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Short communication

Preparative separation of punicalagin from pomegranate husk by high-speed countercurrent chromatography

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Abstract

Punicalagin, the main ingredient of pomegranate (*Punica granatum* L.) husk, is a high molecular weight polyphenolic compound. It has shown remarkable pharmacological activities attributed in the presence of dissociable −OH groups. To isolate punicalagin, previous methods included labor intensive and expensive solid phase extractions by column chromatography (C-18, polyamides, dellulose, Sephadex Lipophilic LH-20, Diaion HP20). High-speed countercurrent chromatography (HSCCC) was used for isolation and purification of punicalagin from pomegranate husk. Using preparative HSCCC about a 350 mg amount of the crude extract was separated, yielding 105 mg of punicalagin at a high-purity of over 92%. Eighty milligrams of gallic acid was simultaneously separated as another product, at a purity of 75%.

Keywords: Countercurrent chromatography; Punicalagin; Pomegranate husk

1. Introduction

Pomegranate (*Punica granatum* L.) husk, a traditional Chinese herbal medicine, is very rich ellagic acid derivatives, such as the ellagitannins (ETs) punicalagin [2,3-(*S*)-hexahydroxydiphenoyl-4,6-(*S*, *S*)-gallagyl-D-glucose], as well as less amounts of punicalin [4,6-(*S*, *S*)-gallagylglucose], gallic acid (GA), ellagic acid (EA) and EA-glycosides (hexoside, pentoside, rhamnoside, etc.) [1–4] (Fig. 1). These ETs are extracted in significant levels into the juice from the husk during industrial hydrostatic processing methods.

Commercial pomegranate juice (PJ) has one of the highest antioxidant activities compared to other fruit juices, red wine and green tea [1]. This can be attributed to its high content of polyphenols including ellagic acid in its free and bound forms, gallotannins and anthocyanins, and other flavonoids [5]. The most abundant of these polyphenols is punicalagin. Since pomegranate-derived foods are widely consumed and there have been conflicting reports regarding the toxicity of punicalagin [6–8], there is a need for in depth in vitro and in vivo studies to determine the biological properties of this compound.

Most studies investigate the physiological effects of pomegranate extracts in vitro and in animals. At present, clinical studies of pomegranate have utilized commercial PJ [9]. Commercial PJ can be obtained by pressing whole pomegranate fruits and its peels, and then the major ingredient punicalagin is extracted into PJ. However, punicalagin levels are widely variable in PJ and range from 0.017 to 2 g/l of PJ depending on the fruit cultivar as well as processing and storage conditions. Therefore, there may be a need to explore standard methods to determine the biological properties of this compound by using high-purity punicalagin.

Unfortunately, present purification methods are always used for preparation of total pomegranate tannins (TPT). Each ingredient of ETs, especially punicalagin isomers cannot be separated and purified, respectively. The successful preparative separation of punicalagin from crude ethanol extract of pomegranate husk by high-speed countercurrent chromatography is described in this paper. Gallic acid was separated as another product simultaneously.

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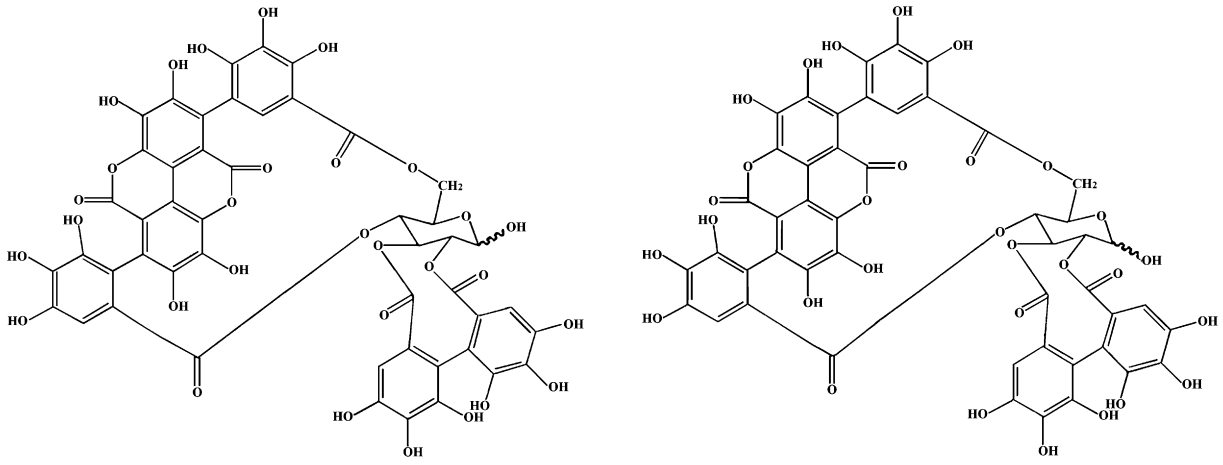
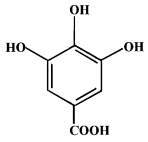


Two isomers of punicalagin (m/z 1083)



Gallic acid (m/z 170)

Fig. 1. Molecular structures of punicalagin and gallic acid.

2. Experimental

2.1. Apparatus

The preparative high-speed countercurrent instrument was used with a Model GS10 multilayer coil planet centrifuge (revolution radius is 8 cm and beta values of the coil is 0.75) equipped with a PTFE multilayer coil of 110 m × 1.6 mm I.D. with a total capacity of 250 ml, which is designed and constructed in Beijing Institute of New Technology Application (Beijing, China).

The HPLC used was a Shimadzu LC-20AVP system with two LC-20AT solvent delivery units, an SPD-20A UV/VIS detector, a CTO-10ASVP column oven (Shimadzu, Kyoto, Japan), a T2000P workstation (Beijing, China) and a reversed phase C18 column (250 mm × 4.6 mm, 5 μm, Diamodsil^{TM}).

The UPLC-ES/MS used was a Waters Ultra Performance LC with Waters Quattro Premier XE and a reversed phase C_{18} column (2.1 mm × 50 mm, 1.7 μm, Acquity UPLC^{TM}).

The mass spectrometer was av600 (Druker, Germany).

2.2. Reagents

All organic solvents used for sample preparation and HSCCC were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Trifluoracetic acid (TFA) used for preparative liquid chromatography was of chromatography grade and purchased from Merck Co. (Hohenbrunn, Germany). Formate used for UPLC-ES/MS was of chromatography grade and purchased from Fisher Scientific Co. (Tustin, CA). Methanol used for HPLC and UPLC-ES/MS analysis was of chromatography grade and purchased from Dima Technology Inc. (USA). Pomegranate husk was purchased from Lintong district in Shanxi Province.

2.3. Preparation of sample

Ten grams of dried pomegranate husk (Lintong, Shanxi, China) was grounded, then 5 g amount of this dried powder was extracted (supersonic) with 60 ml of 40% ethanol for 30 min and the insoluble residue was extracted again under the same condition. Hundred millilitres of liquid was extracted with 100 ml ether and then the lower phase was extracted with 100 ml ethyl acetate. The lower phase was freeze-dried, yielding 1.5 g crude sample in which punicalagin purity is determined by HPLC (Fig. 2).

2.4. Preparation of two-phase solvent and sample solution

The solvent system utilized in the present study was prepared by mixing butyl alcohol–TFA–water (100:1:100, v/v), and thoroughly equilibrating the mixture in a separatory funnel at room temperature, two phase being separated shortly before use.

The sample solution for HSCCC was prepared by dissolving the crude extract in both the upper and lower phase at suitable concentration according to the preparative purpose. The sample solution for HPLC was prepared by dissolving the dried peak fraction containing punicalagin obtained from HSCCC with water at suitable concentration.

2.5. Separation procedure

HSCCC separation was performed as follows: the multiplayer coiled column was first entirely filled with the lower phase. The upper phase was then pumped into the head end

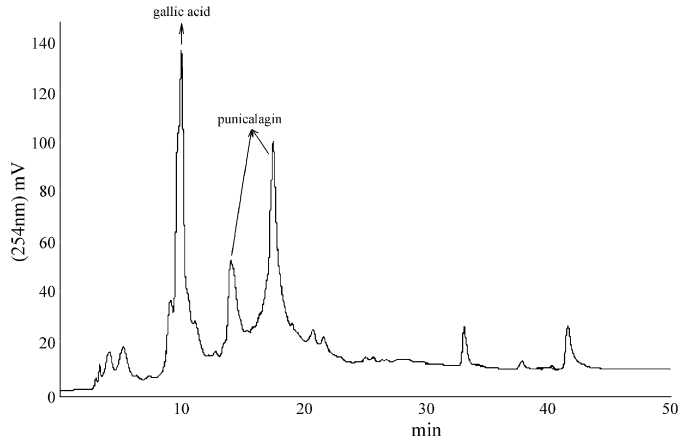


Fig. 2. HPLC analyses of the crude extract from pomegranate husk of punicalagin and gallic acid. HPLC conditions: reversed phase C_{18} column (250 mm × 4.6 mm, 5 μm, Diamodsil™), column temperature: 30 °C. Mobile phase: MeOH (eluent A) and 0.1% (v/v) TFA in water (eluent B). Gradient conditions: A increased from 10 to 70% over a 50 min time period. This was followed by a 10 min re-equilibration flow rate: 1.0 ml/min, monitored at 254 nm.

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 established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram. The retention of the stationary phase was computed from the volume of the stationary phase collected from the column after the separation was completed.

2.6. HPLC analyses of punicalagin and gallic acid

HPLC condition of the punicalagin and gallic acid is as follows: MeOH (eluent A) and 0.1% (v/v) TFA in water (eluent B). Gradient conditions: A increased from 10 to 70% over a 50 min time period. This was followed by a 10 min re-equilibration. Flow rate was 1.0 ml/min, and 2 μl portions were injected into the column. Both punicalagin and gallic acid were detected by absorbance at 254 nm.

2.7. UPLC-ES/MS analyses

Conditions for detection of punicalagin were as follows: solvent A 0.2% HCOOH in water, B methanol; gradient % A in B: initial 80%, 3 min 50%, 8 min 5%, run time 10 min; flow rate: 0.3 ml/min; injection volume 2 μl; MS parameters: ionization mode, electron spray (ES) negative mode; scan range: 200–1200 amu; scan rate: 1 scan/s; cone voltage: 30 V, source temperature: 110 °C. Peak identities were obtained by matching their molecular ions (M–H^+) obtained by ES/MS and tandem MS with the expected theoretical molecular weights from literature data as punicalagin (m/z 1083).

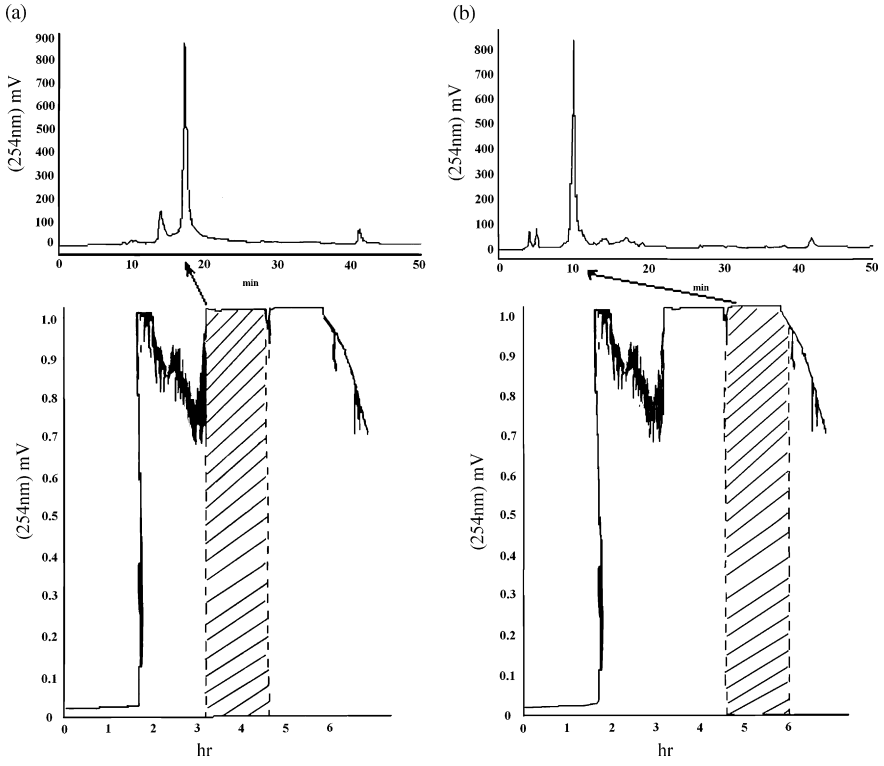


Fig. 3. Chromatogram of punicalagin by high-speed countercurrent chromatography (HSCCC). Solvent system: butyl alcohol–TFA–water (100:1:100); stationary phase: lower aqueous phase; mobile phase: upper organic phase; retention of the stationary' phase: 20.8%; flow rate: 2.0 ml/min; revolution speed: 800 rpm, sample: 350 mg dissolved in 10 ml of upper phase and 10 ml lower phase. HPLC conditions: reversed phase C_{18} column (250 mm × 4.6 mm, 5 μm, Diamodsil™), column temperature: 30 °C. Mobile phase: MeOH (eluent A) and 0.1% (v/v) TFA in water (eluent B). Gradient conditions: A increased from 10 to 70% over a 50 min time period. This was followed by a 10 min re-equilibration. Flow rate: 1.0 ml/min, monitored at 254 nm.

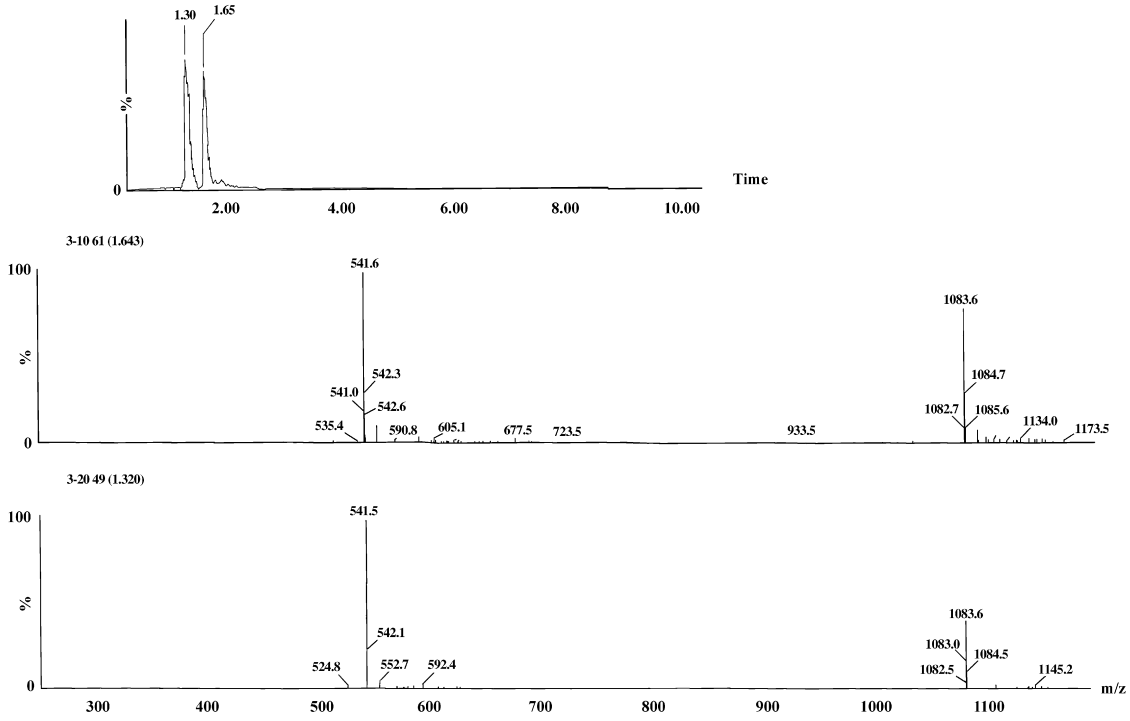


Fig. 4. UPLC-ES/MS spectra of punicalagin anomers, (*M*–H) *m/z* 1083; Conditions for detection of punicalagin: solvent A 0.2% HCOOH in water, B methanol; gradient % A in D: initial 80%, 3 min 50%, 8 min 5%, run time 10 min, flow rate: 0.3 ml/min, injection volume 2 μl; MS parameters: ionization mode, electron spray (ES) negative mode; scan range: 200–1200 amu; scan rate: 1 scan/s; cone voltage: 30 V, source temperature: 110 °C.

3. Results and discussion

The HPLC analyses of the crude extract of pomegranate husk indicated that it contained several compounds as shown in Fig. 2. Punicalagin purity of crude extract is 41% and gallic acid purity of crude extract is 32% based on the ratio of peak area as determined by HPLC.

In order to achieve an efficient resolution of target compound, distribution coefficients of punicalagin in six kinds of two-phase solvent systems were examined by HPLC (Table 1). The solvent system of butyl alcohol–acetone–water (7:2:11) was most suitable. However, the retention of the stationary phase of that system was very low. So, butyl alcohol–TFA–water (100:1:100, v/v) was chosen for punicalagin separation. The retention of the stationary phase was 20.8%.

Fig. 3a and b shows the results obtained from 350 mg of the crude extract of pomegranate husk by preparative HSCCC. The shaded part of the peak was cut and concentrated. This separation yielded 105 mg of punicalagin at 92% purity and 80 mg gallic acid at 75% purity based on HPLC analysis.

Compound identities of punicalagin isomers were confirmed by UPLC-ES/MS analyses (Fig. 4) where two ions at *M*–H *m/z* 1083 accounted for the α- and β-isomers of punicalagin as previously reported [10]. ^{1}H NMR and ^{13}C NMR (600 MHz, acetone d_6) spectra of the punicalagin were similar to those in Ref. [7].

Compound identity of gallic acid was confirmed by HPLC of gallic acid as previously reported [11].

Table 1
The *K* values of punicalagin anomers in different two-phase systems

Two-phase system	K_1	K_2
Butyl alcohol:TFA:water = 100:1:100	0.29	0.32
Butyl alcohol:TFA:water = 50:1:50	0.18	0.2
Butyl alcohol:isopropanol:water = 2:1:3	0.32	0.36
Butyl alcohol:acetone:water = 7:2:11	0.6	0.5
ethyl acetate:MeOH:water = 10:1:10	0.15	0.04
Butyl alcohol:ethyl acetate:water = 4:1:5	0.23	0.28

$K = C_U/C_L$ = Peak area of punicalagin in upper phase/peak area of punicalagin in lower phase. 1—α-isomer. 2—β-isomer.

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