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Short communication

Ultrahigh pressure extraction of lignan compounds from *Dysosma versipellis* and purification by high-speed counter-current chromatography

Qing Zhu[a], Feng Liu[b], Meixia Xu[b], Xiaojing Lin[b], Xiao Wang[b,*]

[a] *Qilu Hospital of Shangdong University, 107 West of Wenhua Street, Jinan, Shandong 250012, China*
[b] *Shandong Analysis and Test Center, Shandong Academy of Sciences, 19 Keyuan Street, Jinan, Shandong 250014, China*

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ABSTRACT

Ultrahigh pressure extraction (UPE) was employed to extract podophyllotoxin and 4′-demethylpodophyllotoxin from *Dysosma versipellis*. The effects of extraction parameters including extraction solvents, pressure, time and solid/liquid ratio were investigated using a High Hydrostatic Pressure Processor. The optimal condition for UPE of the target compounds was 80% methanol, 200 MPa of pressure, 1 min of extraction time and 1:12 (g/mL) of solid/liquid ratio. Podophyllotoxin and 4′-demethylpodophyllotoxin in the crude extract were purified by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of petroleum ether–ethyl acetate–methanol–water (10:10:8:12, v/v), and the fractions were analyzed by HPLC, ESI-MS and ^{1}H NMR. As a result, 73.7 mg podophyllotoxin and 16.5 mg 4′-demethylpodophyllotoxin with purities over 96% were obtained from 260 mg crude sample in one-step separation.

1. Introduction

Dysosma versipellis (Hance) Cheng is a rare and endemic herb to China, mainly distributed in central and eastern parts of China [1]. Research showed that the main bioactive components were podophyllotoxin and its derivatives [2,3]. Podophyllotoxin exhibits significant biological activity such as anti-genital warts [4,5], anti-cancer activity [6] and immunomodulatory activity [7], and its semi-synthetic derivatives including etoposide, teniposide and etopophos are used as important chemotherapies of various cancers [8–10]. Further studies on pharmacological and clinical effects of podophyllotoxins necessitate the development of an efficient preparative separation method of these drugs.

At present, podophyllotoxis are often isolated and purified by some conventional protocols of extraction and separation techniques, such as heat reflux to extract and silica gel column chromatography to isolate. However, these methods are tedious, time-consuming and low efficiency. Ultrahigh pressure extraction is a new technique which developed quickly in recent years. In the process of ultrahigh pressure extraction (UPE), more solvent will enter the inner of cells and more active components will be extracted out of cells more easily [11]. It has been successfully used for extraction of various kinds of natural products with higher product yields and shorter extraction time comparing with conventional extraction methods [11–13]. HSCCC, a liquid–liquid partition chromatography, eliminates irreversible adsorption of samples on solid support in conventional column chromatography and offers excellent recovery of target compounds [14]. Many kinds of natural products have been successfully separated and purified by HSCCC [15–18]. However, no reports have been seen on the extraction and purification of podophyllotoxin and 4′-demethylpodophyllotoxin (Fig. 1) from *D. versipellis* by ultrahigh pressure technique combined with HSCCC. This paper successfully reported the preparative separation of the two compounds and the critical parameters of UPE and HSCCC were also optimized.

2. Experimental

2.1. Reagents and materials

Chromatographic grade methanol (Tedia Company Inc., Fairfield, USA) was used for HPLC analysis. Organic solvents were all of analytical grades (Damao Chemical Factory, Tianjin, China). The water used in solutions and dilutions was treated with a Milli-Q water purification system (Millipore, USA).

The *D. versipellis* was purchased from a local drug store and identified by Dr. J. Li (College of Pharmacy, Shandong University of Traditional Chinese Medicine, Shandong, China). The herbs were powdered and sieved through a 60 mesh screen.

* Corresponding author. Tel.: +86 531 8260 5319; fax: +86 531 8296 4889.
E-mail address: wangxiao2008@yahoo.cn (X. Wang).

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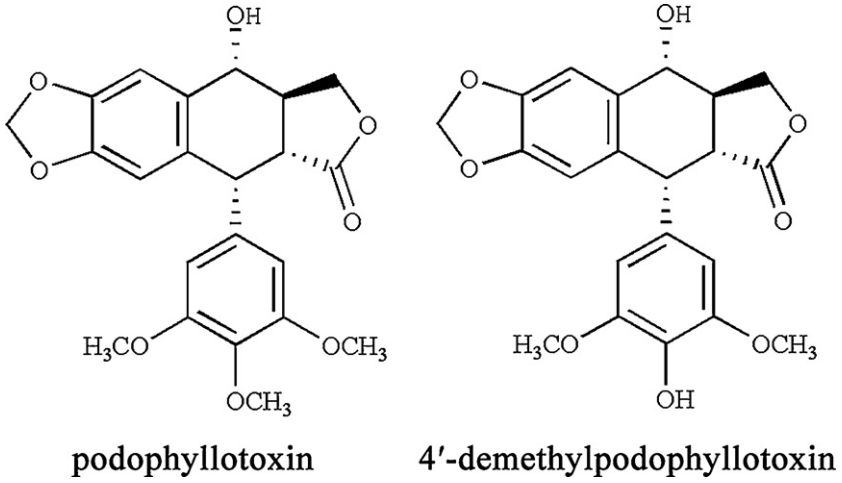

Fig. 1. Chemical structures of podophyllotoxin and 4′-demethylpodophyllotoxin from *Dysosma versipellis*.

2.2. Apparatus

The ultrahigh pressure-assisted extraction was conducted with a High Hydrostatic Pressure Processor (HPP.L3-600, Huataisenmiao Biology Engineering Technology Co. Ltd., Tianjin, China). The pressure ranged from 0 to 900 MPa, and the pressure precision was ±5 MPa.

The HSCCC separation was carried out using a Model GS10A-2 (Beijing Emilion Science & Technology Co., Beijing, China) equipped with a PTFE multilayer coil (1.6 mm i.d., total capacity of 230 mL) and a 10 mL sample loop. The β values of the multilayer column range from 0.5 at internal terminal to 0.8 at the external. A Model NS-1007 constant-flow pump was used to pump the two phases into the column. Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor at 254 nm. A Model 3057 portable recorder was employed to record the chromatogram.

HPLC analysis was carried out on an Agilent 1120 HPLC equipped with G4290A system (Agilent, California, USA).

2.3. Ultrahigh pressure extraction (UPE)

In each test 10.0 g sample powder was extracted with solvent, and the mixture was poured into a plastic bag. The sealed bag was subjected to ultrahigh pressure treatment for a given period. The extraction solution was centrifuged at a speed of 4000 rpm for 10 min, and the extraction was filtered through 0.45 μm membrane. Then the filtrate was injected into the HPLC for further analysis. After the UPE conditions were optimized, 200 g sample powder was extracted under the optimal conditions. After removing methanol, the extract solution was then extracted by ethyl acetate. The ethyl acetate extract was evaporated to dryness and 15.5 g of crude sample was obtained for the further separation of HSCCC.

2.4. Heat reflux extraction

Heat reflux extraction was the conventional extraction method of lignan compounds. An 80% (v/v) methanol solution was selected as solvent. The dried plant (10 g) sample was weighed in a flask, and 120 mL extraction solvent was added. Heat reflux extraction was carried out at 75 °C for 1 h.

2.5. HSCCC separation procedure

In the present study, HSCCC experiment was performed with the two-phase solvent system composed of petroleum ether–ethyl acetate–methanol–water (10:10: 8:12, v/v). The sample solution was prepared by dissolving 260 mg of crude sample in the mixture of upper phase and lower phase (1:1, v/v). HSCCC separation was performed as follows: firstly the multiplayer coiled column was entirely filled with the upper phase (stationary phase). Then the apparatus was rotated at 800 rpm, while the lower phase (mobile phase) was pumped from the head of the column into the end at a flow rate of 2.0 mL/min. After hydrodynamic equilibrium was reached, the sample solution was injected into the column through sample loop. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm and the chromatogram was recorded. Each peak fraction was manually collected according to the UV absorbance profile.

2.6. HPLC analysis and identification of HSCCC fractions

The crude sample and each peak fraction from HSCCC were analyzed by HPLC with a Shim-Pack VP-ODS column (250 mm × 4.6 mm I.D., 5 μm) at room temperature. The mobile phase, a solution of acetonitrile and water (35:65, v/v), was set at a flow-rate of 1.0 mL/min. The effluent was monitored at 215 nm by PAD.

The identification of HSCCC peak fractions was carried out by electrospray ionization mass spectrometry (ESI-MS) on an Agilent 1100/MS-G1946 (Agilent, California, USA) and ^{1}H NMR spectra on a Varian-600 NMR spectrometer (Varian, Palo Alto, USA).

3. Results and discussion

3.1. Optimization of UPE procedure

In order to obtain an optimal extraction condition, four major parameters of UPE including extraction solvents, pressure, time and liquid/solid ratio were optimized. The concentration of target compound was used as the marker for evaluation of extraction efficiency.

The extractions with different concentrations of methanol and ethanol solutions in water (100%, 80%, and 60%) were performed by UPE under the conditions of 2 min and 300 MPa. As shown in Fig. 2(A), the yields of target compounds in methanol system were

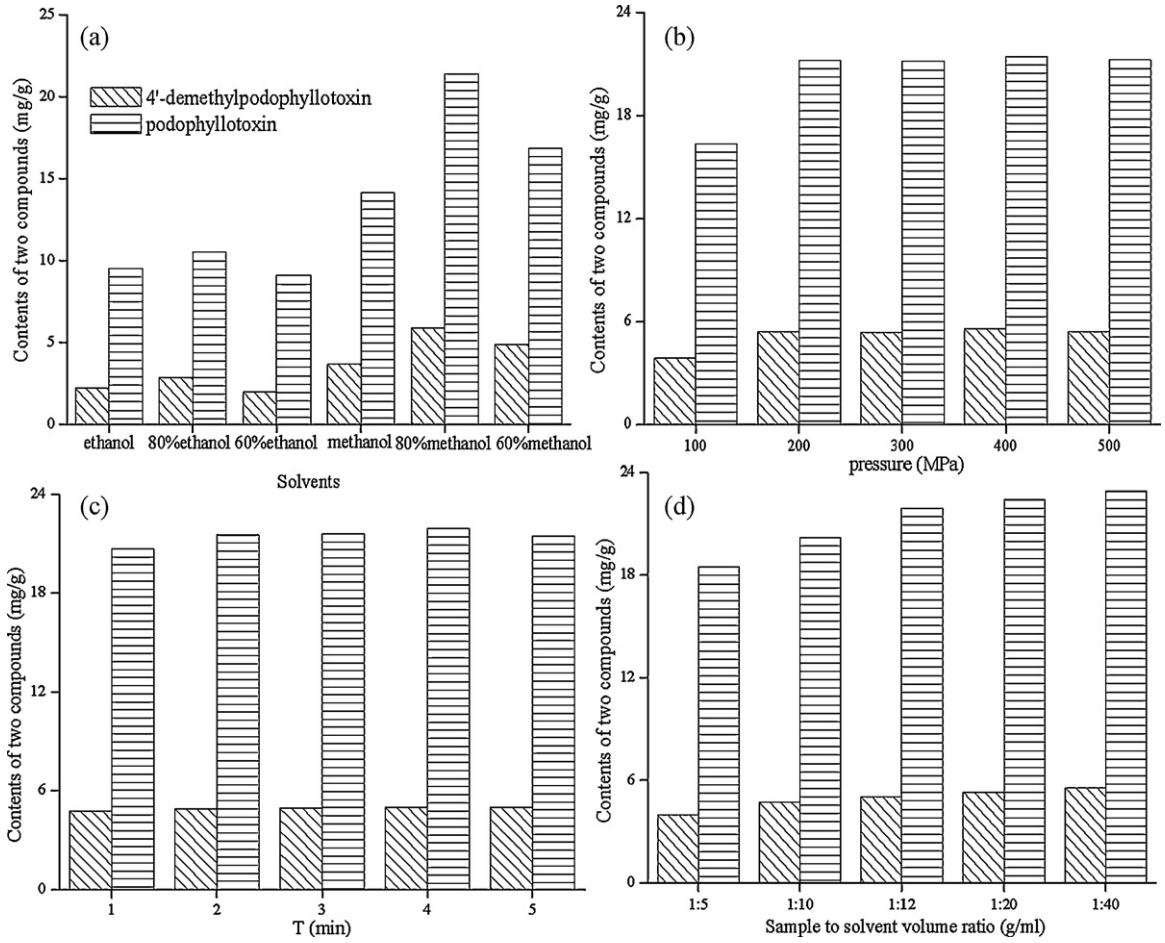

Fig. 2. Effects of solvents (A), extraction pressure (B), extraction time (C) and sample solvent volume ratio (g/mL) (D) on the contents of target compounds by UPE.

better than ethanol. Extraction with 80% methanol gave the best extraction yield and was chosen as the optimized extraction solvent in the following experiments.

The effects of extraction pressure and time were also investigated. As shown in Fig. 2(B), higher pressures led to slightly higher extraction yield. When the extraction pressure was higher than 200 MPa, the extraction yield did not indicate significant increase. From Fig. 2(C), it can be seen that the extraction efficiency of the two target compounds had less significant change when increasing the extraction time at 80% methanol. This is because under high pressure, the different pressures between inner and outer cell membranes is so large that it will lead to instant permeation and the yield could reach the highest value rapidly. Therefore, 200 MPa and 1 min were sufficient for the process of UPE.

The influence of solid/liquid ratio on the extraction yield was evaluated using the following conditions: 80% methanol, 200 MPa of pressure and 1 min of extraction time, and the results were shown in Fig. 2(D). It could be clearly seen that the extraction yield increases with the decrease of solid/liquid ratio. Considering the extraction solvent and processing costs, the best choice of the solid/liquid ratio was 1:12 (g/mL).

The results of the factor experiments showed that the optimum conditions for extraction of the two target compounds by UPE were 80% methanol, 200 MPa of pressure, 1 min of extraction time and 1:12 (g/mL) of solid/liquid ratio. Under the optimum UPE conditions, the extraction yields of 4′-demethylpodophyllotoxin and podophyllotoxin were 5.1 and 21.9 mg/g, respectively. The initial extraction HPLC chromatogram was shown in Fig. 3(A).

To compare UPE with heat reflux extraction, parallel experiment was carried out. The extraction yields of 4′-demethylpodophyllotoxin and podophyllotoxin were 4.2 and 21.1 mg/g by heat reflux extraction and the initial extraction HPLC chromatogram was shown in Fig. 3(B). The results showed that the time of heat reflux extraction is 30 times more than that of UPE. Therefore UPE can greatly reduce the extraction time and have higher product yield in this system.

3.2. Selection of the suitable two-phase solvent system

Successful separation by HSCCC largely depends upon the selection of the suitable two-phase solvent system, which provides an ideal range of the partition coefficient (K_D) for the target compound [19]. In general, the most suitable K_D value of the target compound is close to 1 and the separation factor between the two components ($\alpha = k_2/k_1$, $k_2 > k_1$) should be greater than 1.5. If K_D value is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in loss of peak resolution; if the K_D value is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time [14]. Based on the physicochemical properties of lignan compounds, a series of two-phase solvent systems consisting of petroleum ether, ethyl acetate, methanol and water were tested by changing the volume ratios of the four solvents to obtain the optimum condition. The K_D values of the target compounds in different two-phase solvent systems were measured and summarized in Table 1. According to the K_D values and the separation factors of compound I and

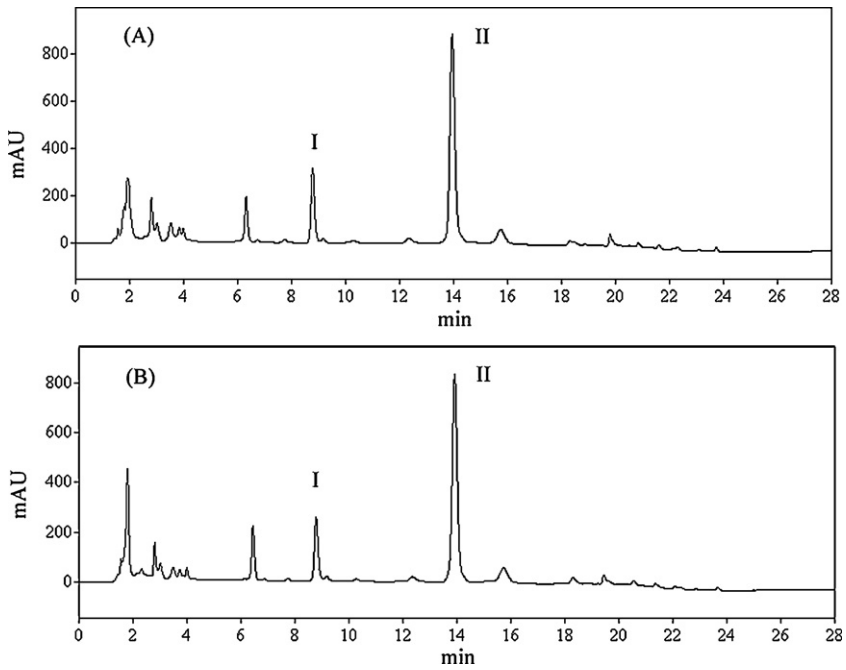

Fig. 3. HPLC chromatograms of the initial extraction samples by UPE (A) and heat reflux extraction (B) from *Dysosma versipellis*. Peak I: 4′-demethylpodophyllotoxin and peak II: podophyllotoxin.

compound II shown, it can be seen that the two-phase solvent system of petroleum ether–ethyl acetate–methanol–water (10:10:8:12, v/v) was most suitable for the separation of the two compounds.

3.3. Purification of 4′-demethylpodophyllotoxin and podophyllotoxin by HSCCC

When the two-phase solvent system composed of petroleum ether–ethyl acetate–methanol–water (10:10:8:12, v/v) was used for the HSCCC separation, several peaks were present in the chromatogram as shown in Fig. 4. After analysis by HPLC, it can be seen that two compounds with high purities were obtained in one-step separation as shown in Fig. 5(B) and (C). As a result, 16.5 mg 4′-demethylpodophyllotoxin (with the purity of 96.1%) and 73.7 mg podophyllotoxin (98.2%) was obtained from 260 mg of crude sample at the total separation time of 120 min.

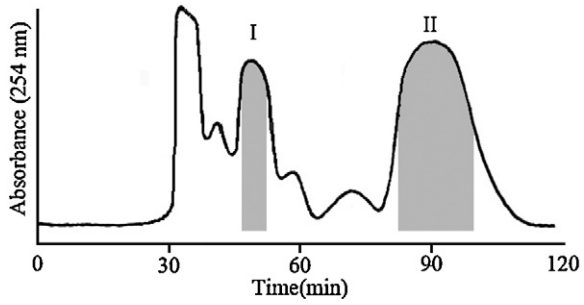

Fig. 4. HSCCC chromatogram of the crude sample extracted from *Dysosma versipellis*. Two-phase solvent system: petroleum ether–ethyl acetate–methanol–water (10:10:8:12, v/v); revolution speed: 800 rpm; flow rate: 2.0 mL/min; sample size: 260 mg; UV detection wavelength: 254 nm; retention of stationary phase: 75%; peak I: 4′-demethylpodophyllotoxin; peak II: podophyllotoxin.

3.4. Identification of purified compounds

Compound I (peak I in Fig. 3): positive ESI-MS *m/z*: 423 $[M+Na]^+$. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.11 (1H, s, 5-H), 6.49 (1H, s, 2′, 6′-H), 6.37 (1H, s, 8-H), 5.97 (2H, s, OCH_2O), 4.73 (d, J = 3.0 Hz, 1-H), 4.71 (1H, d, J = 8.5 Hz, 4-H), 3.73 (3H, s, 3′, 4′, 5′-OCH_3), 2.84 (dd, J = 3.0 Hz, 1.3 Hz, 2-H), 2.77 (1H, m, 3-H). The data were in accordance with data listed in the literature [20], and compound I was identified as 4′-demethylpodophyllotoxin.

Compound II (peak II in Fig. 3): negative ESI-MS *m/z*: 413 $[M-H]^-$. ^{1}H NMR(600 MHz, $CDCl_3$) δ: 7.12 (1H, s, 5-H), 6.50 (1H, s, 2′, 6′-H), 6.45 (1H, s, 8-H), 5.97 (2H, s, OCH_2O), 4.76 (d, J = 3.1 Hz, 1-H), 4.58 (1H, d, J = 8 Hz, 4-H), 3.74 (3H, S, 3′, 4′, 5′-OCH_3), 2.83 (dd, J = 3.0 Hz, 1.3 Hz, 2-H), 2.77 (1H, m, 3-H). The data were in accordance with data listed in the literature [20], and compound II was identified as podophyllotoxin.

Table 1
K_D-values of target compounds measured in petroleum ether–ethyl acetate–methanol–water.

Solvent system	K_D values[a]		Separation factor (α)
	Compound I	Compound II	
10:10:10:10	0.11	0.69	6.3
10:10:9:11	0.26	0.91	3.5
10:10:8:12	0.51	1.36	2.7
10:10:6:14	0.75	2.31	3.1

[a] 1 mL of each phase of the equilibrated two-phase solvent system was added to a 10 mL test tube, and approximately 1 mg of crude extract was added. Then an equal volume of each phase was analyzed by HPLC to obtain K_D. The K_D value was the peak area of the compound in the upper phase divided by that in the lower phase.

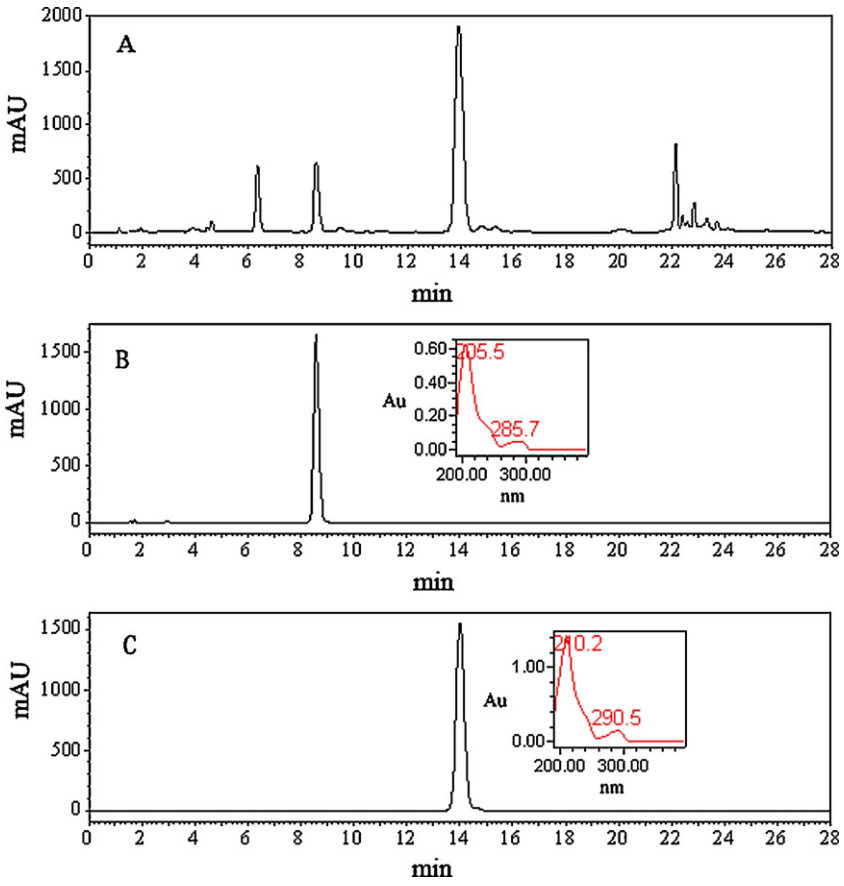

Fig. 5. (A) HPLC chromatograms of the crude sample extract by UPE; (B) HPLC analyses and UV spectrum of 4′-demethylpodophyllotoxin purified with HSCCC; (C) HPLC analyses and UV spectrum of podophyllotoxin purified with HSCCC. Conditions – Shim-pack VP-ODS column (250 mm × 4.6 mm i.d.); column temperature: 25 °C; mobile phase: acetonitrile–water (35:65, v/v); flow-rate: 1.0 mL/min; detection wavelength: 215 nm.

4. Conclusions

Podophyllotoxin and 4′-demethylpodophyllotoxin were successfully extracted and separated from *D. versipellis* by ultrahigh pressure extraction combined with HSCCC technique. Under optimal conditions i.e., 200 MPa of pressure, 80% methanol extraction solution, 1 min of extraction time and 1:12 (g/mL) of solid/liquid ratio, the extraction yields of 4′-demethylpodophyllotoxin and podophyllotoxin were 5.1 and 21.9 mg/g, respectively. At last, two compounds with high purities were obtained by HSCCC with two-phase solvent system composed of petroleum ether–ethyl acetate–methanol–water at volume ratio of 10:10:8:12 (v/v). The study demonstrates that UPE and HSCCC are very useful techniques for the extraction, isolation and purification of podophyllotoxins from plant material.

Acknowledgements

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