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Preparative Isolation and Purification of Linderalactone and Lindenenol from *Radix linderae* by HSCCC

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Abstract: A preparative high speed countercurrent chromatography (HSCCC) method for isolation and purification of sesquiterpene lactone compounds from the Chinese traditional medicinal herb *Radix linderae* (Wuyao in Chinese) was successfully established by using light petroleum-ethyl acetate-methanol-water (5 : 5 : 6 : 4, v/v) as the two phase solvent system. The upper phase of the two phase solvent system was used as the stationary phase of HSCCC. Linderalactone, 40.2 mg and 64.8 mg of lindenenol were obtained from 450 mg of crude extracts in one-step separation. The purity of linderalactone and lindenenol was 99.7% and 98.2%, respectively, as determined by high performance liquid chromatography (HPLC). The structures of linderalactone and lindenenol were identified by ^{1}H-NMR and ^{13}C-NMR.

Keywords: Countercurrent chromatography, HSCCC, *Radix linderae*, Linderalactone, Lindenenol

INTRODUCTION

Radix linderae, the root of *Lindera strychnifolia (Sieb. et Zucc) Vill*, known as Wuyao in Chinese, has been used as a kind of traditional Chinese medicine for centuries and is officially listed in the Chinese Pharmacopoeia.[1] The herb is pungent in flavour, and warm in nature and is often used to treat cold accumulation, distending pain in the chest and abdomen, cold of insufficiency type in the lower abdomen, frequent urination, and other syndromes.[2] Radix Linderae possesses relatively extensive pharmacological activity. Its central

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components are sesquiterpene lactone compounds, such as linderalactone and lindenenol. Linderalactone and lindenenol are also used in the quality control of Wuyao and its products. Therefore, the development of efficient, preparative scale separation and purification methods for these compounds is very important.

HSCCC, being a support free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support.[3] The present paper describes the successful preparative separation and purification of linderalactone and lindenenol from the crude extract of *Radix linderae* by HSCCC. The chemical structures of these compounds are shown in Fig. 1.

EXPERIMENTAL

Reagents and Materials

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

The roots of *Lindera strychnifolia (Sieb. et Zucc) Vill* was purchased from a local drug store and identified by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

Apparatus

The HSCCC instrument employed in the present study is TBE-300A high speed countercurrent chromatography (Shanghai Tauto Biotech Co., Ltd., Shanghai, China), with a 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 was 5 cm, and the β values of the

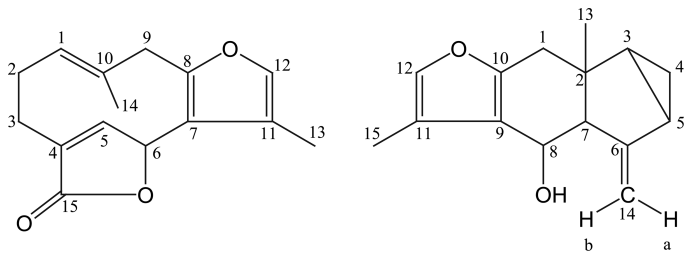

Figure 1. Chemical structures of linderalactone and lindenenol from *Radix linderae.*

multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Instrument Co., Ltd., Beijing, China) was used to control the separation temperature. An ÄKTA prime (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which were used for gradient formation. The data were collected with a Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Co., Ltd., Hangzhou, China).

The HPLC equipment used was an Agilent 1100 HPLC system including a G1311A QuatPump, a G1315B DAD, a Rheodyne 7725i injection valve with a 20 μL loop, a G1332A degasser, and Agilent HPLC workstation.

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

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

Preparation of Crude Sample

The root of *Lindera strychnifolia (Sieb. et Zucc) Vill* was dried to constant weight at 60°C and then pulverized to about 30 mesh with a FZ102 plant disintegrator. The powder of 100 g was extracted with 500 mL of light petroleum (boiling range: 60–90°C) under reflux three times. The extraction time was 2 h, 2 h, and 1 h, respectively. The extracts were combined and then filtrated with a ceramic filter. The filtrate was evaporated by rotary vaporization at 40°C under reduced pressure. About 2.0 g of yellow oil syrup was obtained. It was stored in a refrigerator (−4°C) for the subsequent HSCCC separation.

Selection of the Two Phase Solvent System

Light petroleum-ethyl acetate-methanol-water was used as the two phase solvent system of the HSCCC. The composition of the two phase solvent system was selected according to the partition coefficient (K) of the target compounds of crude sample extracted from *Radix linderae*. The K values were determined by HPLC as follows: a suitable amount of crude sample was dissolved in 5 mL of aqueous phase of the pre-equilibrated two phase solvent system. The solution was determined by HPLC and the peak area was recorded as A_1. Then, equal volume of the organic phase was added to the solution and mixed thoroughly. After the equilibration was established, the aqueous phase was determined by HPLC again and the peak area was

recorded as A_2. The partition coefficient (K) was obtained by the following equation: $K = (A_1 - A_2)/A_2$.

Preparation of Two Phase Solvent System and Sample Solution

Light petroleum-ethyl acetate-methanol-water (5 : 5 : 6 : 4, v/v) 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 thirty minutes prior to use.

The sample solution for HSCCC separation was prepared by dissolving 450 mg of crude extract sample in the mixture of 2 mL of the upper phase and 2 mL of the lower phase of the light petroleum-ethyl acetate-methanol-water system (5 : 5 : 6 : 4, v/v).

HSCCC Separation Procedure

The upper phase (stationary phase) and the lower phase (mobile phase) of the two phase solvent system, light petroleum-ethyl acetate-methanol-water (5 : 5 : 6 : 4, v/v), were pumped into the multiplayer 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}, at the same time, the HSCCC apparatus was run at a revolution speed of 850 rpm. After hydrodynamic equilibrium was reached (about 30 min), the sample solution (450 mg of crude sample dissolved in the mixture of 2 mL of the lower phase and 2 mL of the upper phase) 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 with ÄKTA prime system 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.

HPLC Analysis and Identification of HSCCC Peak Fractions

The crude extract of *Radix Linderae* and each of the peak fractions collected from HSCCC separation were analyzed by HPLC. The HPLC analyses were performed with a Spherigel ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm) at room temperature. The mobile phase was acetonitrile and water in gradient mode as follows: 0–30 min, the volume ratio of acetonitrile was

changed from 30% to 84%. The flow rate of the mobile phase was 1.0 mL min^{-1} and the effluent was continuously monitored at 235 and 254 nm.

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

RESULTS AND DISCUSSION

Optimization of HPLC Conditions

Several elution systems, such as gradient elution of methanol-water, acetonitrile-water, methanol-acetonitrile-water, etc., were employed to analyze the crude extract from *Radix Linderae* by HPLC. When acetonitrile and water were used as the mobile phase in gradient mode (acetonitrile : water (v/v) from 30 : 70 to 84 : 16, in 30 min), each peak had baseline separation. The HPLC chromatogram of the crude extract from *Radix Lindera* is shown in Fig. 2 (A).

Selection of Two Phase Solvent System and Other Conditions of HSCCC

In order to determine the optimal two phase solvent system for the HSCCC separation, a series of experiments were performed in the present study. Light petroleum-ethyl acetate-methanol-water, which could provide a broad range of polarity by modifying the volume ratio of the four solvents, was used as the two phase solvent system to optimize the HSCCC separation condition. The partition coefficients of the compounds of crude sample in the two phase solvent systems are given in Table 1. It can be seen that when light petroleum-ethyl acetate-methanol-water (5 : 5 : 5 : 5, v/v) and (5 : 5 : 6 : 5, v/v) systems were used as the two phase solvent system, the K values of the compounds were very big and these systems were unsuitable for HSCCC separation. When light petroleum-ethyl acetate-methanol-water (5 : 5 : 7 : 4, v/v) and (5 : 5 : 7 : 3, v/v) systems were used as the two phase solvent system, the K values of linderalactone, Un-1, and lindenenol were suitable. But the purity of the obtained compounds was poor. When light petroleum-ethyl acetate-methanol-water (5 : 5 : 6 : 4, v/v) was used as the two phase solvent system, high purity of linderalactone and lindenenol could be obtained and the separation time was acceptable. Therefore, light petroleum-ethyl acetate-methanol-water (5 : 5 : 6 : 4, v/v) was used as the two phase solvent system for HSCCC separation.

The separation and purification of Un-2, Un-3, and Un-4 were also investigated by using light petroleum-ethyl acetate-methanol-water two phase solvent systems. But no satisfactory results were obtained because they are always present in the stationary phase due to the high K values.

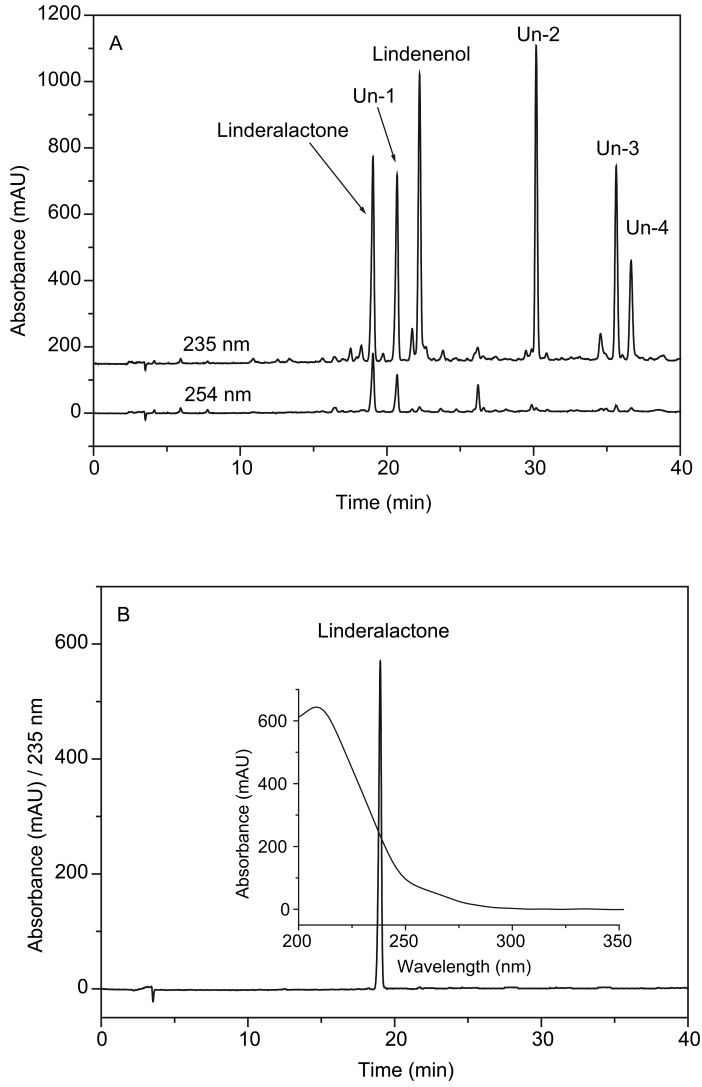

Figure 2. HPLC chromatogram of crude extract from *Radix linderae* and HSCCC peak fractions. Column: SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm); mobile phase: acetonitrile and water in gradient mode (acetonitrile : water (v/v) from 30 : 70 to 84 : 16, in 30 min); flow rate: 1.0 mL min^{-1}; detection wavelength: 235 nm and 254 nm; (A) crude extract from *Radix linderae*; (B) peak I; (C) peak II of Fig. 3.

Although the revolution speed of the apparatus could be regulated with a speed controller in a range between 0 and 1,000 rpm, the speed of 850 rpm was used invariably in the present study. The influence of the flow rate of mobile phase was also investigated. Reducing the flow rate could improve the

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

Light petroleum-ethyl acetate-methanol-water (v/v)	K					
	Linderalactone	Un-1[a]	Lindenenol	Un-2[a]	Un-3[a]	Un-4[a]
5 : 5 : 5 : 5	3.71	12.68	26.38	18.31	—	—
5 : 5 : 6 : 5	2.19	8.65	13.50	16.70	—	—
5 : 5 : 6 : 4	1.72	2.95	4.42	14.40	17.83	20.13
5 : 5 : 7 : 4	1.28	2.10	1.60	9.77	13.49	10.74
5 : 5 : 7 : 3	0.66	1.22	0.30	6.68	10.15	8.66

The symbol "—" means the K values of target compounds is too large that cannot be calculated.

[a]Peak labeled in Fig. 2(A).

reservation of the stationary phase in some degree, but the chromatogram peaks were extended at the same time. Ultimately, a flow rate of 2.0 mL min^{-1} was employed in the experiment.

The crude samples from *Radix linderae* were separated and purified under the optimum HSCCC conditions. The HSCCC chromatogram was shown in Fig. 3. Linderalactone, 40.2 mg, and 64.8 mg of lindenenol were obtained

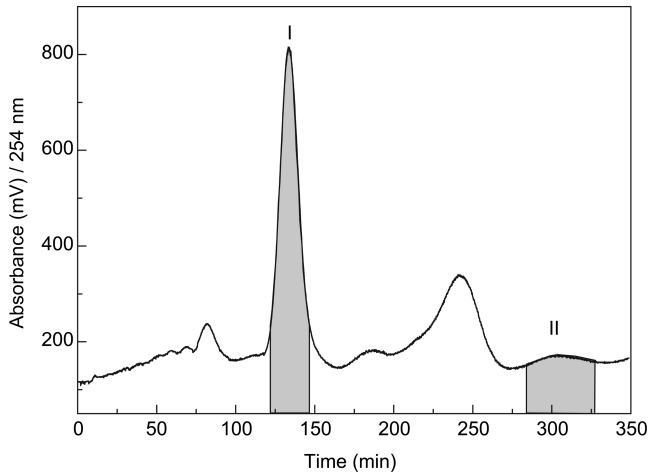

Figure 3. HSCCC chromatogram of crude extract from *Radix linderae*. Two phase solvent system: light petroleum-ethyl acetate-methanol-water (5 : 5 : 6 : 4, v/v); mobile phase: the lower phase; flow rate: 2.0 mL min^{-1}; revolution speed: 850 rpm; sample size: 450 mg crude extract dissolved in 2 mL of the upper phase and 2 mL of the lower phase of the two phase solvent system; separation temperature: 25°C; retention of the stationary phase: 50%. I: linderalactone (collected during 122–147 min); II: lindenenol (collected during 283–327 min).

from 450 mg of the crude sample in a one step separation. The purity of linderalactone and lindenenol was 99.7% and 98.2%, respectively, as determined by HPLC. The chromatograms of HPLC and UV spectra of these compounds are shown in Fig. 2(B–C).

Structural Identification

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

Peak I: ^{1}H-NMR (400 MHz, $CDCl_3$): 4.94 (1H, m, H-1), 2.30–2.37 (2H, m, H-2), 2.30–2.37 (2H, m, H-3), 7.14 (1H, s, H-5), 5.85 (1H, s, H-6), 2.91, 2.94 (2H, d, H-9), 6.82 (1H, s, H-12), 2.11 (3H, s, H-13), 1.27 (3H, s, H-14); ^{13}C-NMR (400 MHZ, $CDCl_3$): 130.34 (C-1), 25.78 (C-2), 26.82 (C-3), 135.41 (C-4), 151.74 (C-5), 74.31 (C-6), 115.34 (C-7), 152.94 (C-8), 40.79 (C-9), 131.08 (C-10), 122.35 (C-11), 137.28 (C-12), 8.47 (C-13), 15.75 (C-14), 173.71 (C-15). Compared with the data given in reference [2], Peak I was identified as linderalactone.

Peak II: ^{1}H-NMR (400 MHz, $CDCl_3$): 2.67, 2.74 (2H, d, H-1), 1.37 (1H, m, H-3), 0.83–0.88 (2H, m, H-4), 1.47 (1H, m, H-5), 2.81 (1H, d, $J = 15.2$ Hz, H-7), 4.49 (1H, t, $J = 9.0$ Hz, H-8), 2.03, (1H, m, H-C_8-OH), 7.06 (1H, s, H-12), 0.85 (3H, s, H-13), 5.12 (1H, s, H-14a), 5.24 (1H, s, H-14b), 2.11 (3H, s, H-15); ^{13}C-NMR (400 MHZ, $CDCl_3$): 38.33 (C-1), 41.28 (C-2), 27.26 (C-3), 16.58 (C-4), 23.05 (C-5), 152.53 (C-6), 68.63 (C-7), 65.10 (C-8), 119.90 (C-9), 149.88 (C-10), 120.67 (C-11), 138.51 (C-12), 18.18 (C-13), 107.96 (C-14), 9.08 (C-15). Peak II was identified as lindenenol according to these ^{1}H-NMR and ^{13}C-NMR data.

CONCLUSION

HSCCC was successfully applied to the separation and purification of sesquiterpene lactone compounds from the light petroleum extract of *Radix Linderae* for the first time. Linderalactone, 40.2 mg, and 64.8 mg of lindenenol were obtained from 450 mg of the crude sample in a one step separation. The results illustrated that, as a preparative separation technique, HSCCC is an effective method for the isolation and purification of bioactive compounds from *Radix Linderae*. HSCCC, thus, provides an attractive method for the preparative scale separation and purification of bioactive components in herbal extracts.

ACKNOWLEDGMENTS

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