rate. The effluent from the outlet of the column was monitored by absorbance at 254 nm and collected into test tubes with a fraction collector for later analysis.

2.6. HPLC analysis

The HPLC system was consisted of two Waters HPLC 510 pumps, a manual injector, a Water 486 UV detector, an ODS column (5 μm, 150 mm×4.6 mm I.D., Beckmann, Germany), and a chromatogram processor (Elite, Dalian, China). The analysis was performed with linear gradient elution, 30 to 80% of methanol in water from 0 to 25 min.

3. Results and discussion

Fig. 1 shows the HSCCC separation of 300 mg of the extract of *E. segittatum* obtained from the small-capacity CCC instrument. The fractions corresponding to three peaks were collected and lyophilized to yield 45 mg of component 1, 103 mg of component 2 and 65 mg of component 3.

The original sample and fractions corresponding to each peak were analyzed by HPLC, the results of which are shown in Fig. 2. The upper chromatogram (Fig. 2A) was obtained from the original extract of *E. segittatum* indicating that the target compound of icariin occupies 28.7% of the dry sample. The lower chromatogram (Fig. 2B) was obtained from the HSCCC fraction corresponding to peak 2 (Fig. 1, shaded) which showed icariin at 86.2% purity. HPLC analysis of fractions corresponding to peaks 1

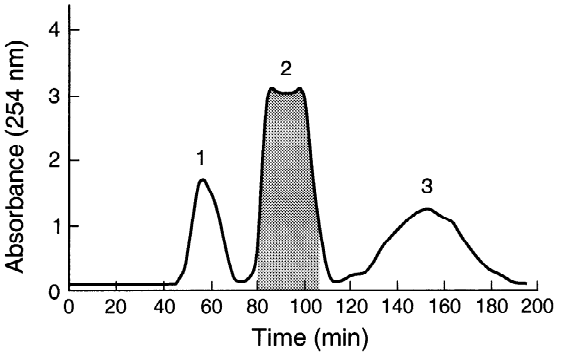

Fig. 1. HSCCC separation of 300 mg of the *E. segittatum* crude extract obtained from the small CCC instrument. Solvent system: *n*-hexane–*n*-butanol–methanol–water (1:4:2:6, v/v); stationary phase: upper phase; flow-rate: 1.5 ml/min; revolution speed: 750 rpm; retention of the stationary phase: 51%.

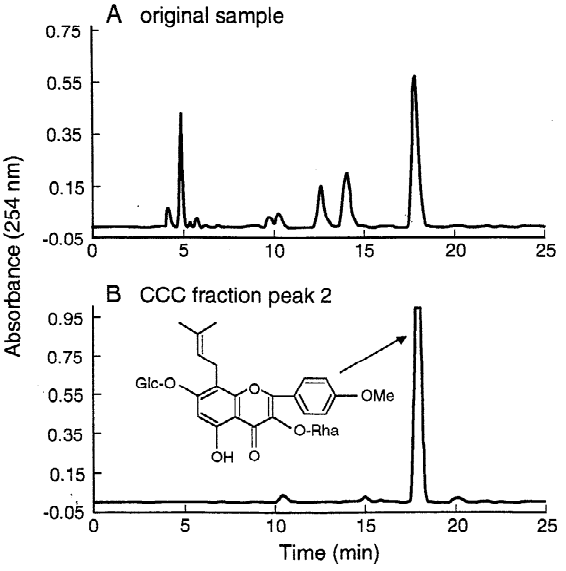

Fig. 2. HPLC analysis of the extract of *E. segittatum*. (A) Original sample and (B) HSCCC fractions corresponding to peak 2 in Fig. 1. ODS column (5 μm, 150 mm×4.6 mm); linear gradient elution: 30 to 80% of methanol in water from 0 to 25 min.

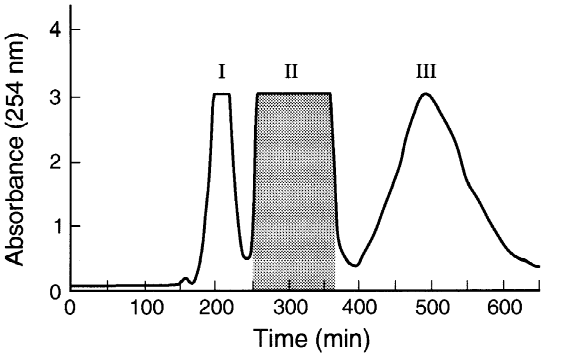

Fig. 3. HSCCC separation of 8 g of the *E. segittatum* crude extract obtained from the triple-column CCC instrument. Solvent system: *n*-hexane–*n*-butanol–methanol–water (1:4:2:6, v/v); stationary phase: upper phase; flow-rate: 5.0 ml/min; revolution speed: 650 rpm; retention of stationary phase: 46%.

and 3 both revealed a mixture of unknown compounds.

A large amount of the extract (8 g) was similarly separated with our triple column CCC instrument. Fig. 3 shows the HSCCC separation which is quite similar to that obtained by the small HSCCC instrument (Fig. 1). The fractions corresponding to peak II (shaded) was collected and lyophilized to produce 2.45 g of icariin with a purity of 85.7%.

Icariin with a purity of over 98% was obtained after recrystallization of these fractions with water.

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