

Original Research Article

Isolation and Purification of Sesquiterpene Lactones from *Ixeris sonchifolia* (Bunge) Hance by High-Speed Counter-Current Chromatography and Semi-Preparative High Performance Liquid Chromatography

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Abstract

Purpose: To isolate and purify sesquiterpene lactones from *Ixeris sonchifolia* (Bunge) Hance by high-speed counter-current chromatography (HSCCC).

Methods: *I. sonchifolia* was extracted with water and then loaded on a glass column (10 × 1500 cm containing 3000g D101 macroporous resin) where various concentrations of aqueous ethanol (0, 10, 30, 50, and 95 %) were used to elute the column successively. The 50 % ethanol fraction was purified by HSCCC using a solvent system comprised of ethyl acetate: n-butanol: methanol: water (4: 6: 1: 20, v/v), and semi-preparative high performance liquid chromatography (HPLC). The chemical structures of the components obtained were further confirmed by high-resolution mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR).

Results: Three compounds, including ixerin Z1 (0.7 mg), ixerin Z (11.4 mg), and 11, 13 α -dihydroixerin Z (8.2 mg), with purity of 96.2, 98.2, and 98.4 %, respectively, were obtained from 200 mg each of the 50 % ethanol fraction.

Conclusion: HSCCC is a rapid and effective method for isolating and purifying sesquiterpene lactones from *I. sonchifolia*.

Keywords: Sesquiterpene lactones, High-speed counter-current chromatography, *Ixeris sonchifolia*, Ixerin, 13 α -Dihydroixerin

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INTRODUCTION

Ixeris sonchifolia (Bunge) Hance, commonly called *kudiezi* in China, which belongs to the family Compositae, is mainly distributed in Northeastern areas of China [1]. It is widely used as a folk medicine in China for its remarkable medical effects, such as dissipating blood stasis, improving microcirculation, and antineoplastic activity [2-5].

The chemical compounds of this plant are sesquiterpene lactones, organic acids, flavonoids, and triterpenes [6-9]. Ixerin Z and 11,13 α -dihydroixerin Z are the major sesquiterpene lactones [10], which have been reported to have various activities for instance, anti-inflammatory, anti-microbial, and anti-tumor activities [11,12]. In order to gain enough reference substance to perform further

investigation on its metabolism, it is important to use an efficient method to isolate and purify ixerin Z and 11,13 α -dihydroixerin Z from their natural sources.

Some conventional techniques including silica gel column chromatography have been successfully used to separate these chemicals from this plant [10]. However, these classic chromatography methods are complicated, inefficient, and time-consuming. Thus, an efficient and low-cost technique to obtain these compounds is required.

In the past few years, high-speed counter-current chromatography (HSCCC), a support-free liquid-liquid partition chromatographic technique, has been reported to eliminate irreversible adsorption of sample onto solid support matrix, stationary-phase deactivation, and contamination, and has made great progress in preparative isolation and purification of active components from natural products [13-15]. Therefore, our aim is to establish a protocol for enriching and isolating sesquiterpene lactones effectively from *I. sonchifolia* by HSCCC and to provide enough reference substance for *in vitro* and *in vivo* studies.

EXPERIMENTAL

Apparatus

The instrument used was a TBE-300B HSCCC (Tauto Biotech, Shanghai, China) equipped with three multi-layer coil separation columns connected in series (total volume of 300 mL) and a 20 mL sample loop. The β value of the preparative column varied from 0.5 at the internal layer to 0.8 at the external layer. The revolution speed of the apparatus was regulated with a speed controller in the range from 0 to 1000 rpm. The HSCCC system was equipped with a TBP-1002 pump (Agilent, Santa Clara, USA), an HX-1050 constant temperature regulator (Beijing Boyikang Lab Implement, Beijing, China), a TBD-2000 UV detector (Agilent, Santa Clara, USA) operating at 254 nm.

The analytical TBE-20A HSCCC instrument (Tauto Biotech, Shanghai, China) used was equipped with three polytetrafluoroethylene preparative coils (diameter of tube, 0.8 mm; total volume, 16 mL). The β value varied from 0.6 at the internal terminal to 0.78 at the external terminal.

HPLC analysis was performed on an Agilent system equipped with ultraviolet detector

(Agilent, Santa Clara, USA). The MS analyses were performed with a LTQ-Orbitrap coupled with an ESI source (Thermo Electron, Bremen, Germany). The structures of the compounds were identified by Bruker Avance 500 NMR (Bruker Biospin, Rheinstetten, Germany).

Reagents and materials

I. sonchifolia was collected from Haicheng city, Liaoning province of China in September 2013. It was authenticated by Professor Jian-Qiu Lu, Center of Scientific Experiment, Beijing University of Chinese Medicine. A voucher specimen (no. 20130901) was deposited in the same department. All organic solvents used for HSCCC separation were of analytical grade (Beijing chemicals works, Beijing, China). Acetonitrile used for HPLC was of HPLC grade (Fisher, NJ, USA), and water was prepared by a Milli-Q water purification system (Millipore, Billerica, MA, USA) in our laboratory. D101 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjin, China).

Preparation of crude extract

The whole herb of *I. sonchifolia* (2 kg) was chopped and extracted with 12 L pure water by refluxing for 1 h, which was repeated three times. The extract was loaded on a glass column (10 \times 1500 cm) containing 3000 g D101 macroporous resin. Various concentrations of aqueous ethanol (0, 10, 30, 50, and 95 %) were used to elute the column successively. The 50 % ethanol fraction was concentrated under reduced pressure to achieve a brown residue.

HSCCC separation and semi-preparative HPLC purification procedures

During HSCCC separation, a two-phase solvent system of ethyl acetate n-butanol methanol water (4 : 6 : 1 : 20, v/v) was chosen to separate the compounds. The multilayer coil column was first completely filled with the upper phase as the stationary phase, and the lower phase was then pumped into the head end of the inlet column at a flow rate of 2.0 mL/min, while the apparatus was rotated at 900 rpm. After hydrodynamic equilibrium was established throughout the coil, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (200 mg of the 50 % ethanol fraction in solvent composed of 10 mL the lower and 10 mL upper phases) was injected into the column through the sample loop. During the separation process, the column temperature was set at 30 °C. The detection wavelength was 254 nm. The effluent was

continuously monitored and the fractions were collected according to the chromatograms obtained.

HPLC analysis and identification of compounds

The 50 % ethanol fraction and each peak fraction from HSCCC were all analyzed by HPLC using a Zorbax SB-C18 column (5 μ m, 250 \times 4.6 mm, Agilent Technologies, Germany). A linear gradient elution of solvent water (A) and acetonitrile (B) was applied with the following program: 0 – 10 min, 5 – 15 % B; 10 – 25 min, 15 – 20 % B; 25 – 35 min, 20 – 25 % B; 35 – 45 min, 25 – 30 % B; 45 – 60 min, 30 – 55 % B; 60 – 75 min, 55 – 100 % B. The flow rate was 1.0 mL/min and the column temperature was at room temperature. The effluents were monitored at 254 nm by a UV detector.

The chemical structures of compounds separated by HSCCC were all analyzed by LTQ-Orbitrap coupled with an ESI source in flow-injection mode. The optimized operating parameters in the negative ion mode were as follows: capillary voltage of 35 V, capillary temperature of 350 $^{\circ}$ C, electrospray voltage of 3.0 kV, sheath gas flow rate of 30 (arbitrary

units), auxiliary gas flow rate of 10 (arbitrary units), and tube lens of 110 V.

RESULTS

Under the HSCCC conditions, fractions A and B were separated from sample in a single HSCCC step (shown in Figure 1) within 240 min. The purities of compound 1 in fractions A was 79.5 % based on HPLC peak area percentage. After crystallization with dichloromethane, compound 1 with higher purity was obtained from fractions A. Fraction B was a mixture of two compounds, which was further purified on semi-preparative HPLC using acetonitrile water (17:83, v/v) at 2.0 mL/min to yield compounds 2 and 3.

The chemical structures of compounds separated by HSCCC were identified according to the HRMS, H-NMR, and C-NMR data. Their chemical structures are displayed in Figure 2. The quantity and purity of the three compounds are shown in Table 1.

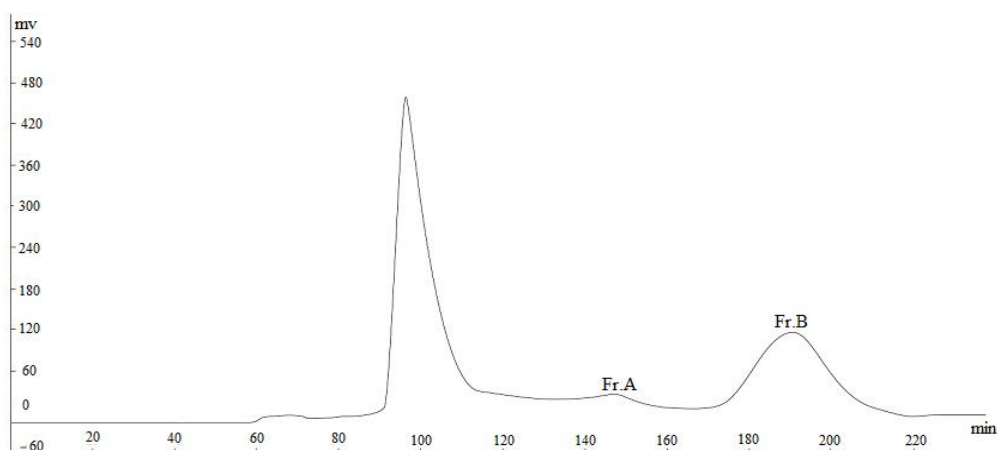


Figure 1: HSCCC chromatogram of crude extract of *Ixeris sonchifolia*

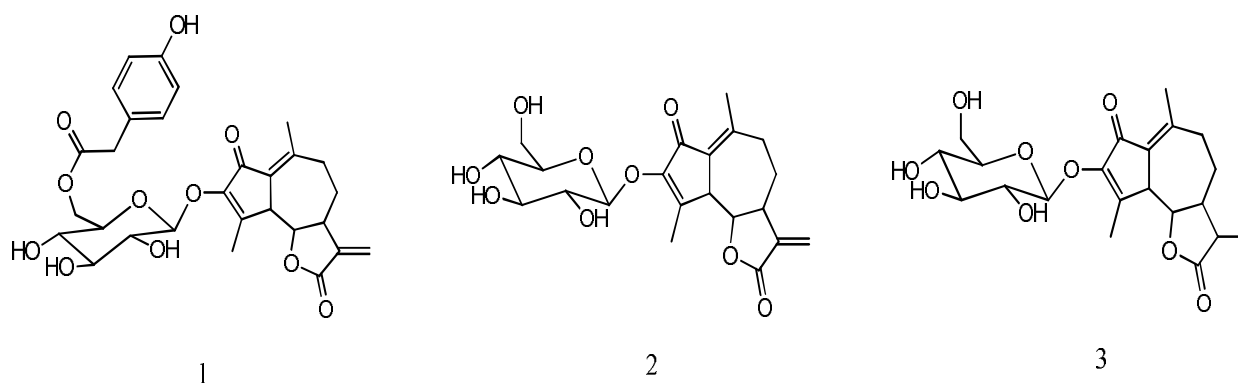


Figure 2: Chemical structures of isolated sesquiterpene lactones

Table 1: Quantity and purity of isolated compounds

Compounds	Content (mg/g)	Purity (%)
Ixerin Z1	3.5	96.2
Ixerin Z	57	98.2
11,13 α -dihydroixerin Z	41	98.4

Compound 1: ESI-MS (m/z): 555.1865 [M-H]⁻ (0.74 ppm, C₂₉H₃₁O₁₁). ¹H-NMR (500 MHz, C₅D₅N) δ : 3.30 (1H, m, H-5), 3.29 (1H, m, H-6), 2.76 (1H, m, H-7), 1.89 (1H, m, H-8), 1.12 (1H, m, H-8), 2.30 (1H, t, H-9), 2.10 (1H, m, H-9), 6.18 (1H, d, J = 3.2 Hz, H-13), 5.35 (1H, d, J = 2.7 Hz, H-13), 2.50 (3H, s, H-14), 2.35 (3H, s, H-15), 6.15 (1H, d, J = 7.8 Hz, H-1'), 4.23 (1H, m, H-2'), 4.30 (1H, m, H-3'), 4.17 (1H, m, H-4'), 4.13 (1H, m, H-5'), 5.02 (1H, m, H-6'), 4.76 (1H, dd, H-6'), 3.72 (2H, d, J = 4.8 Hz, H-2''), 7.33 (2H, d, J = 8.4 Hz, H-4', 8'), 7.13 (2H, d, J = 8.4 Hz, H-5', 7'); ¹³C-NMR (125 MHz, C₅D₅N) δ : 129.7 (C-1), 189.1 (C-2), 153.8 (C-3), 147.2 (C-4), 48.0 (C-5), 85.2 (C-6), 52.5 (C-7), 25.2 (C-8), 37.0 (C-9), 153.1 (C-10), 139.6 (C-11), 169.1 (C-12), 118.0 (C-13), 21.7 (C-14), 15.0 (C-15), 102.0 (C-1'), 75.3 (C-2'), 78.1 (C-3'), 71.2 (C-4'), 75.7 (C-5'), 64.7 (C-6'), 172.1 (C-1''), 40.3 (C-2''), 125.2 (C-3''), 131.0 (C-4''), 116.2 (C-5''), 157.9 (C-6''), 116.2 (C-7''), 131.0 (C-8''). These data are in good agreement with those of ixerin Z1 [16].

Compound 2: ESI-MS (m/z): 421.1506 [M-H]⁻ (3.07 ppm, C₂₁H₂₅O₉). ¹H-NMR (500 MHz, CD₃OD) δ : 3.58 (1H, d, H-5), 3.62 (1H, m, H-6), 3.04 (1H, brt, H-7), 2.27 (1H, m, H-8), 1.42 (1H, d, H-8), 2.63 (1H, t, H-9), 2.42 (1H, m, H-9), 5.56 (1H, d, J = 3.3 Hz, H-13), 6.09 (1H, d, J = 3.3 Hz, H-13), 2.44 (3H, s, H-14), 2.26 (3H, s, H-15), 5.23 (1H, d, J = 7.6 Hz, H-1'), 3.34 (1H, m, H-2'), 3.41 (1H, m, H-3'), 3.36 (1H, m, H-4'), 3.39 (1H, m, H-5'), 3.82 (1H, dd, H-6'), 3.69 (1H, d, H-6'); ¹³C-NMR (125 MHz, CD₃OD) δ : 130.6 (C-1), 191.1 (C-2), 154.5 (C-3), 150.1 (C-4), 53.8 (C-5), 87.1 (C-6), 49.4 (C-7), 25.4 (C-8), 38.1 (C-9), 156.2 (C-10), 140.7 (C-11), 171.3 (C-12), 119.2 (C-13), 22.4 (C-14), 15.4 (C-15), 102.5 (C-1'), 75.5 (C-2'), 78.4 (C-3'), 71.4 (C-4'), 78.0 (C-5'), 62.6 (C-6'). These data are in good agreement with those of ixerin Z [17].

Compound 3: ESI-MS (m/z): 423.1666 [M-H]⁻ (3.88 ppm, C₂₁H₂₇O₉). ¹H-NMR (500 MHz, CD₃OD) δ : 3.47 (1H, d, H-5), 3.66 (1H, m, H-6), 2.09 (1H, brt, H-7), 2.02 (1H, m, H-8), 1.37 (1H, d, H-8), 2.55 (1H, t, H-9), 2.35 (1H, m, H-9), 2.06 (1H, d, H-11), 1.22 (3H, d, J = 6.9 Hz, H-13), 2.44 (3H, s, H-14), 2.23 (3H, s, H-15), 5.22 (1H, d, J = 6.9 Hz, H-1'), 3.41 (1H, m, H-2'), 3.31 (1H, m, H-3'), 3.36 (1H, m, H-4'), 3.39 (1H, m, H-5'), 3.83 (1H, dd, H-6'), 3.69 (1H, d, H-6'); ¹³C-NMR

(125 MHz, CD₃OD) δ : 130.5 (C-1), 191.3 (C-2), 154.4 (C-3), 150.8 (C-4), 49.0 (C-5), 86.8 (C-6), 57.1 (C-7), 26.9 (C-8), 38.4 (C-9), 156.4 (C-10), 42.2 (C-11), 180.3 (C-12), 12.6 (C-13), 22.2 (C-14), 15.4 (C-15), 102.6 (C-1'), 75.5 (C-2'), 78.4 (C-3'), 71.4 (C-4'), 78.0 (C-5'), 62.6 (C-6'). These data are in good agreement with those of 11,13 α -dihydroixerin Z [18].

DISCUSSION

An HPLC method for the determination of the purities of sesquiterpene lactones separated by HSCCC was established at first. In order to obtain an appropriate elution system, mobile systems such as methanol water and acetonitrile water were tested. The result demonstrated that acetonitrile water with a gradient elution mode could afford better resolution of chromatographic peaks among the targets and the other compounds.

The important step in the successful separation of HSCCC is the selection of suitable two-phase solvent system [19]. In our experiments, *n*-butanol methanol water at different volume ratios were first selected as solvent system [20], and tested by analytical HSCCC. The analytes were eluted close to the solvent front with poor separation, whereas an addition of ethyl acetate could yield a suitable separation time and a much better resolution.

Due to the similar structures and largely polarities of ixerin Z and 11,13 α -dihydroixerin Z, it is difficult to separate them from complex system in a single HSCCC procedure with these two-phase solvent systems [21]. Thus semi-preparative HPLC was chosen to isolate and purify the mixture of ixerin Z and 11, 13 α -dihydroixerin Z.

CONCLUSION

A rapid and effective method for the isolation and purification of the major sesquiterpene lactones from *I. sonchifolia*, which could provide reference substances for further metabolism studies, is reported in this work. To the best of our knowledge, this is the first report on separation of sesquiterpene lactones in medical plants using HSCCC method.

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REFERENCES

- Nanjing University of Chinese Medicine. *Encyclopedia of Chinese Materia Medica*, vol.2. Shanghai Science and Technology Press, Shanghai, China; 2006; pp 1783-1785.
- Chen CG, Jia HL, Lv SX, Xu CQ. Protective effect of Kudiezi on acute cerebral ischemic reperfusion injury in rats. *Chin J Clin Pharmacol* 2012; 28: 196-199.
- Lin Y, Hu S, Xu D, Zheng G. Vasodilation effect of water extract from *Ixeris sonchifolia* (Bge.) Hance in rat thoracic aortic rings in vitro. *Chin J Pharmacol Toxicol* 2007; 21: 202-206.
- Su BY, Jung HL, Hae YC, Kwang SI, Song JB, Jae SC, Nam DK. Inhibitory effects of luteolin isolated from *ixeris sonchifolia* hance on the proliferation of hepg2 human hepatocellular carcinoma cells. *Arch Pharmacol Res* 2003; 26(2): 151-156.
- Fukumoto LR, Mazza G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J Agric Food Chem* 2000; 48(8): 3597-3604.
- Zhang WZ, Li XL, Wang MJ, Shi LG, Zhao DF, Zhang SJ. Sesquiterpene lactones from *Ixeris sonchifolia* (Bge.) Hance. *J Asian Nat Prod Res* 2008; 10(8): 753-758.
- Shi P, Zhang Y, Qu H, Fan X. Systematic characterization of secondary metabolites from *Ixeris sonchifolia* by the combined use of HPLC-TOFMS and HPLC-ITMS. *Phytochem Anal* 2011; 22(1): 66-73.
- Feng XZ, Dong M, Gao ZJ, Xu SX. Three new triterpenoid saponins from *Ixeris sonchifolia* and their cytotoxic activity. *Planta Med* 2003; 69(11): 1036-1040.
- Liu Y, Lu JQ, Zhang JY, Wang Q, Wang F, Qiao YJ, Zhang YL. Rapid determination of ten polyphenols in Kudiezi injection using ultra-performance liquid chromatography-tandem mass spectrometry in multiple reaction monitoring mode. *Anal Methods* 2012; 4: 4230-4236.
- Ni R. Preparation of active sesquiterpene lactone glucosides from *Ixeris sonchifolia* (Bge.) Hance and studies on their biotransformation [dissertation]. [Shenyang]: Shenyang Pharmaceutical University, 2006; p 32.
- Zhang YC, Zhou L, Ng KY. Sesquiterpene lactones from *Ixeris sonchifolia* Hance and their cytotoxicities on A549 human non-small cell lung cancer cells. *J Asian Nat Prod Res* 2009; 11(4): 294-298.
- Suh J, Jo Y, Kim ND, Bae SJ, Jung JH, Im KS. Cytotoxic constituents of the leaves of *Ixeris sonchifolia*. *Arch Pharm Res* 2002; 25: 289-292.
- Degenhardt A, Knapp H, Winterhalter P. Rapid isolation of malvidin 3-glucoside from red wine by high speed countercurrent chromatography (HSCCC). *Vitis* 2000; 39(1): 43-44.
- Yang Y, Huang Y, Gu D, Yili A, Sabir G, Aisa H. A. Separation and purification of three flavonoids from *Helichrysum arenarium* (L.) Moench by HSCCC. *Chromatographia* 2009; 69(9/10): 963-967.
- Du Q, Yuan J. Preparation of triterpene saponins from the fruit of *momordica charantia* L. by high speed countercurrent chromatography (HSCCC). *J Liq Chromatogr R T* 2005; 28(11): 1717-1724.
- Feng XZ, Xu SX, Dong M. A new sesquiterpene lactone glucoside from *Ixeris sonchifolia*. *J Asian Nat Prod Res* 2001; 3(4): 247-251.
- Ma JY, Wang ZT, Xu LS, Xu GJ, Kadota S, Namba T. Sesquiterpene lactones from *Ixeris sonchifolia*. *Phytochemistry* 1998; 48(1): 201-203.
- Na Z, Cho JY, Lee HJ, Chung JH, Park KD, Lee YJ, Shin SC, Rim YS, Park HK, Moon JH. Complete ¹H and ¹³C NMR assignments of sesquiterpene glucosides from *Ixeris sonchifolia*. *Magn Reson Chem* 2007; 45(3): 275-278.
- Guzlek H, Wood PL, Janaway L. Performance comparison using the guess mixture to evaluate counter-current chromatography instruments. *J Chromatogr A* 2009; 1216: 4181-4186.
- Cao XL. *High-Speed Counter-Current Chromatography and its Application*, Chemistry Industry Press, Beijing, China; 2005; p 55.
- Su W, Liu Q, Yang Q, Yu JG, Chen XQ. Separation and purification of four compounds from *Desmodium styracifolium* using off-line two-dimensional high-speed counter-current chromatography. *J. Sep. Sci* 2013; 36: 3338-3344