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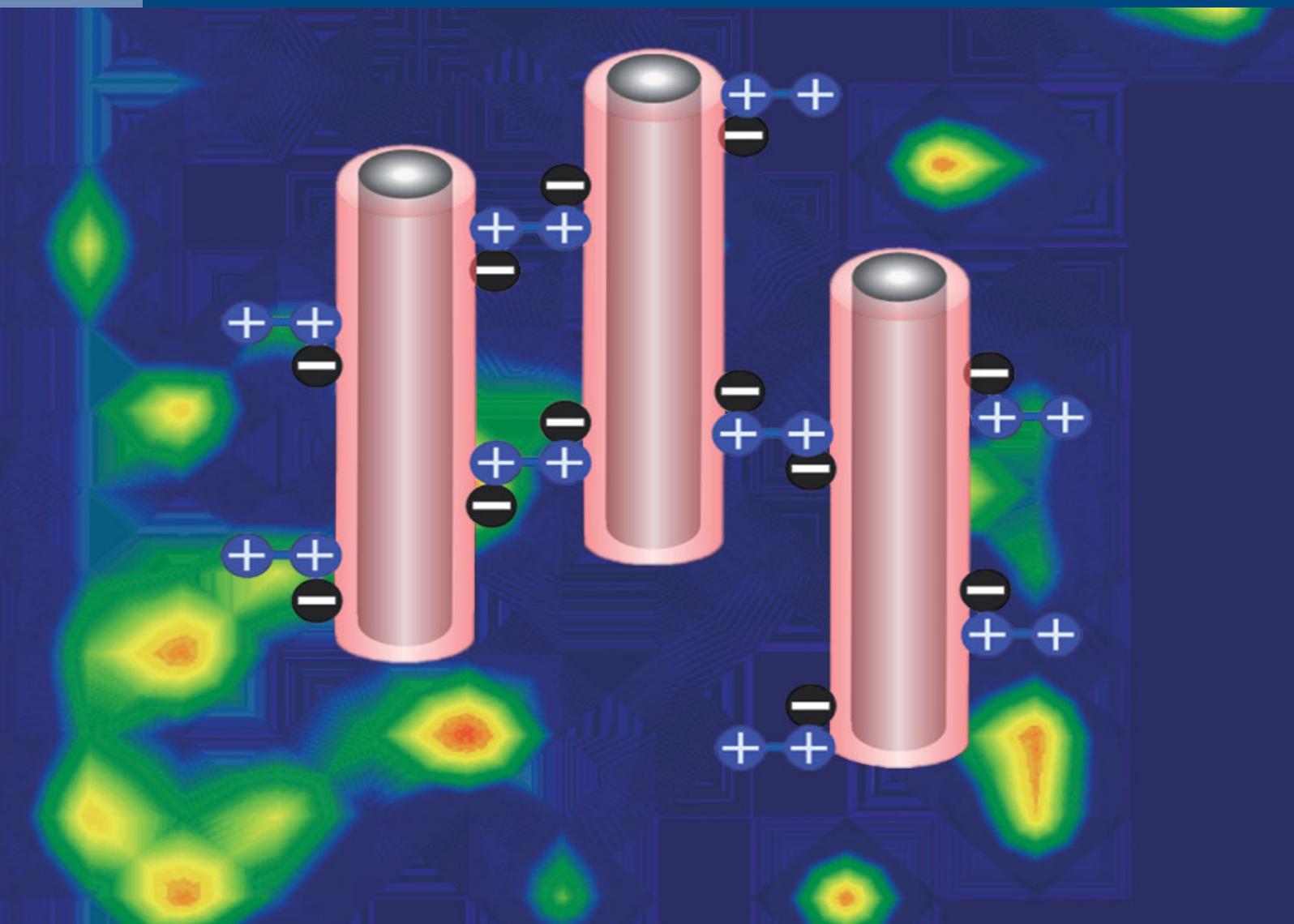
Methods
Chromatography · Electroseparation

Applications
Biomedicine · Foods · Environment

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Research Article

Application of chromatography technology in the separation of active alkaloids from *Hypecoum leptocarpum* and their inhibitory effect on fatty acid synthase

A method that involved the combination of pH-zone-refining counter-current chromatography and semipreparative reversed-phase liquid chromatography has been established for the preparative separation of alkaloids from *Hypecoum leptocarpum*. From 1.2 g of crude sample, 31 mg *N*-feruloyltyramine, 27 mg oxohydrastinine, 47 mg hydroprotopine, 25 mg leptopidine, and 18 mg hypecocarpine have been obtained. The structure of the new compound, hypecocarpine, is confirmed based on the analysis of spectroscopic data, including NMR, UV, and IR spectroscopy and positive electrospray ionization mass spectrometry. The known chemical structures were characterized on the basis of ^{1}H and ^{13}C NMR spectroscopy. The purities of the five alkaloids are all over 92.7% as determined by high-performance liquid chromatography. The alkaloids' cytotoxicity in breast cancer cells is assessed by using a Cell Counting Kit assay and their inhibitory effect on fatty acid synthase expression is assessed by a Western blot assay. These results suggest that leptopidine could suppress growth and induce cytotoxicity in breast cancer cells and that the cytotoxicity of leptopidine may be related to its inhibitory effect on fatty acid synthase expression.

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1 Introduction

Hypecoum leptocarpum, a herbaceous plant of the *Papaveraceae* family, is commonly found in north- and south-west China [1]. The plant is widely used in traditional Tibetan medicine as an antipyretic, analgesic, and anti-inflammatory substance [1], with specific employment found in the treatment of colds, hepatitis, pneumonia, cholecystitis, and food poisoning [2]. Research performed using modern analytical chemistry and natural product extraction techniques have revealed a number of pharmacologically active alkaloids in *H. leptocarpum*, with the majority of compounds being tetrahydroisoquinoline alkaloids such as protopine [3], cryptopine [4], allocryptopine [5], oxohydrastinine [6, 7], leptocarpinine [8], leptopine [9], etc.

Due to their range of biological activities these alkaloids have received considerable attention in pharmacology re-

search. Various studies have shown that these alkaloids have numerous potential biological activities including the inhibition of tumors [10], bacteria [11], viruses [12], inflammation [13], and coagulation [13]. In addition, these alkaloids have potential to treat breast cancer [14], bronchodilators [15], and disease about central nervous system [16, 17].

Considering the beneficial activities of alkaloids extracted from this herb and to control the quality of this traditional Tibetan medicine and their natural products, a large quality of pure compounds is urgently needed. Furthermore, most active studies of the *H. leptocarpum* have been stayed in the pharmacodynamic evaluation level of the crude extracts. However, few studies focused on the small molecular level and the clear targets. Therefore, to separate and prepare the pure alkaloids from this traditional Tibetan medicine is imperative. This paper firstly describes the successful application of pH-zone-refining CCC to separate the alkaloids from *H. leptocarpum*. However, because the alkalinity and hydrophobicities of the two alkaloids, leptopidine, and hypecocarpine (the structure of them see Fig. 1), are similar,

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Abbreviation: FAS, fatty acid synthase

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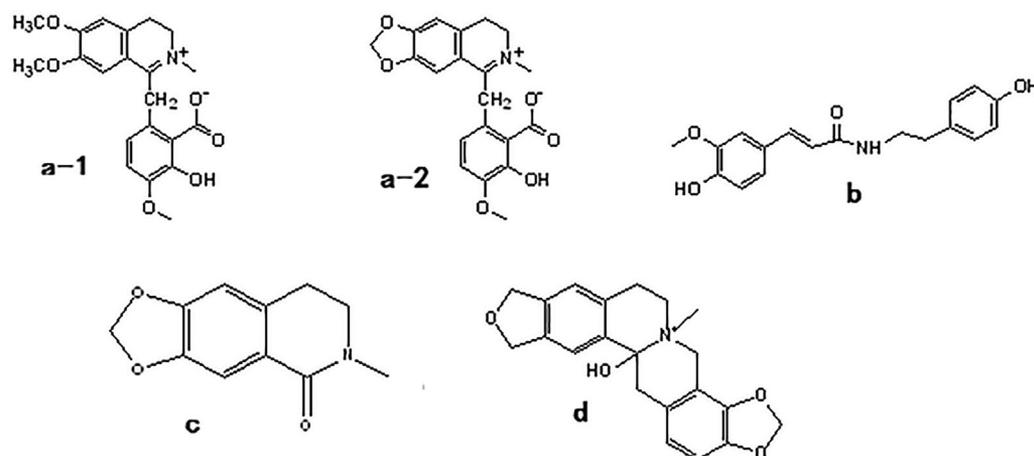
Figure 1. Chemical structures of the five alkaloids.
Hypecocarpine (compound a-1); leptopidine (compound a-2); *N*-feruloyltyramine (compound b); oxohydrastinine (compound c); hydroprotopine (compound d).

it is not easy to separate by pH-zone-refining in one step. Therefore, RPLC was used to separate the two alkaloids. The pure compound, hypecocarpine, was separated from *H. leptocarpum* by this method as a new alkaloid. And no active studies about this kind of alkaloid were reported in the past. In this study, the compounds leptopidine and hypecocarpine were evaluated for its cytotoxicity in breast cancer cells and the inhibitory effect on fatty acid synthase (FAS) expression.

2 Materials and methods

2.1 Reagents and materials

The *H. leptocarpum* was collected from Huzhu County, Qinghai Province, China in 2014, and dried under shade at room temperature. It was identified as *H. leptocarpum* by Engineer Lijuan Mei of the Northwest Institute of Plateau Biology, Chinese Academy of Science. The plants are deposited in the Herbarium of the Northwest Institute of Plateau Biology, Xining, Qinghai Province.

All organic solvents used for sample preparation and HSCCC were of analytical grade and purchased from the Tianjin Chemical Factory (Tianjin, China). Methanol used for HPLC analysis was of chromatographic grade, and was purchased from Yuwang Chemical (Shandong, China). The water used was purified using a PAT-125 (Chengdu Ultra-Technology, Chengdu, Sichuan, China) laboratory ultrapure water system with a 0.45 μm filter.

2.2 Apparatus

HSCCC was performed on a TBE-300C high-speed counter-current chromatography instrument (Shanghai Tauto Biotech, Shanghai, China) with polytetrafluoroethylene, three

preparative coils (tube diameter: 1.6 mm, total volume: 280 mL) and a 20 mL sample loop. The revolution radius or the distance between the holder axis and the central axis of the centrifuge (*R*) was 5 cm. The *β* values of the multi-layer coil ranged 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 HSCCC instrument was assembled by AKTA prime (GE, USA) with a UV absorbance detector to monitor the effluent, an N2000 workstation (Zhejiang University Star Information Technology, Hangzhou, Zhejiang, China) to collect the data, and a HX 1050 constant-temperature module (Beijing Boyikang Laboratory Instrument Company, Beijing, China) for temperature control. The revolution speed of the apparatus can be adjusted from 0 to 1000 rpm.

The HPLC system was an Agilent 1200 system, consisting of a G1354A solvent delivery unit, a G1315B UV-vis photodiode array detector, a G1316A column thermostat, a G1313A autosampler, an XBridge Shield RP 18 analytical column (5 μm, 4.6 × 250 mm), and an Agilent HPLC workstation (Agilent Technologies, USA).

Reversed-phase liquid chromatographic preparation was carried out using a Hanbon RPLC system (Hanbon Science & Technology, Jiangsu, China) equipped with a model N2000 workstation (Zhejiang University, Hangzhou, China), a NU 3000 SERIALS UV/VIS detector, a NP 7000 SERIALS pump, and a Dubhe C_{18} column (20 × 250 mm id, 10 μm) (Hanbon Science & Technology, Jiangsu, China).

2.3 Preparation of the crude sample

A 2.0 kg of the *H. leptocarpum* plants were ground into power and extracted with 95% ethanol three times (12 L × 3). The extraction time for the three extractions was 2, 2, and 1.5 h, respectively. The extracts were combined and evaporated to about 500 mL under vacuum at 60°C. The condensation was

dissolved in 2 L 2% HCl then the acidified aqueous solution was filtered, and extracted using petroleum ether (3 L × 3). The acid solution was successively basified with ammonium hydroxide up to pH 9–10, and extracted with $CHCl_3$, yielding 12.6 g of crude alkaloids that were used for subsequent HSCCC and RPLC separation.

2.4 Preparation of two-phase solvent systems and sample solution

pH-zone-refining CCC separation was performed with the two-phase solvent system composed of *n*-hexane/ethyl acetate/methanol/water (3:5:1:7, v/v/v/v). The two-phase solvent was equilibrated in a separatory funnel, and separated before used. The lower phase (mobile phase) was acidified with HCl to give a 5 mM solution. The upper phase (stationary phase) was rendered basic by addition of TEA to give a 10 mM solution. The sample solution is prepared by dissolving crude sample in the solvent mixture consisting of 10 mL aqueous phase free of retainer acid and 10 mL organic phase containing triethylamine.

2.5 HSCCC separation procedure

The HSCCC was performed as follows: the multilayer column was first completely filled with the upper phase (stationary) using the AKTA prime plus system. Then the apparatus was rotated at 850 rpm (forward direction), and the lower phase was pumped into the column at a flow rate of 1.5 mL/min when the revolution velocity was smooth. After hydrodynamic equilibrium was reached, indicated by the emergence of the mobile phase front, the sample solution was injected into the column through the injection valve. The effluent from the tail end of the column was continuously monitored by a UV detector at 280 nm. Peak fractions were manually collected according to the chromatogram data, and each fraction was evaporated to dryness under reduced pressure and dissolved in methanol for subsequent purity analysis by HPLC. The HCl and traces of triethylamine were removed from target compounds by water washing.

After the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas.

2.6 HPLC analysis of HSCCC and RPLC peak fractions

The crude sample and each peak fraction obtained by HSCCC and RPLC were analyzed by HPLC. The HPLC analysis was performed on an XBridge Shield RP 18, 5 μm, 4.6 × 250 mm analytical column with gradient elution, at a column temperature of 25°C. The mobile phase of methanol/water was used: (methanol: 0–15 min, 20–50%; 15–25 min, 50–80%; 25–35 min, 80–95%). The flow rate and detection wavelength were set at 1.0 mL/min and 280 nm, respectively. The crude

sample and peak fractions separated by HSCCC and RPLC were analyzed by HPLC, and the chromatograms are presented in Fig. 2A and B, respectively.

2.7 Cell line and cultures

The human breast epithelial cell line MDA-MB-231, an estrogen receptor-negative cell derived from a metastatic carcinoma, was used in the study. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were maintained at cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.8 Cell viability assay

Cell viability was assessed by Cell Counting Kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay. Briefly, cell were seeded at a concentration of 10^4 cells/200 μL/well into 96-well plates, and allowed an overnight period for attachment. The medium was removed, and fresh medium along with various concentrations of compound were added to cultures in parallel. Following treatment, drug-free medium (100 μL/well) and 10 μL CCK-8 solution were added and cells were incubated for 1 h at 37°C. The optical density value (absorbance) was measured at 450 nm by a microplate spectrophotometer (Multiskan, MK3). All experiments were performed in four replicates on three separate occasions.

2.9 Immunoblot analysis

Cells were washed three times with ice-cold PBS and harvested in RIPA lysis buffer with 1 mM PMSF, and then lysed for 5 min on ice. The homogenate was centrifuged at 12 000 rpm for 30 min at 4°C and supernatant was collected for FAS analysis. Equal protein extracts were separated using SDS-PAGE. Proteins were electrophoretically transferred to PVDF membranes. Incubation with primary and secondary antibodies was performed in Tris-buffered saline containing 5% nonfat dry milk for 2 h or more. After incubation, membranes were washed in Tris-buffered saline containing 0.1% Tween 20. ECL was used for detection. The relative expression of proteins was according to the reference bands of GAPDH.

3 Result and discussion

3.1 Selection of optimum solvent system for pH-zone-refining CCC

The solvent system used in this study was selected in the typical way developed by Ito [18]. Successful separation by pH-zone-refining CCC largely depends on the selection of

A

B
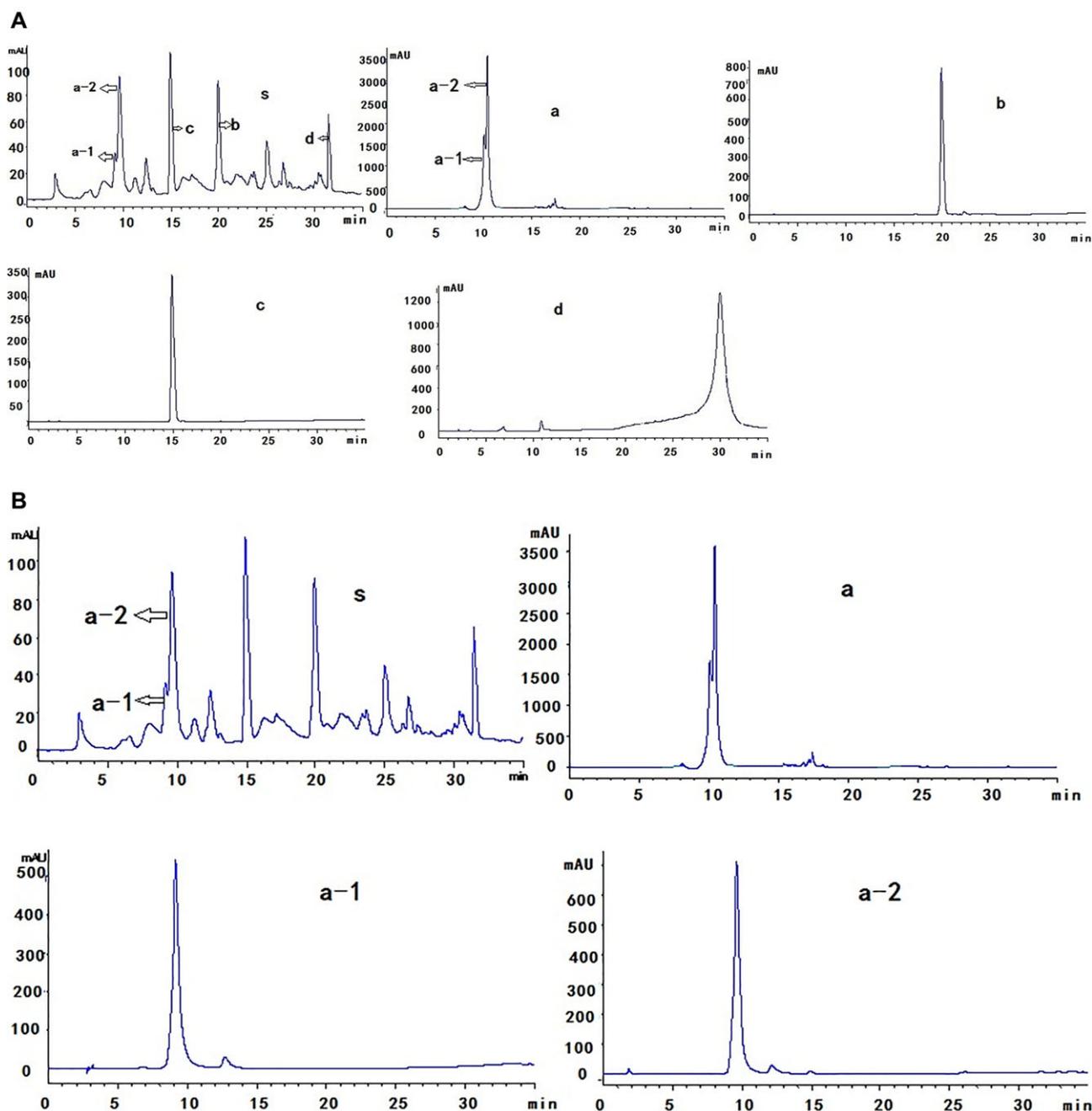
Figure 2. HPLC chromatogram of the crude sample and four collected fractions (a, b, c, and d) separated by HSCCC (2A) and the HPLC chromatogram of the alkaloids separated by RPLC (2B). Conditions: XBridge Shield RP18, 5 μm, 4.6 × 250 mm analytical column; column temperature 25°C; mobile phase: methanol and water (methanol: 0–15 min, 20–50%; 15–25 min, 50–80%; 25–35 min, 80–95%); flow rate 1.0 mL/min; detection wavelength: 280 nm.

suitable solvent system that should have an appropriate partition coefficient (K) values in both basic ($K_{base} >> 1$) and acidic ($K_{acid} << 1$) conditions [19, 20]. According to the rules for the selection of the suitable solvent system described in the related references, the new quaternary biphasic solvent system composed of *n*-hexane/ethyl acetate/methanol/water at different volume ratios (5:5:5:5, 3:5:3:5, 3:4:2:5) was tested. After trying, we found that when the solvent system *n*-hexane/ethyl

acetate/methanol/water at the volume ratio of 3:4:2:5, where triethylamine (10 mM) was added to the upper organic stationary phase as a retainer and hydrochloric acid (5 mM) to the aqueous mobile phase as an eluter, was used as the two-phase solvent system, the alkaloids could be separate well and the separation chromatogram was showed in Fig. 3A. However, the rectangular peaks were too narrow, which means that the alkaloids were eluted too quickly and the purity of the
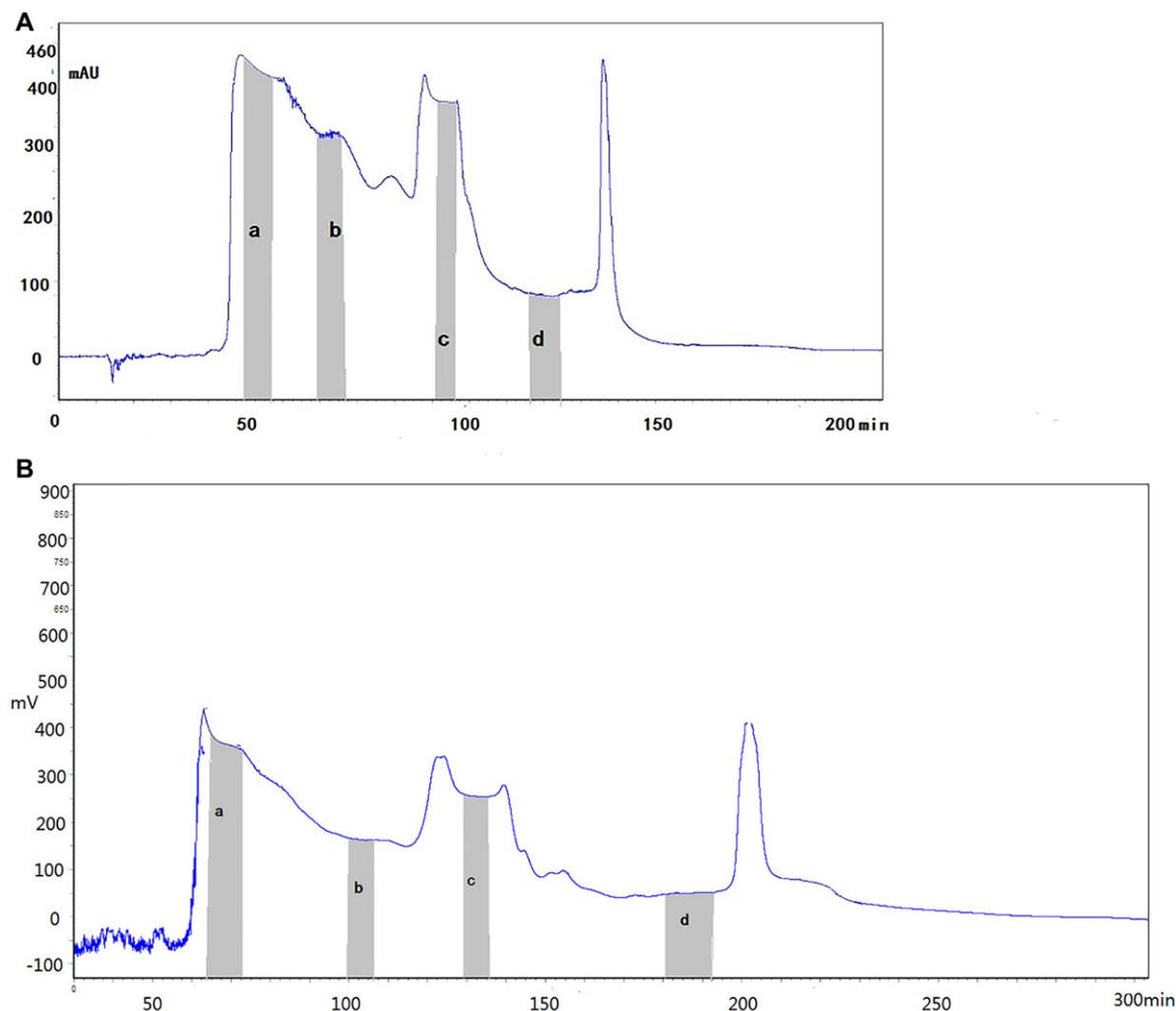
Figure 3. HSCCC chromatogram of the crude sample. Two-phase solvent system: *n*-hexane/ethyl acetate/methanol/water at the volume ratio of (3:4:2:5, v/v/v/v, Fig. 3A) and (3:5:1:7, v/v/v/v, Fig. 3B). The lower phase (mobile phase) was acidified with HCl to give a 5 mM solution. The upper phase (stationary phase) was rendered basic by the addition of TEA to give a 10 mM solution; stationary phase: the upper phase; mobile phase: the lower phase; flow rate: 1.5 mL/min; revolution speed: 850 rpm; detection wavelength: 280 nm; sample size: 1200 mg of the crude sample. The sample solution is prepared by dissolving crude sample in the solvent mixture consisting of 10 mL aqueous phase free of retainer acid and 10 mL organic phase containing triethylamine; separation temperature: 25°C; retention of the stationary phase: 57.8%.

separated alkaloids was not good. According to the rule of the selection of the solvent system, the resolution of the target alkaloids and the purity of them can be improved through increasing the ratio of water and reducing the ratio of methanol. Therefore, *n*-hexane/ethyl acetate/methanol/water (3:5:1:7) was selected for the efficient separation. After tested, it was found that this solvent system where triethylamine (10 mM) was added to the upper organic stationary phase as a retainer and hydrochloric acid (5 mM) to the aqueous mobile phase as an eluter, was suitable for the large-scale separation of alkaloids from *H. leptocarpum*. Figure 3B showed a typical chromatogram for the separation of 1.2 g of the crude extract using pH-zone-refining CCC. The total separation time was only 4 h, and the retention of the stationary phase was about 57.8%. Alkaloids were eluted as an irregular

rectangular peak where four absorbance plateaus (plateaus a, b, c, and d in Fig. 3B) were observed. Based on the HPLC analysis and the elution curve of pH-zone-refining CCC chromatogram, all collected fraction were combined into different fractions. As a result, 31 mg (–)-*N*-methylanadine (fraction b), 27 mg oxohydrastinine (fraction c), 47 mg hydroprotopine (fraction d) with the purity of 97.6, 98.8, and 92.8%, respectively, as determined by HPLC (Fig. 2A) and 58 mg of two other mixed alkaloids (fraction a) were obtained.

3.2 RPLC separation procedure

The two other mixed alkaloids (fraction a) could not been separated once by pH-zone-refining CCC due to their
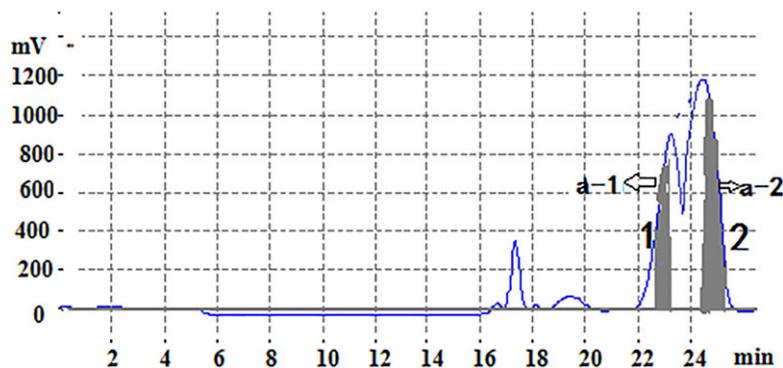
Figure 4. Chromatogram of RPLC. A Dubhe C$_{18}$ column (20 × 250 mm id, 10 μm) (Hanbon Science & Technology, Jiangsu, China) was used in RPLC while the flow rate and detection wavelength were set at 10.0 mL/min and 280 nm, respectively. The mobile phase, a solution of methanol/water was used: (methanol: 0–10 min, 40–42%; 10–30 min, 42–42%).

similar alkaline and hydrophobicity. Then the RPLC was used to separate the two compounds. The RPLC condition was decided according to HPLC results. A Dubhe C$_{18}$ column (20 × 250 mm id, 10 μm; Hanbon Science & Technology, Jiangsu, China) was used in RPLC, and the flow rate and detection wavelength were set at 10.0 mL/min and 280 nm, respectively. The mobile phase, a solution of methanol/water was used: (methanol: 0–10 min, 40–42%; 10–30 min, 42–42%). The chromatogram of the result is shown in Fig. 4.

3.3 Identification of HSCCC and RPLC peak fractions

At first, identification of HSCCC and RPLC peak fractions were carried out by ^{1}H NMR and ^{13}C NMR spectroscopy on a Mercury Plus 600 NMR spectrometer using tetramethylsilane as the internal standard. And the chemical compound b, c, and d were identified according to the related articles. But the component, a-1 and a-2, could not been measured. Then the identification of them was performed on the spectrometers of ESI-MS, DEPT-135, HSQC, and HMBC. Finally, the compound a-1 was identified as a new alkaloid while a-2 was leptopidine. The spectroscopic data of the five compounds are listed below.

3.3.1 Compound a-1

Hypecocarpine was separated as faint yellow powder. Mp. 251–254°C (MeOH). UV λ$_{max}$: 243, 303, 360. IR ν$_{max}$ (cm^{-1}): 3000–3400 (COOH), 1635 (C = O), 1619, 1595, 1507 (phenyl). Positive ESI–MS *m/z*: 386.1596 [M+H]$^+$. Twenty-one signals were observed in its ^{13}C NMR spectrum, of which 14 signals were above 100 ppm. In the C, H-COSY (HSQC) an N-methyl group at δ = 3.75 ppm and three methoxy groups at δ = 56.8 (s, 3H, 6-OCH$_3$), 56.6 (s, 3H, 7-OCH$_3$), and 56.5 ppm (4'-OCH$_3$) could be recognized. The other spectrum data of hypecocarpine resembles closely those of leptopidine [8]. ^{13}C NMR (DEPT-135) signals for three aliphatic C atoms, three methoxy groups, one N-methyl group, four aromatic methines, and ten quaternary C atoms were observed. Two phenyl rings were recognized from the ^{1}H NMR

signals at δ = 6.91 (s, 1H, H-5), 7.17 (s, 1H, H-8), 6.94 (d, 1H, J = 8.3 Hz, H-5'), and 6.68 ppm (d, 1H, J = 8.3 Hz, H-6'). The moiety =N–CH$_2$CH$_2$– could be measured by the HSQC signals at δ = 3.97 and 3.09 (each t, J = 7.5 Hz, H-3, and H-4), and 53.8 (t, C-3), and 26.6 ppm (t, C-4). On the basic of the following correlations in the HMBC the isoquinoline rings were determined: H-8, N-Me, and H-3 with C-1 (178.3 ppm), and H-5 with C-4 (26.6 ppm). The benzyl moiety was confirmed by the HMBC cross-signals of H-6' with C-7', C-2', and C-4', and of H-5' with C-1' and C-3'. Thus, the structure was elucidated. The ^{1}H and ^{13}C NMR spectroscopic data are listed in Table 1. The positive ESI-MS, ^{1}H NMR, ^{13}C NMR, DEPT-135, HSQC, and HMBC spectra are shown in Supporting Information Figs. S5–S10, respectively. And the UV and IR spectra of a-1 are shown in Supporting Information Fig. S11.

3.3.2 Compound a-2

Leptopidine was obtained as yellow powder. Positive ESI–MS *m/z*: 370.1291 [M+H]$^+$; The data of ^{1}H NMR and ^{13}C NMR spectroscopy were listed in Table 1. By comparison with the literature data [8], the compound was identified as leptopidine.

3.3.3 Compound b

Colorless crystals. ^{1}H NMR (600 MHz, CD$_3$OD, δ) feruloyl moiety: 3.91 (3H, s, C$_6$-OCH$_3$), 6.16 (1H, d, J = 15.6 Hz, H-2), 6.82 (1H, d, J = 8.0 Hz, H-8), 7.03 (1H, dd, J = 8.0, 2.0 Hz, H-9), 6.98 (1H, d, J = 2.0 Hz, H-5), 7.43 (1H, d, J = 15.6 Hz, H-3); tyramine moiety: 2.80 (2H, t, J = 6.8 Hz, H-2'), 3.49 (2H, t, J = 6.8 Hz, H-1'), 6.81 (2H, d, J = 8.8 Hz, H-5', and H-7'), 7.05 ppm (2H, d, J = 8.8 Hz, H-4', and H-8'); ^{13}C NMR (600 MHz, CD$_3$OD, δ): 34.77 (C-13), 40.88 (C-α, 55.96 (OCH$_3$), 109.53 (C-3), 114.66 (C-3", C-5"), 115.57 (C-5'), 118.10 (C-2-), 122.18 (C-6'), 126.75 (C-3'), 129.92 (C-2", C-6"), 129.59 (C-1"), 141.05 (C-2), 147.26 (C-4'), 147.86 (C-1'), 155.13 (C-4"), 167.23 ppm (C-1). According to ^{1}H and ^{13}C NMR spectroscopy and comparison with the literature data [21–23], the compound was identified as *N*-feruloyltyramine.

Table 1. ^{1}HNMR and ^{13}CNMR spectroscopic data of the compounds a-1 and a-2.

Position	a-1		a-2	
	δ^{13}C	δ[1]H	δ^{13}C	δ[1]H
1	178.3		178.3	
3	53.8	3.97 (t, 2H, J = 7.5 Hz)	53.6	3.97 (t, 2H, J = 7.5 Hz)
4	26.6	3.09 (t, 2H, J = 7.5 Hz)	27.0	3.09 (t, 2H, J = 7.5 Hz)
4a	134.9		137.2	
5	111.4	6.91 (s, 1H)	108.9	6.85 (s, 1H)
6	156.5		155.1	
7	149.0		148.7	
8	114.5	7.17 (s, 1H)	110.6	7.15 (s, 1H)
8a	120.6		122.4	
1′	130.3		129.3	
2′	118.5		117.7	
3′	154.9		154.7	
4′	149.8		149.7	
5′	114.4	6.94 (d, 1 H, J = 8.3 Hz)	114.4	6.91 (d, 1 H, J = 8.0 Hz)
6′	120.7	6.68 (d, 1H, J = 8.3 Hz)	120.2	6.53 (d, 1H, J = 8.0 Hz)
7′	38.4		38.9	
8′	174.8		174.9	
NCH$_3$	44.3	3.75 (s, 3H)	44.4	3.69 (s, 3H)
6-OCH$_3$	56.8	3.87 (s, 3H)		
7-OCH$_3$	56.6	3.49 (s, 3H)		
4′-OCH$_3$	56.5	3.80 (s, 3H)	56.4	3.81 (s, 3H)
−O−CH$_2$−O−			104.2	6.00 (s, 2H)

3.3.4 Compound c

Colorless crystals. ^{1}H NMR (600 MHz, CDCl$_3$, δ): 3.53 (2H, t, J = 7 Hz, H-3), 2.89 (2H, t, J = 7 Hz, H-4), 6.69 (1H, s, H-5), 7.31 (1H, s, H-8), 5.97 (2H, s, −OCH$_2$O−), 3.08 ppm (3H, s, N−Me). ^{13}C NMR (600 MHz, CDCl$_3$, δ): 164.42 (C-l), 150.17 (C-6), 146.76 (C-7), 133.35 (C-8a), 123.50(C-4a), 108.12 (C-5), 106.75 (C-8), 101.34 (-OCH$_2$O-), 48.14 (C-3), 35.06 (N-Me), 27.95 ppm (C-4). According to ^{1}H and ^{13}C NMR spectroscopic data and comparison with the literature data [8], the compound was identified as oxohydrastinine.

3.3.5 Compound d

Light brown crystals. ^{1}H NMR (600 MHz, CD$_3$OD, δ): 7.14 (1H, s,H-1), 6.76 (1H, s, H-4), 3.03 (3H, s, N−CH$_3$), 3.67 (2H, m, H-8), 6.85 (1H, d, J = 8 Hz, H-11), 6.86 (1H, d, J = 8 Hz, H-12), 4.59 (2H, m, H-13), 5.99 (2H, s, O−CH$_2$−O), 6.02 ppm (2H, s, O−CH$_2$−O). ^{13}C NMR (600 MHz, CD$_3$OD, δ): 106.94 (C-1), 145.53 (C-2), 147.83 (C-3), 110.21 (C-4), 128.01 (C-4a), 25.0 (C-5), 56.25 (C-6), 43.67 (N-CH3), 55.16 (C-8), 123.43 (C-8a), 149.03 (C-9), 150.43 (C-10), 109.52 (C-11), 123.92 (C-12), 125.73 (C-12a), 48.58 (C-13), 178.25 (C-14), 132.7 (C-14a), 103.17 ppm (O−CH$_2$−O), 103.40 ppm (O−CH$_2$−O). According to ^{1}H and ^{13}C NMR spectroscopic data and comparison with the literature data, the compound was identified as hydroprotopine. The protopine has been reported in *H. leptocarpum*. However, in this work hydroprotopine was separated from *H. leptocarpum* because of the condition used in the solvent

system and the two alkaloids could be converted into each other under certain conditions [24].

3.4 Cytotoxicity of compound hypecocarpine and leptopidine in breast cancer cells [25]

To evaluate the cytotoxicity of compound hypecocarpine and leptopidine, human breast cancer cells were incubated with various concentrations of hypecocarpine and leptopidine (0, 2, 4, 6, 8, 10 μg/mL) for 24 and 48 h followed by a CCK-8 assay. As shown in Supporting Information Fig. S12, leptopidine exhibited cytotoxic effects on MDA-MB-231 cell growth in a dose- and time-dependent manner. However, hypecocarpine did not affect cell viability on MDA-MB-231 cells. These results suggested that leptopidine could suppress growth and induce cytotoxicity in breast cancer cells.

3.5 Compound leptopidine downregulated FAS expression in breast cancer cells

Induction of FAS expression is considered one of the most common molecular changes in breast cancers. To further gain a mechanistic insight, we measured the effect of hypecocarpine and leptopidine on expression level of FAS in breast cancer cells. Incubation of MDA-MB-231 cells with the indicated concentrations of leptopidine at 37°C for 24 h resulted in an incubation dose-dependent inhibition of FAS expression (Supporting Information Fig. S13). The results

suggested that the cytotoxicity of leptopidine may be related to its inhibitory effect on FAS expression.

4 Concluding remarks

This study has established an efficient method to separate the two alkaloids with FAS inhibitory and potent anti-cancer activity from crude extract of *H. leptocarpum* by combining pH-zone-refining CCC with RPLC. The pure alkaloids separated from *H. leptocarpum* can be used in the further study of this traditional Tibetan medicine and to find the clear targets in molecular level. The value of *H. leptocarpum* can be greatly increased through extracting and separating the active compounds of alkaloids by this method.

The authors have declared no conflict of interests.

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