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Short communication

The isolation of 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose from *Acer truncatum* Bunge by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) was successfully used for the isolation and purification of 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose from the ethyl acetate extract of the leaves of *Acer truncatum* Bunge using a two-phase system composed of *n*-hexane-ethyl acetate-methanol-water at a volume ratio of (0.25:5:1:5, v/v/v/v) for the first time. Each injection of 80 mg crude extract yielded 7.25 mg of pure 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose. High-performance liquid chromatography (HPLC) analyses of the CCC fraction revealed that the purity of 1,2,3,4,6-penta-*O*-galloyl-beta-D- glucose was over 95%.

Keywords: 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose; *Acer truncatum* Bunge; Counter-current chromatography

1. Introduction

The extract from the leaves of *Acer truncatum* Bunge appeared to be an efficient inhibitor to fatty acid synthase, and exhibited a considerable inhibition against the growth of four kinds of cancer cells [1], *A. truncatum* Bunge is a particular species in north–west China, which has been widely planted for the brilliant autumn colours of its leaves. It has been used as a Chinese folk medicine for the treatment of coronary artery cirrhosis, cerebrovascular diseases, angina pectoris [2] and shows high oxidation resistance [3]. Phytochemical investigations have described that the major active constituents of this plant include tannins [4], chlorogenic acid [5] and flavonoids [6–8].

Tannins are natural products, which are believed to have many physiological activities in various kinds of folk medicines and are well known as one group of the beneficial components in *A. truncatum* Bunge. Tannins represent a highly heterogeneous group of water-soluble polyphenolic compounds divided into condensed tannins and hydrolyzable tannins [9].

1,2,3,4,6-Penta-*O*-galloyl-beta-D-glucose (PGG), whose chemical structure is given in Fig. 1, shows anti-oxidant effects [10–14], anti-mutagenic activity [15], anti-cancer activities [16–18]. It has been shown to block oxidative stress and proliferation of human hepatocellular carcinoma cells, and induce cancer cell apoptosis. It not only inhibits VEGF-induced proliferation and differentiation of human endothelial cells [19], but also inhibits IL-8 gene expression by a mechanism involving its inhibition of NF-κB activation[20]. By all appearances, PGG was a potent chemopreventive agent [21]. Further more, PGG was found to be a major bioactive constituent in the crude extract of *A. truncatum* Bunge. So high-purity preparation of PGG is of great interest.

The preparative separation and purification of tannins from plant materials by conventional methods such as column chromatography and high-performance liquid chromatography

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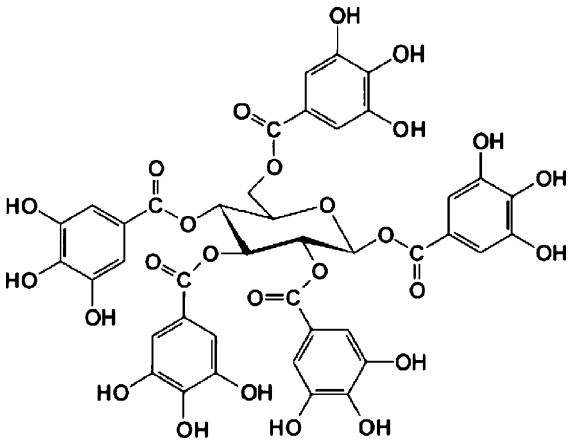

Fig. 1. The chemical structure of 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose.

(HPLC) usually causes irreversible adsorptions onto the column. High-speed counter-current chromatography (HSCCC), being a support free liquid–liquid partition chromatography, can eliminate irreversible adsorption of samples onto the solid support. HSCCC has also been successfully applied to preparative isolations of a wide variety of natural products such as alkaloids [22–24], hydroxyanthraquinones [25,26], flavonoids [27,28], saponins [29], tea catechins and food-related polyphenols [30–33].

The present paper describes the successful preparative separation and purification of PGG from the crude extract of *A. truncatum* Bunge by HSCCC.

2. Experimental

2.1. Apparatus

The preparative HSCCC instrument (Beijing Institute of New Technology Application, Beijing, China) employed in the study was a Model GS10A2 multilayer coil of 110 m × 1.6 mm i.d. with a total capacity of 300 ml. The β values of the multiplayer coil varied from 0.5 to 0.8. Although the revolution speed of the apparatus could 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 study. 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 8823A-UV Monitor (Beijing Institute of New Technology Application) at 280 nm. A manual sample injection valve with a 20 ml loop (Tianjin High New Science Technology Co., Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler, an auto injector, and a photodiode array detector (DAD), and an Agilent 1100 ChemStation software was used for the analysis of PGG in the ethyl acetate extract and fractions collected from the HSCCC separation.

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and were purchased from Beijing Chemical Factory, Beijing, China. Acetonitrile used for HPLC analysis was of chromatographic grade (Fisher), and water used was distilled water.

Leaves of *A. truncatum* Bunge were collected in BaiWang Mountain of China on November 5, 2005.

2.3. Preparation of two-phase solvent system

For the present study, we selected a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water at volume ratios of 0.25:5:1:5 (v/v/v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature. The upper phase (stationary phase) and the lower phase (mobile phase) were separated to be degassed by sonication for 30 min, shortly before use.

2.4. Preparation of crude sample and sample solutions

An amount of 200 g raw leaves of *A. truncatum* Bunge were extracted three times by 50% ethanol (1000 ml for each time) with ultrasonic treatment at room temperature and yielded 50 g of crude extract. Then, the extract was evaporated to dryness under reduced pressure. The obtained residue was dissolved with water. After filtration, the aqueous solution was extracted with water-saturated ethyl acetate three times. The incorporate ethyl acetate extract was further evaporated to dryness under reduced pressure to give the crude sample of *A. truncatum* Bunge, which was stored at room temperature before subjected to HSCCC. The sample solutions were prepared by dissolving the crude extract in the lower phase of the solvent system used for HSCCC separation.

2.5. Measurement of partition cofficient (K)

Approximately 10 mg of each sample was weighed in a 25 ml test tube into which 10 ml of each phase of the pre-equilibrated two-phase solvent system was added. The test tube was capped and shaken vigorously for 1 min, and allowed to stand until it separated completely. An aliquot of 4 ml of each layer was taken out and evaporated separately to dryness in vacuum at 35 °C. The residue was dissolved in 1 ml methanol and filtered through a 0.45 μm filter, then analyzed by LC for determining the partition coefficient (K) of PGG. The K value was expressed as the peak area of target compound in the upper phase divided by that in the lower phase.

2.6. HSCCC separation procedure

The preparative HSCCC separation was performed with a Model GS10A2 HSCCC instrument as follows. The multilayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the column at a flow rate of 2.0 ml/min while the apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium

was achieved, as indicated by a clear mobile phase eluting at the tail outlet, 130 ml of the stationary phase flowed out. Then 20 ml of crude sample solution (4 mg/ml in the lower phase) was injected into the separation tube through the sample port when the recorder was initiated simultaneously. The effluent from the tail end of the column was continuously monitored with a UV detector at 280 nm. Each peak fraction was collected according to the chromatogram. After the separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas.

2.7. HPLC analysis and identification of HSCCC peak fractions

The crude sample and each peak fraction obtained by HSCCC were analyzed by Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler, an auto injector, a photodiode array detector (DAD), and an Agilent 1100 ChemStation software. The analysis was carried out with a Diamonsil C_{18} column (4.6 mm × 250 mm, 5 μm). The binary mobile phase consisted of acetonitrile (solvent A) and water containing 2.5% acetic acid (solvent B). All solvents were filtered through a 0.45 μm filter prior to use. The system was run with a solvent A–solvent B (18:82, v/v). The flow-rate was kept constant at 1.0 ml/min for a total run time of 45 min. The sample injection volume was 10 μl and the effluent was monitored at 280 nm by a DAD.

Identification of HSCCC peak fractions were carried out by MS, ^{1}H NMR and ^{13}C NMR spectra.

3. Results and discussion

3.1. Selection of solvent system

The crude sample of *A. truncatum* Bunge was analyzed by HPLC. The result indicates that the crude sample contains several compounds including PGG and some unknown compounds (see Fig. 3A).

Selection of the two-phase solvent system is the first and a critical step in a HSCCC experiment; a good solvent system can provide an ideal partition coefficient (K) for the target compounds. In our experiment, the measured *K*-values for PGG in different solvent systems are summarized in Table 1. The three systems selected for further evaluation were *n*-butanol-*n*-propyl alcohol-water (2:1:3, v/v/v), *n*-hexane-ethyl acetate-methanol-water (1:5:1:5, v/v/v/v) and *n*-hexane-ethyl acetate-methanol-water (0.25:5:1:5, v/v/v/v). The *K* values of these three systems all fall within the range 0.5–2.0, a range acceptable for HSCCC. Stationary phase retention studies on these three systems were performed using preparative HSCCC and the results are given in Table 2. The system composed of *n*-hexane-ethyl acetate-methanol-water (0.25:5:1:5, v/v/v/v) was able to achieve two-phase equilibrium very quickly and gave the best results.

Table 1
The *K*-values of 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose (PGG) in different two-phase solvent systems used in HSCCC

Solvent system	*K*-value
n-Butanol-*n*-propyl alcohol-water (4:1:5, v/v/v)	6.55
n-Butanol-*n*-propyl alcohol-water (2:1:3, v/v/v)	1.96
Ethyl acetate-methanol-water (5:1:5, v/v/v)	8.91
n-Hexane-ethyl acetate-methanol-water (2:5:1:5, v/v/v/v)	0.08
n-Hexane-ethyl acetate-methanol-water (1:5:2:5, v/v/v/v)	0.23
n-Hexane-ethyl acetate-methanol-water (1:5:1:5, v/v/v/v)	0.55
n-Hexane-ethyl acetate-methanol-water (0.25:5:1:5, v/v/v/v)	1.87

Table 2
The retention of stationary phase of three systems in preparative HSCCC

Solvent system	Retention of stationary phase (ml)
n-Butanol-*n*-propyl alcohol-water (2:1:3, v/v/v)	70
n-Hexane-ethyl acetate-methanol-water (1:5:1:5, v/v/v/v)	95
n-Hexane-ethyl acetate-methanol-water (0.25:5:1:5, v/v/v/v)	170

Stationary phase, upper phase; mobile phase, lower phase; flow-rate, 2.0 ml/min; revolution, 800 rpm.

3.2. HSCCC fractionation of crude sample

With the solvent system composed of *n*-Hexane-ethyl acetate-methanol-water (0.25:5:1:5, v/v/v/v) we investigated the retention of stationary phase while the apparatus was run at a revolution speed of 880 rpm. The measured retention, 175 ml, approaches that at 800 rpm (170 ml). Considering the poor stability at a higher run speed and emulsification as well as the possible damage to the apparatus, the 800 rpm was selected. The retention at flow rate of 2.0 ml/min was comparable to that at 1.5 ml/min, and 2.0 ml/min flow rate was selected as the sample preparation needs longer time at the flow rate of 1.5 ml/min. The crude samples from the leaves of *A. truncatum* Bunge were sep-

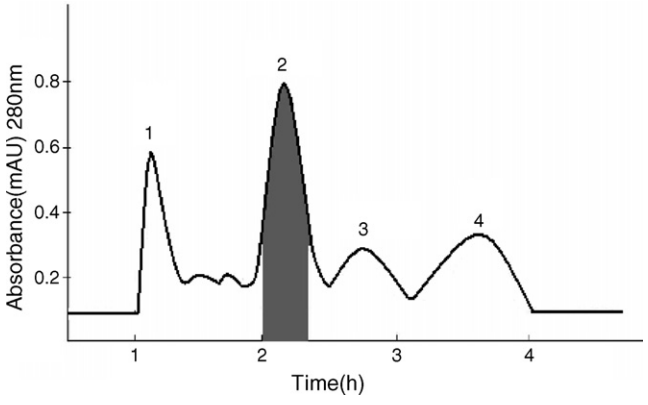

Fig. 2. Preparative HSCCC separation of crude extract from *Acer truncatum* Bunge. Solvent system, *n*-hexane-ethyl acetate-methanol-water (0.25:5:1:5, v/v/v/v); stationary phase, upper phase; mobile phase, lower phase; flow-rate, 2.0 ml/min; sample, 80 mg dissolved in 20 ml of lower phase; revolution speed, 800 rpm; retention of stationary phase, 58%; detection wavelength, 280 nm; peak 1, peak 3, unknown compound; peak 4, quercetin-3-*O*-L-rhamnoside; peak 2 (shaded portion), 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose.

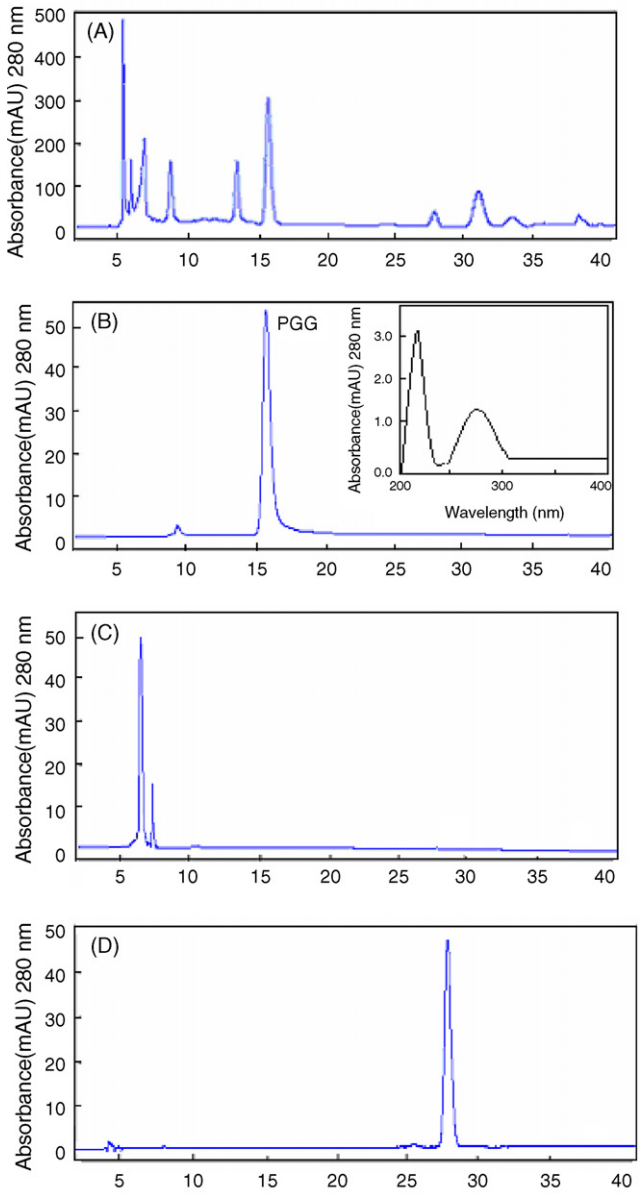

Fig. 3. HPLC analysis of crude sample and the components obtained from HSCCC separation. Experimental conditions: HPLC column, Diamonsil C18 column (4.6 mm × 250 mm, 5 μm); sample inject, 10 μl; column temperature, 25 °C; mobile phase, acetonitrile-2.5% acetic acid (18:82, v/v); flow-rate, 1.0 ml/min; detection wavelength, 280 nm; (A) original sample; (B) HSCCC fraction of peak 2 (1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose); (C) HSCCC fraction of peak 3; (D) HSCCC fraction of peak 4.

arated and purified under the optimum HSCCC conditions. The HSCCC chromatogram is shown in Fig. 2. Under this condition, every time 200 ml of mobile phase is eluted 2 ml of stationary phase flowed out, i.e. lost.

3.3. Purity analyses by HPLC

The HPLC chromatograms of these compounds are shown in Fig. 3. Four fractions were obtained and the yields of fraction 2, 3, and 4 were 7.25, 12.0, and 8.0 mg, respectively. The analysis of these fractions indicated that peak 1 was impure and the purity of peak 3 was 80% and peak 4 was 94%. It should be noted that the peak 2, which was collected according to the shaded part in Fig. 2, contained 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose at over 95% purity as measured from HPLC peak areas (Fig. 3B). The peak 3 was an unknown compound (Fig. 3C) and the peak 4 was quercetin-3-*O*-L-rhamnoside [7] (Fig. 3D). The content of the fraction 2 in the crude samples from the leaves of *A. truncatum* Bunge was 12.03% by the analysis using HPLC. Each injection of 80 mg crude extract yielded 7.25 mg of pure 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose, so the recovery of fraction 2 was 76% instead of complete recovery because the fraction 2 collected only the shaded part of peak 2 giving up the peak beginning and the end parts.

3.4. Confirmation of chemical structure

The structural identification of 1,2,3,4,6-Penta-*O*-galloyl-beta-D-glucose was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra as follows: ESI-MS *m/z*: 941.3 (M + 1), 1881.7 (2M + 1) (Fig. 4B), FAB-MS *m/z*: 939.3 (M − 1) (Fig. 4A); ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 9.3 (15H, s, galloyl-OH), 6.9 (10H, s, galloyl-H), 6.36 (1H, d J = 9.5 Hz, glu-H-1), 5.45 (1H, t, J = 9.5 Hz, glu-H-2), 5.96 (1H, t, J = 9.5 Hz, glu-H-3), 5.94 (1H, t, J = 9.5 Hz, glu-H-4), 4.57 (1H, m glu-H-5), 4.59 (1H, m glu-H-6), 4.30 (1H, dd, J = 3.12 Hz, glu-H-6). ^{13}C NMR (600 MHz, DMSO-d_6) δ ppm: 165.9–164.4 (-C(=O)-), 146.1–145.8 (galloyl-C-3′, C-5′), 140.1–139.2 (galloyl-C-4′), 119.4–117.9 (galloyl-C-1′), 109.5–109.2 (galloyl-C-2′, C-6′), 92.2 (glu-C-1), 72.6∼68.2 (glu-C), 61.9 (glu-C-6). Comparing with the reported

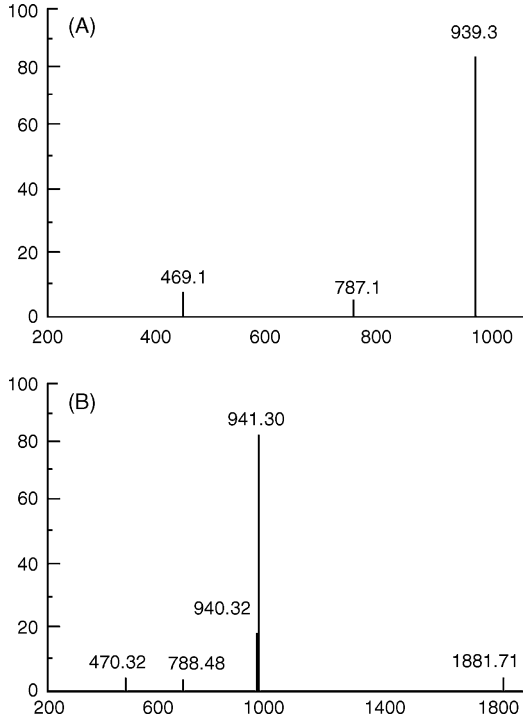

Fig. 4. (A)FAB-MS of 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose; (B) ESI-MS of 1,2,3,4,6-penta-*O*–galloyl -beta-D-glucose. MS conditions, nitrogen gas, 50 unit/min; spray voltage, 3.04.5 kV; capillary temperature, 150275 °C; positive mode.

data, the MS, ^{1}H NMR and ^{13}C NMR data are in agreement with those of 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose [34,35].

4. Conclusion

The above results of our studies clearly demonstrated that single chromatographic separation by preparative HSCCC is able to yield pure 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose from a crude plant extract (Fig. 2). But for HPLC, the crude sample has to be pre-purified before separation using preparative HPLC as we found that the RP-C_{18} column might be polluted with injection of crude sample (0.5 mg/ml × 10 μl) for five times. Compared to preparative HPLC, the method of separating PGG by HSCCC has no irreversible adsorption effects of analytes to solid-phase column material, high sample loading capacity, complete sample recovery and saves time. We believe that the method may be successfully applied to the separation of other polyphenols which have much gallate forms from crude plant extracts by selecting a suitable two-phase solvent system.

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