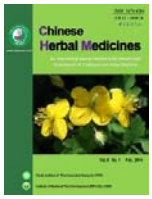

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Original article

Separation of Five Quinolone Alkaloids from Fruits of *Evodia rutaecarpa* by High-speed Counter-current Chromatography

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ABSTRACT

Objective To develop a suitable method for the large-scale separation of quinolone alkaloids from the fruits of *Evodia rutaecarpa* by high-speed counter-current chromatography (HSCCC). **Methods** Two solvent systems were developed for the separation method. The upper phase was used as the stationary phase, and the lower phase was used as the mobile phase at 35 °C with the flow rate of 2 mL/min and rotation speed of 855 r/min. **Results** Using the described method, 1-methyl-2-undecyl-4(1H)-quinolone (**1**), evocarpine (**2**), 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentade-cadienyl-4-(1H)-quinolone (**3**), dihydroevocarpine (**4**), and the mixture (**5**) of 1-methyl-2-[(*Z*)-10-pentadecenyl]-4(1H)-quinolone (V_a) and 1-methyl-2-[(*Z*)-6-pentadecenyl]-4(1H)-quinolone (V_b) could be isolated from a petroleum ether extract. They were identified by ^{1}H-NMR, ^{13}C-NMR, and MS/MS, and the purities were 94.3%, 95.2%, 96.8%, 98.3%, and 96.8%, respectively. **Conclusion** Five quinolone alkaloids from the fruits of *E. rutaecarpa* could be systematically isolated and purified using HSCCC. The presented method is simple and efficient with good potentials on the preparation of reference substances, especially on the quality control of Chinese materia medica.

Key words

dihydroevocarpin; evocarpine; *Evodia rutaecarpa*; high-speed counter-current chromatography; quinolone alkaloids

1. Introduction

The dried fruits of *Evodia rutaecarpa* (Juss.) Benth. (*Wuzhuyu* in Chinese) have been used as Chinese materia medica (CMM) for more than 2000 years and is officially listed in *Chinese Pharmacopoeia 2010* (Pharmacopeia Committee of P. R. China). Alkaloids, containing indole-quinazoline and quinolone alkaloids, are the major active compounds in the fruits of *E. rutaecarpa*.

Recently, some studies indicated that quinolone alkaloids from the fruits of *E. rutaecarpa* had pharmacological effects, including considerable inhibitory effect on

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leukotriene biosynthesis in human granulocytes (Adams et al, 2000) and highly selective antibacterial activity against *Helicobacter pylori* (Hamasaki et al, 2000). It was found that evocarpine (1-methyl-2-[(4*Z*,7*Z*)-4,7-tridecadienyl]-4(1H)-quinolone) and 1-methyl-2-[(6*Z*,9*Z*)-6,9-pentade-cadienyl]-4(1H)-quinolone as blockers of angiotensin II receptor could modulate the blood pressure (Lee et al, 1998). In addition, nine quinolone alkaloids with the inhibitory activity against nuclear factor of activated T cells have also been reported (Jin et al, 2004). Therefore, the separation and analysis of quinolone alkaloids were necessary and of great interest. Moreover, the significance of this report is not only to propose a general procedure of successful isolation and purification of quinolone alkaloids, but also to provide several pure compounds as "marker compounds" to control the quality and to search for the bioactive principles of the herbal products.

Generally speaking, quinolone alkaloids could be isolated from the herbal medicines by using some traditional methods, such as decoction, maceration, percolation, and ultrasonic extraction, followed with separation and purification by silica gel column chromatography (Tang et al, 1996; Wang and Liang, 2004). However, the traditional isolation methods were time-consuming and required the relatively large quantities of polluting solvents, and the purification methods used solid support matrix, easily resulting in irreversible adsorptive sample loss and deactivation. High-speed counter-current chromatography (HSCCC) is a kind of support-free all-liquid partition chromatography which was first invented by Ito (2005) (Han et al, 2010; Hu et al, 2013). The successful application of HSCCC for the separation and purification of different types of natural compounds including flavanoids, organic acids, and alkaloids has been reported before (Chen et al, 2006; Guo et al, 2010; Liu et al, 2013; Tang et al, 2008; Zhao et al, 2012). The isolation of alkaloids from the fruits of *E. rutaecarpa* using HSCCC was reported. Liu et al separated evodiamine, rutaecarpine, and three kinds of quinolone alkaloids using HSCCC with the solvent system of *n*-hexane-ethyl acetate-methanol-water (5:5:7:5) (Liu et al, 2005). While Lu et al (2009) rapidly isolated alkaloids from the fruits of *E. rutaecarpa* using *n*-hexane-ethyl acetate-methanol-water (3:2:3:2), the study mainly focused on the comparison of elution-extrusion and back-extrusion counter-current chromatography, and no compound with enough purity was separated from the crude extract.

In this study, we employed a two-step HSCCC method for preparative separation of the five quinolone alkaloids from the fruit of *E. rutaecarpa*. The chemical structures of 1-methyl-2-undecyl-4(1H)-quinolone (**1**, 25.9 mg), evocarpine (**2**, 66.7 mg), 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1H)-quinolone (**3**, 29.2 mg), dihydroevocarpine (**4**, 28.0 mg), and the mixture (**5**, 6.8 mg) of 1-methyl-2-[(*Z*)-10-pentadecenyl]-4(1H)-quinolone (V_a) and 1-methyl-2-[(*Z*)-6-pentadecenyl]-4(1H)-quinolone (V_b) are shown in Figure 1. Among them, compounds **1** and **4** were successfully separated by HSCCC for the first time.

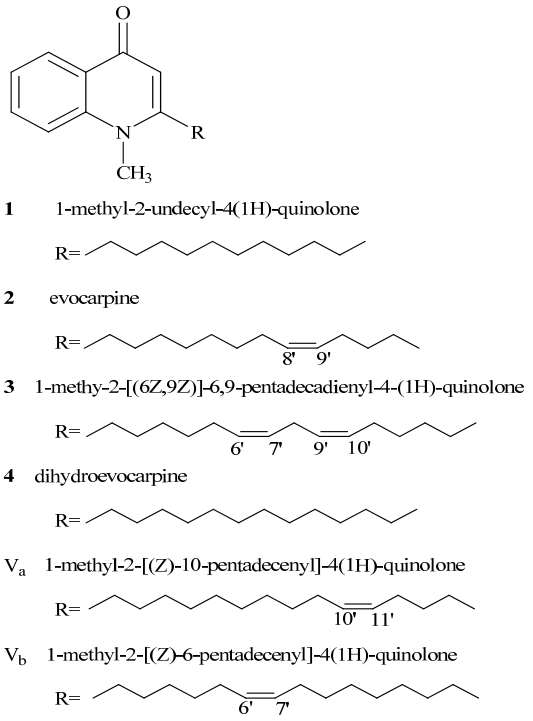

Figure 1 Chemical structures of quinolone alkaloids from fruits of *E. rutaecarpa*

2. Materials and methods

2.1 Reagents and materials

All organic solvents used for the preparation of crude sample and HSCCC separation were of analytical grade and purchased from Jinan Reagent Factory (China).

The acetonitrile of chromatographic grade used for HPLC analysis was purchased from Tedia Co., Inc. (Beijing, China). Distilled water was used throughout the experiment. The dried fruits of *Evodia rutaecarpa* (Juss.) Benth were collected from Hunan province and identified by Prof. Lin Jiang (School of Pharmaceutical Sciences, Sun Yat-sen University). The contents of essential active ingredients were accorded with the requirement of *Chinese Pharmacopoeia 2010* (Pharmacopeia Committee of P. R. China).

2.2 Apparatus

HSCCC was performed using an Mk5 QuikPrep 500 HSCCC Unit (AECS-QuilkPrep Ltd., Britain) with a series of four multilayer coil separation columns (250 mL, 2.16 mm) and a 10 mL sample loop. The revolution radius was 10 cm, and the β values of the preparative column ranged from 0.5 (at the internal terminal) to 0.8 (at the external terminal). The HSCCC system was equipped with Series II Constant Pump (Scientific System Co., USA), Shimadzu UV Absorbance Detector (Hangzhou, China) to monitor the effluent, and N2000 Chromatography Workstation (Zheda Information Project Co., China) to collect the data. The revolution speed of the apparatus could be adjusted from 0 to 860 r/min.

The HPLC system was Waters System, consisting of 1525 Quat Pump, 717 Auto Sampler, 2996 UV-vis Photodiode Array Detector, and Empower Workstation. The chromatographic separations were carried out on a Hypersil BDS C_{18} column (200 mm × 4.6 mm, 5 μm) protected by a guard column (4.0 mm × 3.0 mm, 5 μm).

The LC-MS/MS system consisted of Surveyor MS Pump, Surveyor Autosampler (Thermo Finnigan, USA) and Thermo Finnigan TSQ Quantum Triple Quadrupole Mass Spectrometer (San Jose, USA) equipped with electrospray ionization (ESI) source. Data acquisition was performed with Xcalibur 1.3 software (Thermo Finnigan, USA). The nuclear magnetic resonance (NMR) spectrometer used here was Bruker Avance III 400 MHz Spectrometer (Bruker BioSpin, Germany).

2.3 Preparation of crude extract and sample solution

The dried fruits of *E. rutaecarpa* were ground into powder (about 30 mesh). The powder (75 g) was dipped into 750 mL of anhydrous ethanol for 24 h, and then extracted in an ultrasonic bath (35 ^{o}C, 40 Hz) for 1 h. The extraction procedure was repeated for three times. The paper-filtrated extracts were combined and evaporated to dryness by rotary vaporization at 45 ^{o}C under vacuum, which yielded 5.16 g of dry powder.

The residues were then suspended in distilled water (100 mL) and extracted with light petroleum ether (bp 60–90 ^{o}C, 300 mL, for six times). After the petroleum ether layer was concentrated to dryness, 1.9 g of extract was obtained.

Then the petroleum ether extracts were added into a separation funnel which contained 125 mL of *n*-hexane-ethyl acetate-methanol-water (4:1:4:1) mixture. After shaking repeatedly, the lower phase was separated and evaporated to dryness to yield dried crude extract (0.87 g), which was stored in a refrigerator (4 ^{o}C) for the subsequent HSCCC separation. The sample for HSCCC separation was prepared by dissolving 500 mg of crude extract in 5 mL of the upper phase and 5 mL of the lower phase.

2.4 Preparation of two-phase solvent system

Using *n*-hexane-ethyl acetate-methanol-water (3:2:3:2) as the two-phase solvent system for HSCCC separation. After the mixed solvent was thoroughly equilibrated in a separation funnel at room temperature, the two phases were separated shortly and degassed by sonication for 30 min prior to use.

2.5 HSCCC separation procedure

The HSCCC was performed as follows: the multilayer column was first entirely filled with the upper phase using the Series II constant pump. Then the apparatus was rotated at 855 r/min, while the lower phase was pumped into the column at a flow rate of 2 mL/min when the revolution velocity was smooth. After the mobile phase was emerged, indicating the establishment of hydrodynamic equilibrium, 10 mL of sample solution containing 500 mg of crude extract was injected into the column through the injection loop. The separation temperature was controlled at 35 ^{o}C. The effluent from the tail end of the column was continuously monitored by a UV detector at 335 nm and the chromatogram was recorded simultaneously. Each peak fraction was manually collected according to the chromatographic data and evaporated to dryness under reduced pressure. After the separation, the residuals were dissolved in methanol for subsequent purity analysis by HPLC.

2.6 HPLC analysis and identification of target compounds

The crude extract and each HSCCC peak fraction were analyzed by HPLC. The analysis was accomplished with a Hypersil BDS C_{18} column (200 mm × 4.6 mm, 5 μm) at 25 ^{o}C. Acetonitrile-water system was used as mobile phase in a linear gradient mode as follows: acetonitrile 0–30 min, 40%–50%; 30–35 min, 50%–75%; 35–55 min, 75%–80%; 55–60 min, 80%. The flow-rate of the mobile phase was 1.0 mL/min. The effluents were monitored at 225 nm by a photodiode array detector. The purities of the collected fractions were determined by HPLC based on the peak area of the target species normalized to the sum of all observed peaks. Further identification of HSCCC target fractions was carried out using UV spectrum, ^{1}H-NMR, ^{13}C-NMR, and MS/MS.

3. Results and discussion

3.1 Optimization of HPLC conditions

To obtain good separation results, several elution systems were tested in HPLC separation of sample extract, and the composition and ratios of mobile phase were discussed. When acetonitrile-water was used as the mobile phase in linear gradient mode, the extract was separated efficiently under the optimized conditions. The concentration of compounds **1**–**5** in sample A was 9.6%, 15.8%, 11.3%, 5.9%, and 3.7%, respectively.

3.2 Optimization of two-phase solvent system and other conditions of HSCCC

Since HSCCC is a liquid-liquid partition separation method, the selection of suitable solvent system is the first and most important step in performing preparative HSCCC. The partition coefficient (*K*) is one of the most important parameters in solvent system selection, which should be in the range of 0.5–5 to get an efficient separation and suitable running time. Based on the chemical properties of target compounds with strong hydrophobic group, a series of experiments were undertaken and the *K* values were measured and summarized in Table 1.

Two-phase solvent systems with petroleum ether-methanol were tested first, resulting in small *K* values and poor retention of target compounds in the upper phase. Then,

Table 1 Partition coefficient (*K*) of target components and rutaecarpine in different solvent systems

No.	Solvent systems	Volume ratios	*K*					
			Rutaecarpine	**1**	**2**	**3**	**4**	**5**
1	*n*-hexane-ethanol-water	6:3:1	5.77	12.78	8.27	6.89	5.75	4.71
2	*n*-hexane-ethanol-water	5:4:2	5.79	8.92	4.67	2.62	2.59	1.62
3	*n*-hexane-ethanol-water	5:3:1	3.05	12.62	6.96	5.77	4.94	3.85
4	*n*-hexane-ethanol-water	5:3:2	3.03	3.29	2.01	1.40	1.05	0.15
5	*n*-hexane-ethyl acetate-methanol-water	1:1:1:1	5.72	8.34	10.38	15.26	19.88	21.05
6	*n*-hexane-ethyl acetate-methanol-water	1:1:1.6:1	3.14	3.93	3.71	6.32	7.92	8.23
7	*n*-hexane-ethyl acetate-methanol-water	1:1:2:1	2.63	2.54	3.02	4.10	5.43	5.65
8	*n*-hexane-ethyl acetate-methanol-water	4:3:4:3	2.56	2.50	3.34	4.59	6.02	8.95
9	*n*-hexane-ethyl acetate-methanol-water	11:8:11:8	2.17	2.11	2.77	3.91	4.89	6.60
10	*n*-hexane-ethyl acetate-methanol-water	3:2:3:2	1.68	1.55	2.08	2.95	3.69	5.41
11	*n*-hexane-ethyl acetate-methanol-water	7:4:7:4	1.32	0.93	1.52	1.98	2.36	6.73
12	*n*-hexane-ethyl acetate-methanol-0.1% formic acid	3:2:3:2	2.51	1.85	2.85	4.32	4.67	8.28
13	*n*-hexane-ethyl acetate-methanol-0.2% formic acid	3:2:3:2	1.92	1.56	2.11	3.09	3.43	5.47

the two-phase solvent systems comprising *n*-hexane-ethanol-water (the upper phase used as mobile phase and the lower phase as solid phase) were tested. Although appropriate *K* values were obtained in the ratio of 5:3:2, it was difficult to separate the target compounds from each other because of the low resolution and poor UV detection. Subsequently the solvent systems composed of *n*-hexane-ethyl acetate-methanol-water were used. However, the resulting *K* values were too high (larger than 5), and the long time that the target compounds required for elution resulted in poor resolution. The *K* values could be lessened to some degree by increasing the volume ratio of *n*-hexane and/or methanol. When the volume ratio of *n*-hexane and methanol was increased at the same time, such as 3:2:3:2, better *K* values were got, and finally *K* values were in the suitable range of 0.5–5, though the retention time was a little bit long to be suitable for ordinary separation.

However, from Table 1, it was clear that the polarities of rutaecarpine and compound **1** were similar; for good separation, the partition coefficient ($\alpha = K_1/K_2$, $K_1 > K_2$) ought to be greater than 1.5 in the semi-preparative HSCCC. According to the predicted pK_a values of them, addition of formic acid would improve α value, so the suitable solvent system for the separation of compound **1** could be No. 12 solvent system (3:2:3:2). But using No. 12 solvent system, the *K* values of rutaecarpine and compound **2** were nearly the same, which indicated that they would hardly be separated from each other. As a result, there was no possibility for the separation and purification of these compounds by using a single two-phase solvent system.

For the above difficulties, the separation could be successful only by using two steps and two solvent systems. No. 10 solvent system (3:2:3:2) was chosen as the optimal solvent system for the first step of HSCCC separation of compounds **2**–**5**.

Other conditions such as the revolution speed of separation column, the flow rate of mobile phase, and the temperature were also investigated to improve the retention of the stationary phase. The results indicated that reducing the flow rate and increasing the revolution speed could improve the retention of the stationary phase leading to better resolution. When the flow rate of 2.0 mL/min, revolution speed of 855 r/min, and the separation temperature of 35 ^{o}C were employed in HSCCC separation, the retention percentage of the stationary phase could still be kept at 84%. The sample solution was separated and purified under the optimal HSCCC conditions. The typical HSCCC chromatogram is shown in Figure 2.

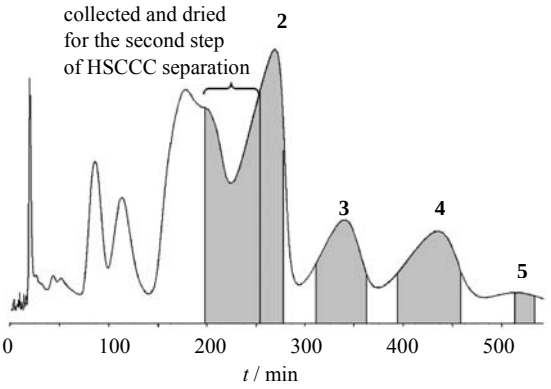

Figure 2 HSCCC of sample A

3.3 HSCCC separation

Because rutaecarpine was a mainly interference to separate compound **1**, the fractions (198–254 min) in the first HSCCC separation, which did not contain rutaecarpine, were collected and yielded sample B (60 mg) for the second step of HSCCC separation. The HPLC of sample B shown in Figure 3A indicated that sample B contained compounds **1** and **2**, and an unknown quinolone alkaloid. To improve the purity of compound **1**, several changes had been made to the existing solvent system. From the data in Table 2, the solvent system with better α value (close to 1.5) and shorter retention time composed of *n*-hexane-ethyl acetate-methanol-water (12:8:11:8) was used for the second step HSCCC separation. The chromatogram of HSCCC separation is shown in Figure 3B. Under this solvent system, 1-methyl-2-undecyl-4(1H)-quinolone was primarily separated from evocarpine.

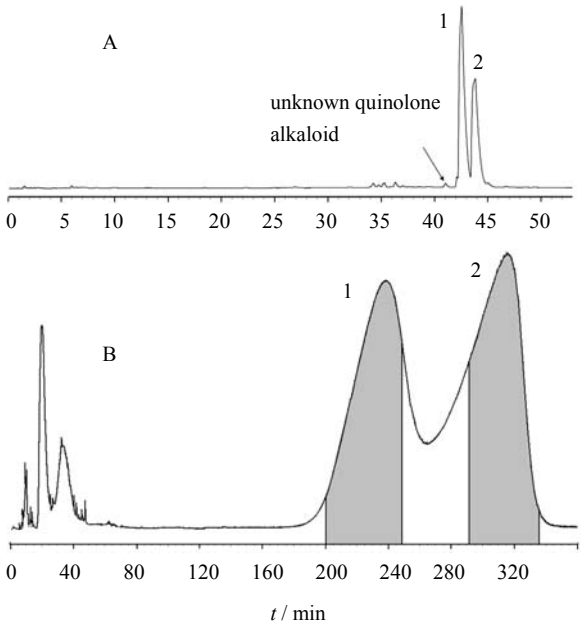

Figure 3 HPLC (A) and HSCCC (B) of sample B

1: 1-methyl-2-undecyl-4(1H)-quinolone 2: evocarpine

Table 2 Partition coefficient (*K*) and separation factor (*α*) of target components in *n*-hexane-ethyl acetate-methanol-water solvent systems

Volume ratios	*K*			*α*	
	Unkown	**1**	**2**	K_{unkomn}/K_1	K_2/K_1
11:8:12:8	1.67	1.26	1.78	1.33	1.41
12:8:11:8	3.30	2.24	3.05	1.47	1.36
12:9:12:8	2.14	1.76	2.33	1.21	1.32
12:8:12:9	2.90	2.24	3.25	1.30	1.45

Five kinds of alkaloids were obtained in the two-step separation and 25.9 mg of compound **1**, 66.7 mg of compound **2** (36.9 mg from the first step and 29.8 mg from the second step), 29.2 mg of compound **3**, 28.0 mg of compound **4,** and 6.8 mg of compound **5** from 500 mg of crude sample were yielded. The recoveries of these compounds were 54.0%, 84.2%, 51.5%, 95.1%, and 36.8%, respectively. The purities were 94.3%, 95.2%, 96.8%, 98.3%, and 96.8%, respectively, as determined by HPLC.

In the present study, we used a two-step separation method to separate quinolone alkaloids from *E. rutaecarpa*. Though the recovery of compound **1** was lower than 55%, the turnout was acceptable. Moreover, the result of this research was different from that of other study (Liu et al, 2005). Instead of compound **1**, compound **4** was found in the crude extract of *E. rutaecarpa* and separated successfully. This might be caused by the different sources of *E. rutaecarpa* used in the experiment. Among the products, since compound **3** was unstable to heat, the temperature should be controlled under 60 ^{o}C. Due to the similar chemical structures of V_a and V_b, it was impossible to separate them by HSCCC, and the traditional method could be used, such as silica gel column chromatography.

3.4 Identification of separation peaks

The chemical structures of peak fractions separated by HSCCC were identified according to their UV, ESI-MS, ^{1}H-NMR, and ^{13}C-NMR data. The data of each peak fraction were as follows.

Compound **1**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.44 (1H, dd, J = 1.5, 8.0 Hz, H-5), 7.66 (1H, m, H-7), 7.49 (1H, d, J = 8.0 Hz, H-8), 7.37 (1H, m, H-6), 6.23 (1H, s, H-3), 3.73 (3H, s, N-CH_3), 2.70 (2H, t, J = 7.8 Hz, H-1′), 1.67 (2H, m, H-2′), 1.51–1.19 (14H, m, H-4′–H-10′), 0.88 (3H, t, J = 6.7 Hz, H-11′). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 177.8 (C-4), 154.8 (C-2), 141.9 (C-8a), 132.0 (C-7), 126.6 (C-5), 126.5 (C-4a), 123.3 (C-6), 115.3 (C-8), 111.1 (C-3), 34.8 (C-1′), 34.1 (N-CH_3), 31.9 (C-9′), 29.6 (C-3′), 29.5 (C-4′), 29.3 (C-4′), 29.3 (C-4′), 28.6 (C-2′), 22.7 (C-10′), 14.1 (C-11′). Compared with the data reported (Tang et al, 1996), compound **1** corresponded to 1-methyl-2-undecyl-4(1H)-quinolone.

Compound **2**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.41 (1H, dd, J = 1.5, 7.8 Hz, H-5), 7.63 (1H, m, H-7), 7.48 (1H, d, J = 8.0 Hz, H-8), 7.33 (1H, m, H-6), 6.19 (1H, s, H-3), 5.35 (2H, m, H-8′, -9′), 3.70 (3H, s, N-CH_3), 2.67 (2H, t, J = 8.0 Hz, H-1′), 2.02 (4H, m, H-7′, -10′), 1.65 (2H, m, H-2′), 1.20–1.44 (12H, m, H-3′, -4′, -5′, -6′, -11′, -12′), 0.90 (3H, t, J = 6.8 Hz, H-13′). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 177.8 (C-4), 154.8 (C-2), 141.9 (C-8a), 132.0 (C-7), 130.0 (C-9′), 129.6 (C-8′), 126.5 (C-4a), 126.4 (C-5), 123.3 (C-6), 115.4 (C-8), 111.0 (C-3), 34.7 (N-CH_3), 34.1 (C-1′), 31.9 (C-11′), 29.6 (C-5′), 29.2 (C-3′), 29.2 (C-4′), 29.1 (C-6′), 28.5 (C-2′), 27.1 (C-7′), 26.9 (C-10′), 22.3 (C-12′), 14.0 (C-13′). Compared with the reference data (Tang et al, 1996; Liu et al, 2005), compound **2** corresponded to evocarpine.

Compound **3**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.41 (1H, dd, J = 1.5, 8.0 Hz, H-5), 7.64 (1H, m, H-7), 7.50 (1H, d, J = 8.0 Hz, H-8), 7.35 (1H, m, H-6), 6.24 (1H, s, H-3), 5.35 (4H, m, H-6′, -7′, -9′, -10′), 3.72 (3H, s, N-CH_3), 2.77 (4H, m, H-1′, -8′), 2.04 (4H, m, H-5′, -11′), 1.67 (s, 1H), 1.38 (12H, m, H-2′, -3′, -4′, -12′, -13′, -14′), 0.88 (3H, t, J = 6.7 Hz, H-15′). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 177.8 (C-4), 155.1 (C-2), 141.8 (C-8a), 132.2 (C-7), 130.3 (C-10′), 129.4 (C-6′), 128.5 (C-9′), 127.7 (C-7′), 126.5 (C-4a), 126.2 (C-5), 123.4 (C-6), 115.5 (C-8), 110.8 (C-3), 34.7 (C-1′), 34.2 (N-CH_3), 31.5 (C-13′), 29.6 (C-12′), 29.3 (C-4′), 28.9 (C-3′), 28.5 (C-2′), 27.2 (C-11′), 27.0 (C-5′), 25.6 (C-8′), 22.5 (C-14′), 14.0 (C-15′). Compared with the data reported (Liu et al, 2005), compound **3** corresponded to 1-methy-2-[(6*Z*,9*Z*)]-6,9-pentadecadienyl-4-(1H)-quinolone.

Compound **4**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.44 (1H, dd, J = 1.5, 7.9 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), 6.24 (1H, s, H-3), 3.74 (3H, s, N-CH_3), 2.73 (2H, t, J = 7.8 Hz, H-1′), 1.67 (2H, m, H-2′), 1.41 (2H, m, H-3′), 1.24–1.32 (18H, m, H-4′–H-12′), 0.88 (3H, t, J = 6.7 Hz, H-13′). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 177.8 (C-4), 154.9 (C-2), 141.9 (C-8a), 132.1 (C-7), 126.6 (C-5), 126.5 (C-4a), 123.3 (C-6), 115.3 (C-8), 111.1 (C-3), 34.8 (C-1′), 34.1 (N-CH_3), 31.9 (C-11′), 29.6 (C-10′), 29.6 (C-9′), 29.6 (C-8′), 29.6 (C-7′), 29.5 (C-6′), 29.3 (C-5′), 29.3 (C-4′),

29.3 (C-3′), 28.6 (C-2′), 22.7 (C-12′), 14.1 (C-13′). Compared with the reference data (Tang et al, 1996), compound **4** corresponded to dihydroevocarpine.

Compound **5**: The ^{1}H-NMR spectrum showed signals of a quinolone skeleton, such as N-methyl group at δ 3.77, conjugated olefinic proton at δ 6.31 (1H, s, 3H), aromatic proton at δ 8.46 (1H, dd, J = 1.5, 7.9 Hz, 5-H), 7.68 (1H, m, 7-H), 7.52 (1H, d, J = 8.0 Hz, 8-H), 7.40 (1H, m, 6-H). However, the ^{13}C-NMR spectrum indicated that it was a mixture of two quinolone alkaloids (V_a + V_b). The ESI-MS/MS spectrum showed $[M + H]^+$ at *m/z* 368 corresponding to the molecular formula $C_{25}H_{37}NO$, and two sets of fragments of *m/z* 324, 270 and *m/z* 268, 214 were observed which might be explained to be originated from two long aliphatic side chains with double bond at positions with four carbon difference. Compared with the reference data (Chu and Sheu, 1996), the compound corresponded to the mixture of 1-methyl-2-[(*Z*)-10-pentadecenyl]-4(1H)-quinolone (V_a) and 1-methyl-2-[(*Z*)- 6-pentadecenyl]-4(1H)-quinolone (V_b).

4. Conclusion

A two-step separation method is developed for the semi-preparative purification of quinolone alkaloids from *E. rutaecarpa* on HSCCC with two solvent systems, *n*-hexane-ethyl acetate-methanol-water (3:2:3:2 and 12:8:11:8). Five kinds of quinolone alkaloids are obtained from 500 mg extract with the purity over 94.3%. The presented method is simple and efficient. Since the lack of standard compounds becomes the main limitation on the research and development of natural products, this method has good potential on the preparation of standards, especially on the quality control of CMM.

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