Separation and Purification of Three Main Lignans from the Anti-Inflammatory Extract of *LIlicium Difengpi* by High-Speed Counter-Current Chromatography

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Abstract. Based on the principle of partition coefficient values (κ) for target compounds and the separation factor (α) between target compounds, the optimal two-phase solvent system composed of n-hexane–ethyl acetate–methanol–water (1:0.3:1:0.2, v/v) was applied for the first time to isolate and purify three main lignans from the anti-inflammatory active extract of bark of *Illicium difengpi*. Using upper phase as the mobile phase in the head to tail elution model, magnolol (297.6 mg), difengpin (15.7 mg), and isodunnianol (17.5 mg), with the purities of more than 95%, were obtained from 800 mg of crude extract in a single operation. Additionally, their structures were identified by MS, ^{1}H-NMR and ^{13}C-NMR.

Introduction

Illicium difengpi is a toxic shrub that grows in Karst region of Guangxi Province, China. Its stem bark is listed in the Chinese Pharmacopeia as a traditional Chinese medicine to dispel wind, eliminate dampness, reduce swelling and relieve pain. The plant also is used to treat rheumatoid arthritis in Zhuang folk medicine [1]. Lignans, which was recognized as main constituents in *Illicium difengpi*, usually have significant anti-inflammatory activity [2]. Our previous study proved that the dichloromethane extract of *Illicium difengpi* exhibited inhibitory activities of mice ear engorement induced by dimethyl benzene. In this study, three lignans were obtained from this extract by high-speech counter-current chromatography, which structures (Fig.1) were identified by spectroscopic analysis (MS, ^{1}H-NMR and ^{13}C-NMR).

peak 1 (magnolol)   peak 2 (difengpin)   peak 3 (isodunnianol)
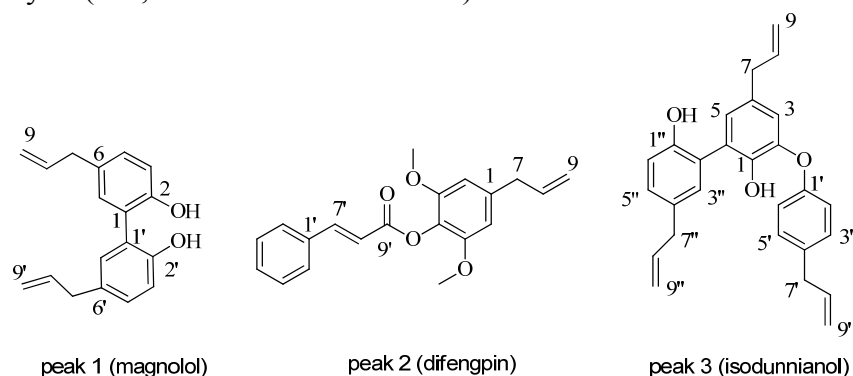
Fig. 1 Chemical structures of three main lignans from *Illicium difengpi*

Experimental

Reagents and Plant Materials. All solvent used for HSCCC are of analytical grade (XiLong Chemical Co., Ltd. China). Acetonitrile used for HPLC analysis is of chromatographic grade (Fisher Scientific, China). 100-200 mesh silica gel was used to enrich active components (Qingdao Haiyang Chemical Co., Ltd. China).

The bark of *Illicium difengpi* was collected in Du'an County, Guangxi Zhuang Autonomous Region, China, and identified by Professor Hui Tang, Guangxi Institute of Botany.

Apparatus. HSCCC (TBE-300C, Tauto Biotech, Shanghai, China) equipped with a three multilayer coil separation column connected in coil (i.d. of the tube, 1.9 mm, total volume, 300mL) and a 20

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mL sample loop. The separation system was also employed a TBP 5002 pump (Tauto Biotech, Shanghai, China), UV2000D detector (Shanghai Sanotac Scientific Instrument Co., Ltd. China), CHF161RA fraction collector (Toyo Seisakusho Kaisha, Ltd. Japan) and Easy Chrom-1000 workstation. The separation temperature was adjusted by DC-0506 low constant temperature bath (Tauto Biotech, Shanghai, China).

The analytical HPLC (LC-2030C 3D, SHIMADZU, Japan) was used for the determination of crude sample and peak fractions from HSCCC. LC-MS-IT-TOF system (Shimadzu, Japan) and Bruker AVANCE III HD 500 MHz NMR system (Bruker Inc., Sweden) was applied to identify the chemical structure of target compounds.

Preparation of Crude Sample. The bark of *Illicium difengpi* (1.5 kg) were smashed and percolated three times with dichloromethane (5L), following by drying under reduced pressure at 60 °C to yield 24 g of crude extract. In order to enrich the anti-inflammatory components, the dried extract was loaded on silica gel column (100-200mesh), and eluted with mixture solvents of petroleum ether and ethyl acetate (30:1, 10:1, v/v) in gradient model. And then fractions (10:1, v/v) were concentrated with rotary vaporization under reduced pressure at 60 °C to obtained 3.8 g of HSCCC sample.

Determination of Partition Coefficient (κ) and Separation Factor (α). According to the golden rules for HSCCC [3], the measurements of partition coefficient and separation factor are most importance steps in selecting two-phase solvent systems. In this paper, the determination of κ-value and α-value were performed by HPLC as follows: 1.0 mg of HSCCC sample was weighted into 25 mL test tube, and dissolved with 5mL each of upper and lower phase from pre-equilibrated biphasic solvent system. Then the test tube was shaken vigorously for several minutes to thoroughly equilibrate the sample in both phases. Subsequently, equal volume (200μl) of upper and lower phases were transferred and evaporated to dryness, separately. The residues were diluted with 200 μl of methanol and analyzed by HPLC to obtain the κ-value of target compound and the α-value between both target compounds.

Preparation of Two-Phase Solvent System and Sample Solution. A two-phase solvent system used in this study was consisted of n-hexane–ethyl acetate–methanol–water at volume rate of 1:0.3:1:0.2. The mixture solvent was thoroughly equilibrated in a separator funnel at room temperature, and separated and degassed by sonication 15 minute before use.

The sample solution was prepared by dissolving crude sample in 5 mL each of both phases of pre-equilibrated biphasic solvent system.

Separation Procedure. The multilayer column was first entirely filled with the upper phase of solvent system as stationary. Then the apparatus was rotated at 800 rpm in forward direction, while the lower phase was pumped into column at a flow rate of 2.0 mL/min from heal to tail. The temperature was set at 20 °C. After hydrodynamic equilibrium was reached, 10 mL of sample solvent included 800 mg of crude sample was injected into column through the injection valve. The effluent was continuously monitored by UV detector at 254 nm and automatically collected in a 25mL test tube for 5 min by fraction collector. When all target compounds had been eluted, the apparatus was stopped and the stationary phase in column was pushed out to calculate the retention rate of stationary phase. Subsequently, the target peak fractions were manually collected and dried under reduced pressure according to the chromatogram (Fig. 2).
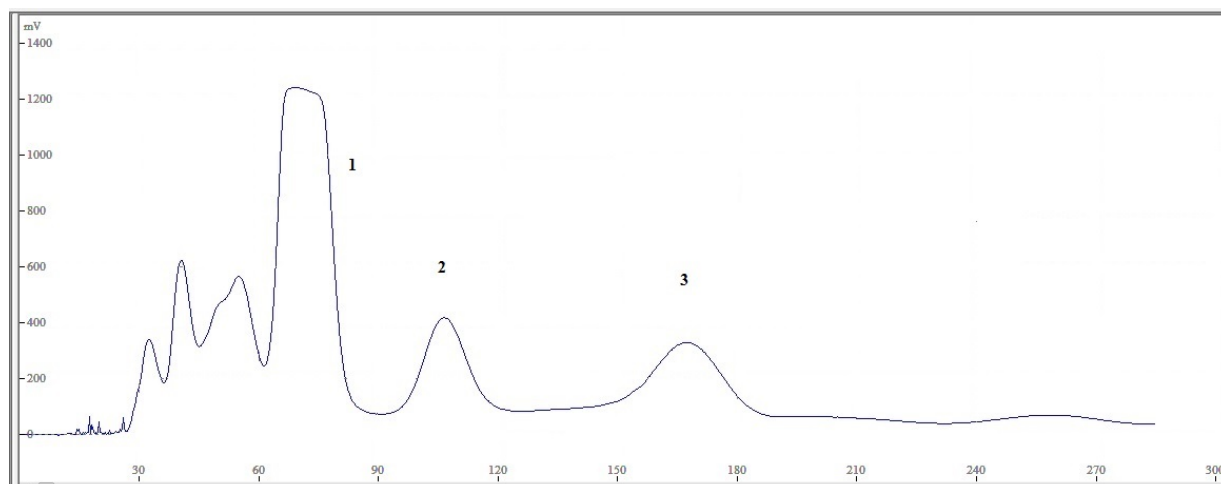
Fig. 2 HSCCC chromatogram of crude extract sample

Analysis and Identification of Target Compounds. The crude sample and each peak fractions were analyzed by HPLC with an Aglient ZORBAX SB-C$_{18}$ (5μm, 4.6 x 250 mm) at 30 °C. The mobile phase composed of acetonitrile and water was used in gradient elution model as follows: acetonitrile: 0-10 min: 45-65%, 10-25 min: 65-85% and 25-30 min, 85%. The flow rate of mobile phase was kept at 1mL/min and the effluent was monitored at 254 nm (Fig. 3).

The target peak fractions were identified by analyzing their spectroscopic data and comparing with published literature.
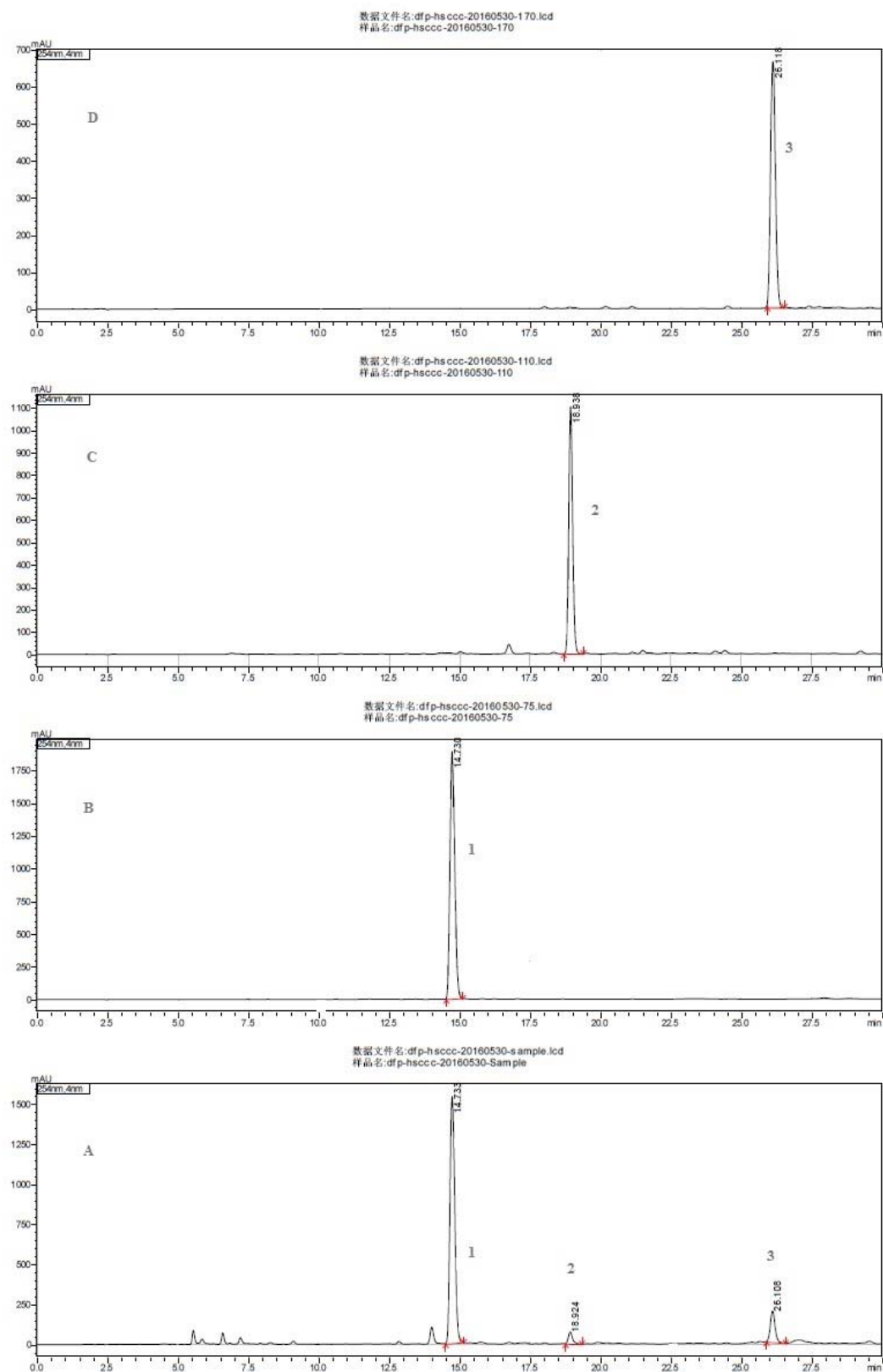
Fig. 3 Chromatograms of crude extract sample and target peak fractions. A: crude extract sample, B: peak 1 (magnolol), C: peak 2 (difengpin), D: peak 3 (isodunnianol)

Results and Discussion

Selection of Two-Phase Solvent System. The successful HSCCC separation acquired a suitable solvent system. Based on the analysis of the chromatographic behavior of magnolol in HSCCC [4,5], n-hexane–ethyl acetate–methanol–water was first considered as biphasic solvent system to

search ideal partition coefficient and separation factor of target compounds in different volume rate of solvent system. Usually, the ideal κ-value was considered in the range of 0.5-2 [6], and α-value ought to be greater than 1.5 ($\alpha = k_1/k_2$, where $k_1 > k_2$). In our study, the screening results presented in Table 1 show that the biphasic solvent systems (1:0.3:1:0.2 and 1:0.6:1.5:0.2, v/v) provided accepted κ-values for target compounds, but the α_{12} value in selected solvent systems indicated the biphasic solvent systems (1:0.3:1:0.2, v/v) had a greater resolution for compounds 1 and 2. Thus this solvent system was applied for the HSCCC separation. Additional, the retention of stationary phase also is an importance influence factor [3], and the retention of 76% was achieved in present study.

Table 1 The partition coefficient and separation factor of three anti-inflammatory compounds in different solvent systems.

n-hexane–ethyl acetate–methanol–water	κ value			α value		
	Compound 1	Compound 2	Compound 3	α_{12}	α_{13}	α_{23}
1:0.1:1:0.2	0.13	0.34	0.53	2.62	4.08	1.56
1:0.2:1:0.2	0.28	0.52	1.04	1.86	3.71	2.00
1:0.3:1:0.2	0.38	0.58	1.12	1.53	2.95	1.93
1:0.2:0.8:0.2	0.28	0.51	1.07	1.82	3.82	2.10
1:0.2:1.3:0.2	0.25	0.42	0.72	1.68	2.88	1.71
1:0.3:1.2:0.2	0.16	0.26	0.41	1.63	2.56	1.58
1:0.4:1.3:0.2	0.32	0.44	0.79	1.38	2.47	1.80
1:0.5:1.4:0.2	0.31	0.43	0.71	1.39	2.29	1.65
1:0.6:1.5:0.2	0.54	0.72	1.45	1.33	2.69	2.01
1:0.7:1.6:0.2	0.23	0.26	0.38	1.13	1.65	1.46

Structure Identification of the HSCCC Peaks

Peak 1: white powder. ESI-MS (*m/z*): 365 [M-H]⁻. ¹H NMR (500 Hz, CDCl₃) *δ:* 7.08 (2H, s, H-4, 4'), 7.06 (2H, s, H-6, 6'), 6.88 (2H, d, *J* = 7.5 Hz, H-3, 3'), 5.96 (2H, m, H-8, 8'), 5.09 (2H, d, *J* = 1.5 Hz, H-9a, 9a'), 5.04 (2H, d, *J* = 1.5 Hz, H-9b, 9b'), 3.34 (4H, d, br, *J* = 4.5 Hz, H-7, 7'). ¹³C-NMR (125 Hz, CDCl₃) *δ:* 151.3 (C-2, 2'), 137.7 (C-8, 8'), 133.2 (C-5, 5'), 131.4 (C-6, 6'), 129.9 (C-4, 4'), 124.6 (C-1, 1'), 116.9 (C-3, 3'), 115.9 (C-9, 9'), 39.5 (C-7, 7'). By comparing with reference data [7], peak 1 was identified as magnolol.

Peak 2: white powder. ESI-MS (*m/z*): 347 [M+Na]⁺. ¹H NMR (500 Hz, CDCl₃) *δ:* 7.88 (1H, d, *J* = 16.0 Hz, H-7'), 7.58 (2H, m, H-2', 6'), 7.40 (3H, m, H-3', 4', 5'), 6.72 (1H, d, *J* = 16.0 Hz, H-8'), 6.49 (2H, s, H-2, 6), 5.99 (1H, m, H-8), 5.17 (1H, d, *J* = 1.5 Hz, H-9a), 5.12 (1H, *J* = 1.5 Hz, H-9b), 3.82 (6H, s, 2xOCH₃). ¹³C-NMR (125 Hz, CDCl₃) *δ:* 164.9 (C-9'), 152.2 (C-3, 5), 146.5 (C-7'), 138.7 (C-1), 137.0 (C-8), 134.5 (C-1'), 130.6 (C-4'), 129.0 (C-3', 5'), 128.4 (C-2', 6'), 127.0 (C-4), 117.1 (C-8'), 116.4 (C-9), 105.3 (C-2, 6), 56.2 (2 x OCH₃), 40.8 (C-7). Compared with the data given in reference [7], peak 2 was identified as difengpin.

Peak 3: white powder. ESI-MS (*m/z*): 397 [M-H]⁻. ¹H NMR (500 Hz, CDCl₃) *δ:* 7.18 (2H, d, *J* = 9.0 Hz, H-3', 5'), 7.13 (1H, dd, *J* = 9.0, 2.0 Hz, H-5"), 7.12 (2H, d, *J* = 2.0 Hz, H-3"), 7.01 (1H, d, *J* = 8.5 Hz, H-6"), 6.99 (2H, d, *J* = 8.5 Hz, H-2', 6'), 6.90 (1H, d, *J* = 2.0 Hz, H-5), 6.72 (1H, d, *J* = 2.0 Hz, H-3), 5.86-6.04 (3H, m, H-8, 8', 8"), 5.02-5.12 (6H, m, H-9, 9' 9"), 3.38 (4H, d, *J* = 6.0 Hz, H-7', 7"), 3.29 (2H, d, *J* = 6.5 Hz, H-7), ¹³C-NMR (125 Hz, CDCl₃) *δ:* 154.6 (C-1'), 151.8 (C-1"), 144.2 (C-2), 141.7 (C-1), 137.8 (C-8'),137.3 (C-8), 137.1 (C-8"), 135.8 (C-4'), 133.3 (C-4"), 132.7 (C-4), 131.0 (C-3"), 130.0 (C-3'), 129.7 (C-5'), 129.7 (C-5"), 126.4 (C-5), 125.6 (C-6), 125.0 (C-2"), 118.5 (C-2'), 118.5 (C-6'), 117.8 (C-3), 117.5 (C-6"), 116.0 (C-9"), 116.0 (C-9'), 115.6 (C-9), 39.5 (C-7), 39.5 (C-7"), 39.4 (C-7'). Comparing the above data with literature [8], peak 3 was identified as isodunnianol.

Conclusions

Three main lignans were isolated from the anti-inflammatory extract of *Illicium difengpi* by one step HSCCC separation. Among of them, isodunnianol was isolated from this plant for the first time. The study results described above showed the biphasic solvent systems (1:0.3:1:0.2, v/v) was very suitable for separation of three compounds. Moreover, the separation method may be applied for the scale-up production.

Acknowledgements

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