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Artigo

EXTRACTION AND ISOLATION OF DICTAMNINE, OBACUNONE AND FRAXINELLONE FROM *Dictamnus dasycarpus* TURCZ. BY SUPERCRITICAL FLUID EXTRACTION AND HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

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Supercritical fluid extraction was used to extract active compounds from the Chinese traditional medicinal *D. dasycarpus* under the pressure of 30 MPa and temperature of 45 ºC. Further separation and purification was established by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (1:0.8:1.3:0.9, volume ratio). The separation yielded a total of 47 mg of dictamnine, 24 mg of obacunone and 83 mg of fraxinellone from 1.0 g of the crude extract in one step separation with the purity of 99.2, 98.4 and 99.0%, respectively, as determined by HPLC. The chemical structures of these compounds were identified by ESI-MS, IR, ^{1}H-NMR and ^{13}C-NMR.

Keywords: *Dictamnus dasycarpus*; dictamnine; obacunone.

INTRODUCTION

D. dasycarpus (Baixian-pi in Chinese, Rutaceae plants) is one of the most popular Chinese traditional medicines and has been used for the treatment of jaundice, rheumatism, cough, headache, colds, and other diseases.[1] Modern pharmacological studies showed that its extract has anti-allergic effect, anti-fungal activity and appetite depressant.[2-4] Numerous constituents were found including limonoids, furoquinoline alkaloids, flavonoids and sesquiterpenes.[5-8] Among them, dictamnine, obacunone and fraxinellone are the most demonstrated representative. Dictamnine has the activity against fungi, cytotoxic effect, anti-platelet aggregation and vasorelaxing effect.[9-11] Obacunone possesses the ability against aberrant crypt foci and carcinoma formation.[12,13] Faxinellone has significant anti-inflammatory, hepatoprotective action and against colon carcinogenesis.[13,14] Their chemical structures of these compounds are shown in Figure 1.

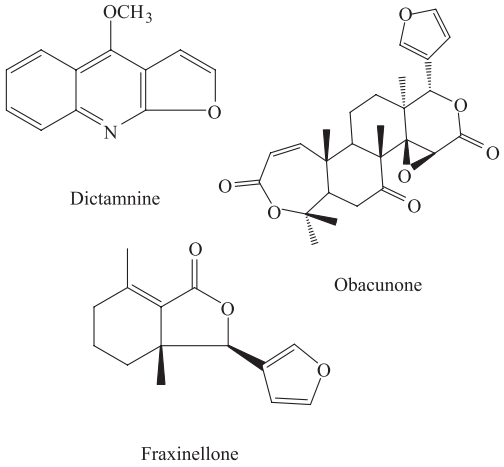

Figure 1. *Chemical structures of three compounds*

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The preparative separation and purification of those compounds from the root bark of *D. dasycarpus* by traditional column methods are tedious and usually require multiple chromatographic steps.[15] Therefore, it is important to develop an efficient method to extract, separate and purify them. Supercritical fluid extraction, for its low solvent consumption and mild conditions, has been used to extract the thermally labile components.[16,17] High-speed counter-current chromatography (HSCCC) is a support free liquid-liquid partition chromatographic technique which eliminates the irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. With a large volume of sample injection, multifarious relatively pure substances can be obtained at one step in large amount. It is especially suitable for separation and purification of active components from natural products.[18-20] However, there are no reports of using SFE to extract and HSCCC to isolate chemical compounds from *D. dasycarpus*. The aim of this paper was to develop an efficient method for the preparative extraction, separation and purification of dictamnine, obacunone and fraxinellone with high purity from *D. dasycarpus* by SFE and HSCCC.

EXPERIMENTAL

Apparatus

HSCCC was carried out using a Model GS10A-2 commercial instrument (Beijing Institute of New Technology Application, China), with a multilayer PTFE coil of 1.6 mm i.d. and 110 m in length with a total capacity of 230 mL. The β values of this preparative column range from 0.5 at the internal to 0.8 at the external (β = *r*/*R*, where *r* is the rotation radius or the distance from the coil to the holder shaft, and *R* (*R* = 8 cm) is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, China). Continuous monitoring of the effluent was carried out with a Model 8823A-UV detector (Beijing Institute of New Technology Applica-

tion, China) and a Model 320 pH meter (Mettler Toledo Instruments, China). A manual sample injection valve with 20 mL loop (Tianjin High New Science Technology Company, China) was used to introduce the sample into the column. A Model 3057 portable recorder (Yokogawa, Sichuan Instrument Factory, China) was used to draw the chromatogram.

The high-performance liquid chromatography (HPLC) used throughout this study consisted of a Waters 996 photodiode array detection (PDA), a Waters 600 Multisolvent Delivery, a Waters 600 system controller, a Waters 600 pump, and a Millennium^{32} workstation (Waters, Milford, USA).

The *Spe-ed*™ supercritical fluid extraction (SFE) system (Applied Separations, Inc., Allentown, PA, USA) was used for extracting the crude extract from the material.

Materials

Carbon dioxide (99.9%) was purchased from Yaotian Gas Company, Jinan, China. petroleum ether (60-90 °C), *n*-Hexane, methanol, ethanol, ethyl acetate were analytical grade (Juye Chemical Factory, Jinan, China). Methanol used for HPLC analysis was of chromatographic grade (Yuwang Special Reagent Factory, Dezhou, China). Reverse osmosis Milli-Q water (Millipore, USA) was used for all solutions and dilutions.

The root bark of *D. dasycarpus* was obtained from a local drug store and identified by Dr. L. Jia (College of Pharmacy, Shandong University of Traditional Chinese Medicine, China). The voucher specimen of this plant (KL 1012) is deposited in the Process Control Research Center of TCM, Shandong Academy of Sciences, Shandong Analysis and Test Center, Jinan, China.

Sample preparation

Air-dried and ground root bark (500 g) was placed into a 1 L extraction vessel and extracted statically for 1 h followed by further 6.5 h of dynamic extraction under the pressure of 30 MPa with a temperature of 45 °C. The flow-rate of carbon dioxide supercritical fluid was set at 2 L/min, and the extract in the supercritical fluid was depressed directly into a separation vessel which yielded 17.3 g of crude extract for further isolation and purification.

Selection of two-phase solvent system

The composition of the two-phase solvent system was selected according to the partition coefficient (K_D) of the target compounds of the samples. The partition coefficient was determined by HPLC as follows: 10 mg of the crude extract was added to a test tube, to which 2 mL of each phase of the two-phase solvent system was added. The test tube was shaken violently for several minutes. Equal volumes of each phase were then analyzed by HPLC to obtain the partition coefficients (K_D). The K_D-value was defined as the peak area of compound in the upper phase divided by the peak area of compound in the lower phase.[21]

Preparation of the two-phase solvent system and sample solution

The HSCCC experiments were performed with a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (1:0.8:1.3:0.9, volume ratio, the same as follows). The solvent system was equilibrated in a separation funnel, and the two phases were separated before use. The upper organic was used as stationary phase and the lower as mobile. The sample solution was prepared by dissolving the crude sample in the mixture solution of organic phase and aqueous phase (1:1) of the solvent system used for HSCCC separation.

HSCCC separation

The multilayer coiled column was first entirely filled with the upper phase, and then the lower phase was pumped into the column at 2.0 mL/min while the column was rotated at 800 rpm in the head to tail elution mode. After hydrodynamic equilibrium was reached as indicated by a clear mobile phase eluting from the tail outlet, the solution of the sample was injected through the injection valve. The effluent from the outlet of the column was continuously monitored by UV detector at 254 nm, and the peak fractions were collected according to the chromatogram. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

Separation and purification by silica gel column

The SFE extract (10 g) was subjected to column chromatography on silica gel, elution with petroleum ether-ethyl acetate (petroleum ether, 5: 1, 4: 1, 3: 1, 2: 1, 3: 2, 1: 1, 1: 2), afforded eight fractions according to TLC and HPLC results. Fraction 2 (5: 1, containing fraxinellone) was repeatedly chromatographed on silica gel with the same solvent to afford fraxinellone. Fraction 5 (2: 1) and 8 (1: 2) afforded dictamnine and obacunone with the same method.

Analysis and identification of HSCCC peak fractions

The crude sample and each purified fraction from the HSCCC were analyzed by HPLC with a Shim-pack VP-ODS column (250 x 4.6 mm, i.d., 5 μm) and column temperature of 25 °C. The mobile phase, a solution of methanol and water (75:25), was set at a flow-rate of 1.0 mL/min. The effluent was monitored by PDA at 240 nm.

The identification of HSCCC peak fractions were performed by mass spectrometry (ESI-MS) with an Agilent 1100/MSD (California, USA), IR spectra with a Bruker Vertex 70 spectrometer and NMR spectra with a Varian-600 spectrometer (Varian, Palo Alto, CA, USA) with $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard.

RESULTS AND DISCUSSION

Optimization of HSCCC conditions

Partition coefficient (K_D) is the most important parameter in solvent system selection. Successful separation by HSCCC needs a suitable K_D-value. Large K_D-value usually tends to produce excessive sample band broadening, while small K_D-value results in a poor peak resolution.[22,23] In this experiment, the solvent system based on *n*-hexane-ethyl acetate-methanol-water at different volume ratios were tested (Table 1). As shown in Table 1, the K_D-value of the compound 2 in the solvent system of *n*-hexane-ethyl acetate-methanol-water (1:0.5:1:0.5) was too small, while it was difficult to separate compound 1 and 3 for the close K_D-value. The K_D-value of *n*-hexane-ethyl acetate-methanol-water (1:1:1:1) was too big and would result in a long separation time. Among these solvents, *n*-hexane-ethyl acetate-methanol-water (1:0.2:1:0.2) and (1:0.8:1.3:0.9) have suitable K_D-value. After trying all the above solvent systems, the solvent system *n*-hexane-ethyl acetate-methanol-water (1:0.8:1.3:0.9) was found to be the best. Figure 3 shows the separation of HSCCC using this solvent system.

Table 1. Partition coefficients (K_D) of compounds 1-3

Solvent system (n-hexane-ethyl acetate-methanol-water)	K_D		
	Compound 1	Compound 2	Compound 3
1:0.2:1:0.2	0.37	1.32	1.28
1:0.5:1:0.5	0.60	1.78	1.76
1:1:1:1	1.26	3.40	4.92
1:0.8:1.3:0.9	0.94	2.75	3.26

The SFE extract (1.0 g) from the root bark of *D. dascarpus* was purified under the optimum HSCCC conditions. The upper phase was used as the stationary phase while the lower phase was used as the mobile phase in the head to tail elution mode. The retention of the stationary phase was 44.6%, and the total separation time was about 8 h. The HSCCC fractions were analyzed by HPLC, and their absorbance was measured at 254 nm to draw the elution curve (Figure 3). Based on the HPLC analysis, three compounds were obtained in one step separation and yielded 47 mg of dictamnine (peak I in Figure 3), 24 mg of obacunone (peak II in Figure 3) and 83 mg of fraxinellone (peak III in Figure 3) with the purity of 99.2, 98.4 and 99.0%, respectively (Figure 2).

Separation with conventional silica gel column

The SFE extract (10 g) was separated and purified by conventional silica gel column and 310 mg of fraxinellone, 153 mg of dictamnine and 68 mg of obacunone were obtained after several separation steps about 80 h. The recovery of fraxinellone, dictamnine and obacunone is about 35, 30 and 27%. However, HSCCC could purify the crude extract in one-step separation with more than 90% recovery. The separation time was only 8 h in each separation run. In comparison with open-column silica gel, HSCCC represents very low solvent consumption for a sample of the size and short separation time used in this experiment.

The structural identification

The structural identification of the three compounds was performed with ESI-MS, IR, ^{1}H and ^{13}C-NMR spectra.

Compound corresponding peak I: ESI-MS (positive mode), *m/z* 200.0 $[M+H]^+$. $IR\nu_{max}^{cm^{-1}}$: 3432, 3002, 1579, 1368, 1121, 1085, 980, 757, 721, 636, 575. Comparing the data of ^{1}H and ^{13}C-NMR with references, the obtained compound was identified as dictamnine.[15,24]

Compound corresponding peak II: ESI-MS (positive mode), *m/z* 454.2 $[M+H]^+$. $IR\nu_{max}^{cm^{-1}}$: 2988, 2947, 1736, 1708, 1281, 1029, 988, 802. Comparing the data of ^{1}H and ^{13}C-NMR with references, the obtained compound was identified as obacunone.[15,24]

Compound corresponding peak III: ESI-MS (positive mode), *m/z* 255.2 $[M+Na]^+$. $IR\nu_{max}^{cm^{-1}}$: 2932, 1743, 1672, 1204, 1161, 1135, 1023, 977, 950, 871, 814, 747, 607. Comparing the data of ^{1}H and ^{13}C-NMR with references, the obtained compound was identified as fraxinellone.[15,24]

CONCLUSION

Three main compounds including dictamnine, obacunone and fraxinellone from the root bark of *D. dasycarpus* were extracted and purified by SFE and HSCCC. With a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (1:0.8:1.3:0.9), 47 mg of dictamnine, 24 mg of obacunone and 83 mg of fraxinellone were obtained from 1.0 g of SFE extract with the purity of 99.2, 98.4 and 99.0%, respectively. The recovery of this separation is over 90%, compared with the conventional silica gel column (less than 35%). The present study demonstrates that SFE and HSCCC are very useful techniques for the extraction, isolation and purification of bioactive natural components.

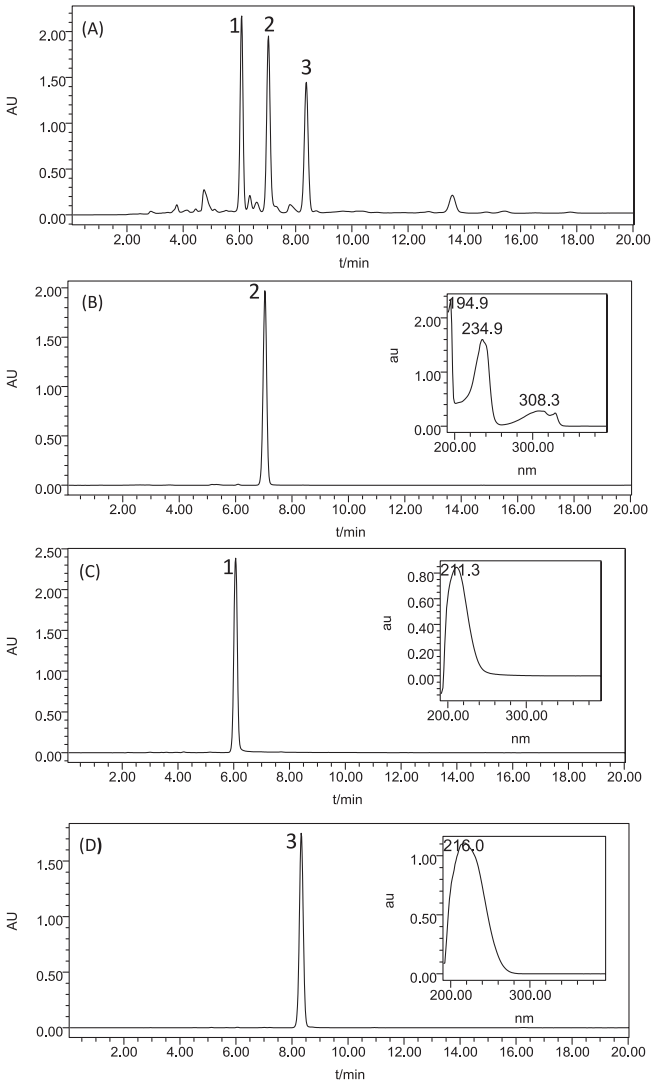

Figure 2. *(A) HPLC chromatograms of the SFE extract from D. dascarpus; (B) HPLC analysis and UV spectrum of the dictamnine (peak I in Figure 3) purified with HSCCC; (C) HPLC analysis and UV spectrum of the obacunone (peak II in Figure 3) purified with HSCCC; (D) HPLC analysis and UV spectrum of the fraxinellone (peak III in Figure 3) purified by HSCCC. Experimental conditions: a Shim-pack VP-ODS column (250 x 4.6 mm, i.d., 5 μm); column temperature: 25 °C; mobile phase: methanol-water (75:25); flow rate: 1.0 mL/min; detection: 240 nm; injection volume: 10 μL*

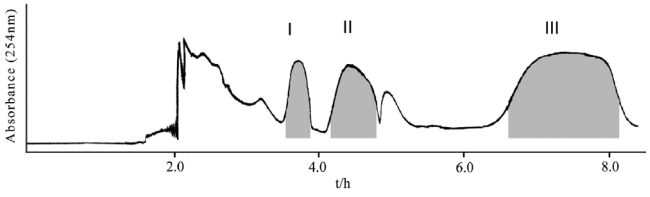

Figure 3. *HSCCC chromatogram of the crude extract. Two-phase solvent system: n-hexane-ethyl acetate-methanol-water (1:0.8:1.3:0.9); mobile phase: the lower phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm; detection wavelength: 254 nm; sample size: 1.0 g; injection volume: 10 mL; retention of stationary phase: 44.6%; I: dictamnine; II: obacunone; III: fraxinellone*

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