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Short Communication

Preparative Isolation and Purification of Deoxypodophyllotoxin from the Rhizomes of *Anthriscus sylvestris* by High-speed Counter-current Chromatography

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A simple and rapid purification method of deoxypodophyllotoxin from the crude methanol extract of rhizomes of *Anthriscus sylvestris* was established using high-speed counter-current chromatography. From the crude extract (288.9 mg), deoxypodophyllotoxin (8.8 mg) was separated using a two-phase solvent system composed of *n*-hexane/ethyl acetate/methanol/water (7:3:5:5, v/v). The final purity of the deoxypodophyllotoxin was determined to be over 98% by ultra-performance liquid chromatography-UV analysis.

Key words: *Anthriscus sylvestris*, deoxypodophyllotoxin, high-speed counter-current chromatography (HSCCC)

The rhizomes of *Anthriscus sylvestris* (*A. sylvestris*) Hoffm. (Umbelliferae) have been used traditionally as an antitussive, an antipyretic, an analgesic, a diuretic, and a cough remedy in Asian countries. Phytochemical investigations of this plant have resulted in the identification of various coumarins, lignans, monoterpenes, and phenylpropanoids [Kozawa *et al*., 1978a; 1978b; Jeong *et al*., 2007]. Of the chemical constituents in this plant, deoxypodophyllotoxin [Noguchi and Kawanami, 1940] (Fig. 1), one of the major secondary metabolites, is an important lignan possessing a wide range of biological activities, including cytotoxic [Kim *et al*., 2002; Masuda *et al*., 2002], anti-platelet aggregation [Chen *et al*., 2000], anti-proliferative [Ikeda *et al*., 1998], anti-viral [Sudo *et al*., 1998], insecticidal [Inamori *et al*., 1985; Gao *et al*., 2004], antiasthmatic [Lin *et al*., 2006], anti-allergic [Lee *et al*., 2004], and anti-oxidant activities [Dall'Acqua *et al*., 2006]. Conventional purification methods of deoxypodophyllotoxin from the rhizomes of *A. sylvestris* utilize solid stationary phases such as silica gels [Van Uden *et al*., 1997; Jeong *et al*., 2007; Yong *et al*., 2009].

High-speed counter-current chromatography (HSCCC) is a liquid-liquid partition chromatographic system [Ito and Bowman, 1970; Conway and Ito, 1984] that relies on distribution of the analytes between two immiscible liquid phases. Currently, HSCCC has been widely used for the separation and purification of various natural and synthetic products [Sutherland and Fisher, 2009] given its theoretical "zero" loss of target compounds on the solid support.

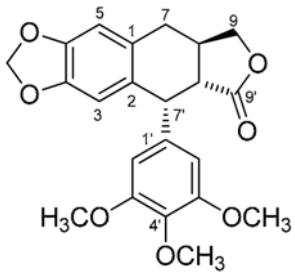

Fig. 1. Structure of deoxypodophyllotoxin.

The purpose of this study was to develop a one-step isolation and purification method for deoxypodophyllotoxin from the rhizomes of *A. sylvestris* using HSCCC.

Preparative HSCCC was carried out with a model TBE-1000A high-speed counter-current chromatography system (Shanghai Tauto Biotech Co. Ltd, Shanghai, China) with three serially connected multilayer coil separation columns (i.d. of tubing=1.6 mm; total volume =1000 mL) and an 80 mL sample loop. The system was equipped with LPLC pumps TBP5002 (Shanghai Tauto Biotech Co. Ltd, Shanghai, China) and Thermo Finnigan

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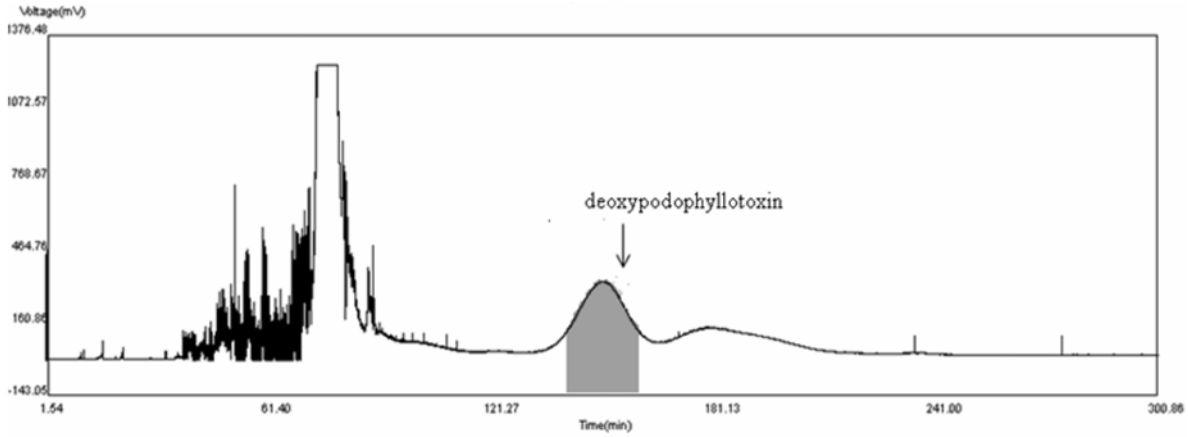

Fig. 2. HSCCC chromatograms of the crude extract from root of *Anthriscus.sylvestris*. Solvent system: *n*-hexane-ethyl acetate-methanol-water (7:3:5:5, v/v/v/v); column volume: 1000 mL; mobile phase: lower phase; flow rate: 5 mL/min; rotation speed: 500 rpm; separation temperature: 25°C; sample amount: 288.9 mg; detection wavelength: 280 nm; Sample size: 288.9 mg of crude sample dissolved in 20 mL of stationary phase and 10 mL mobile phase; Retention percentage of the stationary phase: 60%

SSI 500 UV Detector (Thermo Electron Co. San Jose, CA), Autochro-WIN software (version 2.0, Younglin-Tech, Seoul, Korea). Ultra-performance liquid chromatography (UPLC) was carried out with a Waters ACQUITY UPLC® System with a PDA Detector; column: AcQUITY UPLC®BEH C18 1.7 μm 2.1×50 mm. NMR spectral data were obtained on a Varian UNITY 300 NMR system (Varian, Palo Alto, CA). The HRESIMS were measured on a Waters Q-Tof Premier mass spectrometer. Solvents (methanol, ethyl acetate, chloroform, *n*-hexane) were obtained from SK chemicals (Daejeon, Korea) and distilled prior to use. All UPLC solvents were purchased from SK chemicals. Solvents for NMR (CD_3OD-d_4) were purchased from Cambridge Isotope Laboratories (Andover, MA). Deionized water was used in all solutions and for all dilutions.

The roots of *A. sylvestris* Hoffm. (Lot NO. 55687) were purchased from the Dae-Yeon pharmacy. A voucher specimen (PBC-326) has been deposited at the Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea. The dried rhizomes of *A. sylvestris* (38.0 g) were extracted three times by sonication in 400 mL methanol for 30 min at room temperature. The extracts were then combined and evaporated to dryness under reduced pressure. The crude extract (4.0 g) was used for preparative HSCCC separation.

The composition of the two-phase solvent was selected according to the partition coefficient (*K*) of the target compounds in the crude extract. The partition coefficient for the solvent system was determined by ultra-performance liquid chromatography (UPLC). The crude extract (1.0 mg) was added into 4.0 mL of each two-phase solvent system. Next, the vial was shaken vigorously until the two phases thoroughly equilibrated. After setting, 400 μL of each phase was transferred to new vials and condense under vacuum. The residue was diluted with 200 μL by methanol and analyzed by UPLC. The *K* values were expressed as the peak area of the target compound in the upper phase, divided by that in the lower phase. The two-phase solvent system was composed of *n*-hexane/ethyl acetate/methanol/water at various volume ratios. The solvent mixture was equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use.

The upper (stationary) and lower phase (mobile) were simultaneously pumped into the multilayer-coiled column in a 3:1 volumetric ratio [Slacanin *et al.*, 1989]. When the column was completely filled with the two phases, the apparatus was rotated at 500 rpm; simultaneously, the mobile phase was pumped through the column at a flow-rate of 5.0 mL/min. The temperature was set at 25°C. The sample solution (15 mL) containing the crude MeOH extract (288.9 mg) was loaded onto the HSCCC and the effluent monitored at 280 nm. Fractions were collected every minute and analyzed by UPLC-UV.

The UPLC conditions were as follows: the column used in this study was an AcQUITY UPLC®BEH C18 (1.7 μm 2.1×50 mm, Waters, Menchester, UK). The mobile phase was composed of acetonitrile/water/formic acid and a liner gradient liner gradient (20:80:01-100:0:0.1, v/v) was eluted at a flow-rate of 0.4 mL/min. The UV detector was set at 280 nm. The column temperature was set at 35°C.

Deoxypodophyllotoxin: white needles; HR-ESI-MS *m/z* 399.1446 $[M+H]^+$ (calcd. for $C_{22}H_{23}O_7$, 399.1444);

Table 1. Partition coefficient (*K*) values of deoxypodophyllotoxin in different two-phase solvent systems

Two-phase solvent system	Volume ratio (v/v)	*K* value
n-hexane-ethyl acetate-methanol-water	10:5:5:1	11.76
n-hexane-ethyl acetate-methanol-water	7:3:5:5	1.76
n-hexane-ethyl acetate-methanol-water	35:30:3	0.05
n-hexane-ethyl acetate-methanol-water	35:30:5	0.02

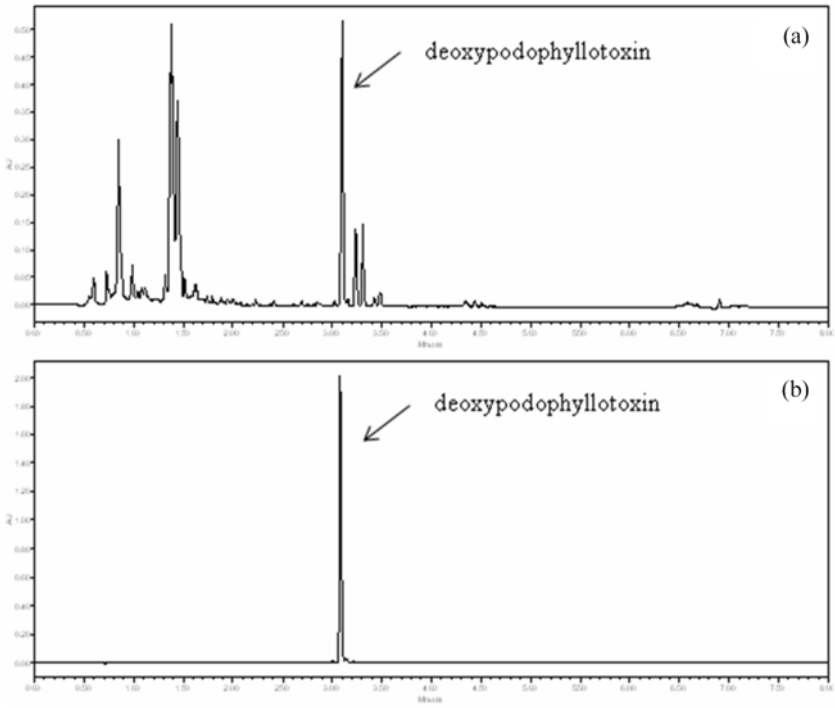

Fig. 3. UPLC chromatograms of the crude extract from the root of *A. sylvestris* and deoxypodophyllotoxin separated from HSCCC. Column, AcQUITY UPLC®BEH C18 1.7 μm 2.1×50 mm; mobile phase, acetonitril-water-formic acid (20:80:0.1-100:0:0.1, gradient); flow rate, 0.4 mL/min; sample volume, 2 μL; UV wavelength, 280 nm; column temperature, 35°C.

^{1}H-NMR (300 MHz, CD_3OD) δ 6.71 (1H, s, H-6), 6.45 (1H, s, H-3), 6.38 (2H, s, H-2', 6'), 5.90 (2H, d, *J*=2.4 Hz, -OCH_2O-), 4.57 (1H, d, *J*=4.8 Hz, H-7'), 4.44 (1H, dd, *J*= 8.4, 6.9 Hz, H-9a), 3.96 (1H, dd, *J*=9.6, 8.4 Hz, H-9b), 3.71 (6H, s, 3', 5'-OCH_3), 3.70 (3H, s, 4'-OCH_3), 3.08 (1H, dd, *J*=14.3, 4.8 Hz, H-7b), 2.88 (1H, dd, *J*=14.3, 4.8 Hz, H-7a), 2.75 (2H, m, H-8, 8'); ^{13}C-NMR (75 MHz, CD_3OD) δ 177.7 (C-9'), 153.7 (C-3', 5'), 148.5 (C-5), 148.1 (C-4), 138.6 (C-1', 4'), 132.0 (C-2), 130.4 (C-1), 111.1 (C-3, 6), 109.5 (C-2', 6'), 102.5 (-OCH_2O-), 73.6 (C-9), 61.1 (4'-OCH_3), 56.5 (3', 5'- OCH_3), 48.3 (C-8'), 45.0 (C-7'), 34.4 (C-8), 33.6 (C-7).

As shown in Fig. 3a, the UPLC analysis of the methanol extract revealed that deoxypodophyllotoxin was one of the major secondary metabolites. In order to develop a simple and rapid isolation and purification method using HSCCC, key conditions were closely studied [Chen and Sutherland, 2006].

The *K* values of deoxypodophyllotoxin were tested in several two-phase solvent systems listed in Table 1. Among them, *n*-hexane/ethyl acetate/methanol/water (7:3:5:5, v/v) was found to be suitable (K=1.76, 0.5<K< 2) solvent system. The rhizomes of *A. sylvestris* (288.9 mg) were injected, and deoxypodophyllotoxin (t_R 132-152 min, 8.8 mg) was successfully isolated (Fig. 2). Identification of peak fraction was performed by interpretation and comparison of previously published ^{1}H- and ^{13}C-NMR spectroscopic data [Fonseca *et al.*, 1980]. The purity of the deoxypodophyllotoxin was determined to be over 98% by UPLC-UV (Fig. 3b).

In the present study, a simple and rapid HSCCC separation method was established for the preparative isolation and purification of deoxypodophyllotoxin from the methanol extract of *A. sylvestris*. Deoxypodophyllotoxin (8.8 mg), with purity over 98%, was successfully purified from the extract (288.9 mg) using HSCCC and a solvent system composed of *n*-hexane/ethyl acetate/methanol/water (7:3:5:5, v/v/v/v). This

study demonstrates that such an HSCCC application could be applied to the preparative purification of deoxypodophyllotoxin from *A. sylvestris*.

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