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Preparative Isolation and Purification of the Lignan Pinoresinol Diglucoside and Liriodendrin from the Bark of *Eucommia Ulmoides Oliv.* by High Speed Countercurrent Chromatography

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P. R. China

Abstract: In this study, we reported a high throughput isolation procedure of pinoresinol diglucoside (PDG) and liriodendrin (SDG) from the traditional Chinese medical plant *Eucommia ulmoides Oliv.* by high speed countercurrent chromatography. The solvent system consisted of n-butanol-acetic acid-water(4:1:5, v/v/v), in which the lower phase was used as the mobile phase at a flow-rate of $2.0\text{mL} \cdot \text{min}^{-1}$ in the head to tail elution mode. The method yielded 21 mg of PDG at 96.7% purity and 16 mg of liriodendrin at 95.5% purity from 200 mg of crude extract in a one step. The purity and identity of the isolated compounds were checked by high performance liquid chromatography (HPLC) analysis, in combination with mass spectrometry (MS), infrared spectrum (IR), and NMR measurements.

Keywords: *Eucommia ulmoides Oliv.*, High speed countercurrent chromatography, HPLC, Pinoresinol diglucoside, Liriodendrin, Lignans

INTRODUCTION

Eucommia ulmoides Oliv. (Duzhong) is one of the longest known tonic herbs in China, Japan, and Korea. Its bark is used to benefit liver and kidney, strengthen tendons and bones, prevent miscarriages, reinforce muscles and lungs, as well as for the treatment of hypertension, and as an anti aging

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agent.^[1,2] Pinoresinol diglucoside (PDG) and liriodendrin (SDG) are the main bioactive lignan components in this plant. They possess anti-hypertensive effects^[3] and analgesic effects in mice and local anaesthesia in guinea pigs.^[4] These two compounds were regarded as reference standards in the quality control of *Duzhong* and its products.^[5] The chemical structures of PDG and liriodendrin are shown in Figure 1.

Conventional chromatographic column methods use solid stationary phases and need multiple chromatography steps. In contrast, high speed countercurrent chromatography (HSCCC), which was first invented by Y. Ito,^[4] is a liquid-liquid partition chromatography with a liquid stationary phase. It eliminated irreversible adsorption of the sample onto the solid support.^[6] In the past 30 years, HSCCC has been employed in separation of active components from many traditional Chinese herbs and other natural products.^[7-11] However, so far, there is no report using HSCCC for the separation and purification of PDG and liriodendrin from *Eucommia ulmoides Oliv.* (*Duzhong*). The purpose of this report was to develop a high throughput and efficient protocols for the preparation of high purity PDG and liriodendrin from *Eucommia ulmoides Oliv.* by HSCCC.

EXPERIMENTAL

Apparatus

Preparative HSCCC was carried out with a TBE-300A high speed countercurrent chromatograph (Tauto Biotechnology Company, Shanghai, China). The apparatus consisted of three multilayer coil separation columns connected in series (inner diameter of tube, 1.6 mm; total volume, 300 mL), and a 20 mL sample loop. The revolution radius was 5 cm, and the β values 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 can be regulated with a speed controller in the range between 0 and 1000 rpm. An optimum speed of 800 rpm was used in the present studies. An HX 1050 constant temperature circulating implement

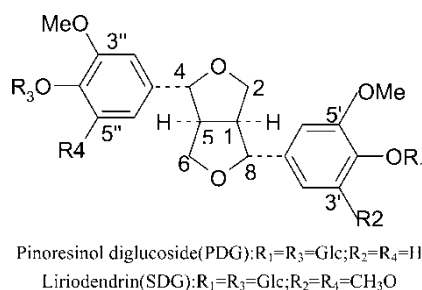


Figure 1. Chemical structures of pinoresinol diglucoside and liriodendrin.

(Beijing Boyi Lab. Instrument. Co. Ltd., Beijing, China) was used to control the separation temperature. The solvent was pumped into the column with a Model NS-1007 constant flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 88230A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 280 nm. The data were collected with an N-2000 Chromatography Workstation (Zhida Information Technique of Zhejiang University, Hangzhou, China).

The HPLC equipment was from Hitachi, including Pump L-7110, UV-VIS and Detector L-7420. The column used was a reversed phase YWG C₁₈. Evaluation and quantification were made on N2000 Chromatography Data Workstation. The NMR spectrometer used here was AVANCE AV 500 (Bruker, Germany); Mass spectrometer was HP5989B (Hewlett-Packard, USA).

Reagents

All solvents used for preparation of the crude extract and HSCCC separation were of analytical grade (Xi'an Reagent Factory, Xi'an, China). Methanol used for HPLC was chromatography grade (Merck Company), and water used was purified water.

The bark of *Eucommia ulmoides* Oliv. was purchased from the Duzhong Developing Centre of Luoyang Country, (Ankang, China) and identified by Professor Yazhou Wang (School of Life Science, Northwest University, Xi'an, China).

Preparation of Crude Extract

The air dried bark of *Eucommia ulmoides* Oliv. (1 kg) was chopped and extracted three times with 60% ethanol at 70°C. The ethanol solution was evaporated until there was no ethanol odour under reduced pressure, and then filtered. The filtrate was applied to a D101 macroporous resin column and eluted successively with water, 10% ethanol, 20% ethanol, 30% ethanol. The 30% ethanol eluate was concentrated and the residue was dissolved in methanol, and then filtered; the solution was evaporated to dryness by rotary vaporization. Totally, 11.35 g of residue was stored in a refrigerator for the subsequent HSCCC separation. The isolation protocol was schematically shown in Figure 2.

Measurement of Partition Coefficient (k)

The two-phase solvent system was selected according to the partition coefficient (k) of the target components. The k values were determined by HPLC analysis. The crude extract of 2 mg was dissolved in 1 mL lower phase. The solution was

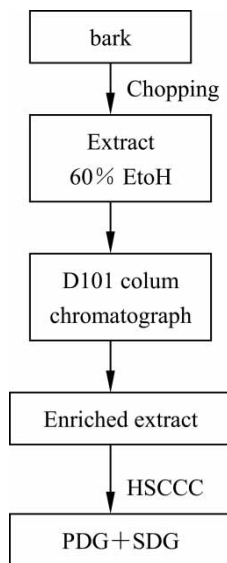


Figure 2. Work-up protocol for the preparation of crude extract.

determined by HPLC. The peak area was recorded as A_1 , and then an equal volume (1 mL) of the upper phase was added to the solution and mixed thoroughly. After partition equilibration, the lower phase solution was determined by HPLC again, and the peak area was recorded as A_2 . The partition coefficient (k) was obtained by the following equation: $k = A_2/A_1 - A_2$.

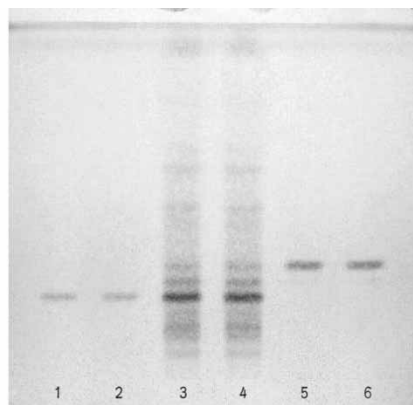
Preparation of the Two-Phase Solvent System and Sample Solution

The two-phase solvent system used for HSCCC separation was composed of n-butanol-acetic acid-water (4:1:5). Here, the upper phase was used as the stationary phase, and the lower phase was used as the mobile phase. Each solvent mixture was thoroughly equilibrated in a separation funnel at room temperature and the two-phases were separated and degassed by sonication for 30 min right before use.

The sample solution was prepared by dissolving 200 mg of the crude extract in 15 mL solution of the upper phase.

HSCCC Separation Procedure

In each separation procedure, the coiled column was, first, entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 800 rpm. The lower phase (mobile phase) was pumped into the column in the head to tail elution mode at a flow rate of 2.0 mL min^{-1} . Then, the mobile phase front



1:PDG;2:PDG;3:the crude extract;4:the crude extract;5:SDG;6:SDG
 Conditions:developer reagent-the lower phase of chloroform-methanol-
 water(65:35:16,v/v/v);
 colour reagent:10% H_2SO_4 -EtOH;TLC plate:1%CMC-Na silica.

Figure 3. Thin layer chromatography (TLC) of the crude extract, PDG and SDG. 1: PDG; 2: PDG; 3: The crude extract; 4: The crude extract; 5: SDG; 6: SDG. Conditions: developer reagent: the lower phase of chloroform-methanol-water (65 : 35 : 16 v/v/v); colour reagent: 10% H_2SO_4 -EtOH; TLC plate: 1% CMC-Na silica.

emerged and hydrodynamic equilibrium was established in the column, and 15 mL of the crude extract was injected through the injection valve. All through the experiment, the separation temperature was controlled at 25°C. The effluent from the tail end of the column was continuously monitored with a UV absorbance detector at 254 nm. The data were collected immediately

Table 1. The K (partition coefficient) values of PDG and liriiodendrin in different solvent systems

Solvent system	K value	
	PDG	Liriiodendrin
Chloroform-methanol-water (4 : 2.2 : 1)	12.6	0.10
chloroform-methanol-water (5 : 5 : 3)	12.5	0.27
chloroform-methanol-water (3 : 1.5 : 1)	4.7	0.13
chloroform-methanol-water (4 : 3 : 2)	9.9	0.15
n-Butanol-acetic acid-water (4 : 1 : 5)	1.0	0.9
n-Butanol-water (1 : 1)	0.76	1.40
n-butanol-methanol-water (5 : 1 : 5)	0.91	1.21
n-Butanol-ethyl acetate-water (4 : 1 : 4)	0.42	1.32

after sample injection. The peak fraction was collected manually according to the obtained chromatogram. Each collection was evaporated under reduced pressure, and dissolved by methanol for subsequent purity analysis.

HPLC Analysis and Identification of HSCCC Peak Fraction

The HPLC analysis was performed with a YWG C₁₈ column (250 mm × 4.6 mm. i.d., 10 μm) at room temperature. The mobile phase was methanol-water (40 : 75, v/v). The effluent was monitored at 254nm and the flow rate was kept at 1.0 mL min⁻¹ constantly.

The structure identification of HSCCC peak fractions was carried out by ¹H-NMR and ¹³C-NMR. ¹H-NMR and ¹³C-NMR spectra were recorded on a Avance AV 500 (Bruker, Germany) with DMSO as solvent.

RESULTS AND DISCUSSION

Thin Layer Chromatography (TLC) of the Crude Extract, Separated PDG and SDG

The crude extract from the bark of *Eucommia ulmoides Oliv.* contained several compounds with very similar chemical structures (see Figure 1). There are two

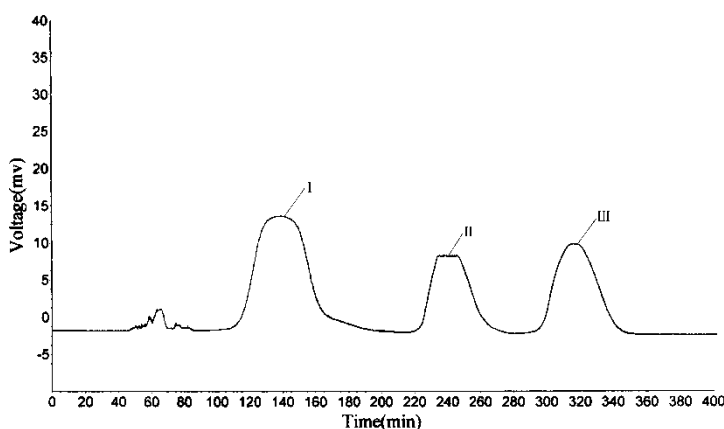


Figure 4. Chromatogram of the crude extract from the bark of *Eucommia ulmoides Oliv.* by HSCCC separation: I = PDG, III = SDG. Conditions: solvent system: n-butanol-acetic acid-water (4 : 1 : 5). Stationary phase: upper organic phase; mobile phase: lower aqueous phase; sample: 200mg dissolved in 15 mL upper phase; flow-rate: 2.0 mL min⁻¹; detection-254 nm; revolution: 800 rpm; retention of stationary phase: 42%.

CH₃O- differences between PDG and SDG. It was difficult to separate these compounds completely using normal column chromatography. The TLC result in Figure 3, also indicated their little difference on R_f values.

Optimization of HSCCC Conditions

The successful separation by HSCCC depends mainly upon the selection of a suitable two-phase solvent system. This means choosing the stationary phase

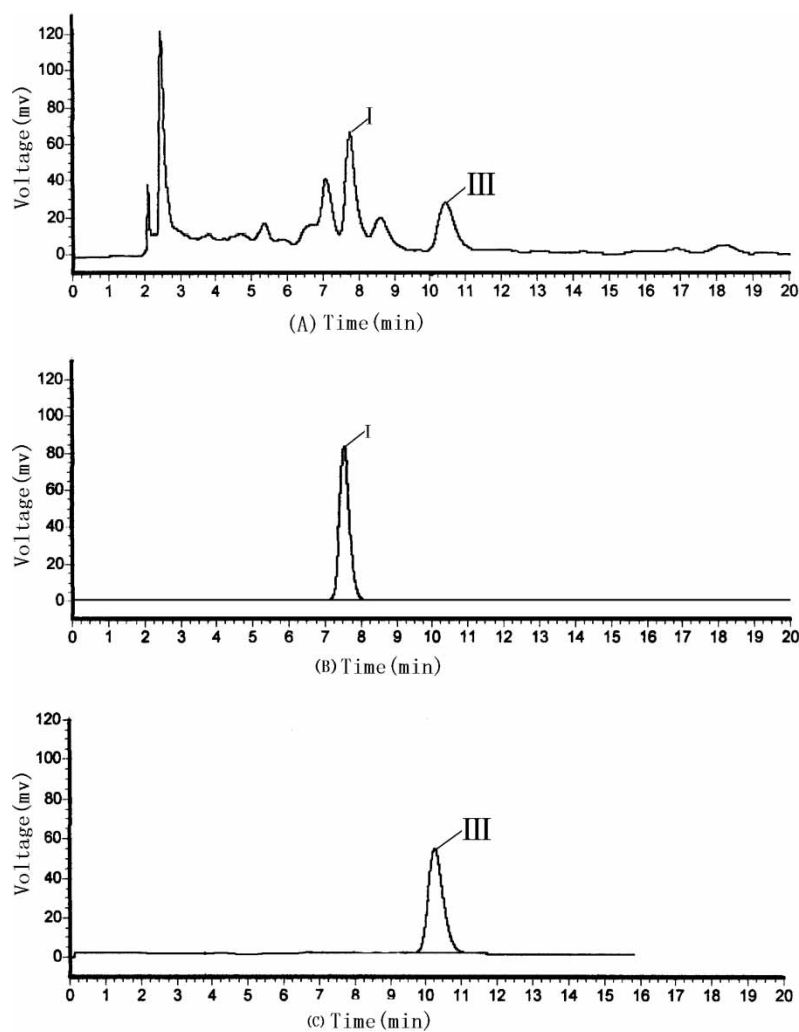


Figure 5. HPLC chromatograms. (A) crude extract from *Eucommia ulmoides* Oliv. (B) HSCCC fraction of peak I (PDG). (C) HSCCC fraction of peak III.

and the mobile phase simultaneously. In order to achieve efficient resolution of target compounds, many different solvent systems were examined and the k values were shown in Table 1. The results indicated that the chloroform-methanol-water solvent system, which had the big k values, could elute PDG and SDG in an excessively broad peak with a long elution time. When *n*-butanol-water (1:1) was used for HSCCC separation, the phase separation of the two-phase solvent system was not ideal, and the stationary phase of HSCCC was seriously lost. We found, however, that acetic acid could improve the phase separation and the maintenance of the stationary phase. So, the two-phase solvent system composed of *n*-butanol-acetic acid-water at a volume ratio of 4:1:5 was found to be the best for the separation of PDG and SDG from the crude sample.

The influence of revolution speed, flow rate of the mobile phase, and temperature on the HSCCC separation were also investigated. The results indicated that when the flow rate, revolution speed, separation temperature, were at 2 mL/min⁻¹, 800 rpm, and 25°C, respectively, retention percentage of the stationary phase could reach 41.8% and good separation results could be obtained. Under the optimum conditions, three major peaks were obtained and yielded 21 mg of peak I, 15 mg of peak II, 16 mg of peak III from 200 mg crude extract. The purity of peak I and peak III determined by HPLC, were 96.7% and 95.5%,

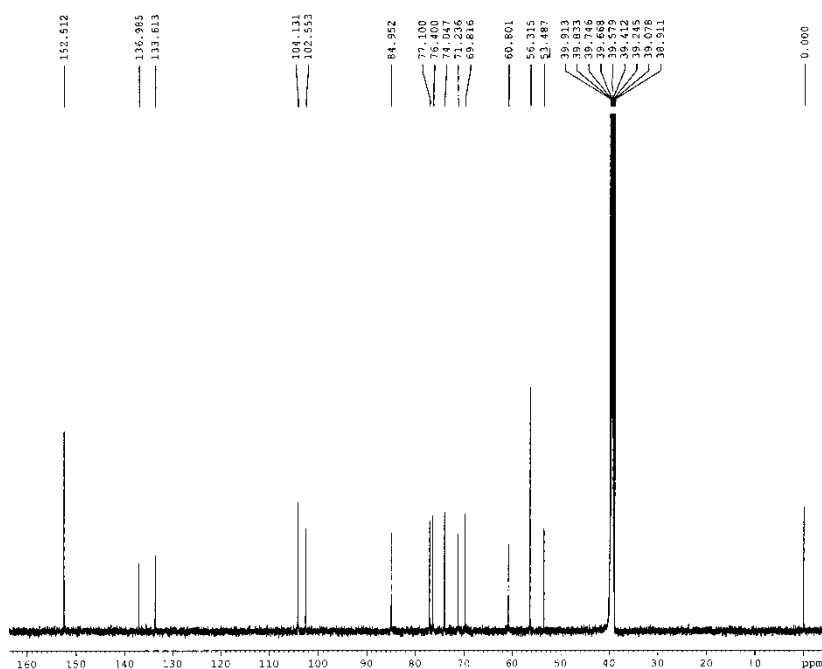


Figure 6. ¹³C-NMR of SDG.

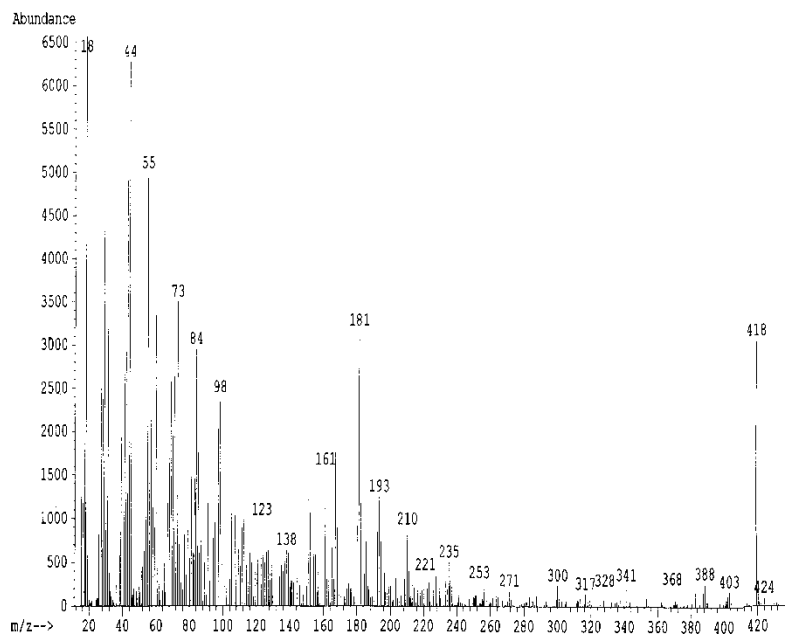


Figure 8. MS of SDG.

respectively. Peak II was a mixture. The HSCCC chromatograms of crude extract from *Eucommi ulmoides Oliv.* was given in Figure 4.

Results of HPLC Analysis and Identification of HSCCC Peak Fraction

Each HSCCC peak fraction was analyzed by HPLC. The purity of peak I and peak II fractions in Figure 4 was 96.7% and 95.5%, respectively. HPLC chromatograms of the crude extract and the HSCCC peak fractions were shown in Figure 5(A), (B), (C).

Identification of peak I was carried out by HPLC, TLC, and IR. According to the literature,^[12] peak I was confirmed as pinoresinol diglucoside (PDG).

The structural identification of peak III in Figure 4 was carried out by MS, ¹H-NMR, and ¹³C-NMR spectra as follows (Figures 6–8): FD-MS m/z : 418 ($M^+ - 2C_6H_{11}O_5 + 2$). ¹H-NMR (500 MHz, DMSO-*d*₆) δ : 6.658 (4H, s, arom.), 3.761 (12H, s, 4XOCH₃), ¹³C-NMR (500 MHz, DMSO-*d*₆): C-1,5 (53.487), C-4,8 (71.236), C-2,6 (84.952); C-1',1'' (133.613), C-6',2'' (104.131), C-5',3'' (152.512), C-4',4'' (136.985), C-3',5'' (152.512), C-2',6'' (104.131); 102.553 (glc-1',1''), 74.047 (glc-2',2''), 76.400 (glc-3',3''), 69.816 (glc-4',4''), 77.100 (glc-5',5''), 60.801 (glc-6',6''). Comparing these data with the literature,^[12] peak III was identified as liriodendrin (syringaresinol diglucoside, SDG).

The results clearly demonstrated that HSCCC is very useful in the preparative separation of PDG and SDG from the crude plant extract of *Eucommi ulmoides Oliv.* It is, also, the first time these two compounds from this plant was isolated by HSCCC. We believe that, the method may be successfully applied for separation of other compounds from the crude extract by selecting a suitable two-phase solvent system.

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