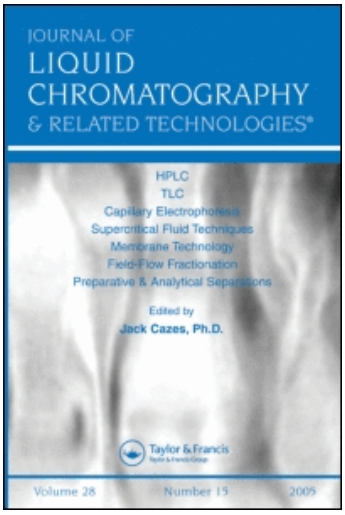

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Preparative Isolation and Purification of Saponin and Flavone Glycoside Compounds from *Clinopodium chinensis (Benth) O. Kuntze* by High-Speed Countercurrent Chromatography

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Abstract: A preparative high-speed countercurrent chromatography (HSCCC) method for isolation and purification of didymin, nairutin, and clinopodiside A from *Clinopodium chinensis (Benth) O. Kuntze* (Duanxueliu in Chinese) was successfully established. The separation was performed in two steps with two different kinds of solvent systems. In the first step, ethyl acetate-1-butanol-water (5:0.8:5, v/v) was used as the two-phase solvent system; nairutin was purified, and didymin and clinopodiside A were eluted together. In the second step, ethyl acetate-methanol-water (5:1:5, v/v) was used as the two-phase solvent system; didymin and clinopodiside A were separated and purified. After two-step separation, 15.2 mg of nairutin, 39.1 mg of clinopodiside A, and 20.6 mg of didymin were obtained from 100 mg of crude extract with purities of 96.5%, 98.4%, and 99.1%, respectively, as determined by HPLC analysis. The chemical structures of the three components were confirmed by ^{1}H NMR and ^{13}C NMR.

Keywords: HSCCC, *Clinopodium chinensis (Benth) O. Kuntze*, Didymin, Nairutin, Clinopodiside A

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INTRODUCTION

Duanxueliu, the aerial parts of *Clinopodium chinensis (Benth) O. Kuntze*, have long been used as an astrictive remedy in traditional Chinese medicine, and is officially listed in the Chinese Pharmacopoeia.[1] Duanxueliu and its products are often used for the treatment of many kinds of hemorrhage, such as dysfunctional uterine bleeding, hysteromyome hemerrhage, postpartum hemerrhage, ectopic pregnancy, hematuria, and epistaxis. Furthermore, it also has good effects on allergic purpura, simple purpura, thrombocytopenia, hemophilia, and diphtheria. Pharmacological studies and clinical practice have demonstrated its astrictive, anti-inflammatory, anti-radiation, and immune activities.[2] Since saponins and flavone glycosidepresent in Duanxueliu, such as nairutin, clinopodiside A, and didymin, are known as the major beneficial components, they have been chosen as "the marker components" for the chemical evaluation or standardization of Duanxueliu and its products. The chemical structures of these components are given in Fig. 1.

Because the compounds with strong polarity tend to be irreversibly absorbed onto solid supports, it is difficult to separate and purify such polar components in studies of natural products. The separation and purification of nairutin, clinopodiside A, and didymin from plant materials of Duanxueliu

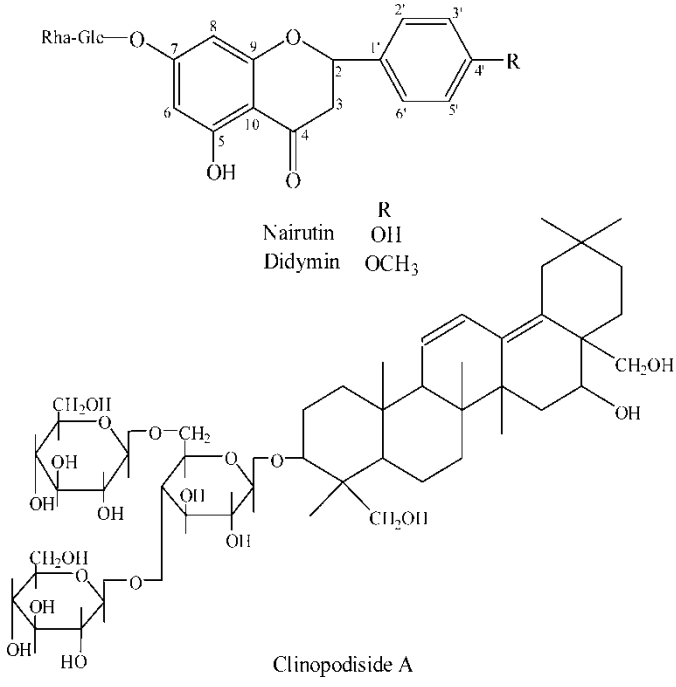

Figure 1. Chemical structures of didymin, nairutin, and clinopodiside A.

was reported using a conventional column method.[3–5] But, the methods are tedious and usually require multiple chromatographic steps, such as column chromatography and preparative HPLC.

High-speed countercurrent chromatography (HSCCC) successfully eliminates irreversible adsorptive loss of samples onto the solid support used in conventional chromatographic columns. So, it has gained great progress in preparation of various reference standards from Chinese traditional medicines and other natural products for pharmacological studies and good manufacturing practice. Many kinds of saponins and flavone glycosides were purified by HSCCC, such as ginsenoside,[6] gastrodin,[7] salidroside,[8] glycyrrhizin,[9] icariin,[10] baicalein,[11] etc.

The present paper establishes a method for the purification of flavone glycosides and saponins from a crude extract of Duanxueliu by a two-step HSCCC. Nairutin was purified in the first step by using ethyl acetate-1-butanol-water (5:0.8:5, v/v) as the two-phase solvent system. Didymin and clinopodiside A were eluted together in this step. In the second step, didymin and clinopodiside A were separated and purified using ethyl acetate-methanol-water (5:1:5, v/v) as the two-phase solvent system. The chemical structures of the three components were confirmed by ^{1}H NMR and ^{13}C NMR.

EXPERIMENTAL

Apparatus

The HSCCC instrument used in this study is a TBE-300A high-speed countercurrent chromatograph (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) with three multilayer coil separation columns connected in series (I.D. of the tubing = 1.6 mm, total volume = 260 mL) and a 20 mL sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1,000 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 system (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurements. It contains a switch valve and a mixer which were used for gradient formation. The data were collected with a Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Company, Hangzhou, China).

The HPLC equipment was an Agilent 1100 HPLC system, including G1311A QuatPump, G1315B UV-Vis photodiode array detector, Rheodyne

7725i injection valve with a 20 μL loop, G1332 degasser and Agilent HPLC workstation (Agilent Technologies, Germany).

A FZ102 plant disintegrator (Taisite Instrument Company, Tianjin, China) was used for disintegration of Duanxueliu samples.

The nuclear magnetic resonance (NMR) spectrometer used here was a Mercury Plus 400 NMR system (Varian Inc., USA).

Reagents and Materials

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

Duanxueliu was purchased from a local drug store and was identified as *Clinopodium chinensis (Benth) O. Kuntze* by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

Preparation of Crude Extract

Duanxueliu was ground to a powder (about 30 mesh) by a disintegrator. 2,000 g of the powder was extracted with 10 L of 80% ethanol for 2 hours under reflux. The extraction procedure was repeated twice. The extracts were combined together. After filtration 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 × 80 cm) of macroporous resin (D-101, 400 g) and washed with water and 50% ethanol. The eluant of the 50% ethanol was concentrated, in vacuum, using a rotary evaporator to give a dried powder (40 g). The powder was stored in a refrigerator (−4°C) for the subsequent HSCCC separation.

Selection of Two-Phase Solvent System

The two-phase solvent system was selected according to the partition coefficients (K) of the target components. The K values were determined by HPLC analysis. About 0.5 mg of the crude extract was gently shaken with 2 mL of each phase of the equilibrated two-phase solvent system in a tube. After settling for some time, the two phases were separated. The concentrations of the target components in both phases were quantitatively analyzed by HPLC and the partition coefficient was calculated from the concentration of the upper phase divided by that of the lower phase.

Preparation of Two-Phase Solvent System and Sample Solution

In the present study, ethyl acetate-1-butanol-water (5:0.8:5, v/v) was used as the two-phase solvent system for the first HSCCC separation step and ethyl acetate-

methanol-water (5:1:5, v/v) for the second HSCCC separation step. Each solvent system was prepared by adding the solvents to a separatory funnel and thoroughly equilibrating at m temperature. The upper phase and the lower phase were separated and degassed by sonication for 30 min shortly before use.

The sample solution was prepared by dissolving the crude extract in the upper phase of ethyl acetate-1-butanol-water (5:0.8:5, v/v) at a suitable concentration.

HSCCC Separation Procedure

A two-step HSCCC separation procedure was employed. In the first separation step, the upper phase (the stationary phase) and the lower phase of ethyl acetate-1-butanol-water (5:0.8:5, v/v) were pumped into the column, simultaneously, at a volume ratio of 45:55. After the column was totally filled, the apparatus was rotated at 700 rpm. At the same time, the lower phase was pumped into the column at a flow rate of 2.0 mL min^{-1}. About half an hour later, hydrodynamic equilibrium was reached, and 5 mL of the sample solution containing 100 mg of the crude extract was injected into the column through the injection valve. All through the experiment, the separation temperature was controlled at 20°C. The effluent from the tail end of the column was continuously monitored with a UV absorbance detector at 254 nm. The data were collected 60 min after sample injection. Each fraction was collected manually according to the obtained chromatogram (Fig. 2-A) and dried by vacuum freeze-drying.

The fraction of peak II in Fig. 2-A (yield 45 mg of dried powder) was separated in the second separation step, using ethyl acetate-methanol-water (5:1:5, v/v) as the two-phase solvent system. The upper phase was used as the stationary phase and the lower phase was used as the mobile phase. The volume ratio of the stationary phase to the mobile phase was 40:60. The revolution speed was 650 rpm and the flow-rate of the mobile phase was 2.0 mL min^{-1}. The separation temperature was 20°C and the detection wavelength was 254 nm. The data were collected 60 min after sample injection. Each peak fraction was collected manually according to the obtained chromatogram (Fig. 2-B) and dried by vacuum freeze-drying.

HPLC Analysis and Identification of HSCCC Peak Fractions

The HPLC analysis was performed with a YWG C_{18} column (200 × 4.6 mm I.D., 10 μm) at room temperature. The mobile phase was methanol and water in gradient mode as follows: 0–60 min, 10% methanol to 100% methanol. The flow rate of the mobile phase was kept at 1.0 mL min^{-1} constantly and the effluent was monitored at 254 nm.

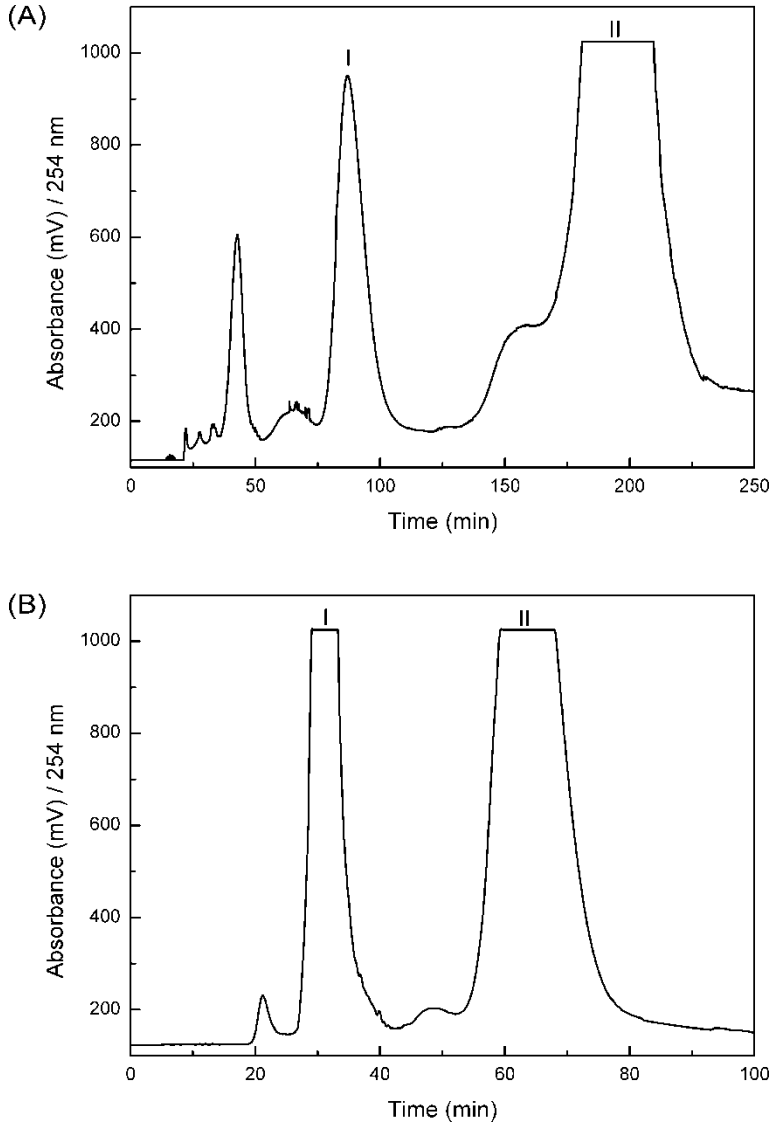

Figure 2. HSCCC chromatogram. (A) First step of HSCCC separation. Two-phase solvent system: ethyl acetate-1-butanol-water (5:0.8:5, v/v); mobile phase: the lower phase; flow rate: 2.0 mL min^{-1}; revolution speed: 700 rpm; detection wavelength: 254 nm; separation temperature: 20°C; sample size: 100 mg of crude sample dissolved in 5 mL of the stationary phase. I: nairutin; II: mixture of didymin and clinopodiside A; (B) Second step of HSCCC separation. Two-phase solvent system: ethyl acetate-methanol-water (5:1:5, v/v); mobile phase: the lower phase; flow rate: 2.0 mL min^{-1}; revolution speed: 650 rpm; detection wavelength: 254 nm; separation temperature: 20°C; sample size: 45 mg of dried powder of Peak II in (A) dissolved in 5 mL of the stationary phase. I: clinopodiside A; II: didymin.

The structure identification of HSCCC peak fractions was carried out by ^{1}H-NMR and ^{13}C-NMR. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Mercury Plus 400 NMR with TMS as internal standard and DMSO-d_6 as solvent.

RESULTS AND DISCUSSION

Optimization of the HPLC Method

As one of the most exact and rapid analytical techniques, HPLC has been used for compositional analysis of crude extract, selection of two-phase solvent systems, and purity analysis of HSCCC peak fractions. In the present study, the partition coefficient determinations and purity examinations of HSCCC peaks were both conducted by HPLC. In order to obtain satisfactory separation results, many different elution solvent and elution modes were tested using a C_{18} column. When methanol-water was used as the mobile phase in a gradient elution mode (0–60 min, 10% methanol to 100% methanol), four major peaks were obtained and each peak achieved baseline separation. The HPLC chromatogram of crude extract from Duanxueliu is shown in Fig. 3-A.

Optimization of HSCCC Conditions

A series of experiments was performed to choose a suitable two-phase solvent system for HSCCC. The following polar two-phase solvent systems were tested: ethyl acetate-water, ethyl acetate-methanol-water, 1-butanol-water, 1-butanol-methanol-water, and ethyl acetate-1-butanol-water, at various volume ratios. The K values of the major components are listed in Table 1. It can be seen that the four major components cannot be purified by a single-step separation. So, a single solvent system cannot be used for a one-step separation. But nairutin can be separated with the other components if ethyl acetate-1-butanol-water (5:0.8:5, v/v) is used as the solvent system. At the same time, didymin and clinopodiside A may be eluted together because their K values are very similar. On the other hand, the K values of didymin and clinopodiside A are in a suitable range (0.2$\sim$5) and the difference is very distinct ($\beta = 3.6$) using ethyl acetate-methanol-water (5:1:5, v/v) as the two-phase solvent system.

So a two-step separation procedure was employed. In the first step, ethyl acetate-1-butanol-water (5:0.8:5, v/v) was used as the two-phase solvent system, and the HSCCC chromatogram is shown in Fig. 2-A. Two major peaks were obtained. Subsequent HPLC analysis showed that peak I was nairutin, and peak II was a mixture of didymin and clinopodiside A (Fig. 3-B). In the next step, peak II was subjected to HSCCC using ethyl

acetate-methanol-water (5:1:5, v/v) as the two-phase solvent system. The two major peaks achieved baseline separation in the second step. The HSCCC chromatogram is given in Fig. 2-B.The HPLC analysis of the two HSCCC peaks indicated that the two peaks were clinopodiside A and didymin.

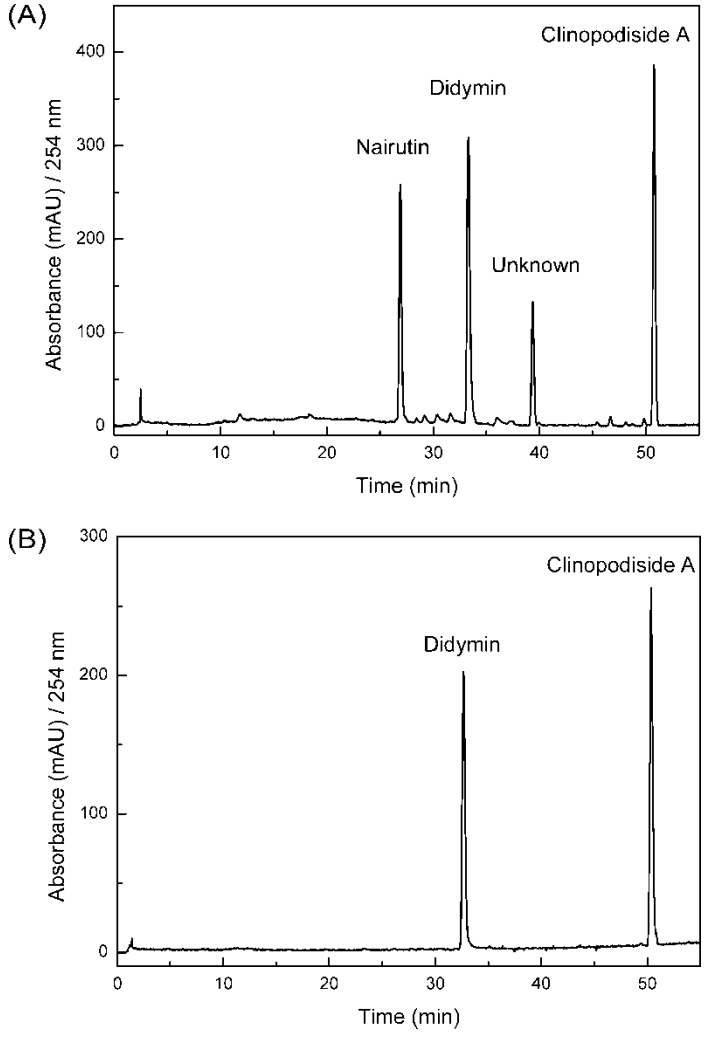

Figure 3. HPLC chromatograms. (A) Crude extract from *Clinopodium chinensis (Benth) O. Kuntze*; (B) Peak II in Fig. 2-A; (C) Peak I in Fig. 2-A; (D) Peak I in Fig. 2-B; (E) Peak II in Fig. 2-B; Column: YWG C_{18} column (200 × 4.6 mm I.D., 10 μm); mobile phase: methanol and water in gradient elution mode (0–60 min, 10% methanol to 100% methanol); flow rate: 1.0 mL min^{-1}; detection wavelength: 254 nm.

(continued)

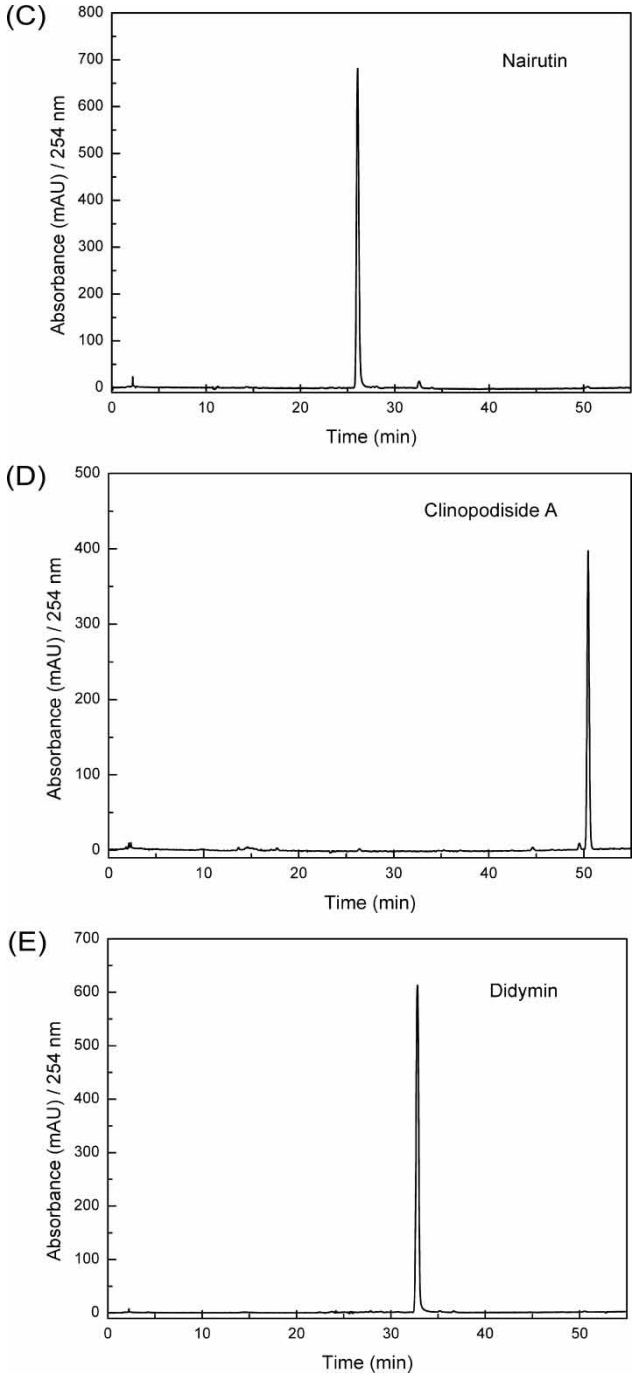

Figure 3. Continued.

Table 1. The K (partition coefficient) values of didymin, nairutin, and clinopodiside A in several different solvent system

	K		
Solvent system	Nairutin	Didymin	Clinopodiside A
Ethyl acetate-water (5:5)	0.22	0.58	0.08
Ethyl acetate-methanol-water (5:1:5)	0.54	1.24	0.34
1-Butanol-water (5:5)	3.38	5.20	15.20
1-Butanol-methanol-water (5:1:5)	2.86	4.14	10.08
Ethyl acetate-1-butanol-water 5:0.5:5	0.80	1.11	0.77
Ethyl acetate-1-butanol-water 5:0.8:5	1.26	3.21	2.66
Ethyl acetate-1-butanol-water 5:1:5	2.18	4.59	3.82

The influence of the revolution speed and the flow rate of the mobile phase on HSCCC separation were also investigated. The result indicated that the retention ratio of the stationary phase was not good below 50%. In order to improve the retention of the stationary phase, different revolution speeds and flow rates were tested. The result indicated that properly decreasing revolution speed and flow rate can yield higher retention ratio of the stationary phase. In the first separation step, the apparatus was rotated at 700 rpm, and in the second separation step, the revolution speed was set at 650 rpm. Under the optimum conditions, three major peaks were obtained which yielded 15.2 mg of nairutin (peak I in Fig. 2-A), 39.1 mg of clinopodiside A (peak I in Fig. 2-B), and 20.6 mg of didymin (peak II in Fig. 2-B) from 100 mg of crude extract. The purities of the three components were 96.5%, 98.4%, and 99.1%, respectively. HPLC chromatograms of the HSCCC peak fractions are shown in Fig. 3 (C-E).

Identification of HSCCC Peak Fraction

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

Fraction of HSCCC peak I in Fig. 2-A: ^{1}H-NMR (400 MHz, DMSO-d_6): δ(ppm): 7.34 (d, 2H, J = 8.5 Hz, H-2′, H-6′), 6.80 (d, 2H, J = 8.5 Hz, H-3′, H-5′), 6.13 (d, 1H, J = 2.2 Hz, H-6), 6.11 (d, 1H, J = 2.2 Hz, H-8), 5.49 (dd, 1H, J = 11.9, 2.8 Hz, H-2), 3.13 (dd, 1H, J = 16.7, 13.0 Hz, H-3a), 2.73 (dd, 1H, J = 16.7, 2.8 Hz, H-3e), 1.07 (d, 3H, J = 6.2 Hz, Rha-H-6); ^{13}C-NMR (100 MHz, DMSO-d_6): δ(ppm): 197.4 (C-4), 165.2 (C-7), 165.2 (C-5), 162.8 (C-9), 158.0 (C-4′), 128.8 (C-1'), 128.7 (C-2'), 128.6 (C-6′), 115.4 (C-3′), 115.3 (C-5′), 103.4 (C-10), 100.7 (Glc-C-1), 99.5 (Rha-C-1), 96.6 (C-6), 95.6 (C-8), 78.7 (C-2), 76.4 (Glc-C-5), 75.7 (Glc-C-3), 73.1 (Glc-C-2), 72.2 (Rha-C-4), 70.8 (Rha-C-2), 70.4 (Glc-C-4), 69.7 (Rha-C-3),

68.5 (Rha-C-5), 66.2 (Glc-C-6), 42.4 (C-3), 18.1 (Rha-C-6). These data were identical with the literature [4], and the peak was identified as nairutin.

Fraction of HSCCC peak I in Fig. 2-B: ^{1}H-NMR (400 MHz, DMSO-d_6): δ(ppm): 0.58∼1.1 (each 3H, 6 × CH_3), 3.37 (1H, m, H-3α), 3.80 (1H, m, H-16α), 3.57, 4.45 (each 1H, J = 11 Hz, H-28), 3.73, 4.24 (each 1H, J = 11 Hz, H-23), 5.56 (1H,dd, J = 4,11 Hz, H-11), 6.40 (1H, d, J = 11 Hz, H-12), 4.32, 4.47, 4.65 (each 1H, d, J = 7.3 Hz); ^{13}C-NMR (100 MHz, DMSO-d_6): δ(ppm): 135.4 (C-13), 132.9 (C-18), 126.5 (C-11), 125.1 (C-12), 103.7 (C-1′), 103.3 (C-1″), 102.1 (C-1‴), 88.7 (C-3), 80.7 (C-4′), 77.1 (C-5′), 76.9 (C-16), 76.8 (C-3′), 76.5 (C-5″), 76.4 (C-5″), 75.2 (C-3″), 75.1 (C-3‴), 75.0 (C-2′), 74.0 (C-2‴), 73.9 (C-2″), 70.9 (C-4‴), 70.2 (C-4″), 69.5 (C-6′), 65.5 (C-23), 62.4 (C-28), 61.8 (C-6‴), 61.2 (C-6″), 53.7 (C-9), 46.3 (C-5), 43.5 (C-17), 43.4 (C-4), 42.8 (C-14), 39.9 (C-8), 39.0 (C-19), 38.8 (C-1), 37.6 (C-10), 35.7 (C-21), 34.5 (C-15), 33.9 (C-30), 32.4 (C-7), 32.1 (C-20), 29.4 (C-22), 25.4 (C-2), 24.5 (C-29), 21.6 (C-27), 18.3 (C-6), 17.4 (C-25), 16.8 (C-26), 12.1 (C-24). Comparing these data with the literature [3], so the peak was identified as clinopodiside A.

Fraction of HSCCC peak II in Fig. 2-B: ^{1}H-NMR (400 MHz, DMSO-d_6): δ(ppm): 12.02 (s, 1H, C_5-OH), 7.47 (d, 2H, J = 8.7 Hz, H-2′, H-6′), 6.98 (d, 2H, J = 7.8 Hz, H-3′, H-5′), 6.13 (s, 2H, H-6, H-8), 5.58 (dd, 1H, J = 12.6, 2.8 Hz, H-2), 4.98 (d, 1H, J = 7.6 Hz, Glc-H-1), 4.52 (brs, 1H, Rha-H-1), 3.77 (s, 3H, -OCH_3), 2.77 (dd, 1H, J = 17, 2.7 Hz, H-3e), 1.07(d, 3H, J = 6.2 Hz, Rha-H-6).^{13}C-NMR (100 MHz, DMSO-d_6): δ(ppm): 197.2 (C-4), 165.3 (C-7), 163.2 (C-5), 162.7 (C-9), 159.7 (C-4′), 130.5 (C-1′), 128.7 (C-2′), 128.5 (C-6′), 114.1 (C-3′), 114.1 (C-5′), 103.4 (C-10), 100.8 (Glc-C-1), 99.5 (Rha-C1), 96.6 (C-6), 95.6 (C-8), 78.5 (C-2), 76.4 (Glc-C-5), 75.7(Glc-C-3), 73.1(Glc-C-2), 72.2 (Rha-C-4), 70.8 (Rha-C-2), 70.4 (Rha-C-3), 69.8 (Glc-C-4), 68.5 (Rha-C-5), 66.1 (Glc-C-6), 55.3 (-OCH_3), 42.0 (C-3), 18.0 (Rha-C-6). According to the literature [5], the peak was confirmed as didymin.

In conclusion, HSCCC was successfully used for the isolation and purification of 3 major components from *Clinopodium chinensis (Benth) O. Kuntze*, which yielded 15.2 mg of nairutin, 39.1 mg of clinopodiside A, and 20.6 mg of didymin from 100 mg of the crude extract after two-step separation.

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