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Short Communication

Preparative isolation of hydrolysable tannins chebulagic acid and chebulinic acid from *Terminalia chebula* by high-speed counter-current chromatography

As a chromatographic column, the high-speed counter-current chromatography system was equipped with a preparative HPLC series, enabling the successful isolation of hydrolysable tannins from the fruits of *Terminalia chebula*, a traditional Chinese medicine. The two-phase solvent system was composed of *n*-hexane-ethyl acetate-methanol-water (1 : 20 : 1 : 20 v/v). As a result, 33.2 mg chebulagic and 15.8 mg chebulinic acids were obtained in one step from 300 mg of crude extract. Their purities were determined by HPLC to be 95.3 and 96.1%, respectively. The chemical structures were identified by their MS and ^{1}H NMR spectra.

Keywords: Chebulagic acid / Chebulinic acid / Counter-current chromatography / Hydrolyzable tannin / *Terminalia chebula*

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1 Introduction

High-speed counter-current chromatography (HSCCC), a support free liquid-liquid partition chromatographic technique, has an excellent sample recovery by eliminating irreversible adsorption of the sample onto a solid support. This method permits direct introduction of crude samples into the column without extra preparation, so it has been successfully applied to isolate and purify a number of natural products [1, 2]. As a developing chromatography technique, the commercial HSCCC system is deficient in the sample injection, detection, and chromatographic signal processing, *etc.* In comparison, the HPLC system has all the advantages over HSCCC, except the HSCCC column itself. A combination of these two techniques may take all the strong points, if HSCCC is used just as a column in the HPLC system. We therefore connected an HSCCC (TBE-300A) with an HPLC (Agilent 1100) series, and tested it in the preparative isolation of two hydrolysable tannins from the fruits of *Terminalia chebula*.

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Abbreviation: HSCCC, high-speed counter-current chromatography

Fruits of *T. chebula* Retzius (*T. chebula* Retz.) (Combretaceae), commonly known as black Myroblans in English, Harad in Hindi, and Hezi in Chinese, indigenous in Pakistan and India, is a popular folk medicine in many Asian and African countries and has been studied for its homeostatic, antitussive, laxative, diuretic, cardiotonic, antibacterial, and anticancer activities [3 – 7]. This herbal medicine is rich in hydrolysable tannins, in which chebulagic acid and chebulinic acid (Fig. 1) have been claimed as the major components [8]. The conventional isolation methods, such as column chromatography and HPLC, are tedious and usually require multiple steps [9, 10]. Furthermore, these tannins are very unstable and easily hydrolysable, which increases the difficulty of isolation. HSCCC has been applied in the preparative isolation of many tannin-like polyphenols, such as flavonoids, catechins, and teaflavines [11 – 13]. However, reports about the hydrolysable galloyl tannin are seldom released, except those involving either acids or *n*-butanol that require a higher temperature of condensation [14, 15].

This paper reports the successful preparative isolation of chebulagic and chebulinic acids with high purities from the fruits of *T. chebula* by the modified HSCCC.

2 Experimental

2.1 Apparatus

The HSCCC instrument employed in the present study is a TBE-300A HSCCC (Tauto Biotechnique Company,

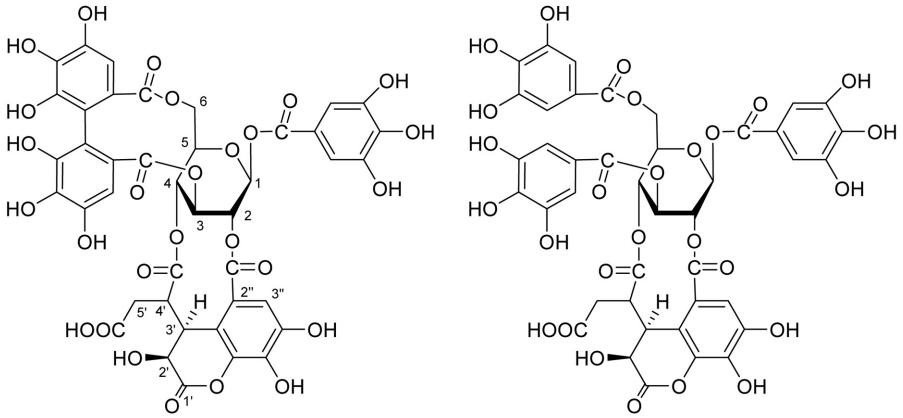

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Figure 1. The chemical structures of chebulagic and chebulinic acids (1 and 2).

Shanghai, China) with three multilayer coil separation columns connected in series (id of the tubing = 1.6 mm, total volume = 260 mL). 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 HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument, Beijing, China) was used to control the separation temperature. As a chromatographic column, the HSCCC system was equipped with a preparative Agilent 1100 HPLC Series (G1361A Prep Pump and G1365B MWD), and a 3725i-038 injector (Rheodyne, USA).

HPLC analysis was carried out on an analytical Agilent 1100 series and an Alltima-C_{18} column (4.6 mm × 250 mm, 5 μm) at room temperature. The Agilent 1100 HPLC system included a G1311A solvent delivery unit, G1315B UV-Vis photodiode array detector, Rheodyne 7725i injection valve with a 20 μL loop, G1332A degasser, and Agilent HPLC workstation.

^{1}H- (400 MHz) NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as the internal standard. Mass spectra were recorded on a VGAuto Spec-3000 spectrometer.

2.2 Reagents

Methanol, ACN, *n*-hexane, ethyl acetate, and phosphoric acid of HPLC grade were purchased from International Laboratory Limited, IL, USA. Distilled water was prepared using a MILLI-Q SP reagent water system, and was distilled twice before use.

The dried fruits of *T. chebula* were purchased from a local drug store and identified by the authors. The identification was confirmed by various chromatographic comparisons with the authentic herb provided by the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), P. R. China. A voucher specimen (CMED-0179) was deposited in the Herbarium of Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong, China.

2.3 Measurement of partition coefficient

Approximately 2 mg of the crude extract was weighed in a 10 mL test tube to which 4.0 mL of each phase of the equilibrated two-phase solvent system was added. The tube was shaken vigorously for 2 min to equilibrate the sample thoroughly with the two phases. Then the upper and lower phases were analyzed by HPLC. The partition coefficients (*K*) were expressed as the peak area of target components in the upper phase divided by that in the lower phase.

2.4 Preparation of two-phase solvent system, the crude extract, and sample solution

Two-phase solvent systems were prepared by adding the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated by shaking repeatedly at room temperature (20°C). The upper and lower phases were separated shortly before use and degassed by sonication for 30 min.

The dried fruits of *T. chebula* were grinded into powder, and 4 g of the powder was added to a bottle and extracted by reflux in 50 mL 60% aqueous methanol for 2 h. The mixture was filtered, and the filtrate was collected. The extract was then concentrated to dryness by rotary vaporization at 50°C under reduced pressure, and a light brown powder (2.8 g) was obtained. This powder (300 mg) was directly dissolved in the two-phase solvent (each phase 3 mL) composed of *n*-hexane-ethyl acetate-methanol-water (1 : 20 : 1 : 20 v/v) for HSCCC isolation.

2.5 HSCCC separation procedure

The whole procedure was carried out as follows: The coil column was first entirely filled with the upper phase of the solvent system by the preparative HPLC pump at a flow rate of 20 mL/min. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 1.5 mL/min. After the mobile phase front emerged and a hydrodynamic equilibrium was established in the column, 6 mL sample solution containing 300 mg of crude extract was injected through the injector. The separation temperature was controlled at 20°C. The effluent from the outlet of the column was continuously monitored at 254 and 216 nm by the Agilent 1100 HPLC UV-Vis detector and ChemStation. Each peak fraction was manually collected according to the chromatogram and evaporated under reduced pressure. The residue was dissolved in methanol for subsequent HPLC analysis.

2.6 HPLC analysis and identification of CCC peak fractions

The crude sample and each HSCCC peak fraction were analyzed by HPLC. The analysis was accomplished with an alltima C_{18} column (250 mm × 4.6 mm id, 5 μm) at room temperature. An ACN-0.1% aqueous phosphoric acid system was used as the mobile phase in a gradient mode as follows: 17 : 83 → 23 : 77 in 30 min. The flow rate was 1.0 mL/min, and the effluents were monitored at 216 nm by a photodiode array detector.

The HSCCC peak fraction was identified by MS and ^{1}H-(400 MHz) NMR spectra. The UV spectra of the isolated HSCCC peaks were taken by the Agilent 1100 ChemStation.

3 Results and discussion

3.1 Selection of two-phase solvent system and other conditions of HSCCC

In HSCCC separation, a suitable two-phase solvent system was critical for a successful isolation and separation. To avoid using acids and *n*-butanol in our experiment, several solvent systems were evaluated and the partition coefficients were measured and listed in Table 1. According to the *K* values, the results indicated that the solvent systems of ethyl acetate-water (1 : 1 v/v) and ethyl acetate-methanol-water at the volume ratios of 5 : 1 : 5, 8 : 1 : 5, and 11 : 1 : 5 v/v had unsuitable *K* values, which contributed to the poor resolution. The solvent system comprising hexane-ethyl acetate-MeOH-water showed a trend to give suitable *K* values, when the volume ratios changed from 1 : 5 : 1 : 5 to 1 : 20 : 1 : 20 v/v. Finally, the ratio of 1 : 20 : 1 : 20 v/v was proved most suitable for our experiment.

The effect of the flow rate of the mobile phase, the separation temperature and the revolution speed were also investigated. The result indicated that a slow flow rate could produce a good separation, but would require more time and more mobile phase, and the chromatogram peak would be broadened. Considering these aspects, the flow rate was selected to be 1.5 mL/min in the present study. The temperature has a significant effect on the *K* values, the retention of the stationary phase and the mutual solubility of the two phases. After testing at 15, 20, 25, 30, 35, and 40°C, the best result was obtained at the separation temperature of 20°C. The revolution speed has a great impact on the retention of the stationary phase as high rotary speed increases the retention of the stationary phase. In our experiment, the revolution speed was set at 800 rpm.

Under the optimized conditions, two fractions were obtained in one-step elution (HSCCC chromatogram is shown in Fig. 2), and the retention of the stationary phase was 60.6%. After vacuum drying, the obtained fractions produced 33.2 mg chebulagic acid and 15.8 mg chebulinic acid with purities of 95.3 and 96.1% determined by HPLC (shown in Fig. 3B and C). The recoveries of chebulagic acid and chebulinic acid were 89.3 and 88.2%, respectively (Fig. 3).

3.2 Structural identification

The identification of the obtained materials was carried out by MS and ^{1}H NMR spectra as follows.

Peak I: negative FAB-MS *m/z*: 953 $[M-H]^+$, 1045 [M + glycerol-H]$^+$. It showed the molecular weight was 954, which was in agreement with the molecular formula $C_{41}H_{30}O_{27}$.

Table 1. The partition coefficients (*K*) of chebulinic acid and chebulagic acid in different solvent systems

Solvent system		Chebulagic acid, K_1	Chebulinic acid, K_2
Ethyl acetate-water	1 : 1	2.51	–
Ethyl acetate-MeOH-water	5 : 1 : 5	7.02	0.011
Ethyl acetate-MeOH-water	8 : 1 : 5	6.23	0.112
Ethyl acetate-MeOH-water	11 : 1 : 5	5.32	0.22
Hexane-ethyl acetate-MeOH-water	1 : 5 : 1 : 5	0.02	0.23
Hexane-ethyl acetate-MeOH-water	1 : 10 : 1 : 10	0.08	0.32
Hexane-ethyl acetate-MeOH-water	1 : 20 : 1 : 20	0.65	1.20

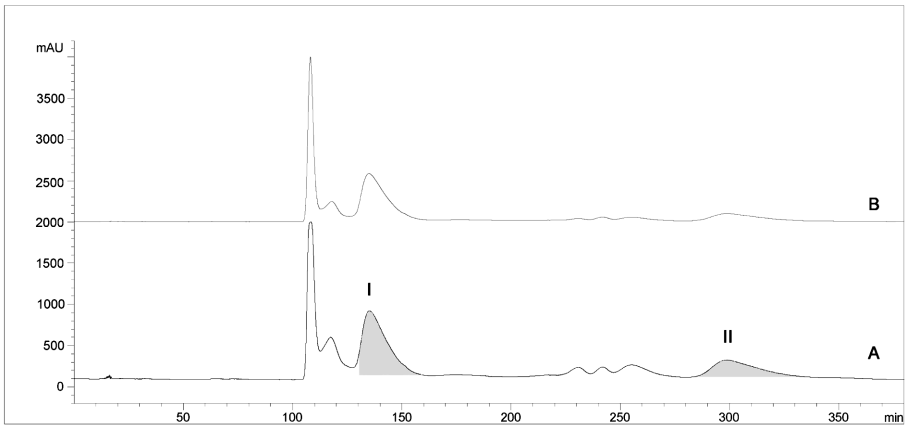

Figure 2. Chromatogram of the crude extract by preparative HSCCC. Solvent system: hexane-ethyl acetate-MeOH-water (1:20:1:20 v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.5 mL/min; revolution speed: 800 rpm; separation temperature: 20°C; sample size: 300 mg; sample loop: 6 mL; detection wavelength: (A) 216 nm, (B) 254 nm; retention of the stationary phase: 60.6%.

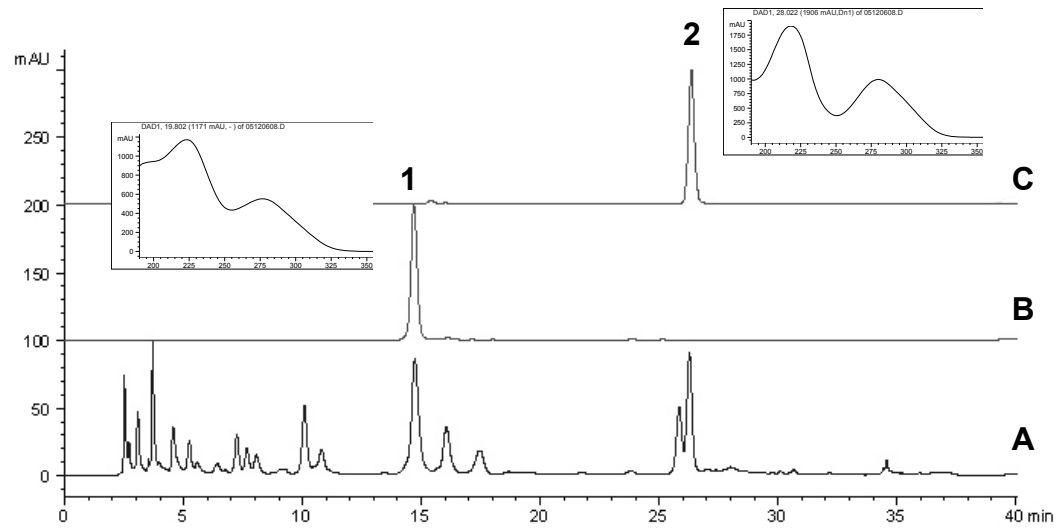

Figure 3. HPLC chromatograms of the crude extract and the fractions obtained by HSCCC. Column: RP alltima C_{18} column (250 mm × 4.6 mm id, 5 μm); mobile phase: ACN-0.1% aqueous phosphoric acid system (17:83 → 23:77 in 30 min); flow rate: 1.0 mL/min; UV wavelength: 216 nm; (A) crude extract; (B) fraction "I" obtained by HSCCC; (C) fraction "II" obtained by HSCCC; peak 1: chebulagic acid; peak 2: chebulinic acid, and their respective UV spectra.

Peak I: ^{1}H NMR (400 MHz, acetone-d_6 + D_2O): δ 7.52 (1H, s, che H-3′), 7.18 (2H, s, H-galloyl), 7.05 and 6.68 (each 1H, s, H-HHDP), 6.53 (1H, s, glc H-1), 5.92 (1H, br s, glc H-3), 5.52 (1H, br s, glc H-2), 5.25 (1H, d, *J* = 3 Hz, glc H-4), 5.10 (1H, dd, *J* = 7, 1.5 Hz, glc H-3′), 4.91 (1H, d, *J* = 7 Hz, che H-2′), 4.86 (1H, br t, *J* = 9 Hz, glc H-5), 4.75 (1H, d, *J* = 7 Hz, glc H-6), 4.44 (1H, dd, *J* = 11, 8 Hz, glc H-6), 3.84 (1H, dd, *J* = 4, 11.2 Hz, che H-4′), 2.18 (2H, m, che H_2-5′).

Peak II: negative FAB-MS *m/z*: 955 $[M-H]^+$, 1047 [M + glycerol-H]$^+$. It showed the molecular weight was 956, which was in agreement with the molecular formula $C_{41}H_{32}O_{27}$. ^{1}H NMR (400 MHz, acetone-d_6 + D_2O): δ 7.59 (1H, s, che H-3′), 7.30, 7.22, and 7.11 (each 2H, s, H-galloyls), 6.59 (1H, d, *J* = 2.4 Hz, glc H-1), 6.34 (1H, br s, glc H-3), 5.53 (1H, br s, glc H-2), 5.20 (1H, m, glc H-3′), 5.15 (1H, d, *J* = 3 Hz, glc H-4), 4.95 (1H, d, *J* = 7 Hz, che H-2′), 4.93 (1H, m, glc H-5), 4.70 (2H, m, glc H_2-6), 3.97 (1H, m, che H-4′), 2.28 (2H, m, che H_2-5′). By comparing with the published data in [9] and [16], peaks I and II were identified as chebulagic acid, and chebulinic acid, respectively.

3.3 The HSCCC system

The HSCCC series, coupled with HPLC control, permits the selection of the UV detection wavelength at the maximum absorption peak of the target compounds. In this study, the maximum UV absorption of two hydrolysable tannins was about 216 nm. The normal HSCCC system is equipped with aptotic UV detection such as 254 nm,

which presents a poor monitoring effect on the elution of chebulinic acid (fr. II) as shown in Fig. 2B. By comparison, the modified UV detection at 216 nm brings a better monitoring effect (Fig. 2A).

The isolation of these two hydrolysable tannins with high purities was successfully performed using HSCCC in one step after an injection of a simply prepared crude extract. This makes these instable tannins very easily available, avoiding any traditional repeated column chromatography. Being acid-free and time-saving, without sample loss caused by absorption, this method has considerable reproducibility. The advantages of HSCCC were fully incarnated in this case of preparative isolation of two tannins from the fruits of *T. chebula*.

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