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Preparative isolation and purification of three flavonoids from the Chinese medicinal plant *Epimedium koreamum* Nakai by high-speed counter-current chromatography

Renmin Liu[a,b], Aifeng Li[b], Ailing Sun[b], Jichun Cui[b], Lingyi Kong[a,*]

[a] *Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing 210009, China*
[b] *College of Chemistry and Chemical Engineering, Liaocheng University, Liaocheng 252059, China*

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Abstract

A preparative high-speed counter-current chromatography (HSCCC) method for isolation and purification of flavonoids from the Chinese medicinal plant *Epimedium koreamum* Nakai was successfully established by using chloroform–methanol–water (4:3.5:2, v/v) as the two-phase solvent system. The method yielded 11.4 mg of epimedokoreanoside I, 46.5 mg of icariin and 17.7 mg of icariside II from 200 mg of the crude sample in one-step separation with the purity of 98.2%, 99.7% and 98.5%, respectively, as determined by high-performance liquid chromatography (HPLC). The structures of the flavonoids were identified by ^{1}H NMR and ^{13}C NMR.

Keywords: Counter-current chromatography; *Epimedium koreamum* Nakai; Epimedokoreanoside I; Icariin; Icariside II

1. Introduction

Epimedium koreamum Nakai, Chaoxian Yinyanghuo in Chinese, is one of the herbs that were classified as invigorator in traditional Chinese herbal medicine. As one of the most centuries-old Chinese herbs, it is officially listed in the Chinese Pharmacopoeia [1]. It has been used to treat various kinds of disorders such as hypertension, coronary heart disease, osteoporosis, menopause syndrome, breast lump, rheumatism, arthritis, neurasthenic, bronchitis, and hypogonadism [2]. Pharmacological studies and clinical practice demonstrated that its extract has anticancer, anti-AIDS, antibacterial, antiphlogistic, antitussive, and expectorant effects [3]. The active constituents of the herb are flavonoids (chemical structures shown in Fig. 1), mainly icariin which significantly dilates coronary arteries, decreases myocardial oxygen consumption, improves microcirculation, and enhances immune function [4].

The separation and purification of icariin from the extract of *Epimedium segittatum* using HSCCC was recently reported [5], which only obtain icarrin and the purity of icariin was only 86.2% before recrystallization. In present paper, a more efficient HSCCC method for preparative separation and purification of flavonoids from the different medicinal plant *E. koreamum* Nakai was developed. Three kinds of flavonoids including epimedokoreanoside I, icariin and icariside II were obtained in one-step separation with the purity of 98.2%, 99.7% and 98.5%, respectively.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study is TBE-300A high-speed counter-current chromatography

* Corresponding author. Tel.: +86 25 85391289.
E-mail address: lykong@jlonline.com (L. Kong).

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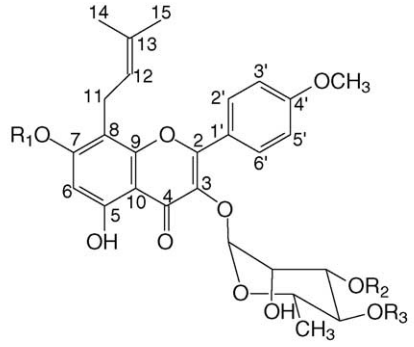

	R_1	R_2	R_3
Epimedokoreanoside I:	Glc	β-(6-Ac)Glc	Ac
Icariin:	Glc	H	H
Icariside II:	H	H	H

Fig. 1. Chemical structures of flavonoids from *Epimedium koreamum* Nakai.

(Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop.

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 can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature. An ÄKTA prime (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which can be used for gradient formation. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Company, Hangzhou, China).

The HPLC equipment used was Agilentl 100 system including a G1311A QuatPump, a G1315B UV–vis photodiode array detector, a 7725i injection valve with a 20 μl loop, a G1332A degasser and Agilent HPLC workstation.

Nuclear magnetic resonance (NMR) spectrometer used here was Mercury Plus 400 NMR (Varian Inc., USA).

AFZ102 plant disintegrator (Taisite Instrument Company, Tianjin, China) was used for disintegration of *E. koreamum* Nakai sample.

2.2. Reagents and materials

All organic solvents used for preparation of crude extract and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). D-101 macroporous resin (Chemical Plant of Nankai University, Tianjin) was used for sample preparation. Acetonitrile used for HPLC was of chromatographic grade (Yucheng Chemical Factory, Yucheng, China), and water was distilled water. [$^2H_6$]Dimethyl sulfoxide (DMSO-d_6) was used as the solvent for NMR determination.

E. koreamum Nakai was purchased from a local drug store and identified by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.3. Preparation of crude extract

E. koreamum Nakai was ground to powder (about 30 mesh) by a disintegrator. The powder (1000 g) was extracted with 10 l of 70% ethanol for 2 h under reflux. The extraction procedure was repeated twice. The extracts were combined together. After filtrated with cotton, the filtrate was concentrated to remove ethanol in vacuum to give water fluid. The water fluid was subjected to a glass column (5 cm × 80 cm) of macroporous resin (D-101, 400 g) and washed with water, 70% ethanol, respectively. The eluant of 70% ethanol (5 l) was concentrated in vacuum using a rotary evaporator to give dried powder (11.5 g). The dried powder was stored in a refrigerator (−4 °C) for the subsequent HSCCC separation.

2.4. Selection of two-phase solvent system

The two-phase solvent system was selected according to the partition coefficient (K) of each target component. The K-values were determined by HPLC as follows: suitable amount of crude extract was dissolved in the lower phase of the two-phase solvent system. The solution was then determined by HPLC. The peak area was recorded as A_1. Then equal volume of the upper phase was added to the solution and mixed thoroughly. After partition equilibration was reached, the lower phase solution was determined by HPLC again, and the peak area was recorded as A_2. The K-values were calculated according to the following equation: $K = (A_1 - A_2)/A_2$.

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

In the present study, the two-phase solvent system composed of chloroform–methanol–water at volume ratio of 4:3.5:2 was used for HSCCC separation. Each solvent was added to a separatory funnel and thoroughly equilibrated at room temperature. The upper phase and the lower phase were separated and degassed by sonication for 30 min shortly before use.

The sample solution for HSCCC separation was prepared by dissolving 200 mg of the dried powder of crude extracts in the 5 ml of the upper phase of the two-phase solvent system.

2.6. HSCCC separation procedure

The upper phase and the lower phase of chloroform–methanol–water (4:3.5:2, v/v) were pumped into the

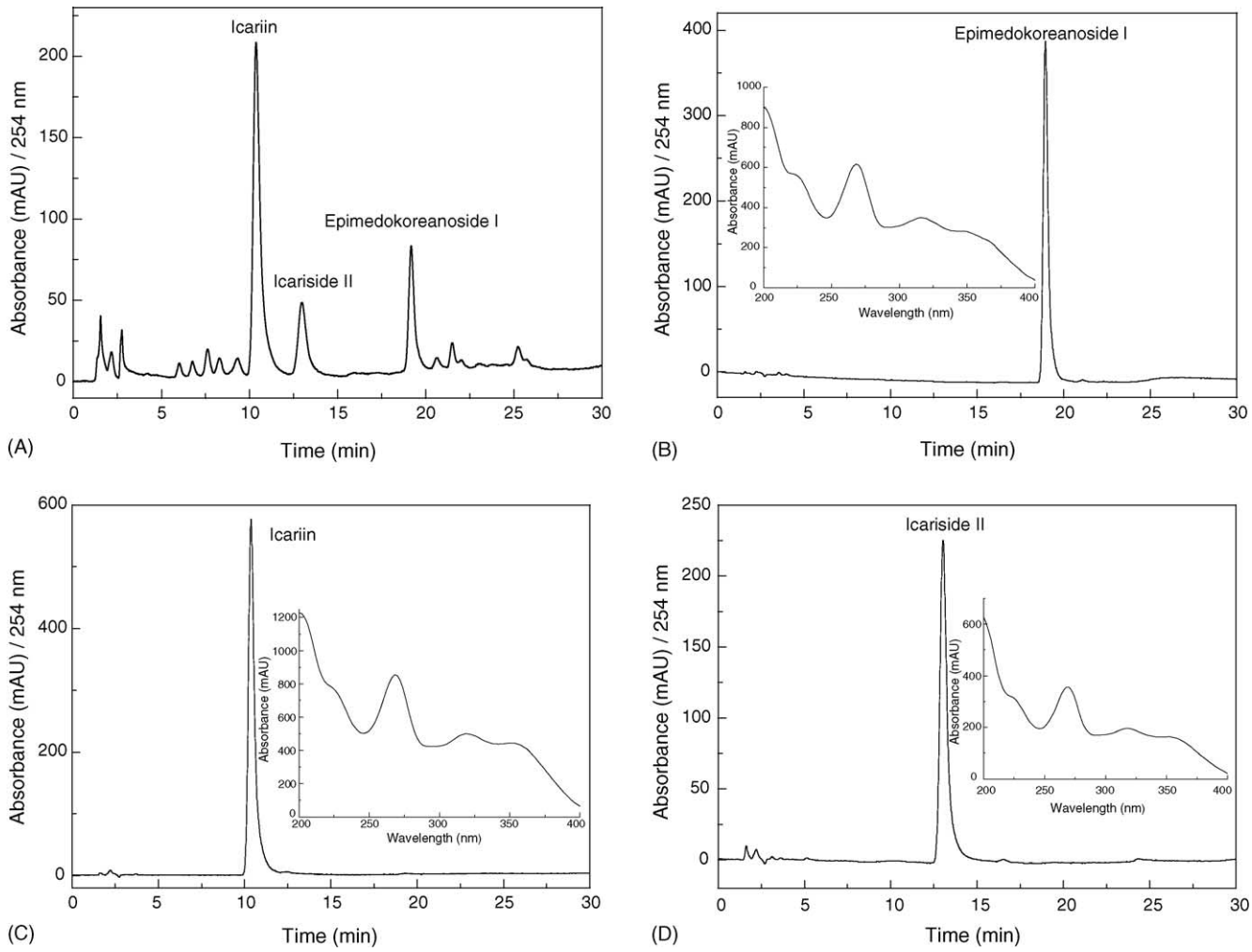

Fig. 2. HPLC chromatogram of crude extract from *Epimedium koreamum* Nakai and HSCCC peak fractions. Conditions: column: reversed phase YWG C_{18} column (200 mm × 4.6 mm i.d., 10 μm); mobile phase: acetonitrile and water in gradient mode (acetonitrile: 0–12 min, 28%; 12–20 min, 28–35%; 20–30 min, 35%); flow-rate: 1.0 ml min^{-1}; detection wavelength: 254 nm. (A) Crude extract from *Epimedium koreamum* Nakai; (B–D) HSCCC peak fractions I–III in Fig. 3.

multilayer-coiled column simultaneously by using ÄKTA prime system, according to the volume ratio of 50:50. After the column was totally filled with the two phases, only the lower phase was pumped at a flow-rate of 2.0 ml min^{-1}; and at the same time, the HSCCC apparatus was run at a revolution speed of 900 rpm. After hydrodynamic equilibrium was reached (about half an hour), 5 ml of the sample solution containing 200 mg of the crude extract was introduced into the column through the injection valve. All through the experiment the separation temperature was controlled at 25 °C. The effluent from the tail end of the column was continuously monitored with ÄKTA prime system at 254 nm and the chromatogram was recorded immediately after the sample injection. Each peak fraction was collected manually according to the obtained chromatogram and each collection was evaporated under reduced pressure and dissolved by methanol for subsequent purity analysis by HPLC.

2.7. HPLC analysis and identification of HSCCC peak fractions

The HPLC analysis was performed with a reversed-phase YWG C_{18} column (200 mm × 4.6 mm i.d., 10 μm) at room temperature. The mobile phase was acetonitrile–water in gradient mode as follows: 0–12 min, 28% acetonitrile; 12–20 min, 28% to 35% acetonitrile; 20–30 min, 35% acetonitrile. The effluent was monitored at 254 nm and the flow-rate was at 1.0 ml min^{-1} constantly.

The structure identification of each HSCCC peak fraction was carried out by ^{1}H NMR and ^{13}C NMR. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Mercury Plus 400 NMR.

3. Results and discussion

The crude extracts of *E. koreamum* Nakai were analyzed by HPLC and the chromatogram was given in Fig. 2A.

Table 1
The *K*-values of the target components in different solvent systems

Solvent systems	*K*		
	Epimedokoreanoside I	Icariin	Icariside II
Ethyl acetate–water (5:5, v/v)	1.97	1.49	0.22
Ethyl acetate–methanol–water (5:1:5, v/v)	1.84	1.17	0.31
Ethyl acetate–methanol–water (5:2:5, v/v)	1.35	0.94	0.46
Ethyl acetate–methanol–water (5:3:5, v/v)	1.18	0.68	1.00
Chloroform–methanol–water (4:2.5:2, v/v)	2.62	4.70	8.71
Chloroform–methanol–water (4:3:2, v/v)	1.70	2.70	4.95
Chloroform–methanol–water (4:3.5:2, v/v)	1.47	2.22	3.85
Chloroform–methanol–water (4:4:2, v/v)	1.23	1.52	2.33

The selection of the two-phase solvent system is the most important step in performing HSCCC. The two-phase solvent system was selected according to the *K*-values of each target component. In order to achieve efficient resolution of target compounds, the *K*-values of the target component in different solvent systems were determined by HPLC and the *K*-values were summarized in Table 1. The two-phase solvent systems listed in Table 1 were also tested in HSCCC separation. The purity of the compounds obtained by HSCCC was determined by HPLC. The results indicated that when ethyl acetate-water (5:5, v/v) was used as the two-phase solvent system, the purity of icariside II was very low, only 68.2%. When ethyl acetate–methanol–water (5:1:5, v/v) and ethyl acetate–methanol–water (5:3:5, v/v) were used for HSCCC separation, only icariin with the purity of 98% could be obtained. The purity of icariside II and epimedokoreanoside I was lower than 80%. When ethyl acetate–methanol–water (5:2:5, v/v) was used as the two-phase solvent system, no pure compound could be obtained. When chloroform–methanol–water (4:2.5:2, v/v) was used as the two-phase solvent system, a very long time was required for separation. When chloroform–methanol–water (4:3:2, v/v) was used, the three compounds can be well separated, but the separation time for icariside II was long and the peak broadened seriously. When chloroform–methanol–water (4:3.5:2, v/v) was used, three peaks were well separated and the separation time was also acceptable. When chloroform–methanol–water (4:4:2, v/v) was used, the three compounds could not be separated well and the purity of them became poor. So, chloroform–methanol–water (4:3.5:2, v/v) was used as the two-phase solvent system of HSCCC.

The influence of revolution speed, flow-rate of the mobile phase, and temperature on HSCCC peak resolution were also investigated. The results indicated that when the flow-rate was 2.0 ml min^{-1}, revolution speed was 900 rpm, and separation temperature was 25 °C, retention percentage of the stationary phase was 50%, good separation results can be obtained. Under the optimum conditions, three major components were obtained and yielded 11.4 mg of epimedokoreanoside I (peak I, collected during 168–188 min), 46.5 mg of icariin (peak II, collected during 221–271 min), and 17.7 mg of icariside II (peak III, collected during 342–384 min) from 200 mg of crude extracts. The HSCCC chromatogram of crude extracts from *E. koreamum* Nakai was given in Fig. 3.

Each HSCCC peak fraction was analyzed by HPLC. The purity of peak I, peak II and peak III fraction in Fig. 3 was 98.2%, 99.7% and 98.5%, respectively. HPLC chromatograms and UV spectra of the HSCCC peak fractions were shown in Fig. 2B–D.

Identification of each HSCCC fraction was carried out by ^{1}H NMR and ^{13}C NMR.

Peak I: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.63 (1H, s, H-6), 3.52 (2H, m, H-11, overlapped), 5.14 (1H, t, J = 8.0 Hz, H-12), 1.59 (3H, s, H-14), 1.67 (3H, s, H-15), 12.48 (1H, s, 5-OH), 7.88 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.14 (2H, d, J = 8.8 Hz, H-3′, 5′), 3.87 (3H, s, 4′-OCH_3), 5.35 (1H, br s, H-$1_{3\text{-O-Rha}}$), 0.70 (3H, d, J = 6.4 Hz, H-6_{Rha}), 1.94 (3H, s, 4_{Rham}-OAcMe), 4.16 (1H, d, J = 7.2 Hz, H-$1_{term,Glc}$), 1.97 (3H, s, $6_{term,Glc}$-OAcMe), 4.98 (1H, d, J = 7.6 Hz, H-$1_{7\text{-O-Glc}}$), 3.00–5.40 (sugar protons). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 157.5 (C-2), 133.9 (C-3), 178.2 (C-4), 160.7 (C-5), 98.4 (C-6), 161.8 (C-7), 108.6 (C-8), 153.2 (C-9), 105.7 (C-10), 122.2

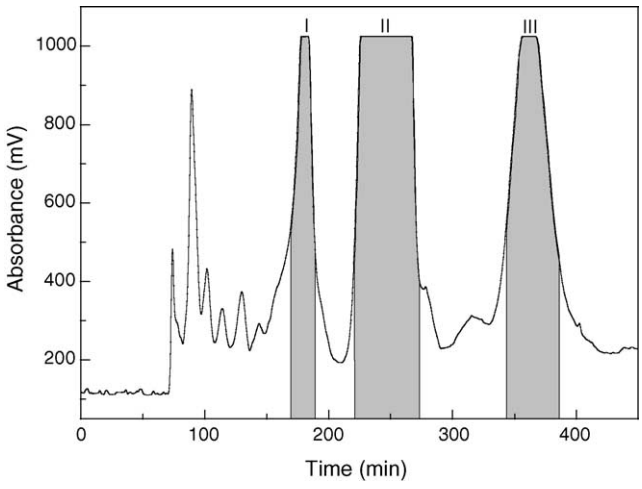

Fig. 3. HSCCC chromatogram of crude extract from *Epimedium koreamum* Nakai. Two-phase solvent system: chloroform–methanol–water (4:3.5:2, v/v); mobile phase: the lower phase; flow-rate: 2.0 ml min^{-1}; revolution speed: 900 rpm; detection wavelength: 254 nm; sample size: 200 mg of crude sample dissolved in 4 ml of the upper phase; separation temperature: 25 °C; retention percentage of the stationary phase: 50%.

(C-1′), 130.7 (C-2′), 114.3 (C-3′), 159.2 (C-4′), 114.3 (C-5′), 130.7 (C-6′), 21.8 (C-11), 122.2 (C-12), 131.4 (C-13), 25.6 (C-14), 18.0 (C-15), 55.7 (4′-OMe), 101.1 (C-1″$_{3\text{-O-Rha}}$), 70.0 (C-2″), 77.3 (C-3″), 71.2 (C-4″), 68.5 (C-5″), 17.1 (C-6″), 170.6 (4″-acetyl), 20.8 (CH_3CO), 105.7 (C-1‴$_{term,Glc}$), 73.5 (C-2‴), 76.7 (C-3‴), 70.4 (C-4‴), 73.8 (C-5‴), 64.0 (C-6‴), 169.9 (6‴-acetyl), 20.7 (CH_3CO), 100.7 (C-1⁗$_{7\text{-O-Glc}}$), 73.8 (C-2⁗), 76.7 (C-3⁗), 69.8 (C-4⁗), 76.9 (C-5⁗), 60.7 (C-6⁗). Comparing the data with the literature [6], peak I was identified as epimedokoreanoside I.

Peak II: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.60 (1H, s, H-6), 3.56 (2H, m, H-11, overlapped), 5.12 (1H, t, J = 7.2 Hz, H-12), 1.57 (3H, s, H-14), 1.65 (3H, s, H-15), 12.52 (1H, s, 5-OH), 7.86 (2H, d, J = 9.2 Hz, H-2′, 6′), 7.10 (2H, d, J = 9.2 Hz, H-3′, 5′), 3.82 (3H, s, 4′-OCH_3), 5.25 (1H, d, J = 1.2, H-1$_{3\text{-O-Rha}}$), 0.76 (3H, d, J = 6.0 Hz, H-6$_{Rha}$), 4.98 (1H, d, J = 7.6 Hz, H-l$_{7\text{-O-Glc}}$), 3.00–5.44 (sugar protons), ^{13}C NMR (DMSO-d_6, 100 MHz): δ 157.8 (C-2), 134.9 (C-3), 178.6 (C-4), 160.8 (C-5), 98.5 (C-6), 161.8 (C-7), 108.8 (C-8), 153.4 (C-9), 106.0 (C-10), 122.5 (C-1′), 130.9 (C-2′), 114.4 (C-3′), 159.4 (C-4′), 114.4 (C-5′), 130.9 (C-6′), 21.7 (C-11), 122.3 (C-12), 131.7 (C-13), 25.8 (C-14), 17.7 (C-15), 55.8 (4′-OMe), 102.2 (C-1″$_{3\text{-O-Rha}}$), 70.6 (C-2″), 71.0 (C-3″), 70.0 (C-4″), 70.4 (C-5″), 18.2 (C-6″), 100.8 (C-1‴$_{7\text{-O-Glc}}$), 73.7 (C-2‴), 76.8 (C-3‴), 71.4 (C-4‴), 77.4 (C-5‴), 61.0 (C-6‴). Peak II was identified as icariin according to the literature [7].

Peak III: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.63 (1H, s, H-6), 3.50 (2H, m, H-11, overlapped), 5.15 (1H, t, J = 5.8 Hz, H-12), 1.59 (3H, s, H-14), 1.68 (3H, s, H-15), 12.50 (1H, s, 5-OH), 7.91 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.17 (2H, d, J = 8.8 Hz, H-3′, 5′), 3.82 (3H, s, 4′-OCH_3), 5.40 (1H, br s, H-1$_{3\text{-O-Rha}}$), 0.72 (3H, d, J = 6.0 Hz, H-6$_{Rha}$), 3.00∼5.40 (sugar protons). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 157.5 (C-2), 133.7 (C-3), 178.3(C-4), 160.6 (C-5), 98.2 (C-6), 161.7 (C-7), 108.5 (C-8), 153.1 (C-9), 105.6 (C-10), 122.1 (C-1′), 130.8 (C-2′), 114.3 (C-3′), 159.2 (C-4′), 114.3 (C-5′), 130.8 (C-6′), 20.8 (C-11), 122.1 (C-12), 131.3 (C-13), 25.5 (C-14), 17.9 (C-15), 55.6 (4′-OMe), 101.0 (C-1″$_{3\text{-O-Rha}}$), 69.8 (C-2″), 70.0 (C-3″), 71.0 (C-4″), 70.4 (C-5″), 17.1 (C-6″). Comparing the data with the literature [7]. Peak III was identified as icariside II.

4. Conclusion

Three main bioactive flavonoids (epimedokoreanoside I, icariin and icariside II) of the traditional Chinese medicinal herb, *E. koreamum* Nakai, were successfully purified with chloroform–methanol–water (4:3.5:2, v/v) as the two-phase solvent system of HSCCC. From 200 mg of crude extract, 11.4 mg of epimedokoreanoside I, 46.5 mg of icariin and 17.7 mg of icariside II were obtained with the purity of 98.2%, 99.7% and 98.5%, respectively, after one-step separation.

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