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Preparative isolation and purification of coumarins from *Cnidium monnieri* (L.) *Cusson* by high-speed counter-current chromatography

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Abstract

A high-speed counter-current chromatography (HSCCC) method for separation and purification of coumarins from *Cnidium monnieri* (L.) *Cusson* was developed by using stepwise elution with a pair of two-phase solvent system composed of light petroleum–ethyl acetate–methanol–water at volume ratios of 5:5:5:5, 5:5:6:4 and 5:5:6.5:3.5. Five kinds of coumarins were obtained and yielded 7.6 mg of xanthotoxin(I), 7.6 mg of isopimpinellin(II), 9.7 mg of bergapten(III), 60.5 mg of imperatorin(IV), 50.6 mg of osthole(V) and 10.2 mg of one unknown compound from 150 mg crude sample. The purity of these compounds was 95.0%, 99.6%, 99.7%, 100%, 100% and 98.1%, respectively, as determined by HPLC. Their structures were identified by ^{1}H NMR.

Keywords: Counter-current chromatography; Preparative chromatography; *Cnidium monnieri* (L.) *Cusson*; Osthole; Imperatorin; Bergapten; Xanthotoxin; Isopimpinellin

1. Introduction

The fruit of *Cnidium monnieri* (L.) *Cusson*, known as Shechuangzi in Chinese, has many beneficial effects in anti-epiphyte, anti-virus, anti-osteoporosis, anti-tumour and anti-AIDS. It is also an important traditional medicinal, perennial herb in the treatment of skin disease and gynecopathy [1]. The active constituents of this herb are coumarins [2]. The chemical structures of these compounds are given in Fig. 1. Osthole and imperatorin are the major compounds present in Shechuangzi. They are often used as standards in the quality control of Shechuangzi products [3].

High-speed counter-current chromatography (HSCCC) is a support-free liquid–liquid partition chromatographic technique, so the irreversible adsorbing effects on stationary-phase material and artifact formation can be eliminated [4]. It has been widely used for the preparative separation of natural products such as traditional Chinese medicinal herbs [5–9]. In this paper, separation of crude sample from *C. Cusson* using HSCCC is described. The upper phase of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) was used as the stationary phase. The lower phase of light petroleum–ethyl acetate–methanol–water at different volume ratios was used as the mobile phase in stepwise elution. Five kinds of major coumarins including xanthotoxin(I), isopimpinellin(II), bergapten(III), imperatorin(IV), osthole(V) and one unknown compound were obtained in a one-step separation.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China) with three

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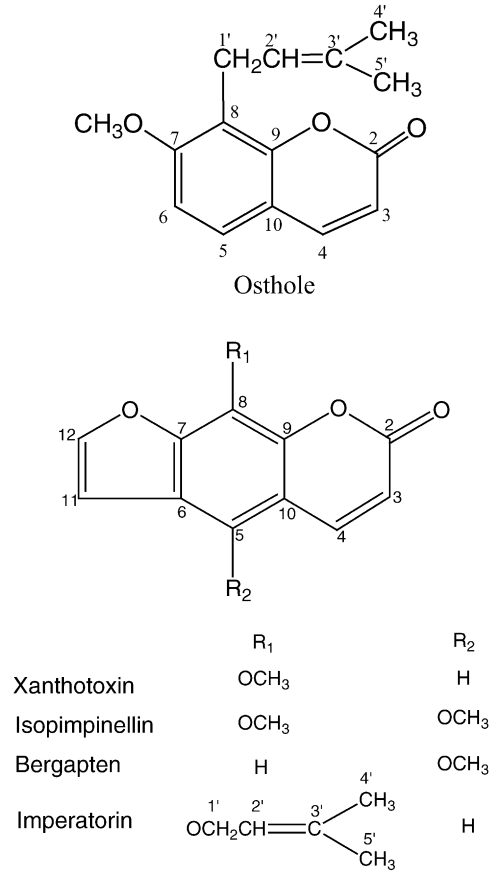

Fig. 1. The chemical structures of coumarins in *Cnidium monnieri* (L.) *Cusson*.

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 rpm and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Co. Ltd., 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 Co. Ltd., Hangzhou, China).

The HPLC equipment used was Agilent 1100 HPLC system including a G1311A solvent delivery unit, G1315B UV–vis photodiode array detector, Rheodyne 7725i injection valve with a 20 μl loop, G1332A degasser and Agilent HPLC workstation.

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

2.2. Reagents and materials

All organic solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was chromatographic grade (Yucheng Chemical plant, Yucheng, China), and water used was distilled water.

The dried seeds of *C. Cusson* were purchased from a local drug store and identified by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.3. Preparation of sample

One kilogram of dried powder of *C. Cusson* was marinated with 3000 ml of 95% ethanol for 24 h for three times. The extracts were combined and evaporated to 300 ml by rotary vaporization at 40 °C under reduced pressure. The enriched extract was extracted with light petroleum four times (200 ml each time). Then the light petroleum solutions were combined and evaporated to 50 ml and frozen under −4 °C for 24 h. The deposit was separated and dried. 10.06 g of crude sample was obtained. It was stored in a refrigerator for subsequent HSCCC separation.

2.4. Selection of the two-phase solvent systems

Light petroleum (boiling point range: 60–90 °C)–ethyl acetate–methanol–water two-phase system was used as solvent system. The composition of the two-phase system was selected according to the partition coefficient (*K*) of target compounds of crude sample from *C. Cusson*. The partition coefficients were determined by HPLC as follows: about 0.1 mg of crude sample was added to a test tube, to which 2 ml of each phase of the two-phase solvent system was added. The test tube was shaken violently for several minutes. Then the upper and lower phases were analyzed by HPLC. The partition coefficients of all components in sample were obtained according to the peak areas.

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

Light petroleum–ethyl acetate–methanol–water solvent systems with the volume ratios of 5:5:5:5, 5:5:6:4 and 5:5:6.5:3.5 were prepared by adding the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated by shaking repeatedly. Then upper and lower phases were separated and degassed by sonication for 30 min prior to use.

One hundred and fifty milligrams of crude extract sample was dissolved in 5 ml of the upper phase of light petroleum–ethyl acetate–methanol–water system (5:5:5:5, v/v).

2.6. Separation procedure

The whole procedure was carried out as follows: the upper phase (stationary phase) and the lower phase (mobile phase) of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) were pumped into the multilayer-coiled column simultaneously by using ÄKTA prime system, according to the volume ratio of 60:40. When the column was

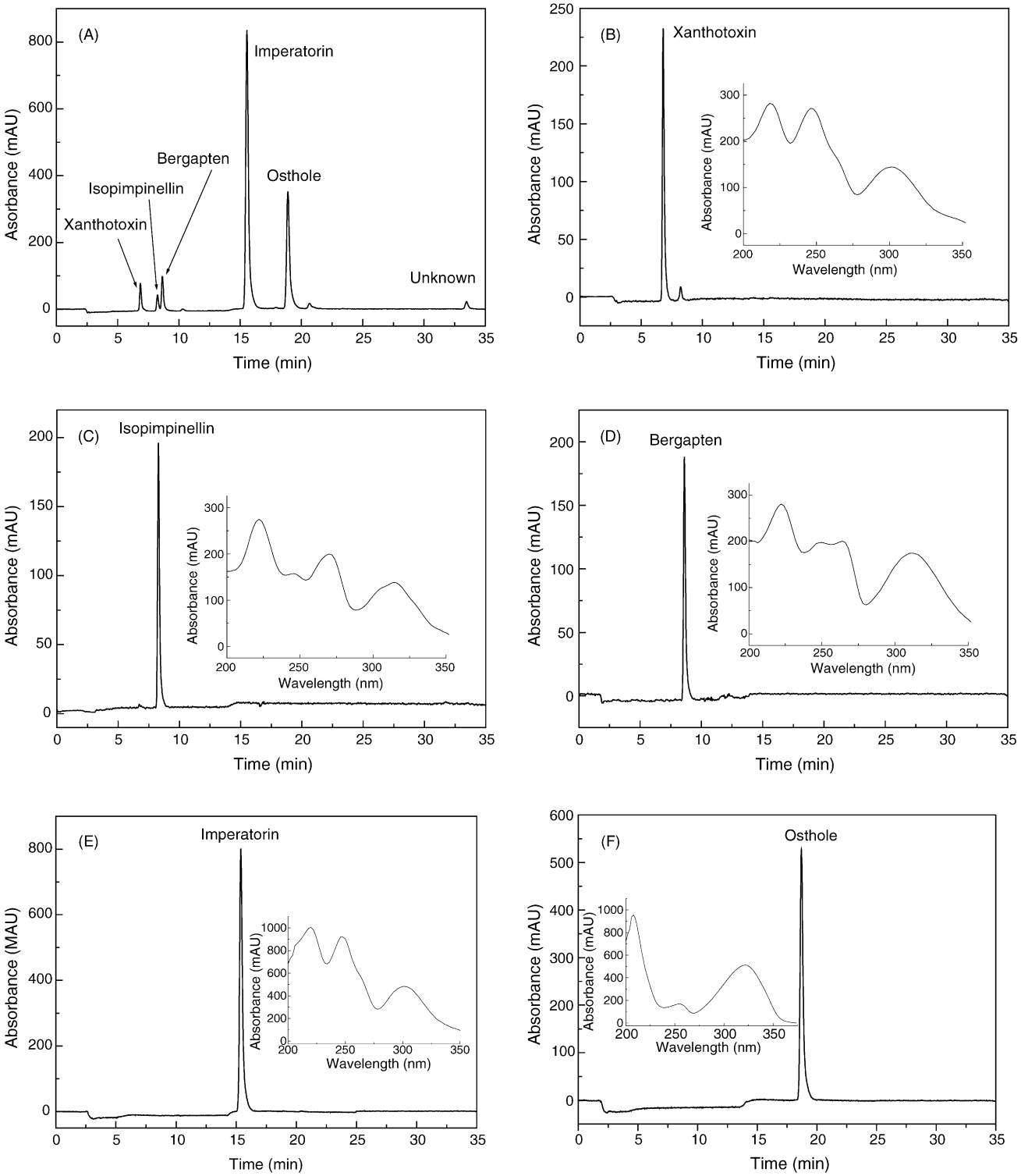

Fig. 2. HPLC chromatograms of crude extract and HSCCC peak fractions. (A) Crude extract from *Cnidium monnieri* (L.) *Cusson*; (B–G) HSCCC peak fraction I–VI in Fig. 3. Column: SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm); column temperature: 25 °C; mobile phase: methanol–acetonitrile–water in gradient mode (30:30:40 → 50:30:20 in 30 min). Flow rate: 1.0 ml min^{-1}, detection wavelength: 254 nm.

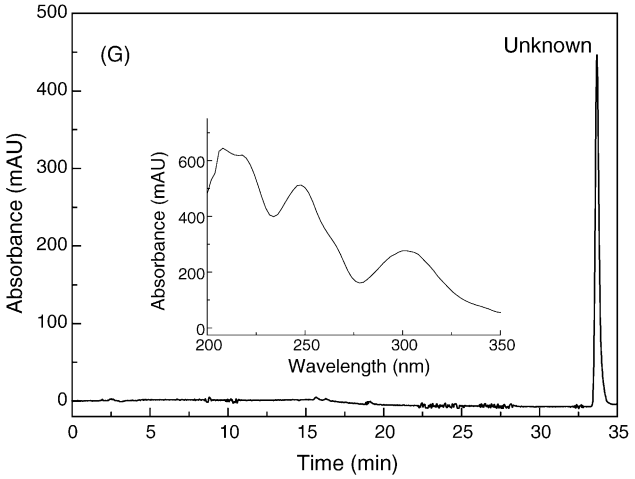

Fig. 2. (*Continued*).

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 later), the sample solution was injected into the separation column. At the same time, the stepwise elution began. In the first 150 min, the lower phase of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) was pumped. In second 100 min, the lower phase of light petroleum–ethyl acetate–methanol–water (5:5:6:4, v/v) was pumped, and then the lower phase of light petroleum–ethyl acetate–methanol–water (5:5:6.5:3.5, v/v). The separation temperature was controlled at 35 °C. The effluent from the outlet of the column was continuously monitored at 254 nm. Each peak fraction was manually collected according to the chromatogram and evaporated under reduced pressure. The residuals were dissolved in methanol for subsequent HPLC analysis.

2.7. HPLC analysis and identification of HSCCC peak fractions

The crude sample and each HSCCC peak fraction were analyzed by HPLC. The analysis was accomplished with a SPHERIGEL ODSC$_{18}$ column (250 mm × 4.6 mm i.d., 5 μm) at room temperature. Methanol–acetonitrile–water system was used as mobile phase in gradient mode as follows: 30:30:40 → 50:30:20 in 30 min. The flow rate was 1.0 ml min^{-1}, and the effluents were monitored at 254 nm by a photodiode array detector.

Identification of HSCCC peak fraction was performed by ^{1}H NMR. The UV spectra were taken from the HPLC three-dimensional spectrum of absorbance versus time and wavelength.

3. Results and discussion

3.1. Optimization of HPLC conditions

Several elution systems were tested in HPLC separation of crude sample, such as gradient elution of methanol–water, acetonitrile–water, methanol–0.1% H_3PO_4, acetonitrile–0.1% H_3PO_4, methanol–1% NaH_2PO_4, acetonitrile–1% NaH_2PO_4, methanol–acetonitrile–water, etc. The results indicated that good separation results could be obtained when using methanol–acetonitrile–water system as the mobile phase in gradient elution mode (30:30:40 → 50:30:20 in 30 min).

The crude sample and peak fractions separated by HSCCC were analyzed by HPLC. The chromatograms are shown in Fig. 2.

3.2. Selection of two-phase solvent system and other conditions of HSCCC

A series of experiments were performed to optimize the two-phase solvent system for HSCCC separation. The partition coefficients of the target compounds in different solvent systems were determined by HPLC as given in Section 2.4 and the results are shown in Table 1. According to the *K* values shown in Table 1, some solvent systems were tested. The results indicated that when light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) was used as the solvent system, every target compound can be separated, but the separation time was very long and the peaks of imperatorin and osthole were wide. When other solvent systems were used for HSCCC separation, the anterior peaks could not be separated. So stepwise elution was used in the HSCCC separation. When the upper phase of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) was used as the stationary phase, and the lower phases of light petroleum–ethyl acetate–methanol–water system at the volume ratio of 5:5:5:5, 5:5:6:4 and 5:5:6.5:3.5 were used as the mobile phase in stepwise elution mode, good separation results can be obtained.

The influence of the flow rate of mobile phase, the separation temperature and revolution speed were also investigated. The results indicated that reducing flow speed can improve separation in some degree, but at the same time, the chromatogram peaks were extended. Considering the two aspects, the flow rate of the mobile phase was set at 2 ml min^{-1} in this experiment. The temperature has significant effect on *K* (partition coefficient), the retention percentage of the stationary phase and the mutual solvency of the two-phase. After tested at 20 °C, 25 °C, 30 °C, 35 °C and 40 °C, it can be seen that good results can be obtained when the separation temperature was controlled at 35 °C. The rotary speed of the separation coil tube has great influence on the retention percentage of the stationary phase. Expediting the rotary speed can increase the retention of the stationary phase. In this experiment, all separations were performed at 900 rpm.

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

Light petroleum–ethyl acetate–methanol–water (v/v)	*K*				
	Xanthotoxin	Isopimpinellin	Bergapten	Imperatorin	Osthole
5:5:5:5	1.49	1.95	3.33	6.52	9.91
5:5:5.5:4.5	0.72	0.86	1.57	2.98	4.51
5:5:6:4	0.53	0.62	0.93	1.71	2.78
5:5:6.5:3.5	0.49	0.55	0.79	1.30	2.19
5:5:7:3	0.22	0.25	0.37	0.65	1.06

The crude samples from *C. Cusson* were separated and purified under the optimum HSCCC conditions. The HSCCC chromatogram is shown in Fig. 3. Five kinds of coumarins were obtained and yielded 7.6 mg of xanthotoxin(I), 7.6 mg of isopimpinellin(II), 9.7 mg of bergapten(III), 60.5 mg of imperatorin(IV), 50.6 mg of osthole(V) and 10.2 mg of one unknown compound from 150 mg crude sample. The purity of these compounds was 95.0%, 99.6%, 99.7%, 100%, 100% and 98.1%, respectively, as determined by HPLC. The chromatograms of HPLC and UV spectra of these compounds are shown in Fig. 2.

3.3. The structural identification

The chemical structure of each peak fraction of HSCCC was identified according to its ^{1}H NMR data:

- *Peak I*: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.30 (3H, s, –OCH_3), 6.38 (1H, d, J = 9.6 Hz, H-3), 6.82 (1H, d, J = 2.4 Hz, H-11), 7.36 (1H, s, H-5), 7.69 (1H, d, J = 2.4 Hz, H-12), 7.77 (1H, d, J = 9.6 Hz, H-4). Comparing the above date with reference [10], peak I was identified as xanthotoxin.

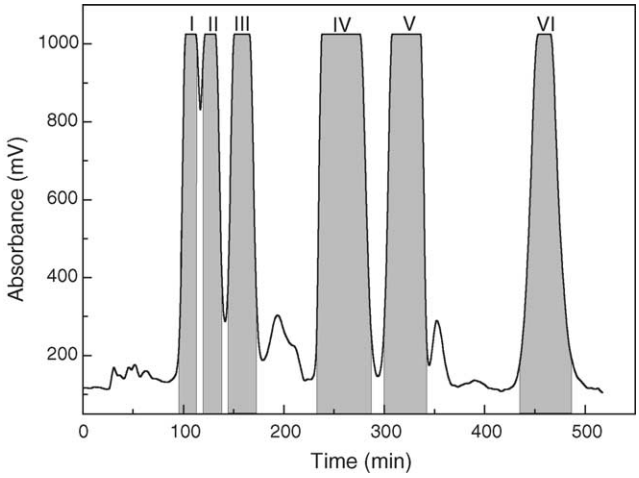

Fig. 3. HSCCC chromatogram of extract of *Cnidium monnieri* (L.) *Cusson*. Solvent system: light petroleum–ethyl acetate–methanol–water (5:5:5:5, 5:5:6:4, 5:5:6.5:3.5, v/v) in stepwise elution; stationary phase: upper organic phase of 5:5:5:5; mobile phase: lower aqueous phase of 5:5:5:5 in 0–100 min, lower aqueous phase of 5:5:6:4 in 100–250 min, lower aqueous phase of 5:5:6.5:3.5 after 250 min; flow rate: 2.0 ml min^{-1}; revolution speed: 900 rpm; retention of the stationary phase: about 60%.

- *Peak II*: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.17 (6H, s, 2× OCH_3), 6.29 (1H, d, J = 10 Hz, H-3), 7.0 (1H, d, J = 2.4 Hz, H-11), 7.63 (1H, d, J = 2.8 Hz, H-12), 8.13 (1H, d, J = 9.6 Hz, H-4). Comparing the above date with reference [11], the obtained product was confirmed as isopimpinellin.
- *Peak III*: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.27 (3H, s, –OCH_3), 6.27 (1H, d, J = 10 Hz, H-3), 7.02 (1H, d, J = 2 Hz, H-11), 7.13(1H, s, H-8), 7.59 (1H, d, J = 2.4 Hz, H-12), 8.16 (1H, d, J = 9.6 Hz, H-4). Comparing the above date with reference [12], the obtained product was identified as bergapten.
- *Peak IV*: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.72, 1.74 (6H, s, 2× CH_3), 5.00 (2H, d, J = 7.2 Hz, H-1′), 5.61 (1H, m, H-2′), 6.37 (1H, d, J = 10 Hz, H-3), 6.82 (1H, d, J = 2.0 Hz, H-11), 7.36 (1H, s, H-5), 7.69 (1H, d, J = 2.0 Hz, H-12), 7.77 (1H, d, J = 9.6 Hz, H-4). Comparing the above date with reference [13], the obtained product was identified as imperatorin.
- *Peak V*: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.67 (3H, s, H-5′), 1.84 (3H, s, H-4′), 3.49 (2H, d, J = 7.2 Hz, H-1′), 3.92 (3H, s, –OCH_3), 5.22 (1H, m, H-2′), 6.23 (1H, d, J = 9.6 Hz, H-3), 6.84 (1H, d, J = 8.4 Hz, H-6), 7.29 (1H, d, J = 8.4 Hz, H-5), 7.62 (1H, d, J = 9.6 Hz, H-4). Comparing the above date with reference [14], the obtained product was confirmed as osthole.

In conclusion, the result of our studies demonstrated that HSCCC is a powerful tool for isolation and purification of coumarins from *C. Cusson*

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