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One-step Separation and Purification of Three Lignans and One Flavonol from *Sinopodophyllum emodi* by Medium-pressure Liquid Chromatography and High-speed Counter-current Chromatography

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ABSTRACT:

Introduction – Lignans and flavonols are the primary constituents of *Sinopodophyllum emodi* and have been used as cathartic, anthelmintic, chemotherapeutic and anti-hypertensive compounds. Although these compounds have been isolated, there have been no reports on the separation of 4′-demethyl podophyllotoxin, podophyllotoxin, deoxypodophyllotoxin and kaempferol in one step by medium-pressure liquid chromatography (MPLC) and high-speed counter-current chromatography (HSCCC).

Objective – Development of an efficient method for the preparative separation and purification of three lignans and one flavonol from *S. emodi*.

Methods – The precipitate of crude extracts was first separated by MPLC into four parts, numbered GJ-1, GJ-2, GJ-3 and GJ-4. GJ-1 was separated and purified by HSCCC using a solvent system composed of *n*-hexane:ethyl acetate:methanol:water (1.75:1.5:1:0.75, v/v/v/v). The purities of the target compounds were assessed using high-performance liquid chromatography (HPLC) and chemical structures were identified by ^{1}H-NMR and ^{13}C-NMR.

Results – The HSCCC and MPLC methods were successfully used for the preparative separation and purification of 4′-demethyl podophyllotoxin (8.5 mg, 92.4%), podophyllotoxin (40.1 mg, 92.1%), deoxypodophyllotoxin (4.6 mg, 98.1%), and kaempferol (1.6 mg, 96.7%) from a 100 mg sample.

Conclusion – Three lignans (4′-demethyl podophyllotoxin, podophyllotoxin, deoxypodophyllotoxin) and one flavonol (kaempferol) were successfully isolated by HSCCC and MPLC in one step.

Keywords: high-speed counter-current chromatography; medium-pressure liquid chromatography; one-step; 4′-demethylpodophyllotoxin; podophyllotoxin; deoxypodophyllotoxin; kaempferol; *Sinopodophyllum emodi*

Introduction

Sinopodophyllum emodi (Wall.) Ying, mainly distributed in the Himalayan region and the Americas, is a member of the genus *Podophyllum* of the Berberidaceae family and has a long history of medicinal use in China for the treatment of cathartics and anthelmintics (Sudo *et al*., 1998; Zhao *et al*., 2001; Zhao *et al*., 2011). The major bioactive constituents of *S. emodi* are lignans and flavonols, such as 4′-demethyl podophyllotoxin, podophyllotoxin, deoxypodophyllotoxin, dehydropodophyllotoxin, 4-demethyl-podophyllotoxin, 3′, 4′-demethylene-podophyllotoxin, podophyllotoxin-7′-*O*-β-D-glucopyranoside, 4-demethyl podophyllotoxin-7′-*O*-β-D-glucopyranoside, quercetin and kaempferol (Sun *et al*., 2011), which have been used extensively as supplements for chemotherapeutic cancer and hypertension treatments (Zhang and Ou, 1995; Izadifar and Baik, 2008). Because of their significant utility, the chemical and pharmacological properties of these components warrant greater attention, and an effective method for the separation and purification of these compounds from natural sources would be very valuable.

High-speed counter-current chromatography (HSCCC) is a liquid–liquid partition chromatographic technology (Ito, 1984, 2005) that does not require a solid sorbent on which irreversible adsorption will occur. Consequently, it provides excellent sample recovery. It is very suitable for separation of active compounds from traditional Chinese herbs and other natural products (Ha and Kim, 2009; Jia *et al*., 2012; Xu *et al*., 2012). It has been

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reported that podophyllotoxin, 4′-demethyl podophyllotoxin and kaempferol could be obtained from *Podophyllum emodi* and deoxypodophyllotoxin from the rhizomes of *Anthriscus sylvestris* by HSCCC, respectively (Zhang and Ou, 1995; Quan *et al*., 2010). However, there have been no publications concerning the one-step isolation of these four compounds from *S. emodi*.

The present paper describes a convenient method to separate 4′-demethyl podophyllotoxin, podophyllotoxin, deoxypodophyllotoxin and kaempferol by HSCCC and MPLC from *S. emodi*. The isolation conditions were optimised after investigation of the effects of a two-phase solvent system, flow rate and revolution speed. The chemical structures of target compounds are shown in Fig. 1. The purity of these compounds was confirmed by high-performance liquid chromatography (HPLC), and the compound chemical structures were elucidated by ^{1}H-NMR and ^{13}C-NMR.

Experimental

Reagents and materials

All organic solvents used for the crude extract preparation and the HSCCC separation were analytical grade (Tianjin Fuyu Chemical Co., Tianjin, China). Methanol used for HPLC was chromatographic grade (Shandong Yuwang Chemical Ltd, Dezhou, Shandong, China). Dimethyl sulphoxide-d_6 was used as the deuterated solvent for the NMR determination (Sigma-Aldrich Ltd, Shanghai, China). *Sinopodophyllum emodi* was collected in Qinghai Province, China, in June 2010; and dried in the shade at room temperature. The species was identified by Professor Shilong Chen (Northwest Institute of Plateau Biology, Chinese Academy of Sciences). Voucher specimens were deposited in the archives of the Northwest Institute of Plateau Biology (HNWP), Chinese Academy of Sciences (Chen *et al*. 5082).

Apparatus

The HSCCC instrument employed in the present study was a TBE-300B instrument (Tauto Biotech, Shanghai, China) with three multilayer coil separation columns connected in series (i.d. 2.6 mm, total volume is 300 mL), and a 20 mL sample loop. The revolution radius was 5 cm, and the β values ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge) of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus was regulated with a speed controller in the range of 0 to 1000 rpm. The HSCCC system was equipped with a model TBP-5002 constant-flow pump (Tauto Biotech, Shanghai, China), a model UV500 detector (Tauto Biotech, Shanghai, China) operating at 292 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted using a DC-0506 constant-temperature circulating bath (Shanghai Shunyuhengping Science Instruments Co. Ltd, Shanghai, China).

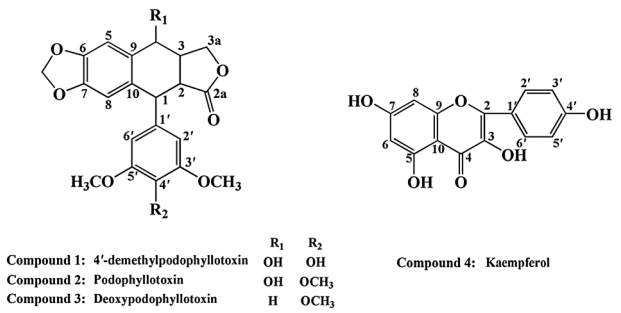

Figure 1. Chemical structures of four target compounds from *Sinopodophyllum emodi*.

The HPLC analysis was performed on an Agilent 1260 system (Agilent Technologies Co. Ltd, Palo Alto, CA, USA). The system was equipped with the following components: a G1311C solvent delivery unit; G1315D UV–vis photodiode array detector; G1316A column thermostat; G1329B autosampler; Eclipse XDB-C_{18} (250 mm × 4.6 mm i.d., 5 μm) analytical column and an Agilent HPLC workstation.

The MPLC system was equipped with a Model 605 pump, a C-660 fraction collector, a C-635 UV absorbance detector, and a GS50-500 glass column (50 × 500 mm), which was pre-fitted with a glass guard column (15 × 150 mm) packed with YMC C_{18} ODS (50 μm).

The NMR determination was performed on a Mercury Plus-400 NMR spectrometer (Varian Co. Ltd, Palo Alto, CA, USA).

Preparation of the crude extract from *S. emodi*

Rhizomes were separated from the whole plants, and 1 kg of dried rhizomes was ground into powder, and extracted three times with 40 L of 70% ethanol at 70°C. The solid:liquid ratios for the three extractions were 1:20, 1:10 and 1:10, while the extraction times for the three extractions were 2 h, 1 h and 1 h. All filtrates were combined and then concentrated by rotary evaporation at 60°C under reduced pressure. A total of 1 L residual liquid was cooled at 0–4°C and then 30 g of precipitate was obtained via filtration for subsequent isolation and purification.

MPLC separation

Thirty grams of precipitate were dissolved in 50 mL of 80% ethanol, and adsorbed on the MPLC column. Before use, the column was washed with distilled water at a volume that was four times the column volume; the column was then eluted by ethanol:water solutions (20:80, 40:60, 60:40, 90:10, v/v). Each part of the eluent was analysed using HPLC and combined into four fractions, named GJ-1, GJ-2, GJ-3 and GJ-4. Each fraction was then evaporated to dryness by rotary evaporation at 65°C under reduced pressure and subsequently stored in a refrigerator at 4°C. From the UV spectra it was observed that lignans were mainly distributed in GJ-1 but most of the flavonols and a few of the lignans were distributed in the other fractions. Thus, 2.7 g of GJ-1 was chosen for further HSCCC separation and purification (Fig. 2).

HSCCC separation

Selection of two-phase solvent system. The two-phase solvent system was selected according to the partition coefficient (K) of the target components of the sample. The K values were determined using HPLC analysis as follows. A suitable amount of sample was added into a series of solvent systems, followed by shaking of the solution. Equal volumes of the upper and lower phases were then evaporated to dryness separately. Residues

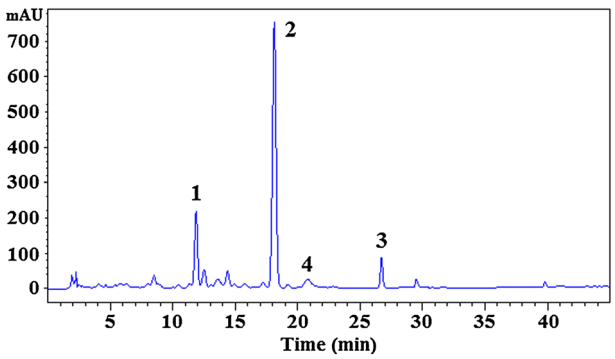

Figure 2. The HPLC chromatogram of the GJ-1 sample. Numbering of the peaks is the same as for the compounds shown in Fig. 1.

were then diluted into 4 mL methanol and analysed by HPLC. The *K* value was defined as follows: the peak area of the compound in the upper phase, divided by the peak area in the lower phase.

Preparation of two-phase solvent system and sample solution. The present study used a two-phase solvent system composed of *n*-hexane:ethyl acetate:methanol:water (1.75:1.5:1:0.75, v/v/v/v). Each solvent was added to a separatory funnel and was then thoroughly equilibrated at room temperature. The upper phase and the lower phase were subsequently separated and degassed via ultrasonic bath for 30 min shortly before use.

The HSCCC separation sample solution was prepared by dissolving 100 mg of GJ-1 sample in 10 mL of the lower phase of the two-phase solvent system.

Separation procedure. The HSCCC separation was performed as follows. First, the multiple coiled column was filled entirely with the upper phase. Then, the lower phase was pumped into the head end of the column at a flow rate of 1.8 mL/min while the apparatus was run at a revolution speed of 900 rpm and a temperature of 25°C. After the system reached hydrodynamic equilibrium, which was indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected via an injection valve. Throughout, the column tail-end effluent was continuously monitored at 292 nm using a UV detector. Following the sample injection, the data were collected immediately. The fractions were manually collected according to the chromatogram data, and then evaporated under reduced pressure and dissolved in methanol for subsequent purity analysis with HPLC.

HPLC analysis of the GJ-1 sample and HSCCC peak fractions. The GJ-1 sample, as well as each peak fraction obtained by HSCCC, was analysed by HPLC. The HPLC analysis was performed on an Eclipse XDB-C_{18}-column (250 mm × 4.6 mm i.d., 5 μm). Baseline separation could be achieved when water (eluent A) and methanol (eluent B) were used as the mobile phase in the gradient elution mode (0 min, 45% B; 5 min, 50% B; 20 min, 55% B; 30 min, 75% B; 35 min, 85% B; 95 min, 95% B), while the other parameters were controlled as follows: flow rate of the mobile phase was 1.0 mL/min, the detection wavelength was 292 nm, the column temperature was 25°C and the injection volume was 10 μL. The HPLC chromatogram of the GJ-1 sample is shown in Fig. 2.

Results and Discussion

Optimisation of HSCCC conditions

It has been demonstrated that the most important step in the design of an HSCCC separation protocol is the selection of a solvent system that can provide an ideal partition coefficient (*K*) for the target compounds, and the choice of a suitable biphasic system is guided by the chemical nature of the target compounds. Generally, an ideal partition coefficient (*K*) of the target compounds should be in the range of 0.2–5.0 (Xie *et al*., 2010). A *K* value that is too small results in the solute eluting closer to the solvent front and gives lower resolution. However, a *K* value that is too large tends to give better resolution but broader peaks due to a longer elution time.

According to the polarity and general chemical properties of the GJ-1 sample, several solvent systems composed of *n*-hexane, methanol and water were first tested. When *n*-hexane, methanol and water at 1:1:1 (v/v/v) was employed, the *K* values of the four target compounds were too small. This indicated that the target compounds were mainly distributed in the upper phase. To decrease the polarity difference between the upper and the lower phase, a higher volume ratio of methanol was added. Unfortunately, the *K* values of the target compounds were still below 0.2. When *n*-hexane:ethyl acetate:methanol:water (1:1:1:1, v/v/v/v) was tested, the *K* values of the four compounds were all between 0.2–5.0. It was found that ethyl acetate could regulate the distribution better than methanol. To obtain a suitable running time, an appropriate retention of stationary phase and an efficient separation, the ratio of *n*-hexane and water was changed concurrently. For the *n*-hexane:ethyl acetate:methanol:water system at 2.3:1:1:2.3 (v/v/v/v), the *K* value of compound **1** was too small while for *n*-hexane:ethyl acetate:methanol:water system at 4:1:1:4 (v/v/v/v), the *K* value of compound **4** was too large. Therefore, this change was not pursued further. The volume ratio of *n*-hexane and ethyl acetate was then increased starting from *n*-hexane:ethyl acetate:methanol:water (1:1:1:1, v/v/v/v). The results showed that *n*-hexane:ethyl acetate:methanol:water (1.5:1.5:1:1, v/v/v/v) yield suitable *K* values. Finally, a 'fine tuning' step was conducted by increasing *n*-hexane and decreasing water starting from *n*-hexane:ethyl acetate:methanol:water (1.5:1.5:1:1, v/v/v/v), and suitable *K* values were also produced. Results are summarised in Table 1. Based on the above, *n*-hexane:ethyl acetate:methanol:water at 1:1:1:1, 1.5:1.5:1:1, and 1.75:1.5:1:0.75 (v/v/v/v) were chosen for the following experiments.

The performance of the above three solvent systems (with suitable *K* values) was evaluated in terms of separation time, stationary phase retention and purities of target compounds (Table 2). The HSCCC chromatograms are shown in Fig. 3. Except for the solvent systems, the other parameters were kept the same: flow rate, 1.8 mL/min; revolution speed, 900 rpm; column temperature, 25°C. Results showed that *n*-hexane:ethyl acetate:methanol:water (1.75:1.5:1:0.75, v/v/v/v) produced the best separation as it provided higher compound purity and shorter separation time. Thus, it was selected as the solvent system.

In addition to the solvent system, the effects of the mobile phase flow rate on the separation were studied (Table 3). A preliminary test was conducted at three flow rates, 1.2, 1.8 and 2.4 mL/min, and it was determined that the former rate gave a higher retention of the stationary phase and better sample

Table 1. *K* values of the target compounds in different solvent systems

No.	*n*-Hexane:ethyl acetate:methanol:water (v/v/v/v)	Partition coefficient (*K*)			
		Compound **1**	Compound **2**	Compound **3**	Compound **4**
1	1:0:1:1	0.021	0.027	0.047	0.098
2	1:0:2:1	0.054	0.071	0.080	0.103
3	1:1:1:1	0.240	0.370	0.670	0.840
4	4:1:1:4	0.400	0.620	1.020	50.000
5	2.3:1:1:2.3	0.090	0.290	0.500	0.780
6	1.5:1.5:1:1	0.310	0.510	0.750	0.970
7	1.75:1.5:1:0.75	0.210	0.340	0.430	0.640

Table 2. Comparison of separation time, stationary phase retention and purities of the four target compounds under different solvent system

No.	*n*-Hexane:ethyl acetate:methanol:water (v/v/v/v)	Separation time(min)	Retention (%)	Purity (%)			
				Compound **1**	Compound **2**	Compound **3**	Compound **4**
3	1:1:1:1	140	67	77.2	85.7	80.9	59.0
6	1.5:1.5:1:1	180	62	88.5	85.7	77.7	86.1
7	1.75:1.5:1:0.75	100	65	86.6	87.2	80.1	85.6

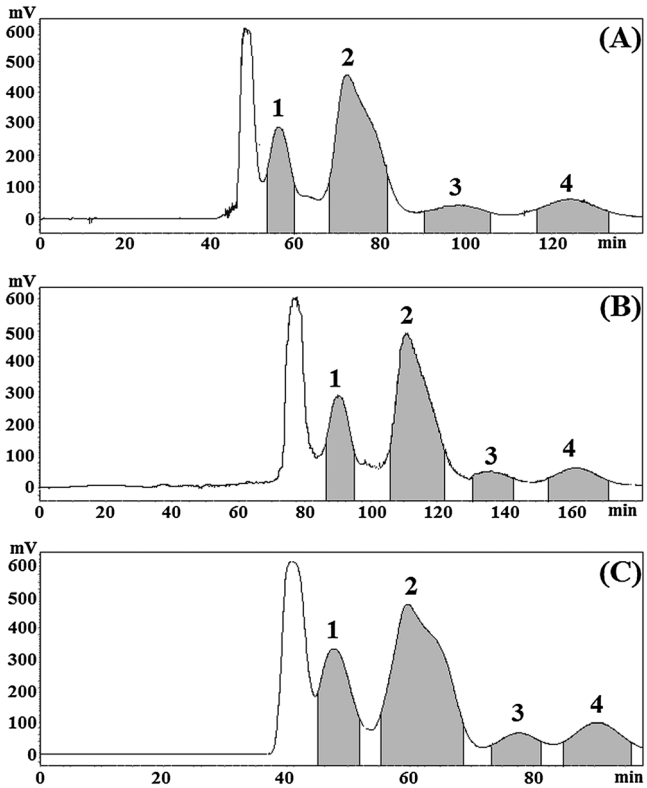

Figure 3. The HSCCC chromatograms of the GJ-1 sample from *Sinopodophyllum emodi*. Solvent system: (A) *n*-hexane:ethyl acetate:methanol:water (1:1:1:1, v/v/v/v); (B) *n*-hexane:ethyl acetate:methanol:water (1.5:1.5:1:1, v/v/v/v); (C) *n*-hexane:ethyl acetate:methanol:water (1.75:1.5:1:0.75, v/v/v/v). Stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 1.8 mL/min; revolution speed: 900 rpm; sample: 100 mg of sample dissolved in 10 mL of the lower phase. Numbering of the peaks is the same as for the compounds shown in Fig. 1.

separation but with a longer separation time, while the latter was the opposite. Taking the above observations into consideration, a flow rate of 1.8 mL/min was selected (Fig. 3C).

Under the selected conditions, 8.5 mg of compound **1**, 40.1 mg of **2**, 4.6 mg of **3** and 1.6 mg of **4** were obtained from the 100 mg sample in a one-step separation. The HPLC analysis showed that the purities of the target compounds were 92.4%, 92.1%, 98.1% and 96.7%, for 4′-demethyl podophyllotoxin, podophyllotoxin, deoxypodophyllotoxin, and kaempferol. Fig. 4 shows the HPLC analysis and UV spectra of the target compounds.

Identification of the separated peaks

Each peak fraction separated by HSCCC was structurally identified by ^{1}H-NMR and ^{13}C-NMR data, and the results are listed below.

Compound 1. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.10 (1H, s, H-5), 6.47 (1H, s, H-8), 6.29 (2H, s, H-2′, 6′), 6.00 (1H, s, OCH_2O), 5.96 (1H, s, OCH_2O), 4.61 (1H, d, J = 9.2 Hz, H-4), 4.46 (2H, m, H-1, 3aα), 4.10 (1H, t, J = 8.8, 10 Hz), 3.63 (6H, s, 3′, 5′-OCH_3), 3.08 (1H, m, H-2), 2.66 (1H, m, H-3). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 174.7 (C-2a), 147.1 (C-3′, 5′), 146.5 (C-6), 146.4 (C-7), 135.0 (C-1′), 134.8 (C-4′), 131.1 (C-9), 131.0 (C-10), 109.1 (C-8), 108.8 (C-2′, 6′), 106.3 (C-5), 100.9 (OCH_2O), 71.0 (C-3a), 70.6 (C-4), 56.1 (3′, 5′-OCH_3), 44.2 (C-2), 43.2 (C-1), 39.5 (C-3). Comparison of the above data with those in the literature (Liao *et al*., 2002) allowed the identification of compound **1** as 4′-demethyl podophyllotoxin.

Compound 2. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.10 (1H, s, H-5), 6.46 (1H, s, H-8), 6.32 (2H, s, H-2′, 6′), 5.97 (2H, ABq, J = 0.8, 9.2 Hz, OCH_2O), 4.60 (1H, d, J = 9.6 Hz, H-4), 4.49 (2H, m, H-1, 3aα), 4.08 (m, 1H, H-3aβ), 3.63 (6H, s, 3′, 5′-OCH_3), 3.61 (3H, s, 4′-OCH_3), 3.16 (1H, m, H-2), 2.49 (1H, m, H-3). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 174.7 (C-2a), 151.9 (C-3′, 5′), 146.6 (C-6), 146.5 (C-7), 136.6 (C-4′), 135.5 (C-1′), 132.6 (C-10), 130.7 (C-9), 109.1 (C-8), 108.3 (C-2′, 6′), 106.3 (C-5), 101.0 (OCH_2O), 71.1 (C-3a), 70.6 (C-4), 59.9 (4′-OCH_3), 55.9 (3′, 5′-OCH_3), 44.1 (C-2), 43.4 (C-1), 40.1 (C-3). Comparison of the above data with those in the literature (Zhao *et al*., 2008) allowed the identification of compound **2** as podophyllotoxin.

Compound 3. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 6.81 (1H, s, H-5), 6.50 (1H, s, H-8), 6.28 (2H, s, H-2′, 6′), 5.96 (1H, s, OCH_2O), 5.94 (1H, s, OCH_2O), 4.50 (1H, d, J = 5.6 Hz, H-1), 4.40 (1H, m, H-3aα), 3.94 (1H,

Table 3. Comparison of separation time, stationary phase retention and purities of the four target compounds under different flow rate

Flow rate (mL/min)	Revolution speed (rpm)	Separation time(min)	Retention (%)	Purity (%)			
				Compound **1**	Compound **2**	Compound **3**	Compound **4**
1.2	900	130	70	92.6	92.9	98.4	97.1
1.8	900	100	65	91.6	90.2	88.7	89.6
2.4	900	74	62	89.2	88.3	72.8	79.4

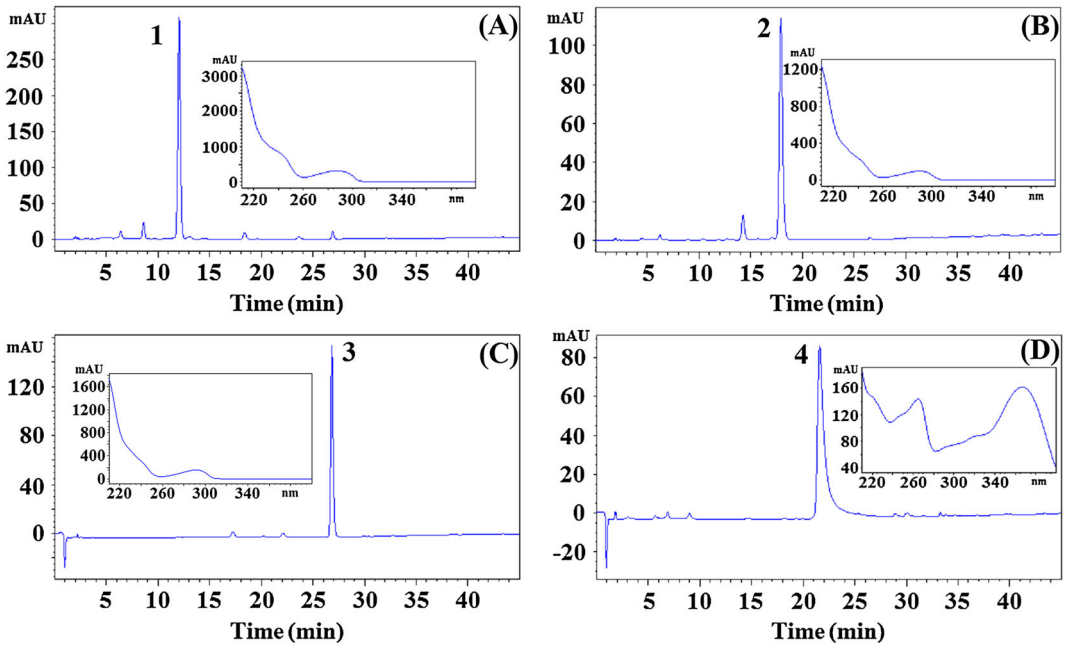

Figure 4. HPLC chromatograms and UV spectra of the isolated compounds. Numbering of the compounds is the same as for the compounds shown in Fig. 1.

dd, $J = 8.4$, 8.0 Hz, H-3aβ), 3.62 (6H, s, 3′, 5′-OCH_3), 3.60 (3H, s, 4′-OCH_3), 3.00 (2H, m, H-2, 4α), 2.76 (1H, m, H-4β), 2.62 (1H, m, H-3). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 175.0 (C-2a), 151.9 (C-3′, 5′), 146.3 (C-6), 145.9 (C-7), 137.0 (C-4′), 136.2 (C-1′), 130.6 (C-9), 129.1 (C-10), 109.9 (C-8), 108.5 (C-5), 108.1 (C-2′, 6′), 100.9 (OCH_2O), 71.6 (C-3a), 59.9 (4′-OCH_3), 55.7 (3′, 5′-OCH_3), 46.0 (C-2), 43.0 (C-1), 32.5 (C-4), 32.0 (C-3). Comparison of the above data with hose in the literature (Jackson *et al*., 1984; Liao *et al*., 2002) allowed the identification of compound **3** as deoxypodophyllotoxin.

Compound 4. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 12.48 (1H, s, 5-OH), 8.03 (2H, d, $J = 8.4$Hz, H-2′, 6′), 6.91 (2H, d, $J = 8.4$ Hz, H-3′, 5′), 6.42 (1H, s, H-8), 6.17 (1H, s, H-6). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 175.9 (C-4), 164.3 (C-7), 160.7 (C-5), 159.2 (C-4′), 156.2 (C-9), 146.7 (C-2), 135.7 (C-3), 129.2 (C-2′, 6′), 121.7 (C-1′), 115.4 (C-3′, 5′), 102.9 (C-10), 98.3 (C-6), 93.5 (C-8). Comparison of the above data with those in the literature (Hu *et al*., 2003) allowed the identification of compound **4** as kaempferol.

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