Journal of Chromatography B, 990 (2015) 111–117

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short Communication

Preparative isolation and purification of urolithins from the intestinal metabolites of pomegranate ellagitannins by high-speed counter-current chromatography

Wenhua Zhao[a], Yuji Wang[a], Weijia Hao[a], Hua Yang[a], Xueying Song[a], Ming Zhao[a,b,*], Shiqi Peng[a,*]

[a] Beijing area major laboratory of peptide and small molecular drugs; Engineering Research Center of Endogenous Prophylactic of Ministry of Education of China; Beijing Laboratory of Biomedical Materials; College of Pharmaceutical Sciences, of Capital Medical University, Beijing 100069, China
[b] Faculty of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan

ARTICLE INFO

Article history:
Received 11 December 2014
Accepted 27 March 2015
Available online 3 April 2015

Keywords:
Pomegranate husk
Urolithins
High-speed counter-current chromatography (HSCCC)
Intestinal metabolites
Anti-oxidant

ABSTRACT

Urolithins were separated from the intestinal metabolites of pomegranate ellagitannins by high-speed counter current chromatography in two steps using two solvent systems composed of *n*-hexane-ethyl acetate-methanol-acetic acid-water (2.5:2:0.25:5, v/v/v/v/v) and *n*-hexane-ethyl acetate-methanol-acetic acid-water (2.5:0. 8:0.25:5, v/v/v/v/v) for the first time. Each injection of 100 mg extract yielded 21 mg of pure urolithin A and 10 mg of pure urolithin B. High-performance liquid chromatography analyses revealed that the purity of urolithin A and urolihtin B was over 98.5%. The structures of urolithin A and urolitihn B were identified by high resolution-MS, NMR and single crystal x-ray analysis. Urolithins reduced the oxidative stress status in colon cancer by decreasing the intracellular ROS and malondialdehyde levels, and increasing SOD activity in H_2O_2 treated Caco-2 cells.

1. Introduction

Pomegranate (*Punica granatum L.*) husk possesses anti-carcinogenic [1], anti-inflammatory [2], antimicrobial [3], anti-diabetic [4], antioxidant [5] and anti-plasmodial activities [6]. These pharmacology actions were attributed to the ellagitannins [7,8]. Of note, it was reported that pomegranate ellagitannins absorption is very few, and the metabolites analysis is well established that pomegranate ellagitannins are mainly metabolized to urolithins in the colon. The major metabolites in the gut are urolithin A and urolithin B by sequential removal of hydroxyls [9]. It was reported that urolithins can be absorbed, and, afterwards, reach different tissues in the body and offer anti-oxidation [10,11], anti-inflammatory [12], anti-carcinogenic [13] and anti-microbial actions[14,15] *in vitro*. For now, the biological activity *in vivo* becomes the focus of research. So, to providing abundant urolithins for animal experiment is of great importance. However, the commercial products are too expensive, and more notably, the synthetic urolithins could contain trace amount of metals [16], the copper may present in the final formulation, and as a consequence, dramatically influence the results. So, the preparation of high-purity and abundant urolithins from the intestinal extracts of pomegranate ellagitannins is of great interest.

High-speed counter-current chromatography (HSCCC) has been successfully used in separation and purification of bioactive compounds from natural products such as, food-related polyphenols [17,18], flavonoids [19,20], cyanidin 3-glucoside [21], phthalide [22], tea catechins [23,24], oils [25] and fatty acids[26]. Being a continuous liquid–liquid partition chromatography and the excellent sample recovery, HSCCC provides an optimal choice for the preparation of the bioactive compounds from the physiological metabolites such as intestinal metabolites.

The present study described the successful preparative separation and purification of urolithins from the intestinal extracts of pomegranate ellagitannins by HSCCC. And the antioxidant activity of urolithins in Caco-2 cells was also detected.

* Corresponding author. Tel./fax: +86 10 8391 1528.
E-mail addresses: mingzhao@bjmu.edu.cn (M. Zhao), sqpeng@bjmu.edu.cn (S. Peng).

http://dx.doi.org/10.1016/j.jchromb.2015.03.024
1570-0232/

2. Experimental

2.1. Apparatus

The preparative HSCCC instrument employed in the study was TBE-300 high-speed counter-current chromatograph (Tauto Bio technique, Shanghai, China) with multilayer coil and separation columns connected in series (i.d. of the tubing = 1.5 mm), and a total volume of 300 mL. The β values varied from 0.5 to 0.8. The speed of the apparatus was 800 rpm. The solvent was pumped into the column with a Tauto TBP-5A constant-flow pump (Tauto Biotech, Shanghai, China). Continuous monitoring of the effluent was achieved with a Waters 2487 (USA) Dual λ Absorbance Detector (Waters, USA). A manual sample injection valve with a 20 mL loop (Tianjin High New Science Technology Co, China) was used to introduce the sample into the column.

An Agilent Technology 1200 Series HPLC system (Agilent, USA) equipped with an auto injector, a quaternary pump, a degasser, a thermostatic auto-sampler, and a photodiode array detector (DAD), and an Agilent 1200 Chem Station software was used for the analysis of each compound in the intestinal extracts of pomegranate ellagitannins and fractions collected from the HSCCC separation. X-ray diffraction (Gemini A ULTRA Single Crystal Diffractometer, USA) was analyzed using the Bruker AXS D8 Advance, X-ray diffractometer with Cu Kα— targets at a scanning rate of 0.010 2θ/s, applying 40 kV, 40 mA.

2.2. Reagents

All solvents used for HSCCC were of analytical grade and were purchased from Beijing Chemical Factory, China. Acetonitrile used for HPLC analysis was of chromatographic grade (Fisher). The dried pomegranate husk was purchased from Tongrentang drugstore, Beijing, China.

2.3. Preparation of two-phase solvent system

The two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-acetic acid-water at volume ratios of 2.5:2:0.25:5(v/v/v/v) and 2.5:0. 8:0.25:5 (v/v/v/v/v) were used for HSCCC separation. The upper phase and the lower phase were separated to be degassed by sonication for 30 min before use.

2.4. Preparation of intestinal metabolites of pomegranate ellagitannins

2.4.1. Preparation of pomegranate ellagitannins extracts

An amount of 200 g raw husk of pomegranate was extracted three times by 50% ethanol (1000 mL for each time) with ultrasonic treatment at room temperature and yielded 63 g of crude extract. Then, the extract was evaporated to dryness under reduced pressure. The obtained residue was dissolved in water. After filtration, the aqueous solution was extracted with water-saturated ethyl acetate for three times. The incorporate ethyl acetate extract (12 g) was further evaporated to dryness under reduced pressure to give the crude sample of pomegranate ellagitannins extract for further use.

2.4.2. Preparation of intestinal bacteria culture solution

The anaerobic medium broth for intestinal bacteria culture was prepared as follows: The anaerobic medium mixed with fresh feces of rats were cultured under an anaerobic condition at 37 °C for 30 min, and then the intestinal bacteria culture solution was prepared for further use.

2.4.3. Preparation of intestinal bacteria culture solution of pomegranate

Pomegranate husk extract (5 g) was weighed and dissolved in 50% methanol (50 mL). Pomegranate husk extract (10 mL) was added into the intestinal bacteria solution (100 mL), and then incubated at 37 °C for 72 h. After the addition of methanol (100 mL) and 2% formic acid, the incubation samples were centrifuged at 10,000 × g for 15 min. The supernatant was dried under nitrogen flow before subjected to HSCCC. The sample solutions were prepared by dissolving the intestinal metabolites extract in the lower phase of the solvent system used for HSCCC separation.

2.5. Measurement of partition coefficient (K)

Successful separation by HSCCC largely depends on the selection of suitable two-phase solvent system. Approximately 10 mg of each sample was weighed in a test tube into which 10 mL of each phase of the pre-equilibrated two-phase solvent system was added. The test tube was shaken for 1 min and placed stand until it separated completely. The 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 methanol and filtered through a 0.45 μm filter. The *K* value was calculated as the peak area of target compound in the upper phase divided by that in the lower phase by LC.

2.6. HSCCC separation procedure

The preparative HSCCC separation was performed as follows: The separation multilayer coiled column was first entirely filled with the upper organic phase (stationary phase). Then the apparatus was rotated at a revolution speed of 800 rpm, while the lower phase (mobile phase) was pumped into the head end of the column at a flow rate of 2.0 mL/min. After hydrodynamic equilibrium was achieved, as indicated by the clear mobile phase front emerged, the sample was injected into the separation column through the injection valve. The effluent from the outlet of the column was continuously monitored with the UV detector at 230 nm. Each peak fraction was manually collected according to the chromatogram and evaporated under reduced pressure.

2.7. HPLC analysis and structural assign of the fractions

The HPLC analysis of each peak fraction obtained by HSCCC were performed by Agilent Technology 1200 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 1200 Chem Station software. The analysis was carried out with a Waters Symmetry$^{R}C_{18}$ column (4.6mm × 250 mm, 5 μm) at room temperature. The binary mobile phase consisted of acetonitrile (solvent A) and water containing 0.4% 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 (0 min,5:95,v/v; 10 min, 8:92,v/v; 15 min, 15:85,v/v; 30 min, 20:80,v/v; 70 min, 50:50,v/v; 71 min, 95:5,v/v; 80 min, 95:5,v/v; 81 min, 5:95,v/v; stop time: 120 min) and the effluent was monitored at 230 nm.

Identification of HSCCC peak fractions was carried out by high resolution MS, ^{1}HNMR, ^{13}CNMR and X-ray spectra.

2.8. Measurement of intracellular levels of ROS, MDA and SOD

The antioxidant activity was evaluated by measuring the intracellular ROS, SOD and MDA level in Caco-2 cells.

ROS content was determined by the DCFH-DA method. Briefly, Caco-2 cells were seeded onto a 96-well culture plate. After