Isolation of deoxypodophyllotoxin and podophyllotoxin from Juniperus sabina by high speed counter current chromatography

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Aislamiento de deoxipodofilotoxina y podofilotoxina de Juniperus sabina mediante cromatografía contracorriente de alta velocidad

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RESUMEN

La deoxipodofilotoxina y la podofilotoxina se conocen por sus excelentes actividades antitumorales contrarias a la proliferación tumoral y por eso se necesita urgentemente una gran cantidad de compuestos puros como ejemplos de los múltiples estudios in vivo e in vitro. En este informe, se ha establecido un método rápido y eficaz de separación y purificación de la deoxipodofilotoxina y de la podofilotoxina del extracto crudo de *Juniperus sabina* usando cromatografía contracorriente de alta velocidad (HSCCC). La HSCCC se realizaba con un sistema de dos fases de disolvente que comprende acetato de etilo-n-hexano-metanol-agua (3:5:3:5, *v/v*) a la velocidad de flujo de 2 mL/min a la velocidad de 850 rpm. 34.8 mg de deoxipodofilotoxina y 7.9 mg de podofilotoxina se obtenían a partir de 200 mg de muestra cruda con una pureza del 96,5% y del 94.4%, respectivamente, determinados mediante cromatografía líquida de alta presión (HPLC).

Palabras clave: *Juniperus sabina*; HSCCC; deoxipodofilotoxina; podofilotoxina.

SUMMARY

Deoxypodophyllotoxin and podophyllotoxin are known for their excellent anti-proliferative and anti-tumor activities, therefore large amount of pure compounds is urgently needed as authentic standards for various in vivo and in vitro studies. In this paper, an effective, rapid separation and purification method of deoxypodophyllotoxin and podophyllotoxin from the crude extract of Juniperus sabina was established using high speed counter current chromatography (HSCCC). HSCCC was performed with a two phase solvent system comprising of n-hexane-ethyl acetate-methanol-water (3:5:3:5, v/v) at the flow rate of 2 mL/min at the speed of 850 rpm. 34.8 mg of deoxypodophyllotoxin and 7.9 mg of podophyllotoxin were obtained from 200 mg crude sample with a purity of 96.5% and 94.4%, respectively, as determined by high performance liquid chromatography (HPLC).

Keywords: Juniperus sabina; HSCCC; deoxypodophyllotoxin; podophyllotoxin**.**

RESUM

La deoxipodofilotoxina i la podofilotoxina es coneixen per les seves excel·lents activitats antitumorals en contra de la proliferació tumoral i per això es necessita urgentment una gran quantitat d compostos purs com eixamples dels múltiples estudis in vivo e in vitro. En aquest paper s'ha establert un mètode ràpid i eficaç de separació i purificació de la deoxipodofilotoxina i de la podofilotoxina del extret cru de *Juniperus sabina* fent servir la cromatografia contracorrent d´alta velocitat (HSCCC). La HSCCC es realitzava amb un sistema de dues fases de dissolvent format per acetat d'etil-n-hexà-metanol-aigua (3:5:3:5, *v/v*) a la velocitat de fluix de 2 mL/min a la velocitat de 850 rpm. 34.8 mg de deoxipodofilotoxina i 7.9 mg de podofilotoxina s'obtenien a partir de 200 mg de mostra crua amb una puresa del 96,5% i del 94.4%, respectivament, per mitja de la cromatografia líquida d'alta pressió (HPLC).

Paraules clau: *Juniperus sabina*; HSCCC; deoxipodofilotoxina; podofilotoxina.

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INTRODUCTION

Podophyllotoxin and deoxypodophyllotoxin, two naturally occurring cyclolignan, are well known cytotoxic natural products that possess potential anti-proliferative effect and anti-tumor activity [1]. The application of podophyllotoxin cured almost all the warts completely in less time than other strategies and with fewer side effects. Podophyllotoxin and analogous compounds are also active against cytomegalovirus and Sindbis virus. Semi synthetic derivatives of podophyllotoxin are widely used as anticancer drugs and showed good clinical effects against several types of neoplasms [2].

Juniperus sabina L.(also called Sabina vugaris Antoine) (Cupressaceae Sabina), which a shrub growing in the mountains of central and southern Europe and central Asia [3]. It is well known to study on the essential oil which was used to stimulate indolent ulcers, cure chronic gout and rheumatism [4]. Recently many compounds have been found from the non-essential oil fractions of this plant, such as monoterpenes, sabinyl acetates, flavonoids, sesquiterpenes etc [5,6]. HSCCC, being as a support-free liquid-liquid partition chromatography, provided an advantage over the conventional column chromatography by eliminating irreversible adsorption of sample onto the solid support [7]. Currently, HSCCC has been widely used for the separation and purification of various natural and synthetic products [8]. In the present study, an effective HSCCC method was established for the separation and purification of podophyllotoxin and deoxypodophyllotoxin from *Juniperus sabina (Fig. 1).*

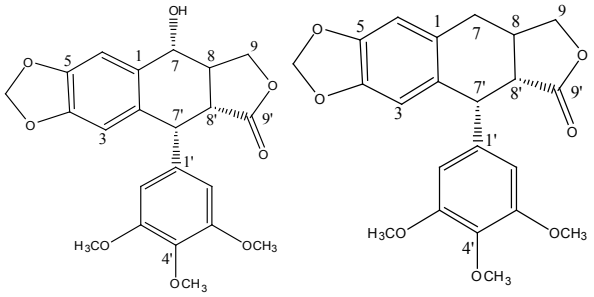

Fig. 1. *Chemical structures for podophyllotoxin (A) and deoxypodophyllotoxin (B).*

MATERIALS AND METHODS

All solvents used for the preparation of extract and for HSCCC separation were of analytical grade (Baishi Chemical Co. Ltd, China). Methanol and acetonitrile used for HPLC were HPLC grade (Dikma Technologies Inc., USA). The leaves of Juniperus sabina were obtained from Nanshan Mountain of Urumuqi, Xinjiang, China, and authenticated by Prof. Guanmian Shen (Xinjiang Institute of Ecology and Geography Chinese Academy of Sciences).

Preparation of Sample

The *Juniperus* sabina leaves (3 kg) were extracted with 70 % Ethanol (10 L × 3, each 1 h) at 80oC. The extracts were then combined, filtered and evaporated to dryness by rotary evaporation at 55oC under reduced pressure. The concentrated residue (700 g) was suspended with distilled water and extracted successively with Petroleum ether, Chloroform and Ethyl acetate. The Ethyl acetate extract was concentrated under reduced pressure delivering 20 g residue. The residue (20 g) was dissolved in Methanol, and subjected to a Sephadex LH 20 column (3×130 cm), eluted with the Methanol at a flow rate of 2 mL/min. The effluents were collected and concentrated under reduced pressure. They were then subjected for subsequent HPLC analysis. According to the results of HPLC analysis, fraction 2 (F2) yielded 2.5 g of crude sample, which was used for preparative HSCCC separation.

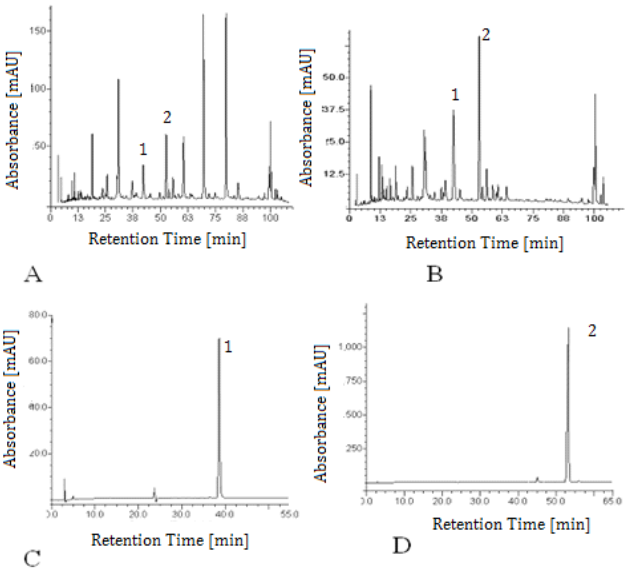

Fig. 2. *HPLC chromatograms for ethyl acetate extract (A), F2 (B), Podophyllotoxin (C) and Deoxypodophyllotoxin (D).*

HPLC Analysis

HPLC (DIONEX, USA) equipment used was a DIONEX system including a P680 pump, an ASI-100 Automated sample injector, a TCC-100 temperature controlled column compartment, and an UVD170U detector. The analysis was carried out with a C18 column (4.6×250mm, 5μm GL Sciences Inc., Japan). Evaluation and quantification were made on a Chromeleon WorkStation. The binary mobile phase consisted of methanol (A) and 0.2 % formic acid (B). The elution was performed with a liner gradient mode as follows: A-B (15:85, v/v) in 20 min, then to A-B (35:65, v/v) in 40 min, then to A-B (55:45, v/v) in 30 min, then to A-B (75:25, v/v) in 30 min, and finally to A-B (15:85, v/v) in 6 min. The effluent was monitored at 254 detection wave length. The sample injection volume was 10 μL and the column temperature was set at 35oC. The flow rate of the mobile phase was 1.0 mL/min.

HSCCC Analysis

HSCCC was carried out using a model of TBE-300A high speed counter current chromatographic instrument (Shanghai, Tauto Biotech, China) with a total volume of 290 mL three polytetrafluoroethylene multilayer coil (tube ID: 2.6 mm). The β-value (β = 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 distances between the holder axis and central axis of the centrifuge) varied from 0.5 (internal) to 0.8 (external). The solvent was pumped into the column with a model

of P230 constant flow pump (Dalian Elite Analytical Instruments Co., Ltd, China). The effluent was continuously monitored by 8823A-UV detector at 254 nm (Beijing Binta Instrument Technology Co., Ltd., China), and the results were recorded by N2000 workstation (Zhejiang University, Hangzhou, China). The sample was injected into the column manually by a 20 mL injection loop.

HSCCC separation procedure

In the present study, series of two phase solvent systems including n-hexane–ethyl acetate–methanol–water of various ratios were tested for their partition abilities. A series of solvent systems with different ratios were prepared and mixed. After being equilibrated for 10 min, the two phase solvents were separated completely; each layer was taken out and analyzed by HPLC. The K-values of target components were calculated according to the equation K = AU/AL, where AU and AL are the peak areas of target compounds in the upper phase and lower phase respectively.

In HSCCC separation, the coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 850 r/min, while the lower phase was pumped into the column at a flow rate of 2 mL/min. The temperature was set at 25oC. After the mobile phase front emerged and hydrodynamic equilibrium was reached in the column, F2 dissolved in 20 mL mobile phase was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. The crude extract and each HSCCC peak fraction were analyzed by HPLC.

RESULTS AND DISCUSSION

HPLC analysis

Ethyl acetate crude extract and F2 were analyzed by HPLC, and chromatograms obtained are shown in Fig. 2. Methanol and 0.2 % formic acid were used as the mobile phase in a linear gradient mode, the extract was separated efficiently under the optimized conditions.

Optimization of two phase solvent system of HSCCC

The separation by HSCCC depends largely on a suitable two phase solvent system that provides an ideal partition coefficient (0.5<K<2.5) for the target compound and are a short settling time [9]. The solute with a smaller K value will be eluted closer to the solvent front with low peak resolution while the solute with a larger K value tends to give better resolution but with broader and more dilute peaks [10]. In this experiment, according to the characters of the targeted compound and related reports [11], several solvent systems were tested, and the results are shown in Table 1. The K values (0.55 and 1.97) in solvent system 3 was moderate which achieved good resolution and acceptable separation time (Table 1). Therefore, solvent system composing of n-hexane-ethylacetate-methanol-water (3:5:3:5) was selected as the solvent system for HSCCC separation. Under the optimized conditions, two fractions (Podophyllotoxin and deoxypodophyllotoxin) were obtained in one-step elution (Fig. 3). When the flow rate of 2.0 mL/min, revolution speed of 850 r/min, and the separation temperature of 25oC were employed in HSCCC separation, the retention percentage of the stationary phase could will be kept at 52.3 %.

Table 1: *K values of the targeted fractions from Juniperus Sabina measured in different solvent systems.*

No.	Solution system (v/v/v/v)	K* values	
		Podophyllotoxin	deoxypodophyllotoxin.
1	n-hexane-ethylacetate-methanol-water (1:1:1:1)	0.16	0.92
2	n-hexane-ethylacetate-methanol-water (2:5:2:5)	0.63	3.86
3	n-hexane-ethylacetate-methanol-water (3:5:3:5)	0.55	1.97
4	n-hexane-ethylacetate-methanol-water (4:5:4:5)	0.47	2.56

K*= solute concentration in the upper mobile phase divided by that in the lower stationary phase.

Purity determination and identification of the collected fractions

Podophyllotoxin and deoxypodophyllotoxin were obtained in the two-steps separation and analyzed by HPLC. According to the HPLC result, 34.8 mg of deoxypodophyllotoxin, 7.9 mg of podophyllotoxin (shadow time range in preparative HSCCC to collect) from 200 mg crude sample were yielded with the purity of 96.5 % and 94.4 %, respectively. The fractions collected from preparative HSCCC separation were dried at 42oC under vacuum with a rotary evaporator, and then subjected to NMR analysis. The HSCCC chromatogram was shown in Fig. 3.

Podophyllotoxin (1): white needles; ^{1}H NMR ($CDCl_3$, 600 MHz) δ ppm: 6.67 (1H, s, H-6), 6.52 (1H, s, H-3), 6.35 (2H, s, H-2', 6'), 5.95 (2H, d, *J* = 12.0 Hz, -OCH_2O-), 4.60 (1H, s, H-7'), 4.46 (1H, S, H-9a), 3.92 (1H, s, H-9b), 3.81 (3H, s, 4'-OCH_3), 3.75 (6H, s, 3',5'-OCH_3), 3.08 (1H, d, *J* = 12.0 Hz, H-7b), 2.77 (1H, m, H-7a), 2.74 (2H, m, H-8, 8'). ^{13}C NMR ($CDCl_3$, 150 MHz) δ ppm: 175.1 (C-9'), 152.7 (C-3', 5'), 147.2 (C-5), 146.9 (C-4), 137.2 (C-4'), 136.5 (C-1'), 130.8 (C-1), 128.5 (C-2), 110.7 (C-3), 108.7 (C-6), 108.4 (C-2',6'), 101.4 (-OCH_2O-), 72.3 (C-9), 61.0 (4'-OCH_3), 56.4 (3',5'- OCH_3), 47.7 (C-8'), 43.9 (C-7'), 33.3 (C-7), 32.9 (C-8). The ^{1}H NMR and ^{13}C NMR profiles of podophyllotoxin matched with the reported NMR data [11,12].

Deoxypodophyllotoxin white needles; ^{1}H NMR ($CDCl_3$, 600 MHz) δ ppm: 7.02 (1H, s, H-6), 6.43 (2H, s, H-2', 6'), 6.36 (1H, s, H-3), 5.93 (2H, d, *J* = 18.0 Hz, -OCH_2O-), 4.49 (1H, dd, *J* = 3.6, 7.2 Hz, H-7'), 4.48 (1H, S, H-9a), 3.83 (3H, s, 4'-OCH_3), 3.80 (6H, s, 3',5'-OCH_3), 3.21 (1H, dd, *J* = 9.0 Hz ,5.4 Hz, H-7b), 2.74 (2H, m, H-8,8'). 2.18 (1H, d, *J* = 7.8 Hz ,H-7a), ^{13}C NMR ($CDCl_3$, 150 MHz) δ ppm: 178.0 (C-9'), 153.8 (C-3', 5'), 147.6 (C-4), 147.3 (C-5), 139.5 (C-1'), 137.3 (C-4'), 132.2 (C-1), 130.8 (C-2), 109.5 (C-3), 105.8 (C-2', 6'), 105.6 (C-6), 101.4 (-OCH_2O-), 70.0 (C-9), 69.7 (C-7), 61.1 (4'- OCH_3), 56.4 (3',5'- OCH_3), 45.6 (C-8'), 44.2 (C-7'), 42.9 (C-8). The ^{1}H NMR and ^{13}C NMR profiles of deoxypodophyllotoxin matched with the reported NMR data [8].

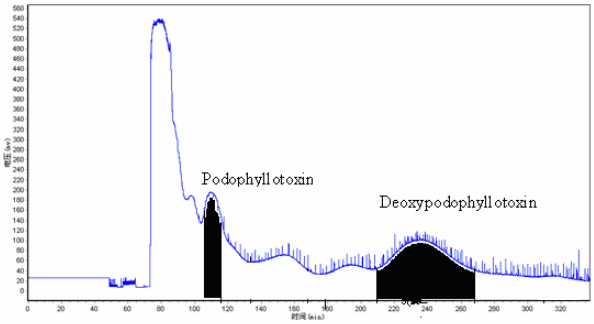

Fig. 3. *Separation of podophyllotoxin and deoxypodophyllotoxin from Juniperus Sabina by HSCCC.*

CONCLUSION

Podophyllotoxin is a naturally occurring lignan with important antineoplastic and antiviral properties and supported by detailed understanding of their mechanism of action, and facilitated by chemical manipulations that have amplified their bioactivity. Deoxypodophyllotoxin, an analogue of podophyllotoxin, is well known for its antitumor, antiviral, and anti-inflammatory activities. In the past few years, extensive structural modifications of podophyllotoxin have led to the development of several clinically valuable anticancer drugs. These drugs are presently in clinical use for the treatment of testicular carcinoma, acute leukemia and lymphoma. In this study, a HSCCC method was successfully developed for the preparative separation and purification of podophyllotoxin and deoxypodophyllotoxin from *Juniperus sabina* in one step. This method was simple, fast, convenient and appropriate to isolate pure compounds as reference substances for related research on deoxypodophyllotoxin and podophyllotoxin.

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