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Preparative counter-current chromatography isolation of liensinine and its analogues from embryo of the seed of *Nelumbo nucifera* GAERTN. using upright coil planet centrifuge with four multilayer coils connected in series

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Abstract

Preparative counter-current chromatography (CCC) isolation of liensinine and its analogues, isoliensinine and neferine from embryo of the seed of *Nelumbo nucifera* GAERTN. has been successfully performed for the first time using upright coil planet centrifuge with four multilayer coils connected in series with 1600 mL capacity. Two kinds of two-phase solvent systems were applied to preparative CCC isolation. The first was the system composed of light petroleum (b.p. 60–90 °C)–ethyl acetate–tetrachloromethane–chloroform–methanol–water (1:1:4:4:6:2, v/v) which was very suitable for fast and small-scale CCC isolation. The second was the system composed of ethyl acetate–tetrachloromethane–methanol–water (1:6:4:1, v/v), which was the optimum for large-scale CCC isolation. Using the first system, 1102 mg of the crude alkaloid was purified in one-step separation of 150 min, yielding 350 mg neferine, 100 mg isoliensinine and 95 mg liensinine with over 95% purity. While using the second solvent system, 5850 mg of the crude alkaloid was purified in one-step separation of 9 h, yielding 2545 mg neferine, 698 mg isoliensinine and 650 mg liensinine with over 97% purity. Structures of the compounds were identified by electrospray ionization multiple mass spectrometry, one- and two-dimensional NMR.

Keywords: Counter-current chromatography; Preparative chromatography; *Nelumbo nucifera*; Plant materials; Pharmaceutical analysis; Liensinine; Isoliensinine; Neferine; Alkaloids

1. Introduction

Nelumbo nucifera GAERTN. is a perennial aquatic crop grown and consumed over the world, especially in India, China, Japan, Korea, South East Asia, Russia and some countries in Africa. It is not only as an ornamental plant, but also for a dietary staple in eastern Asia, particularly in China. Almost all parts of *N. nucifera* GAERTN., i.e., leaves, flowers, seeds and rhizomes, are utilized but rhizomes hold the largest market share. Moreover, these parts are popularly used in the oriental medicine for long times [1–3]. The embryo of the seed of *N. nucifera* GAERTN., a traditional Chinese drug "Lien Tze Hsin", is primarily used for nervous disorders, insomnia, high fevers with restlessness and hypertension. In the past, many studies are focused on the isolation and pharmacology of its alkaloids components [4–11].

Liensinine [4] and its analogues, isoliensinine [5] and neferine [6] (see Fig. 1), are three main phenolic alkaloids components of embryo of the seed of *N. nucifera* GAERTN. [12]. Since the first isolation of liensinine in 1962 [4], the three alkaloids have received considerable attention because of their reputation of chemical and biological properties. However, their pharmacological studies often suffer from the limits of sample purity and sources. To obtain pure compounds by conventional separation methods, such as column

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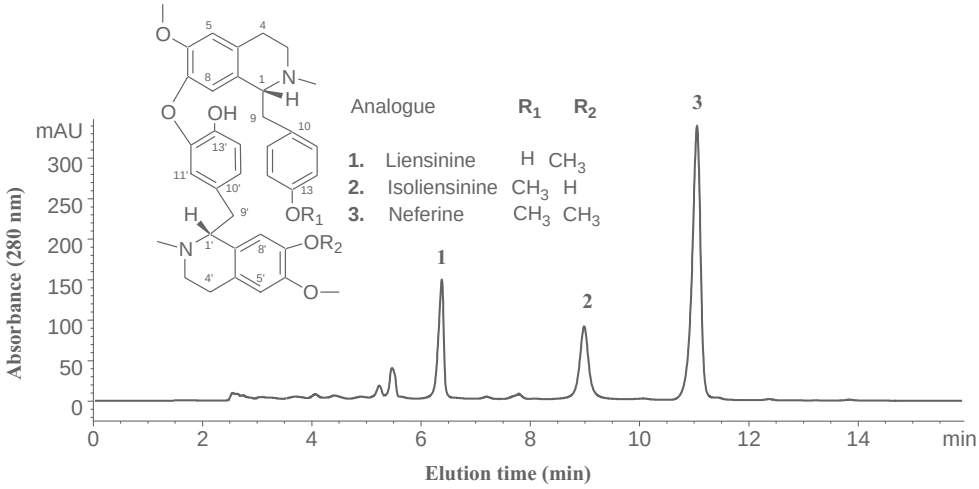

Fig. 1. HPLC analysis of crude alkaloids from the embryo of the seeds of *N. nucifera* GAERTN. as well as the structures of liensinine and its analogues: (**1**) liensinine, (**2**) isoliensinine, (**3**) neferine. Conditions—column, YMC-Pack ODS-A (150 mm × 4.6 mm i.d., 5 μm, 120 Å); column temperature, 25 °C; mobile phase, A (acetonitrile) and B (0.2% triethylamine aqueous solution) at the gradient: A from 40 to 80% and B from 60 to 20% for 15 min; flow rate, 0.8 mL/min; detection, 280 nm; sample concentration, 0.8 μg/μL; injection volume, 15 μL.

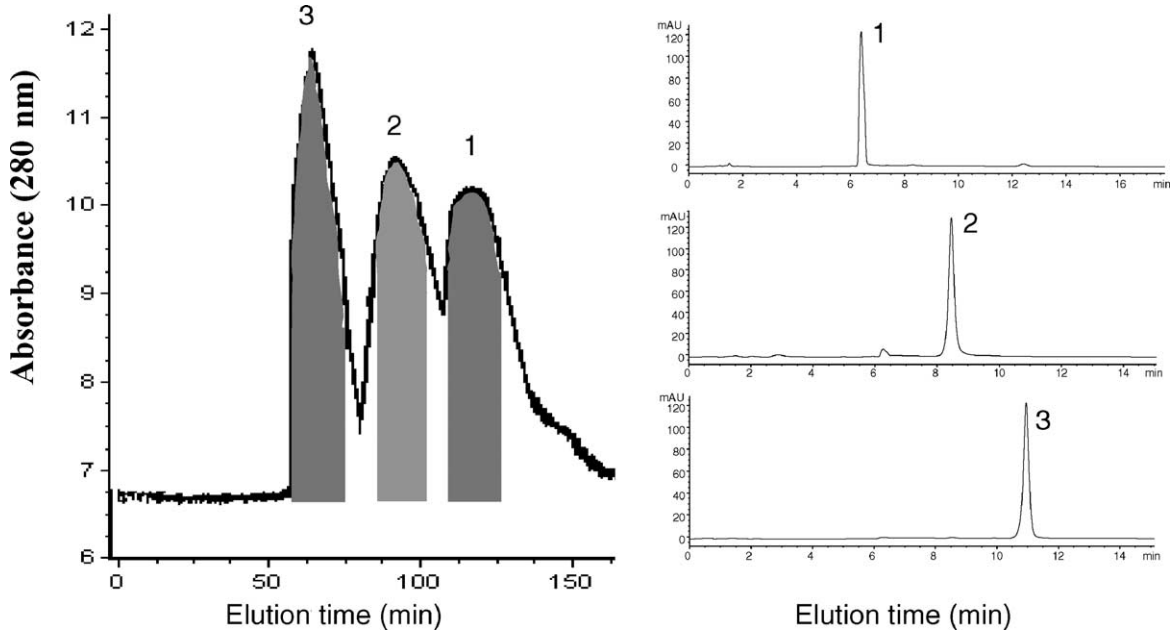

Fig. 2. Preparation CCC separation of the crude alkaloids from *N. nucifera* GAERTN. and HPLC analyses corresponding to the CCC peak fractions: (**1**) liensinine, (**2**) isoliensinine, (**3**) neferine. CCC separation conditions: column, multilayer coil of 4.0 mm i.d. PTFE tube with a total capacity of 1600 mL; rotary speed, 450 rpm; column temperature, 35 °C; solvent system, light petroleum (b.p., 60–90 °C)–ethyl acetate–tetrachloromethane–chloroform–methanol–water (1:1:4:4:6:1, v/v); mobile phase, lower phase; flow rate, 5 mL/min; detection, 280 nm; sample size, 1102 mg dissolved in 9 mL upper phase and 9 mL lower phase; retention of the stationary phase, 80.63%; purity: (**1**) 97.45%, (**2**) 95.32%, (**3**) 98.43%. For HPLC conditions, see Section 2.

chromatography (CC) and thin-layer chromatography (TLC) [6–9] is very difficult because of their structure similarity and unstable chemical properties. Counter-current chromatography (CCC) is a unique liquid–liquid partition chromatography without use of solid support matrix [13]. Therefore, it eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks, and contamination. The method has been successfully applied to the analysis and separation of various natural and synthetic products [14–16]. Recently, we have developed a versatile type-J CCC with four upright multilayer coil columns arranged symmetrically around the centrifuge axis [17]. Our primary experiments have demonstrated the upright CCC apparatus is very useful

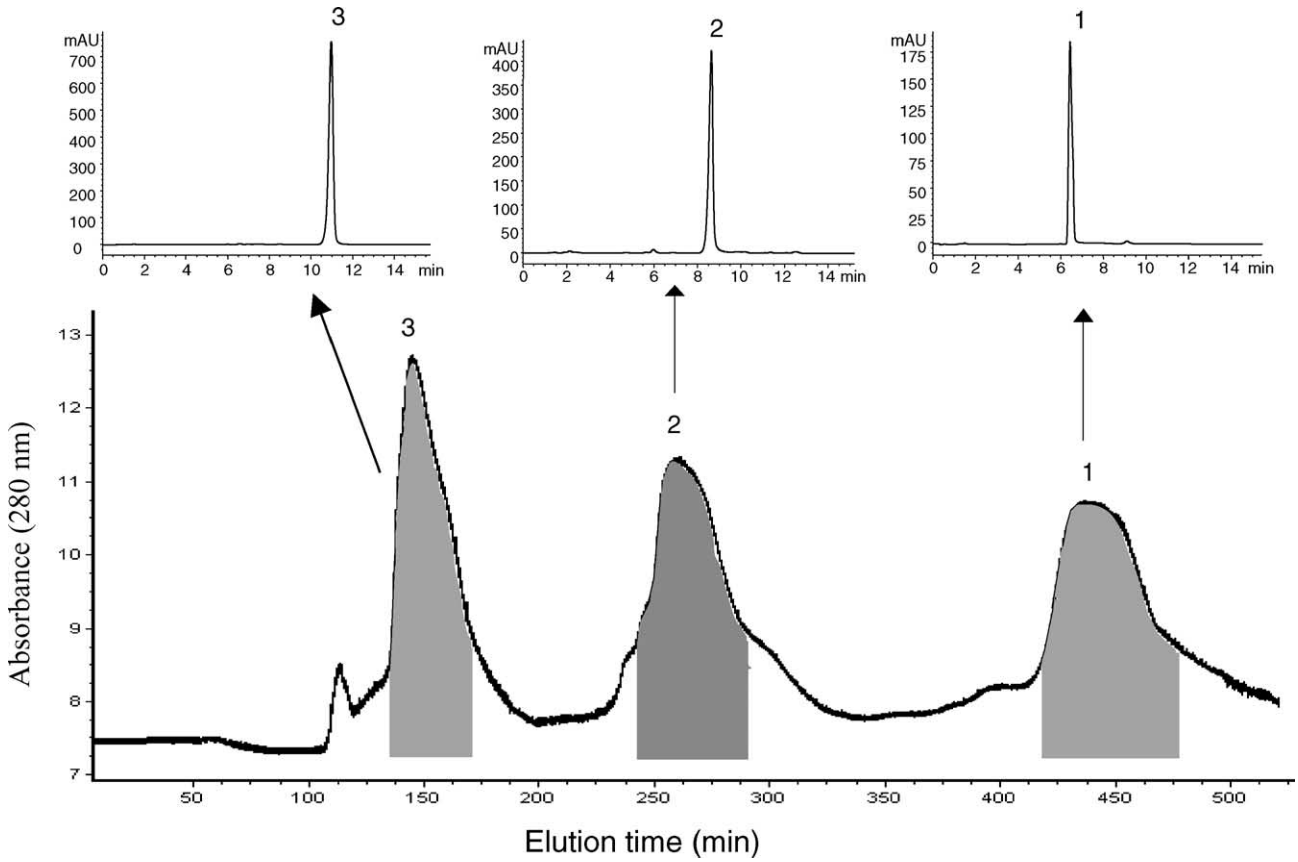

Fig. 3. Preparation CCC separation of the crude alkaloids from *N. nucifera* GAERTN. and HPLC analyses corresponding to the CCC peak fractions: (**1**) liensinine, (**2**) isoliensinine, (**3**) neferine. CCC separation conditions: column, multilayer coil of 4.0 mm i.d. PTFE tube with a total capacity of 1600 mL; rotary speed, 450 rpm; column temperature, 35 °C; solvent system, ethyl acetate–tetrachloromethane–methanol–water (1:6:4:1, v/v); mobile phase, lower phase; flow rate, 5 mL/min; detection, 280 nm; sample size, 5850 mg dissolved in 15 mL upper phase and 15 mL lower phase; retention of the stationary phase, 83.75%; purity: (**1**) 98.52%, (**2**) 97.12%, (**3**) 99.54%. For HPLC conditions, see Section 2.

for large-scale isolation and purification of crude extracts of natural products or synthetic compounds. So far, no report has been published on the use of CCC for the isolation and purification of liensinine and its analogues. The purpose of this study, therefore, is to develop a CCC method for the preparative isolation and purification of liensinine and its analogues from embryo of the seed of *N. nucifera* GAERTN.

2. Experimental

2.1. Apparatus

The CCC isolation and purification of liensinine and its analogues from embryo of the seed of *N. nucifera* GAERTN. was performed by upright coil planet centrifuge with four multilayer coils connected in series. Its design principle and dimensions were described in the literature [17]. The upright CCC apparatus holds four identical multilayer coil columns in the symmetrical positions around the rotary frame at distance of 9 cm from the central axis of the centrifuge to maintain perfect balance of centrifuge system without the use of a counterweight. Each separation column was made by winding a single piece of 4 mm i.d. and 1 mm wall thickness polytetrafluroethylene (PTFE) tubing directly onto the holder hub of 5 cm diameter forming three layers of right-handed and left-handed coils alternating in each layer between a pair of flanges spaced 35 cm apart. The β-value (ratio of helical radius of the coil and revolution radius) of the multilayer coil varies from 0.28 at the internal terminal to 0.48 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). These multilayer coils are connected in series on the rotary frame using a flow tube (PTFE, 1.6 mm i.d. and 0.7 mm wall thickness) to give a total capacity of 1600 mL while the unique gear arrangement on the rotary frame establishes a twist-free mechanism of the flow tubes so that continuous elution can be performed without the use of rotary seal.

The apparatus can be operated up to maximum speed of 800 rpm with a speed Sunwind control unit (Shenduo Electric Corp., Shanghai, China) and up to 60 °C with a temperature control unit. In addition, this CCC system

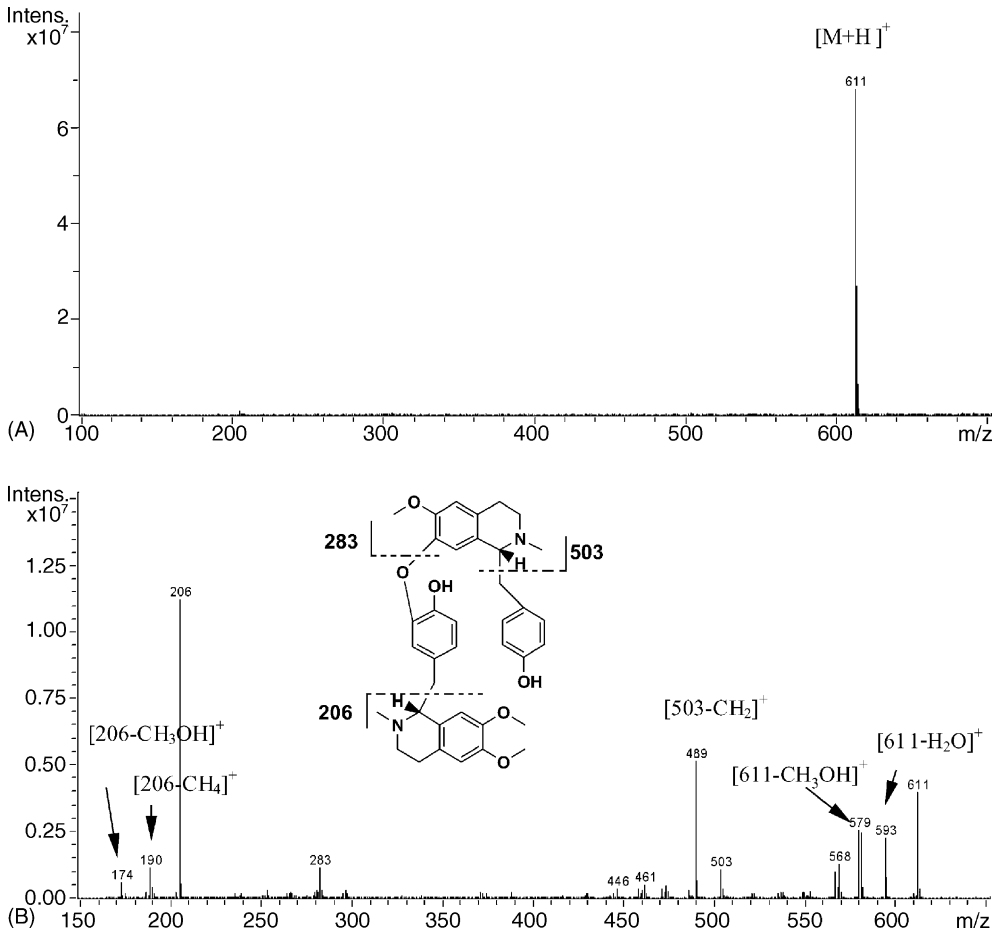

Fig. 4. Electrospray positive ion mass spectra of liensinine: (A) MS, (B) MS–MS spectrum (*m/z* 611, →).

is equipped with a Type-J-W metering pump (Zhijiang Petroleum Equipment, Hangzhou, China), a HD-9704 UV spectrometer operating at 254 and 280 nm, Shimadzu C-R1B Chomatopac recorder, BSZ-100 fraction collector, a sample injection valve with a 30 mL sample loop and NT2000 data analysis system (Institute of Automation Engineering, Zhejiang University, Hangzhou, China).

The high-performance liquid chromatography (HPLC) used was Agilent 1100 system including G1312A BinPump, G1314A variable-wavelength detector (VWD), a model 7725 injection valve with 20 μL loop, a PT100 column oven and Agilent ShemStation for LC.

2.2. Reagents

All organic solvents used for CCC were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, Bedford, MA, USA) was used for all solutions and dilutions. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany.

The embryo of the seed of *N. nucifera* GAERTN. was purchased from Haoyouduo Supermarket, Hangzhou, China. A voucher specimen with reference number 010709 is kept in the Institute of Organic and Pharmaceutical Chemistry, Zhejiang University.

2.3. Preparation of crude alkaloids

Dried and powdered embryo of the seed of *N. nucifera* GAERTN. (12 kg) was extracted three times with 95% aq. ethanol (10 L × 3). Then, the extract solutions were combined and evaporated under reduced pressure and 40 °C to about 500 mL. The concentrated solution was dissolved in 4 L 1.5% hydrochloric and filtrated by use of 102-type filter paper (Xinhua Paper, Hangzhou, China). Next, 10% aqua ammonia was added into the filtrated solution until the pH of the solution reached 8.5. As a result, 85 g of crude alkaloids were collected as yellow precipitates.

2.4. Preparation of two-phase solvent system and sample solutions

The two-phase solvent system used was composed of light petroleum (b.p. 60–90 °C)–ethyl acetate–tetrachloromethane–chloroform–methanol–water at various volume ra-

Table 1
^{1}H NMR and ^{1}H–^{1}H COSY spectra of liensinine (**1**)

Atom	^{1}H NMR δ (ppm)	Mutiplicity	COSY correlation with
1	3.5	1H, d, $J = 8.30$ Hz	H-9
2-CH_3	2.58	3H, s	
3ax	2.74	1H, m	H-3eq, H-4
3eq	3.19	1H, m	H-3ax, H-4
4	2.84	2H, m	H-3
5	6.67	1H, s	
6-OCH_3	3.88	3H, s	
8	6.45	1H, s	
9ax	2.69	1H, m	H-9eq, H-1
9eq	3.22	1H, m	H-1,H-9ax
11	6.98	1H, d, $J = 8.15$	H-12
12	6.79	1H, d, $J = 8.15$	H-11
14	6.79	1H, d, $J = 8.15$	H-15
15	6.98	1H, d, $J = 8.15$	H-14
1′	3.69	1H, d, $J = 9.00$ Hz	H-9′
2′-CH_3	2.54	3H, s	
3′ax	3	1H, m	H-3′eq, H-4′
3′-eq	3.46	1H, m	H-4′, H-3′ax
4′ax	2.59	1H, m	H-4′eq, H-3′
4′eq	3.01	1H, m	H-3′, H-4′ax
5′	6.58	1H, s	
6′-OCH_3	3.84	3H, s	
7′-OCH_3	3.43	3H, s	
8′	5.71	1H, s	
9′ax	2.62	1H, m	H-9′eq, H-1′
9′eq	3.13	1H, m	H-9′ax, H-1′
11′	6.81	1H, s	
14′	6.72	1H, d, $J = 8.15$	H-15′
15′	6.39	1H, d, $J = 8.15$	H-14′

tios. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. For the present preparative CCC separation, the total volume of the prepared two phases each time is 6 L.

The sample solutions were prepared by dissolving the crude alkaloids in a solvent mixture consisting of equal volumes of both upper and lower phases at suitable concentration according to the preparative scale of CCC separation.

2.5. Separation procedure

Preparative CCC was performed as follows: the four upright multilayer coil columns connected in series were first entirely filled with upper phase as stationary phase, and then the sample solution was injected through the sample port and the lower phase as a mobile phase was pumped in head-to-tail elution mode at flow rate of 5 mL/min while the column was rotated at 450 rpm. The effluent was monitored on-line at 280 nm and automatically collected in 20 mL test tube per 3 min using a BSZ-100 fraction collector. Peak fractions were collected according to the elution profile and HPLC detection.

Table 2
^{13}C NMR and 2D NMR spectra data of liensinine (**1**)

Atom	^{13}C NMR	DEPT[a]	HMQC correlation with δ_H (ppm)	HMBC correlation with
1	65.604	CH	3.5	H9, 2-CH_3
2-CH_3	42.709	CH_3	2.58	H-1, H-3
3	48.143	CH_2	3.19,2.74	H-4, 2-CH_3
4	26.57	CH_2	2.84	H-3, H-5
4a	129.773	q[b]		H-8, H-4
5	112.17	CH	6.67	
6	148.223	q		H-5, H-8, 6-OCH_3
6-OCH_3	55.985	CH_3	3.88	
7	143.378	q		H-5, H-8
8	118.027	CH	6.45	H-1
8a	130.671	q		H-1, H-8
9	40.087	CH_2	3.22,2.69	H-1, H-11, H-15
10	130.725	q		H-9, H-11, H-15
11	130.992	CH	6.98	H-12, H-15
12	116.921	CH	6.79	H-11, H-14
13	155.755	q		H-11, H-15, H-12, H-14
14	116.921	CH	6.79	H-12, H-15
15	130.992	CH	6.98	H-11, H-14
1′	64.655	CH	3.69	H-9′, 2′-CH_3
2′-CH_3	40.435	CH_3	2.54	H-3', H-1′
3′	44.237	CH_2	3.46,3.00	H-4′, 2′-CH_3
4′	21.705	CH_2	3.01,2.59	H-5′
4′a	123.427	q		H-4′, H-3′,H-8′
5′	111.349	CH	6.58	
6′	147.75	q		H-5′, H-8′, 6′-OCH_3
6′-OCH_3	55.826	CH_3	3.84	
7′	146.307	q		H-5′, H-8′, 7′-OCH_3
7′-OCH_3	55.423	CH_3	3.43	
8′	111.474	CH	5.71	H-1′
8′a	126.654	q		H-5′, H-1′, H-9′
9′	42.546	CH_2	3.13,2.62	H-1′, H-11′, H-15′
10′	130.725	q		H-11′, H-14′, H-9′
11′	121.827	CH	6.81	H-15′, H-9′
12′	144.101	q		H-11′, H-14′
13′	146.462	q		H-14′, H-15′, H-11′
14′	115.377	CH	6.72	
15′	127.496	CH	6.39	H-11′, H-9′

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

2.6. HPLC analysis and identification of CCC peak fractions

HPLC analyses of the crude alkaloids and CCC peak fractions were performed with an YMC-Pack ODS-A column (150 mm × 4.6 mm i.d., 5 μm, 120 Å). The mobile phase was A (acetonitrile) and B (0.2% triethylamine aqueous solution) at the gradient: A from 40 to 80% and B from 60 to 20% for 15 min. The flow-rate was 0.8 mL/min, and the effluent was monitored at 280 nm.

Identification of the CCC peak fraction was carried out by electrospray ionization (ESI) multiple mass spectrometry (MS^n), one- and two-dimensional nuclear magnetic resonance (1D and 2D NMR) including ^{1}H NMR, ^{13}C NMR, DEPT 135, DEPT 90, ^{1}H–^{1}H COSY, HMQC, HMBC spectra. Positive ESI-MS^n analyses were performed using Bruker

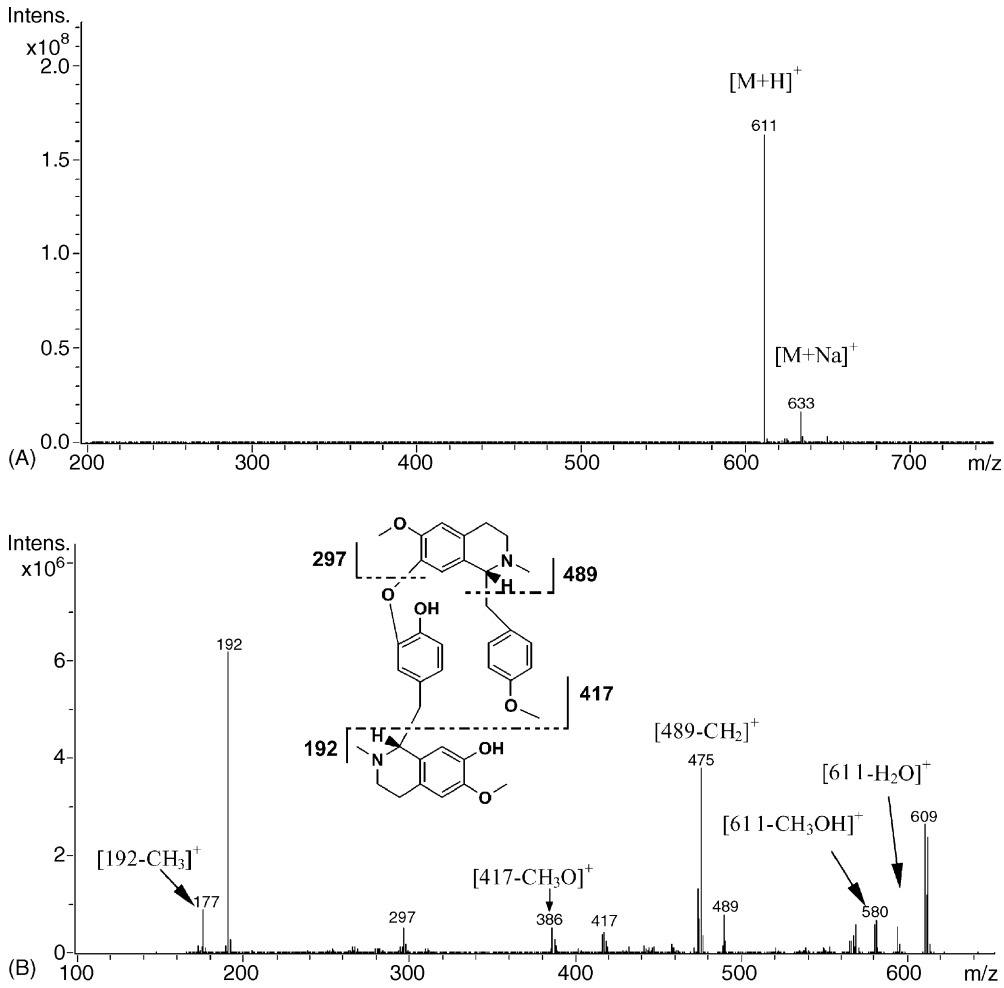

Fig. 5. Electrospray positive ion mass spectra of isoliensinine: (A) MS, (B) MS–MS spectrum (*m/z* 611, →).

Esquire 3000 plus spectrometer with an ESI interface. MS^n analyses of selected ions were performed in the ion trap by collision-induced dissociation (CID) with helium. 1D and 2D NMR experiments were carried out using a Bruker Advanced DMX 500 NMR spectrometer with chloroform ($CDCl_3$) as solvent and tetramethylsilane (TMS) as internal standard.

3. Results and discussion

3.1. HPLC analyses and preparative CCC separation

The crude alkaloids obtained from embryo of the seed of *N. nucifera* GAERTN. were first analyzed by HPLC. The result (Fig. 1) indicated that several compounds including liensinine (about 15.4%), isoliensinine (about 15.9%), neferine (about 47.6%) and some unknown compounds were contained.

Successful CCC separation depends on the correct of the solvent system. Generally speaking, the two-phase solvent system should satisfy the following requirements: (1) no decomposition or denaturation of the sample; (2) sufficient sample solubility; (3) suitable partition coefficient values; (4) satisfactory retention of the stationary phase [15]. Therefore, a series of experiments to investigate the solubility of crude alkaloids in various solvents, such as light petroleum, ethyl acetate, tetracholoromethane, chloroform, meathanol, dimethyl sulfoxide and water, have been performed and found that the present crude alkaloids have lipophilic physical properties and may be sufficiently partitioned in two-phase solvents with medium polarity. Thus the two-phase solvent systems composed of light petroleum (b.p. 60–90 °C)–ethyl acetate–tetrachloromethane–chloroform–methanol–water at various volume ratios were used as start solvents. In order to achieve an efficient resolution of target compound, 500 mg each time of crude alkaloids dissolved in 5 mL of upper phase and 5 mL of lower phase were used and the two-phase solvent systems with volume ratios of 1:1:2:6:6:2, 1:1:3:5:6:2, 1:1:4:4:6:2, 1:1:8:0:6:1, and 0:1:6:0:4:1, have been examined using the present CCC apparatus with

Table 3
^{1}H NMR and ^{1}H-^{1}H COSY spectra of isoliensinine (**2**)

Atom	^{1}H NMR δ (ppm)	Mutiplicity	COSY correlation with
1	3.66	1H, m	H-9
2-CH_3	2.39	3H, s	
3ax	2.64	1H, m	H-3eq, H-4
3eq	3.08	1H, m	H-4, H-3ax
4ax	2.47	1H, m	H-4eq, H-3
4eq	2.7	1H, m	H-4ax, H-3
5	6.47	1H, s	
6-OCH_3	3.81	3H, s	
8	6.34	1H,s	
9ax	2.77	1H, m	H-9eq,H-1
9eq	3.03	1H, m	H-9ax, H-1
11	6.9	1H, d, $J = 8.42$	H-12
12	6.7	1H, d, $J = 8.42$	H-11
13-OCH_3	3.73	3H, s	
14	6.7	1H, d, $J = 8.42$	H-15
15	6.9	1H, d, $J = 8.42$	H-14
1′	3.57	1H, m	H-9′
2′-CH_3	2.51	3H, s	
3′ax	2.78	1H, m	H-3′eq, H-4′
3′eq	3.16	1H, m	H-4′, H-3′ax
4′ax	2.66	1H, m	H-4′eq, H-3′
4′eq	2.87	1H, m	H-4′ax, H-3′
5′	6.64	1H, s	
6′-OCH_3	3.79	3H, s	
8′	6.3	1H, s	
9′ax	2.8	1H, m	H-9′eq, H-1′
9′eq	2.93	1H, m	H-9′ax, H-1′
11′	6.46	1H, s	
14′	6.81	1H, d, $J = 8.16$	H-15′
15′	6.72	1H, $J = 8.16$	H-14′

Table 4
^{13}C-MR and 2D NMR spectra of isoliensinine (**2**)

Atom	^{13}C NMR	DEPT[a]	HMQC correlation with δ_H (ppm)	HMBC correlation with
1	64.569	CH	3.66	H-8, 2-CH_3, H-9
2-CH_3	42.692	CH_3	2.39	
3	47.468	CH_2	3.08, 2.64	2-CH_3
4	25.562	CH_2	2.70, 2.47	H-5, H-3
4a	130.034	q[b]		H-5
5	110.64	CH	6.47	
6	145.254	q		H-5, 6-OCH_3
6-OCH_3	55.982	CH_3	3.81	
7	143.568	q		H-5, H-8
8	113.869	CH	6.34	
8a	125.601	q		H-8, H-4
9	39.339	CH_2	3.03, 2.77	H-11, H-15
10	131.436	q		H-12, H-14
11	130.503	CH	6.9	H-9, H-12
12	113.572	CH	6.7	H-11
13	157.867	q		13-OCH_3, H-11,H-15,H-12,H-14
13-OCH_3	55.206	CH_3	3.73	
14	113.572	CH	6.7	H-15
15	130.503	CH	6.9	H-9, H-14
1′	64.418	CH	3.57	H-8′ 2′-CH_3, H-9′
2′-CH_3	42.748	CH_3	2.51	
3′	46.952	CH_2	3.16, 2.78	2′-CH_3
4′	26.115	CH_2	2.87, 2.66	H-3′, H-5′
4′a	130.699	q		H-1′, H-5′
5′	112.455	CH	6.64	H-4′eq
6′	149.09	q		H-5′, H-8′, 6′-OCH_3
6′-OCH_3	55.821	CH_3	3.79	
7′	142.866	q		H-5′, H-8′
8′	120.067	CH	6.3	
8′a	130.383	q		H-1′, H-5′
9′	40.679	CH_2	2.93, 2.80	H-11′, H-15′
10′	131.81	q		H-14′
11′	119.085	CH	6.46	H-15′, H-9′
12′	144.577	q		H-11′, H-14′
13′	145.254	q		H-15′, H-11′
14′	115.427	CH	6.81	
15′	125.239	CH	6.72	H-11′

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

1600 mL of total capacity. The results were found that the solvent systems with volume ratios of 1:1:4:4:6:2 and 0:1:6:0:4:1 were the optimum for the separation of liensinine and its analogues although when using other solvent systems including the volume ratios of 1:1:2:6:2, 1:1:3:5:6:2 and 1:1:8:6:2, the retention level of stationary phase may be up to 75% and the crude alkaloids can be partly resolved. Using the solvent systems with the volume ratios of 1:1:2:6:2, 1:1:3:5:6:2, the partition efficiencies and resolutions are relative low as well as the short elution time (less than 120 min), which is maybe partly due to that the two kind of solvent systems have stronger polarities than other solvent systems. Using the two-phase solvent system with volume ratio of 1:1:4:4:6:2, the retention of the stationary phase in multiplayer coil column was up to 80% and three alkaloids were efficiently resolved in short time (150 min). However, due to short elution time and close peak resolution, the system could not resolve large quantity of alkaloids. Using the volume ratio of two-phase solvent system was 0:1:6:0:4:1, the three alkaloids were separated at higher resolution although the elution time was relatively long (9 h). Because of high retention of stationary phase (84%) and high resolution, the system could be use to resolve large quantity of alkaloids. Using the system with the volume ratio of 1:1:8:0:6:1, the retention of stationary phase (76%) is lower and the elution time (11 h) is longer than the system with the volume ratio of 0:1:6:0:4:1. Therefore, two kinds of solvent systems with the volume ratios of 1:1:4:4:6:2 and 0:1:6:0:4:1 were selected and applied to preparative and large-scale CCC separation respectively.

Fig. 2 shows the preparative CCC separation of 1102 mg of the crude alkaloids using the solvent system composed of light petroleum (b.p. 60–90 °C)–ethyl acetate–tetrachloromethane–chloroform–methanol–water (1:1:4:4:6:2, v/v) and HPLC analyses corresponding to the CCC

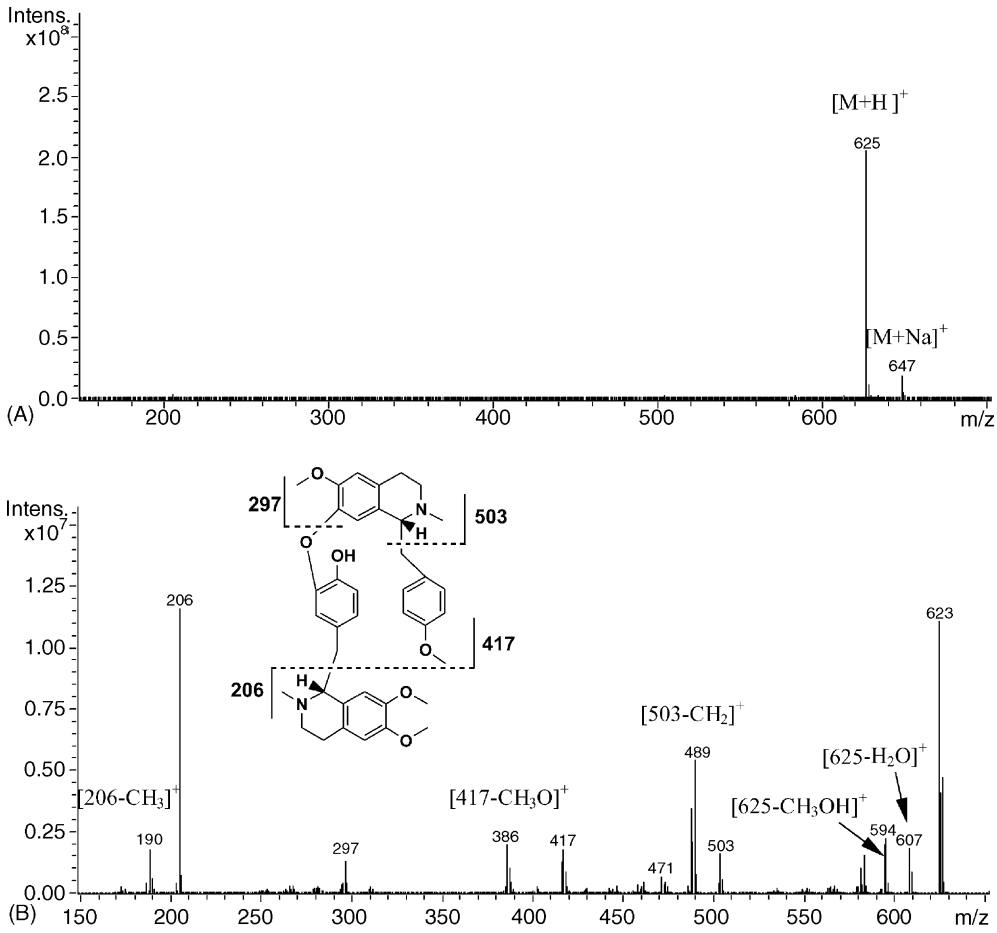

Fig. 6. Electrospray positive ion mass spectra of neferine: (A) MS, (B) MS–MS spectrum (*m/z* 625, →).

peaks of liensinine, isoliensinine and neferine. In order to save solvents and time, the other eluting compounds after the target substances were removed by pumping out the stationary phase instead of eluting them with the mobile phase because of the stationary phase was used only once. After the separation the CCC peak fractions were collected respectively according to CCC profile and further HPLC analysis. As a result, 350 mg neferine, 100 mg isoliensinine and 95 mg liensinine with over 95% purity were obtained in one-step separation of 150 min, which clearly indicated the two-phase system was very efficient for the fast CCC separation of the phenolic alkaloids.

Fig. 3 shows the preparative CCC separation of 5850 mg of the crude alkaloids using the solvent system composed of ethyl acetate–tetrachloromethane–methanol–water (1:6:4:1, v/v) and HPLC analyses corresponding to the CCC peaks of liensinine, isoliensinine and neferine. As a result, 2545 mg neferine, 698 mg isoliensinine and 650 mg liensinine were obtained at more than 97% purity in one step separation of 9 h, which indicated that the two-phase system was very suitable for the large-scale CCC separation of the alkaloids.

3.2. Structural identification of isolated compounds

The structures of all compounds corresponding to the CCC peak 1, 2 and 3 fractions were identified by ESI-MS^n, 1D and 2D NMR spectra. Wu et al. [18] and other early studies [4–11] reported the electron impact ionization (EI), ^{1}H and ^{13}C NMR data of liensinine, isoliensinine and neferine, however, there are no full 1D and 2D data and ESI-MS^n data for these compounds. Here we give for the first time ESI-MS^n data and 2D NMR-based unambiguous assignments for all the protons and carbons of the three compounds.

Liensinine (**1**) is a white amorphous powder with UV (λ_{max}^{MeOH}) absorption at 212 and 278 nm. The positive ESI-MS spectrum of compound showed the characteristic ion at *m/z* 611 due to $[M + H]^+$ (Fig. 4A). The MS^2 spectrum of the precursor ion *m/z* 611 exhibited intensive characteristic fragments which are elucidated in Fig. 4B. 1D and 2D NMR data are shown in Tables 1 and 2. Our 1D data are identical to the 1D data of Wu et al. [18].

Isoiensinine (**2**) is pale yellow amorphous powder with UV (λ_{max}^{MeOH}) absorption at 212 and 280 nm. The positive ESI-MS spectrum of compound showed the $[M + H]^+$ ion

Table 5
^{1}H NMR and ^{1}H–^{1}H COSY spectra of neferine (**3**)

Atom	^{1}H NMR δ (ppm)	Mutiplicity	COSY correlation with
1	3.62	1H, m	H-9
2-CH_3	2.48	3H, s	
3ax	2.73	1H, m	H-3eq
3eq	3.13	1H, m	H-3ax, H-4
4ax	2.62	1H, m	H-4eq, H-3
4eq	2.79	1H, m	H-4ax, H-3
5	6.61	1H, s	
6-OCH_3	3.79	3H, s	
8	6.34	1H, s	
9ax	2.77	1H, m	H-9eq, H-1
9eq	2.98	1H, m	H-9ax, H-1
11	6.89	1H, s	H-12
12	6.67	1H, d, $J = 7.06$	H-11
13-OCH_3	3.7	3H, s	
14	6.67	1H, d, $J = 7.06$	H-15
15	6.89	1H,d, $J = 7.06$	H-14
1′	3.62	1H, m	H-9′
2′-CH_3	2.45	3H, s	
3′ax	2.73	1H, m	H-3′eq, H-4′
3′eq	3.08	1H, m	H-3′ax, H-4′
4′ax	2.59	1H, m	H-4′eq, H-3′
4′eq	2.78	1H, m	H-4′ax, H-3′
5′	6.49	1H, s	
6′-OCH_3	3.76	3H, s	
7′-OCH_3	3.52	3H, s	
8′	5.97	1H, s	
9′ax	2.67	1H, m	H-9′eq, H-1′
9′eq	3.06	1H, m	H-9′ax, H-1′
11′	6.51	1H, s	
14′	6.82	1H, d, $J = 6.68$	H-15′
15′	6.66	1H, d, $J = 6.68$	H-14′

Table 6
^{13}C NMR and 2D NMR spectra of neferine (**3**)

Atom	^{13}C NMR δ (ppm)	DEPT[a]	HMQC correlation with δ_H (ppm)	HMBC correlation with
1	64.48	CH	3.62	2-CH_3, H-8
2-CH_3	42.766	CH_3	2.48	
3	47.275	CH_2	3.13, 2.73	2-CH_3
4	26.194	CH_2	2.79, 2.62	H-3, H-5
4a	130.466	q[b]		H-5
5	112.421	CH	6.61	
6	149.064	q		H-5, H-6, H-8
6-OCH_3	55.794	CH_3	3.79	
7	142.893	q		H-5, H-8
8	120.094	CH	6.34	
8a	130.979	q		H-8, H-5, H-4
9	39.94	CH_2	2.98, 2.77	H-15, H-11
10	131.506	q		H-12, H14, H-9
11	130.555	CH	6.89	H-12,H-9, H-15
12	113.515	CH	6.67	H-11, H-14
13	157.877	q		H-11, H-15, H-12, H-14, 13-OCH_3
13-OCH_3	55.191	CH_3	3.7	
14	113.515	CH	6.67	H-12, H-15
15	130.555	CH	6.89	H-14, H-9, H-11
1′	64.834	CH	3.62	2′-CH_3, H-8′
2′-CH_3	42.521	CH_3	2.45	
3′	46.644	CH_2	3.08, 2.73	2′-CH_3
4′	25.214	CH_2	2.78, 2.59	H-5′
4′a	129.07	q		H-5′, H-8′
5′	111.211	CH	6.49	
6′	147.298	q		H-5′, H-8′, H6′-OCH_3
6′-OCH_3	55.919	CH_3	3.76	
7′	146.367	q		H-5′, 7′-OCH_3, H-8′
7′-OCH_3	55.537	CH_3	3.52	
8′	111.066	CH	5.97	
8′a	125.653	q		H-8′
9′	40.706	CH_2	3.06, 2.67	H-15′, H-11′
10′	131.72	q		H-11′, H-15′, H-9′
11′	119.265	CH	6.51	H-9′, H-15′
12′	144.995	q		H-14′
13′	145.715	q		H-15′, H-11′
14′	115.879	CH	6.82	
15′	125.299	CH	6.66	H-9′, H-11′

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

at *m*/*z* 611 (Fig. 5A). The MS^2 spectrum of the precusor ion *m*/*z* 611 exhibited intensive characteristic fragments which are illustrated in Fig. 5B. 1D and 2D NMR data are shown in Tables 3 and 4.

Neferine (**3**) is white amorphous powder with UV (λ_{max}^{MeOH}) absorption at 212 and 282 nm. The positive ESI-MS spectrum of compound showed the intensive $[M + H]^+$ ion at *m*/*z* 625 (Fig. 6A). The MS^2 spectrum of the precursor ion *m*/*z* 625 exhibited intensive characteristic fragments which are elucidated in Fig. 6B. 1D and 2D NMR data are shown in Tables 5 and 6.

4. Conclusions

In conclusion, preparative CCC isolation of liensinine and its analogues, isoliensinine and neferine from embryo of the seed of *N. nucifera* GAERTN. was successfully performed with two kinds of two-phase solvent systems using upright coil planet centrifuge with four multilayer coils connected in series. The first solvent system has larger polarity and was efficient for fast and small-scale isolation while the second solvent system has smaller polarity and was suitable for large-scale isolation. These results clearly demonstrated the present CCC method is powerful for the separation of liensinine and its analogues from crude alkaloids of embryo of the seed of *N. nucifera* GAERTN.

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