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Preparative isolation and purification of alkaloids from the Chinese medicinal herb *Evodia rutaecarpa* (Juss.) Benth by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water system (5:5:7:5, v/v) was applied to the isolation and purification of alkaloids from the Chinese medicinal plant *Evodia rutaecarpa* (Juss.) Benth. Five kinds of alkaloids were obtained and yielded 28 mg of evodiamine (**I**), 19 mg of rutaecarpine (**II**), 21 mg of evocarpine (**III**), 16 mg of 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1*H*)-quinolone (**IV**), 12 mg of 1-methyl-2-dodecyl-4-(1*H*)-quinolone (**V**) from 180 mg of crude extract in a one-step separation, with the purity of 98.7%, 98.4%, 96.9%, 98.0%, 97.2%, respectively, as determined by high performance liquid chromatography (HPLC). The structures of these compounds were identified by ^{1}H NMR and ^{13}C NMR.

Keywords: *Evodia rutaecarpa* (Juss.) Benth; HSCCC; Evodiamine; Rutaecarpine; Evocarpine; 1-Methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1*H*)-quinolone; 1-Methyl-2-dodecyl-4-(1*H*)-quinolone

1. Introduction

Wu-zhu-yu, the dried fruit of *Evodia rutaecarpa* (Juss.) Benth (*E. rutaecarpa*), is a well-known traditional Chinese medicine and officially listed in the Chinese Pharmacopoeia [1], and has been used for a long time in Chinese medical practice. Wu-zhu-yu is used as a remedy for gastrointestinal disorders (abdominal pain, dysentery), headache, amenorrhea, and postpartum hemorrhage [2]. It has also been claimed to have a remarkable central stimulant effect, a transient hypertensive effect [2,3], and positive inotropic and chronotropic effects [4]. In phytochemical studies, a wide variety of compounds including alkaloids were found in the fruits of this plant. Alkaloids, including evodiamine and rutaecarpine, are the major active compounds present in Wu-zhu-yu. Evodiamine and rutaecarpine are often used as standards in the quality control of Wu-zhu-yu products [5]. Here, some of these alkaloids are separated by high-speed counter-current chromatography (HSCCC). The two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (5:5:7:5, v/v) was applied to the separation and purification of alkaloids from the extract of Wu-zhu-yu. Five kinds of major alkaloids including evodiamine (**I**), rutaecarpine (**II**), evocarpine (**III**), 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1*H*)-quinolone (**IV**) and 1-methyl-2-dodecyl-4-(1*H*)-quinolone (**V**) were obtained in one-step separation. The chemical structures of these alkaloids are shown in Fig. 1.

2. Experimental

2.1. Apparatus

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

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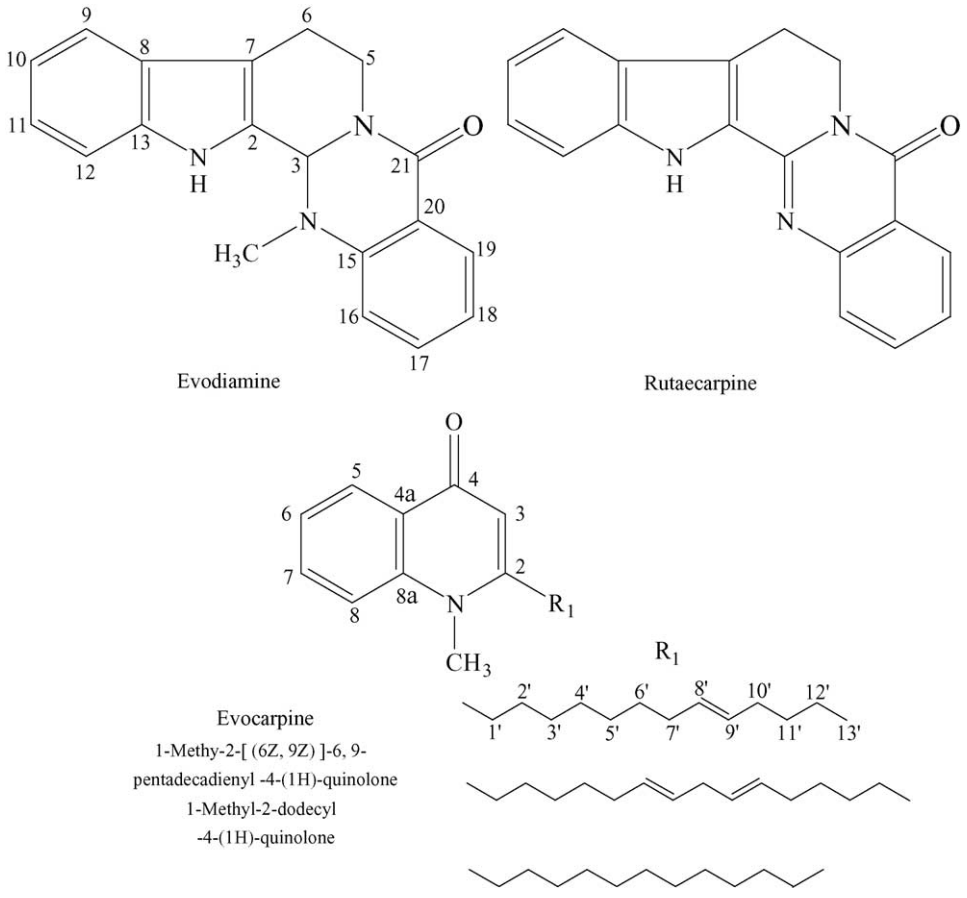

Fig. 1. Chemical structures of alkaloids from *E. rutaecarpa*.

(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 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 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 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature. A ÄKTA prime system (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 were 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 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.

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

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

2.2. Reagents and materials

All 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 Factory, Yucheng, China), and water used was distilled water.

Wu-zhu-yu was purchased from a local drug store and was identified as the dried fruits of *E. rutaecarpa* by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.3. Preparation of crude sample

Preparation of crude sample was carried out as following. The dried fruits of *E. rutaecarpa* were ground to powder (about 40 mesh) by using the FZ102 plant disintegrator. The powder (100 g) was dipped in 800 ml of ethyl acetate

for 30 min, and then extracted by ultrasonic for 45 min. The extraction procedure was repeated twice. The extracts were combined together and evaporated to dryness by rotary vaporization under reduced pressure. Crude sample (6.2 g) were obtained. It was stored in a refrigerator (4 °C) for further use.

2.4. Selection of the two-phase solvent systems

n-Hexane–ethyl acetate–methanol–water was used as the two-phase solvent system. The composition of the two-phase was selected according to the partition coefficient (*K*) of target compounds of crude sample from Wu-zhu-yu. The partition coefficients were determined by HPLC as follows: about 0.5 mg of crude sample was added to a test tube, to which 5 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 phase 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 solutions

n-Hexane–ethyl acetate–methanol–water solvent system (5:5:7:5) was prepared by adding all the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated by shaking repeatedly. Then the upper phase and the lower phase were separated and degassed by sonication for 30 min prior to use.

The sample solution for HSCCC separation was prepared by dissolving 180 mg of crude extract sample in the mixture of 2 ml of the upper phase and 2 ml of the lower phase of *n*-hexane–ethyl acetate–methanol–water system (5:5:7:5, v/v).

2.6. Separation procedure

The upper phase (stationary phase) and the lower phase (mobile phase) of *n*-hexane–ethyl acetate–methanol–water (5:5:7:5, v/v) were pumped into the multilayer-coiled column simultaneously by using ÄKTA prime system, according to the volume ratio of 50:50. When 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 later), the sample solution was injected into the separation column. The separation temperature was controlled at 25 °C. The effluent from the outlet of the column was continuously monitored at 254 nm. The chromatogram was recorded 50 min after sample injection. 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 ODS C_{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: 15:38:47 in 0–22 min, 45:38:17 in 22–65 min. The flow-rate of the mobile phase was 1.0 ml min^{-1}. The effluents were monitored at 254 nm by a photodiode array detector.

Identification of the HSCCC peak fraction was performed 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

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–acetonitrile–water, etc. The results indicated that when methanol–acetonitrile–water was used as the mobile phase in gradient mode (15:38:47 in 0–22 min; 45:38:17 in 22–65 min), good separation results could be obtained. The crude sample and peak fractions separated by HSCCC were analyzed by HPLC under the optimum conditions. The chromatograms were shown in Fig. 2. The content of evodiamine, rutaecarpine, evocarpine, 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1*H*)-quinolone and 1-methyl-2-dodecyl-4-(1*H*)-quinolone in the crude extracts was 16.56%, 12.62%, 13.71%, 9.31% and 6.93%, respectively.

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. Ethyl acetate–water, ethyl acetate–methanol–water, and *n*-hexane–ethyl acetate–methanol–water were tested as the two-phase solvent system. When ethyl acetate–water was used as the two-phase solvent system, the target compounds mainly partitioned in the upper phase. By adding methanol to ethyl acetate–water, the partition could be improved. But the phase separation could not be achieved when the amount of methanol is high (ethyl acetate–methanol–water (5:4:5, v/v)). So, ethyl acetate–water and ethyl acetate–methanol–water were unsuitable for separation and purification of the alkaloids in Wu-zhu-yu. The addition of *n*-hexane to ethyl acetate–methanol–water system could improve the phase separation. The partition coefficients of the target compounds in *n*-hexane–ethyl acetate–methanol–water two-phase solvent systems were determined by HPLC as given in Section 2.4 and the results were shown in Table 1. In

HSCCC separation, the ideal *K*-values are better in the range of 0.5–5. If the *K*-values are too bigger than 5, the separation time will be too long and the HSCCC peak will be extended seriously. According to the *K*-values shown in Table 1, *n*-hexane–ethyl acetate–methanol–water (5:5:6:5, v/v) and *n*-hexane–ethyl acetate–methanol–water (5:5:7:5, v/v) were tested in HSCCC separation. When *n*-hexane–ethyl acetate–methanol–water (5:5:6:5, v/v) was used as the two-phase solvent system, the separation time for 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1*H*)-quinolone and

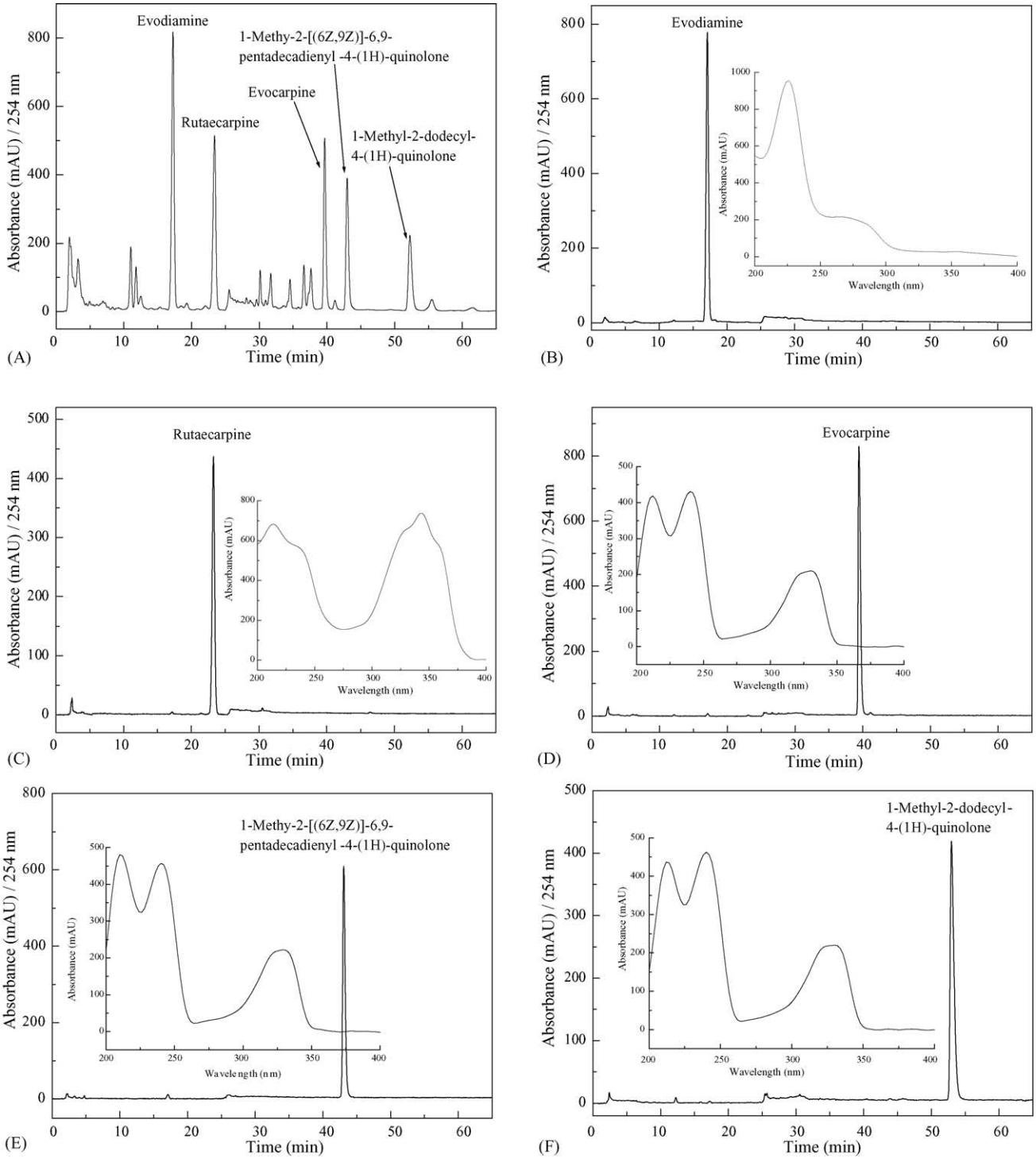

Fig. 2. HPLC chromatograms of crude extract from Wu-zhu-yu and HSCCC peak fractions. Column: SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm); mobile phase: methanol–acetonitrile–water (15:38:47 in 0–22 min; 45:38:17 in 22–65 min); flow rate: 1.0 ml min^{-1}; detection wavelength: 254 nm; (A) crude extract from Wu-zhu-yu; (B)–(F) peaks I–V in Fig. 3.

Table 1
The *K*-values of the target components in *n*-hexane–ethyl acetate–methanol–water solvent systems

Solvent system (v/v)	*K*-values				
	(**I**)	(**II**)	(**III**)	(**IV**)	(**V**)
5:5:5:5	3.05	6.32	9.21	11.93	14.05
5:5:6:5	1.90	3.60	5.26	6.95	8.72
5:5:7:5	1.09	2.23	3.21	4.35	5.60

1-methyl-2-dodecyl-4-(1*H*)-quinolone were too long. When *n*-hexane–ethyl acetate–methanol–water (5:5:7:5, v/v) was used as the two-phase solvent system, good separation results could be obtained and the separation time was acceptable.

Other conditions such as the revolution speed of the separation column, the flow rate of the mobile phase, and the temperature were also investigated. When the flow rate of 2.0 ml min^{-1}, revolution speed of 900 rpm and the separation temperature of 25 °C were employed in HSCCC separation, the retention percentage of the stationary phase could still be kept at 50% when the stationary and mobile phases were pumped into the column at the volume ratio of 50:50. The crude samples from Wu-zhu-yu were separated and purified under the optimum HSCCC conditions. The typical HSCCC chromatogram was shown in Fig. 3. Five kinds of alkaloids were obtained in one-step separation and yielded 28 mg of evodiamine (**I**), 19 mg of rutaecarpine (**II**), 21 mg of evocarpine (**III**), 16 mg of 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1*H*)-quinolone (**IV**) and 12 mg of 1-methyl-2-dodecyl-4-(1*H*)-quinolone (**V**) from 180 mg of crude sample. The recovery of these compounds was 93.9%, 83.6%, 85.1%, 95.5% and 96.2%, respectively. The purity was 98.7%, 98.4%, 96.9%, 98.0%, 97.2%, respectively, as determined by HPLC. The chromatograms of HPLC and UV spectra of these compounds were shown in Fig. 2.

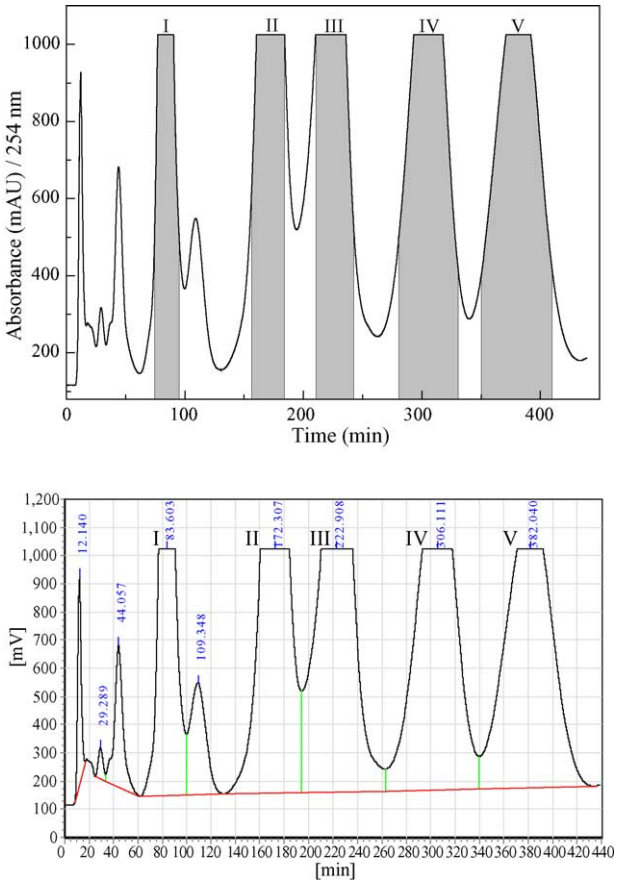

Fig. 3. HSCCC chromatogram of crude extract from Wu-zhu-yu. Two-phase solvent system: *n*-hexane–ethyl acetate–methanol–water (5:5:7:5, v/v); mobile phase: the lower phase; flow rate: 2.0 ml min^{-1}; revolution speed: 900 rpm; detection wavelength: 254 nm; sample size: 180 mg of crude sample dissolved in 2 ml of the upper phase and 2 ml of the lower phase; separation temperature: 25 °C; retention percentage of the stationary phase: 50%.

3.3. The structural identification

The chemical structure of each peak fraction of HSCCC was identified according to its ^{1}H NMR data and ^{13}C NMR data.

Peak I: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.54 (3H, s, N–CH_3), 3.01 (2H, m, H-6), 3.32, 4.90 (1H, each, m, H-5), 5.95 (1H, s, H-3), 7.16–8.17 (8H, m, Ar-H), 8.34(1H, br, N–H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 125.3 (C-2), 68.4 (C-3), 39.1 (C-5), 19.3 (C-6), 117.8 (C-7), 121.9 (C-8), 117.8 (C-9), 121.9(C-10), 118.7 (C-11), 110.8 (C-12), 136.4 (C-13), 150.1 (C-15), 122.3 (C-16), 132.4 (C-17), 128.4 (C-18), 128.2 (C-19), 120.3 (C-20), 164.5 (C-21), 36.4 (N–CH_3). Compared with the data given in [6], peak I corresponded to evodiamine.

Peak II: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.25 (2H, t, H-6), 4.63 (2H, t, H-5), 7.12–7.18 (7H, m, Ar-H), 8.33–8.36 (1H, m, Ar-H), 9.64 (1H, br, s, N–H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 127.7 (C-2), 145.5 (C-3), 41.7 (C-5), 20.1 (C-6), 118.7 (C-7), 121.7 (C-8), 120.4 (C-9), 121.0 (C-10), 120.4 (C-11), 112.6 (C-12), 138.8 (C-13), 147.7 (C-15), 126.0 (C-16), 134.9 (C-17), 127.1 (C-18), 126.8 (C-19), 121.0 (C-20), 162.0 (C-21). Compared with the data given in [7], peak II corresponded to rutaecarpine.

Peak III: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.45 (1H, dd, J = 1.6, 8.0 Hz, H-5), 7.67 (1H, m, H-7), 7.51 (1H, d, J = 8.0 Hz, H-8), 7.38 (1H, m, H-6), 6.26 (1H, s, H-3), 5.37 (2H, m, H-8′, -9′), 3.75 (3H, s, N–CH_3), 2.74 (2H, t, J = 8.0 Hz, H-1′), 2.02 (4H, m, H-7′, -10′), 1.70 (2H, m, H-2′), 1.25–1.46 (12H, m, H-3′, -4′, -5′, -6′, -11′, -12′), 0.93 (3H, t, J = 7.0 Hz, H-13′). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 154.9 (C-2), 111.1 (C-3), 177.9 (C-4), 126.7 (C-4a), 126.5 (C-5), 123.4 (C-6), 132.1 (C-7), 115.3 (C-8), 141.9 (C-8a), 34.8 (N–CH_3), 34.1 (C-1′), 28.6 (C-2′), 29.2 (C-3′), 29.2 (C-4′), 29.6 (C-5′), 29.1 (C-6′), 27.1 (C-7′), 129.6 (C-8′), 130.0 (C-9′), 26.9 (C-10′), 31.9 (C-11′), 22.3 (C-12′), 14.0 (C-13′). Compared with the data given in [8], peak III corresponded to evocarpine.

Peak IV: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.45 (1H, dd, J = 1.6, 8.0 Hz, H-5), 7.66 (1H, m, H-7), 7.51 (1H, d, J = 8.0 Hz, H-8), 7.38 (1H, m, H-6), 6.25 (1H, s, H-3), 5.40

(4H, m, H-6′, -7′, -9′, -10′), 3.74 (3H, s, N–CH_3), 2.70 (4H, m, H-1′, -8′), 2.08 (4H, m, H-5′, -11′), 1.40 (12H, m, H-2′, -3′, -4′, -12′, -13′, -14′), 0.89 (3H, t, J = 6.8 Hz, H-15′). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 154.6 (C-2), 111.1 (C-3), 177.8 (C-4), 126.6 (C-4a), 126.4 (C-5), 123.3 (C-6), 132.0 (C-7), 115.3 (C-8), 141.8 (C-8a), 34.1 (N–CH_3), 34.7 (C-1′), 28.5 (C-2′), 28.9 (C-3′), 29.3 (C-4′), 27.0 (C-5′), 129.4 (C-6′), 127.6 (C-7′), 25.6 (C-8′), 128.5 (C-9′), 130.3 (C-10′), 27.1 (C-11′), 29.3 (C-12′), 31.4 (C-13′), 22.5 (C-14′), 14.0 (C-15′). Compared with the data given in [9], peak IV corresponded to 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1*H*)-quinolone.

Peak V: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.45 (1H, dd, J = 1.6, 8.0 Hz, H-5), 7.67 (1H, m, H-7), 7.52 (1H, d, J = 8.0 Hz, H-8), 7.38 (1H, m, H-6), 6.26 (1H, s, H-3), 3.75 (3H, s, N–CH_3), 2.73 (2H, t, J = 7.8 Hz, H-1′), 1.68 (2H, m, H-2′), 1.42 (2H, m, H-3′), 1.25–1.33 (16H, m, H-4′–H-11′), 0.88 (3H, t, J = 6.8 Hz, H-12′). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 155.8 (C-2), 111.1 (C-3), 177.9 (C-4), 126.5 (C-4a), 126.7 (C-5), 123.4 (C-6), 132.1 (C-7), 115.3 (C-8), 141.9 (C-8a), 34.1 (N–CH_3), 34.8 (C-1′), 28.6 (C-2′), 29.3 (C-3′), 29.3 (C-4′), 29.3 (C-5′), 29.4 (C-6′), 29.5 (C-7′), 29.6 (C-8′), 29.6 (C-9′), 31.9 (C-10′), 22.6 (C-11′), 14.1 (C-12′). Compared with the data given in [16], peak V corresponded to 1-methyl-2-dodecyl-4-(1*H*)-quinolone.

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