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Short communication

Preparative isolation and purification of celastrol from *Celastrus orbiculatus* Thunb. by a new counter-current chromatography method with an upright coil planet centrifuge

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Abstract

A new counter-current chromatography (CCC) method with an upright coil planet centrifuge, which holds four identical multilayer coil columns in the symmetrical positions around the centrifuge axis, was applied to the isolation and purification of celastrol from the roots of *Celastrus orbiculatus* Thunb. The crude celastrol was obtained by elution with light petroleum from ethanol extracts using 15 cm × 5 cm i.d. silica gel flash chromatography. Preparative CCC with a two-phase system composed of light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–methanol–water (1:1:8:6:1, v/v) was successfully performed, yielding 798 mg celastrol at 99.5% purity from 1020 mg of the crude sample in one step separation.

Keywords: Counter-current chromatography; Preparative chromatography; *Celastrus orbiculatus*; Plant materials; Celastrol; Triterpenoids

1. Introduction

Celastrus orbiculatus Thunb. is a perennial creeping planet, belonging to the family celastraceae. Its roots have long been used as a traditional herb medicine to treat fever, chills, joint pain, edema, rheumatoid arthritis and bacterial infection in Chinese folk medicine [1]. The pharmaceutical studies and clinical practice have demonstrated its sesquiterpenes and triterpenes, including celastrol, possess notable antibacterial, antitumor, insect antifeedant and cytoxic activities [2].

Celastrol is the main triterpenoid component of *C. orbiculatus* Thunb. which has been tested and verified to be of antitumor activity, antitumor promoting activity, and inhibitory activity on IL-1 release in the LPS (lipopolysaccharide)-stimulated human peripheral mononuclear cells [3,4]. Recently, celastrol, regarded as a potent antioxidant and anti-inflammatory drug, was used as a hopeful treatment for Alzheimer's disease (AD) [5]. In addition, celastrol showed the most potent inhibitory activity in the reporter gene expression, with an IC_{50} value of 0.27 μM [6].

In view of the wide biologically activities, the preparation of celastrol with high purity has been of much interest to pharmaceutical chemists. The preparative separation and purification of celastrol from plant materials by conventional methods is tedious and usually requires multiple chromatography steps, such as column chromatography (CC) and thin-layer chromatography (TLC) [7,8]. To obtain highly pure celastrol is very difficult because of its unstable properties and irreversible adsorption on solid support column chromatography. Counter-current chromatography (CCC) is a unique liquid–liquid partition chromatography method that uses no solid support matrix. Therefore, it eliminates irreversible absorptive loss of samples onto the solid support matrix used in conventional chromatography. The method has been successfully applied to the analysis and separation of various natural products [9–12]. Recently, we have developed a new CCC method with an upright coil planet centrifuge, which holds four identical multilayer coil columns in the symmetrical positions around the centrifuge axis. Our primary experiments have demonstrated that the CCC apparatus is most suitable for large-scale preparative isolation and purification from crude extracts

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of natural products at a relatively low rotary speed. So far, no report has been published on the use of CCC for the isolation and purification of celastrol from *C. orbiculatus* Thunb. The purpose of this study, therefore, was to develop a method for the isolation and purification of celastrol from *C. orbiculatus* Thunb. by CCC.

2. Experimental

2.1. Apparatus

The isolation and purification of celastrol from *C. orbiculatus* Thunb. was performed by a new CCC method with an upright coil planet centrifuge designed by ourselves. Its general design principle is shown in Fig. 1A where four upright cylindrical column holders are symmetrically arranged around the centrifuge axis, similar to the type-J HSCCC with three horizontal multilayer coils connected in series [13]. Each holder undergoes an identical synchronous planetary motion: revolution around the centrifuge axis and rotation about its own axis at the same angular velocity in the same direction as indicated by arrows. These holders are connected in series with flow tubes. All constructs ensure that the system permits the effluent flow in and out the system through the rotating columns without the use of a rotary seal which would become a source of leakage and contamination.

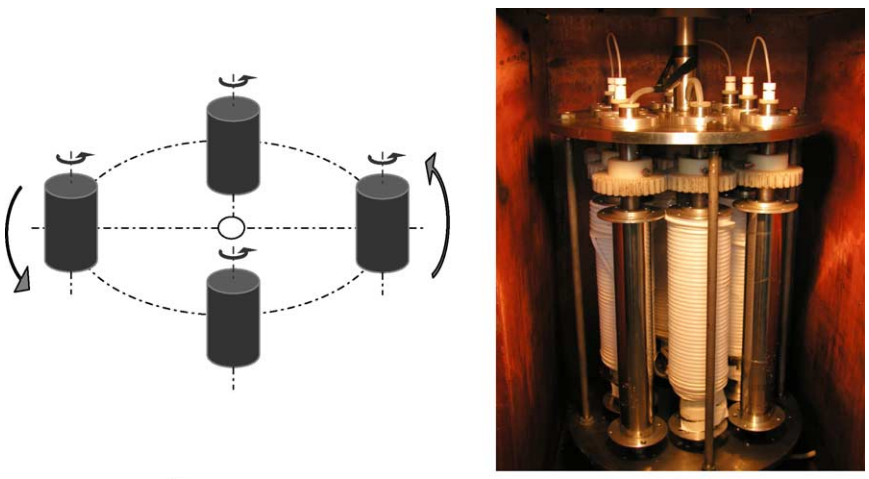
The fabricated CCC apparatus (see Fig. 1B) holds four identical multilayer coils in the symmetrical positions around the rotary frame at distance of 9 cm from the central axis of the centrifuge to maintain perfect balance of centrifuge system without the use of a counterweight. Each separation column was made by winding a single piece of 4 mm i.d. and 1 mm wall thickness polytetrafluroethylene (PTFE) tubing directly onto the holder hub of 5 cm diameter, forming three layers of right-handed and left-handed coils alternating in each layer between a pair of flanges spaced 35 cm apart. The β-value (ratio of helical radius of the coil and revolution radius) of the multilayer coil varies from 0.28 at the internal terminal to 0.48 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). These multilayer coils are connected in series on the rotary frame using a flow tube (PTFE, 1.6 mm i.d. and 0.7 mm wall thickness) to give a total capacity of 1600 ml while the unique gear arrangement on the rotary frame establishes a twist-free mechanism of the flow tubes so that continuous elution can be performed without the use of rotary seal.

The apparatus can be operated up to maximum speed of 800 rpm with a speed Sunwind control unit (Shenduo Electric Corp., Shanghai, China) and up to 60 °C with a temperature control unit. In addition, this CCC system is equipped with a Type-J-W metering pump (Zhijiang Petroleum Equipment, Hangzhou, China), a HD-9704 UV spectrometer operating at 254 nm and 280 nm, Shimadzu C-R1B Chomatopac recorder, BSZ-100 fraction collector, a sample injection valve with a 30 ml sample loop and NT2000 data analysis system (Institute of Automation Engineering, Zhejiang University, Hangzhou, China).

The high-performance liquid chromatography (HPLC) system used was an Agilent 1100 system including G1312A BinPump, G1314A variable-wavelength detector (VWD), a model 7725 injection valve with 20 μl loop, a PT100 column oven and an Agilent ChemStation for LC. The column used was a reversed-phase C_{18} column (YMC-PACK ODS-A, 150 mm × 4.6 mm i.d., 5 μm, 120 Å).

2.2. Reagents

All organic solvents used for CCC were of analytical grade and purchased from Huadong Chemicals Hangzhou, China. Reverse osmosis Milli-Q water (18 Ω) (Millipore, Bedford, MA, USA) was used for all solutions and dilutions.



(A) (B)

Fig. 1. (A) The design principle and (B) the photograph of the present CCC apparatus.

Methanol used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany.

The roots of *C. orbiculatus* Thunb. were purchased from Huadong Medicinal Corporation. A voucher specimen with reference number 021208 is kept in the Institute of Organic and Pharmaceutical Chemistry, Zhejiang University.

2.3. Preparation of crude celastrol

Dried and powdered raw roots of *C. orbiculatus* Thunb. (15 kg) were extracted three times with 95% ethanol. Then, the extract was combined and removed solvent under reduced pressure and 40 °C, which yielded 1.5 kg syrup. The syrup was redissolved in light petroleum (bp 60–90 °C), ethyl acetate, methanol, successively. By combining each extract and remove solvent under reduced pressure, four fractions were obtained, including 200 g light petroleum extracts, 250 g ethyl acetate extracts, 300 g methanol extracts and 200 g undissolved residue. In this work, 100 g light petroleum extract was taken and grossly separated by 15 cm × 5 cm i.d. silica gel flash chromatography (200–300 mesh) and eluted with light petroleum (bp 60–90 °C)–ethyl acetate (1:0, 1:0.25, 1:0.5, 1:1 and 0:1, v/v), which resulted in five fractions. The second fraction was used as crude celastrol source.

2.4. Preparation of two-phase solvent system and sample solutions

The two-phase solvent system used was composed of light petroleum–ethyl acetate–tetrachloromethane–methanol–water at various volume ratios, The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the upper phase and equal lower phase.

2.5. Separation procedure

Preparative CCC was performed as follows: the four upright multilayer coil columns connected in series were first entirely filled with upper phase as stationary phase, and then 15 ml sample solution containing 1020 mg of the crude celastrol was injected through the sample port and the lower organic phase as mobile phase was pumped into the head of the column at a flow rate of 4.0 ml/min while the column was rotated at 400 rpm. The effluent from the tail end of the column was monitored with a UV detector at 280 nm and automatically collected in 40 ml test tube per 5 min using a BSZ-100 fraction collector. Peak fractions were collected according to the elution profile and HPLC detection.

2.6. HPLC analysis and identification of CCC peak fractions

The crude sample and each CCC peak fraction were analyzed by HPLC. The analyses were performed with an YMC-Pack ODS-A column (150 mm × 4.6 mm i.d., 5 μm, 120 Å. The mobile phase was acetonitrile–0.005 M orthophosphoric acid (85:15, v/v). The flow-rate was 1.0 ml/min, and the effluent was monitored at 230 nm.

Identification of the CCC peak fraction was carried out by mass spectrometry (MS) on a Bruker Esquire 3000 plus spectrometer, one- and two-dimensional NMR spectrometry on a Bruker Advanced DMX 500 NMR spectrometer.

3. Results and discussion

Fig. 2 shows HPLC analysis of the crude celastrol from the roots of *C. orbiculatus* Thunb. eluted with light petroleum (bp 60–90 °C) by silica gel column flash chromatography as well as the chemical structure of celastrol. The Result indicated that it contained several compounds including celastrol at 85% purity and some unknown compounds.

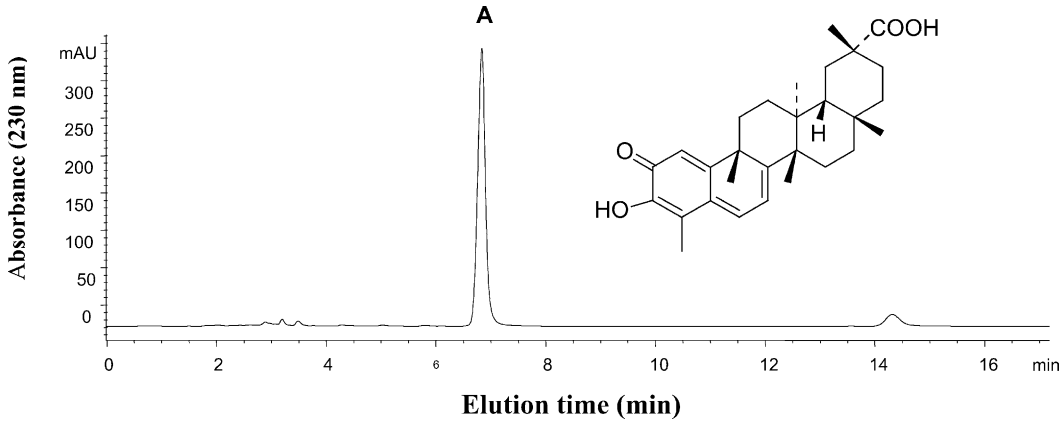


Fig. 2. Chromatogram of crude celastrol from *Celastrus orbiculatus* Thunb. by HPLC analysis as well as the chemical structure of celastrol; A, celastrol. Conditions: column, YMC-Pack ODS-A (150 mm × 4.6 mm i.d., 5 μm, 120 Å); column temperature, 25 °C; mobile phase, acetonitrile and 0.005 M orthophosphoric acid (85:15, v/v); flow rate, 1 ml/min; detection, 230 nm.

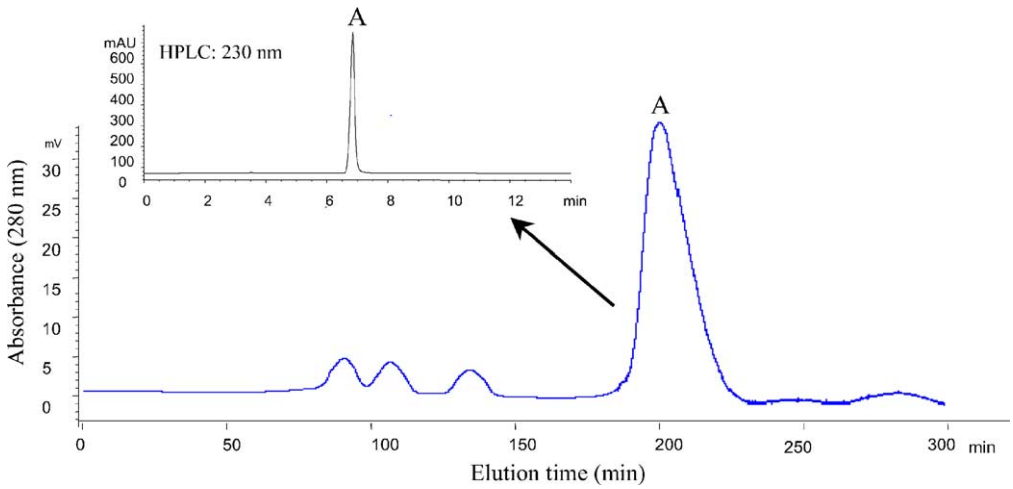


Fig. 3. Preparation CCC separation of the crude celastrol from *Celastrus orbiculatus* Thunb. and HPLC analysis corresponding to the celastrol peak; A, celastrol. CCC separation conditions: column, multilayer coil of 4.0 mm i.d. PTFE tube with a total capacity of 1600 ml; rotary speed, 400 rpm; column temperature, 35 °C; solvent system, light petroleum (bp 60–90 °C) ethyl acetate tetrachloromethane methanol water (1:1:8:6:1, v/v); mobile phase, lower phase; flow rate, 4 ml/min; detection, 280 nm; sample size, 1020 mg; retention of the stationary phase, 78.1%. HPLC analysis conditions: column, YMC-Pack ODS-A (150 mm × 4.6 mm i.d., 5 μm, 120 Å); column temperature, 25 °C; mobile phase, acetonitrile and 0.005 M orthophosphoric acid (85:15, v/v); flow rate, 1 ml/min; detection, 230 nm.

Meanwhile, this also demonstrates that the silica gel flash chromatography is suitable for the primary fractionation of crude celastrol from the roots extracts of *C. orbiculatus* Thunb.

In order to achieve an efficient resolution of target compound, the two-phase solvent systems of light petroleum–ethyl acetate–tetrachloromethane–methanol–water at various volume ratios, i.e., 1:1:8:6:1, 0:2.5:15:10:2.5, 2.5:0:15:10:2.5, were examined using the present CCC apparatus. The result indicated that the volume ratio of 1:1:8:6:1 was the optimum for the separation of celastrol.

Fig. 3 shows the preparative CCC separation of 1020 mg of the crude celastrol sample using the solvent system composed of light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–methanol–water (1:1:8:6:1, v/v) and HPLC analysis corresponding to the celastrol peak of CCC. In order to save solvents and time, the other eluting compounds after the target substance were removed by pumping out. As a result, 798 mg celastrol at 99.5% purity was obtained. The structural identification of the celastrol fraction was carried out by MS, one- and two-dimensional NMR.

In conclusion, large-scale preparative isolation and purification of celastrol from the roots of *C. orbiculatus* Thunb. was successfully performed using a new countercurrent chromatography with upright coil planet centrifuge which holds four identical multilayer coil columns in the symmetrical positions around the centrifuge axis, and yielded 798 mg celastrol at 99.5% purity from 1020 mg of crude sample in one step separation. The present study indicated that the CCC apparatus is very practical for the preparative separation of celastrol from *C. orbiculatus* Thunb.

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