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Activity guided fractionation of pomegranate extract and its antioxidant, antidiabetic and antineurodegenerative properties

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ABSTRACT

Pomegranate (*Punica granatum* L.) peel is a rich source of polyphenols, especially tannins, and concentrations of these compounds may be influenced by extraction processes. This study was aimed to examine phenolic composition and biological activities of pomegranate peel 70% ethanolic extract and its fractions (petroleum ether, ethyl acetate, butanol and water) obtained by liquid/liquid extractions. Fractions were analyzed for polyphenolic content and the highest total phenolic content was obtained in ethyl acetate fraction (200.9 mg gallic acid equivalent/g). This fraction had the highest amounts of gallic acid and ellagic acid, while water fraction contained the highest contents of punicalin and punicalagin. Antioxidant activity was evaluated by four different methods, and butanol and ethyl acetate fractions were the most active in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assays, whereas water fraction showed the strongest activity in ferric reducing antioxidant power (FRAP) and β-carotene tests. All fractions showed better inhibitory effect on α-glucosidase, with inhibition concentration IC_{50} = 0.26–4.57 μg/mL, than α-amylase (IC_{50} = 23.6–284.3 μg/mL), and the most active was ethyl acetate fraction. Antineurodegenerative activity was tested using acetylcholinesterase (AChE) and tyrosinase (TYR) inhibitory activity assays, and the most effective inhibitor of both enzymes was 70% ethanolic extract. Pomegranate peel could be considered as a valuable agro-industrial waste product which could be used as low-cost natural source of biologically active compounds.

1. Introduction

Punica granatum L., commonly known as pomegranate, is significant plant in food industry but is also considered as an ancient, medicinal as well as mystical plant. It is used in traditional systems such as Chinese, Ayurvedic, Unani, and Egyptian medicine and all parts of the tree, i.e. fruit, seed, peel, stem barks, leaves or roots have particular application (Akhtar et al., 2015). Today it is cultivated over the Mediterranean region, India and drier areas of Southeast Asia, America and tropical Africa (Jurenka, 2008). Recent studies have shown that the extracts prepared from different parts of the pomegranate plant possess diverse biological activities, among them antimicrobial, antiviral, antioxidant, cancer preventive, antiinflammatory, cardioprotective, and antidiabetic effects (Akhtar et al., 2015; Baliga et al., 2013; Fawole et al., 2012; Ismail et al., 2012). Therapeutic potential of pomegranate is associated with its diverse phytochemicals such as tannins, ellagic acid, gallic acid, catechins, flavonoids, anthocyanins (Akhtar et al., 2015; BenSaad et al., 2017; Sood and Gupta, 2015).

Pomegranate fruit is widely consumed as fresh or is processed within the various products such as juices, jams or jellies. The juice processing generates waste in the form of peel and seeds. However, in food industry by-products are nowadays considered as a source of nutritionally valuable components (Galanakis, 2012). Several studies have shown that pomegranate peel contained higher amounts of biologically active compounds in comparison with the edible pulp (Akhtar et al., 2015; Salgado et al., 2012; Masci et al., 2016). Additionally, the safety of pomegranate and some of its constituents have been studied experimentally and also clinically, and no evidence for toxic effects on body organs such as heart, liver, or kidney was observed (Cerda et al., 2003; Aviram et al., 2004).

In recent years, utilization of agricultural and food processing by-products has been received much attention. Among plant originated wastes, fruits and vegetables by-products are the most widely investigated (Galanakis, 2012). The main focus of researches is the recovery of valuable compounds and their implementation as a functional food. Recovery of target compounds is conducted in five stages which

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include macroscopic pre-treatment, separation of macro- and micromolecules, extraction, purification, and product formation (Galanakis, 2013). For these purposes, conventional processing technologies such as wet milling, thermal drying, alcohol precipitation, solid-liquid extraction, adsorption, and spray or freeze drying are the most common and widely used methods. Over the last years, some new technologies such as cold plasma treatment, high hydrostatic pressure, pulsed electric fields, nanotechnology, are investigated for their possible application in food industry in order to overcome some disadvantages of conventional techniques. Ultrasound waves have been successfully applied for the extraction of bioactive compounds, especially polyphenols and carotenoids, from plant raw material or by-products (Roselló-Soto et al., 2015).

Nevertheless, despite to the several problems that are raised in utilization of conventional technologies, they are still widely accepted since they are considered as safe and cheap. Polyphenols from pomegranate peel may be effectively recovered by classical solid-liquid extraction method using aqueous-alcoholic mixture (Masci et al., 2016; Çam and Hişil, 2010). However, due to diverse phytochemical profile, other compounds such as sugars, dietary fibers or organic acids could be also co-extracted, therefore additional fractionation of hydro-alcoholic extract with other solvents is necessary. The aim of this work was to study the phenolic composition and antioxidant, antidiabetic and antineurodegenerative properties of 70% ethanol extract and its fractions obtained from peel of wild growing pomegranate.

2. Material and methods

2.1. Chemicals

Folin-Ciocalteu reagent, punicalagin standard, 2(3)-t-butyl-4-hydroxyanisole (BHA), 3.5-di-tert-butyl-4 hydroxytoluene (BHT), 2.2-dyphenyl-1-picrylhydrazyl (DPPH), 2.2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), 2.4.6-tripyridyl-*s*-triazine (TPTZ), α-glucosidase from baker's yeast (type I), α-amylase from porcine pancreas, 5.5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylcholinesterase from *Electrophorus electricus* (AChE), acetylcholine iodide, galanthamine hydrobromide from *Lycoris* sp., kojic acid, tyrosinase from mushroom, and 3.4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from Sigma-Aldrich (Steinheim, Germany). Tween 40 and linoleic acid were purchased from Acros Organics (Belgium). Acetonitrile (Merck, Germany) was of HPLC grade and ultra pure water was prepared using a Milli-Q purification system (Millipore, France). Punicalin standard was obtained from Phytolab (Germany), while ellagic and gallic acid were from Extrasynthese (Cedex, France). All other chemicals were of analytical grade and used without further purification.

2.2. Plant material

Fruits of wild growing pomegranate were collected in a full ripening stage at natural locality (village Do, Bosnia and Herzegovina) during November 2015. Peels were separated manually from the seeds, cut into small pieces, air-dried for 4–6 days at room temperature and then grounded using a laboratory mill to obtain pomegranate peel (PP) powder.

2.3. Preparation of fractions

PP powder (200 g) was extracted with 70% ethanol. Extraction (1:10, w/v) was performed by maceration during 24 h at room temperature with continuous stirring at 100 rpm. The extract was concentrated in vacuum (Buchi rotavapor R-114) to yield reddish brown residue (50.5 g) which was suspended in 100 mL water. Successive extraction with petroleum ether (6 × 50 mL), ethyl acetate (3 × 100 mL), and *n*-butanol (3 × 50 mL) afforded fractions which were filtered and evaporated under reduced pressure to get 0.8 g of petroleum ether, 4.3 g of ethyl acetate, 8.7 g of *n*-butanol, and 36.3 g of water fractions. All samples were stored at +4° C for further experiments.

2.4. Determination of total phenolic content

The total phenolic content of extracts was measured using spectrophotometric method (Singleton and Rossi, 1965). The reaction mixture was prepared by mixing 100 µL of methanolic solution of sample (100, 200 and 500 µg/mL) and 500 µL of 10% Folin-Ciocalteu reagent, and after six minutes 400 µL of 7.5% Na_2CO_3 was added. Distilled water was used as blank, while control was prepared to contain distilled water instead of sample. Absorbance was recorded at 740 nm after two hours incubation at room temperature. The results were expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g). All experiments were repeated at three times.

2.5. HPLC analysis

Analyses were carried out on Agilent 1200 RR HPLC instrument (Agilent, Waldbronn, Germany), equiped with DAD detector, using reverse phase Zorbax SB-C18 (Agilent) analytical column (150 mm × 4.6 mm i.d., 5 µm particle size), and the column temperature was maintained at 25 °C. The mobile phase A was 1% v/v solution of orthophosphoric acid in water, while mobile phase B was acetonitrile. Gradient elution was applied according to the following scheme: 0–5 min, 98–90% A; 5–15 min, 90% A; 15–20 min, 90–85% A; 20–25 min, 85–70% A; 25–30 min, 70–40% A; 30–34 min, 40–0% A, with post-time of 2 min and the flow rate of 1 mL/min. The injection volume was 3 µL. Detection wavelengths were set at 260 and 320 nm. The amounts of the individual compounds were calculated using calibration curves of ellagic and gallic acids, punicalagin and punicalin. The results are presented as milligrams per gram of dry extract (mg/g).

2.6. Evaluation of antioxidant activity

2.6.1. DPPH assay

The scavenging activity of extracts was evaluated using 2.2-dyphenyl-1-picrylhydrazyl (DPPH) assay (Blois, 1958) with slight modifications. 100 µL of sample solutions in methanol (concentrations of 100, 200 and 500 µg/mL) and 900 µL of methanolic solution of DPPH (40 µg/mL) were mixed. BHA, BHT and ascorbic acid in concentrations of 50 and 100 µg/mL were used as positive controls (standards). Methanol was used as a blank, while control was prepared to contain methanol instead of sample/standard. Each blank, samples and standards' absorbance were measured in triplicate. Absorbance of the reaction mixture was measured after 30 min in the dark at room temperature at 517 nm. The decrease of absorption of DPPH radical at 517 nm was calculated using equation:

$$\text{Inhibition of DPPH radical (\%)} = [(A_C - A_S)/A_C] \times 100 \quad (1)$$

where A_c is the absorbance of control (without test sample) and A_s is the absorbance of the test samples at different concentrations.

2.6.2. ABTS assay

The scavenging activity of extracts was evaluated using ABTS assay using procedure of Miller et al. (1993) with some modifications. Stock $ABTS^+$ solution (7 mM) was prepared 12–16 h before experiment in 2.46 mM potassium-persulfate and stored in the dark at room temperature, and then diluted by distilled water to obtain an absorbance of working solution 0.700 ± 0.020 at 734 nm. Sample solutions in methanol (25 µL) in concentrations of 100, 200 and 500 µg/mL were mixed with 1 mL of working $ABTS^+$ solution and incubated for 30 min at 30 °C. The same procedure was applied for positive controls BHA,