Food and Chemical Toxicology 55 (2013) 470–475

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (*Punica granatum*) leaves

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ARTICLE INFO

Article history:
Received 6 December 2012
Accepted 24 January 2013
Available online 1 February 2013

Keywords:
Punica granatum
Anti-inflammatory
Antioxidant
Cytotoxic
Cholinesterase
Extraction

ABSTRACT

This study evaluated antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of extracts with different polarities (hexane, dichloromethane, ethyl acetate, ethanol and methanol) obtained from *Punica granatum* leaves. Total phenolics (8.8–127.3 mg gallic acid equivalent/g dry weight), flavonoids (1.2–76.9 mg quercetin equivalent/g dry weight), tannins (63.7–260.8 mg catechin equivalent/kg dry weight) and anthocyanins (0.41–3.73 mg cyanidin-3-glucoside equivalent/g dry weight) of different extracts were evaluated. The methanolic extract presented a good IC50 by DPPH and ABTS assays (5.62 and 1.31 mg/l respectively). The strongest 5-lipoxygenase (5-LOX), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition activities were obtained for the ethanol extract (IC50 values of 6.20, 14.83 and 2.65 mg/l, respectively) and the best cytotoxic activity against MCF-7 cells was obtained for the methanol extract (IC50 = 31 mg/l). These important biological activities showed that *P. granatum* leaves could be a potential source of the active molecules intended for applications in pharmaceutical industry, but only after additional *in vivo* experiments.

1. Introduction

Oxidative stress is defined as the imbalance between pro-oxidants and antioxidants, in favour of the former. Numerous studies have shown that the oxidative stress contributes significantly to the development and the progression of wide variety of diseases such as diabetes, cancer and neurodegenerative, cardiovascular, and pulmonary diseases (Toullec et al., 2010). Christen (2000) reported that free radicals are likely an important factor in the pathogenesis of Alzheimer disease (AD). The brains of AD patients present several signs of free radical attacks such lipid peroxidation, protein oxidation, damage to mitochondrial and nuclear DNA. Also, the crucial role of superoxide production in the pathogenesis of diabetes was pointed out (Brownlee, 2001). New clinical and epidemiological data consider the oxidative stress as an important determinant in the development of chronic inflammation and cancer through activation of variety of transcriptional factors (Reuter et al., 2010; Toullec et al., 2010). Epidemiological studies reported also that antioxidants play a key role in protecting against oxidative damage. Thus, antioxidants presents in fruit and vegetable were well recommended to reduce cardiovascular and neurodegenerative risks (McCall et al., 2011). These positive health effects were attributed to a variety of compounds such as phenolics and terpenoids. In recent years, considerable attention has been directed to study these active compounds from medicinal plants for various pharmacological purposes (Dorman et al., 2004).

The different parts of pomegranate (*Punica granatum* L.) have been known as a reservoir of bioactive compounds with potential biological activities. Pomegranate, especially the leaves of pomegranate, decreased the dyslipidemia of obesity and cardiovascular risk factors (Lei et al., 2007). Anti-parasitic, anti-microbial and antioxidant activities of pomegranate leaves extracts were reported (Egharevba et al., 2010; El-Shennawy et al., 2010 and Wang et al., 2013). Several papers were reported on the ability of pomegranate leaves extracts to fight obesity (Al-Muammar and Fozia Khan, 2012), cancer and other human diseases (Lansky and Newman, 2007). Phenolics in pomegranate leaves are thought to contribute in their health benefits (Lan et al., 2009). To our knowledge, no study in literature for anti-inflammatory, anti-cholinesterase inhibitory and cytotoxic activities of *P. granatum* leaves.

The objectives of this study were to determine the quantification of the main classes of polyphenolic compounds of pomegranate leaves extracts prepared with different polarity solvents and to compare antioxidant, anti-cholinesterase, anti-inflammatory and cytotoxic activities of different extracts.

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http://dx.doi.org/10.1016/j.fct.2013.01.036

2. Materials and methods

2.1. Chemicals used

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma, Aldrich, Fluka (Saint-Quentin France).

2.2. Plant sample

The leaves of *P. granatum*, belonging to Lythraceae family (ex Punicaceae), were collected in May 2012. Gabsi pomegranate variety was studied in this work and belongs to pomegranate germoplasm, which is located at the experimental field station of Zerkine in the province of Gabès, south of Tunisia. Gabsi variety was the best cultivated variety in Tunisia (Mars and Marrakchi, 1999).

2.3. Preparation of the extracts

Air-dried pomegranate leaves were ground to fine powder and successively extracted with solvents of increasing polarity (hexane, dichloromethane, ethyl acetate, ethanol and then methanol). Thus, 10 g of leaves powder were placed in hexane (100 ml) for 16 h under frequent agitation at ambient pressure and temperature. The mixture was filtered using Wattman paper (GF/A, 110 mm) and the experience was repeated twice. The solvent was evaporated using a rotary evaporator under vacuum at 35 °C. Then, the firstly extracted powder was extracted again with dichloromethane under the same conditions as with hexane. The same procedure was applied for the following solvents: ethyl acetate, ethanol and methanol. Extracts were kept in amber vials and stored at 4 °C for further analysis.

2.4. Quantification of total phenolic content

The total phenolics of each extract were determined by the Folin–Ciocalteu method (Georgé et al., 2005). Briefly, the diluted solution of each extract (0.5 ml) was mixed with Folin–Ciocalteu reagent (0.2 N, 2.5 ml), rested at room temperature for 5 min and then sodium carbonate solution (75 g l^{-1} in water, 2 ml) was added. After 1 h of incubation, the absorbance was measured at 765 nm against water blank. A standard calibration curve was plotted using gallic acid (0–300 mg/L). Results were expressed as mg of gallic acid equivalents (GAE) per g of dw.

2.5. Quantification of total flavonoids content

The total flavonoids content were estimated according to the Arvouet-Grand et al. method (1994) using a microplate reader. To 96 well plate, 100 µl of each variety extract was mixed with a solution (100 µl) of aluminium trichloride ($AlCl_3$) in methanol (2%). The absorbance of the mixture was measured at 510 nm against a reagent blank of methanol (100 µl) and plant extract (100 µl) without $AlCl_3$. Different concentrations of quercetin solution were used for calibrations and results were expressed as mg of quercetin equivalents (QE) per g of dw.

2.6. Quantification of total condensed tannin content

The condensed tannin content of pomegranate flower extract was determined by the vanillin method (Naczk et al., 2000) with modifications: 50 µl of extract solution was mixed with 150 µL of vanillin (1% in 7 M H_2SO_4) in an ice bath and then incubated at 25 °C. After 15 min, the absorbance of the solution was read at 500 nm. Results were expressed as mg catechin equivalents (CE) per g of dw from a calibration curve.

2.7. Quantification of total anthocyanins content

Total anthocyanin contents were determined by a pH differential method (Cheng and Breen, 1991) using two buffers: hydrochloric acid–potassium chloride (pH 1.0, 0.2 M) and acetic acid-sodium acetate (pH 4.5, 1 M) using 96-well plates. 20 µl of pomegranate extract was mixed with 180 µl of corresponding buffers and the absorbance was measured at 510 and 700 nm after 15 min of incubation. Absorbance (A) was calculated using $A = [(A_{510} - A_{700})\ \text{pH1.0} - (A_{510} - A_{700})\ \text{pH4.5}]$ using a molar extinction coefficient of 29600. The final results were expressed as mg cyanidin-3-glucoside equivalent (C3GE) per g of dw.

2.8. Determination of DPPH radical scavenging activity

Antioxidant scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by Blois (1958) with some modifications: 20 µl of various dilutions of the test materials (pure antioxidants or plant extracts) were mixed with 180 µl of a 0.2 mM methanolic DPPH solution. After an incubation period of 30 min at 25 °C, the absorbance at 520 nm and the wavelength of maximum absorbance of DPPH, were recorded as $A_{(\text{sample})}$. A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as $A_{(\text{blank})}$. The free radical-scavenging activity of each solution was then calculated as percentage of inhibition according to the following equation:

$$\%\ \text{inhibition} = 100\ (A_{(\text{blank})} - A_{(\text{sample})})/A_{(\text{blank})}.$$

Extracts' antioxidant activity was expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration. Quercetin was used as a standard. All measurements were performed in triplicate.

2.9. Determination of ABTS radical-scavenging activity

The radical scavenging capacity of the samples for 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonate (ABTS) radical cation was determined as described by Re et al. (1999). ABTS was generated by mixing a 7 mM stock solution of ABTS at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4 and 154 mM NaCl) with 2.5 mM potassium persulfate (final concentration) followed by storage in the dark at room temperature for 16 h before use. The mixture was diluted with ethanol to give an absorbance of 0.70 ± 0.02 units at 734 nm using spectrophotometer. For each sample, diluted methanol solution of the sample (20 µl) was allowed to react with fresh ABTS solution (180 µl), and then the absorbance was measured 6 min after initial mixing. Quercetin was used as a standard and the capacity of free radical scavenging was expressed by IC_{50} (mg/l) values calculated denote the concentration required to scavenge 50% of ABTS radicals. The capacity of free radical scavenging IC_{50} was determined using the same previously described equation for the DPPH method. All measurements were performed in triplicate.

2.10. Anti-inflammatory activity

The anti-inflammatory activity of pomegranate leaves was determined on Soybean lipoxygenase as described by Bylac and Racine (2003) with modifications. Various concentrations of 20 µl of pomegranate leaf extracts was mixed individually with sodium phosphate buffer (pH 7.4) containing 5-LOX and 60 µl of linoleic acid (3.5 mM), yielding a final volume of 1 ml. However the blank does not contain the substrate, but will be added 30 µL of buffer solution. All extracts were re-suspended in the DMSO followed by dilution in the buffer so that the DMSO does not exceed 1%. The mixture was incubated at 25 °C for 10 min, and the absorbance was determined at 234 nm. The absorption change with the conversion of linoleic acid to 13-hydroperoxyoctadeca-9,11-dienoate (characterized by the appearance of the conjugated diene at 234 nm) was flowed for 10 min at 25 °C. Nordihydroguaiaretic acid (NDGA) was used as positive control. The percentage of enzyme activity was plotted against concentration of the leaf extract. The IC_{50} value is the concentration of the flower extract that caused 50% enzyme inhibition.

2.11. Anti-cholinesterase activity

Cholinesterase (ChE) inhibitory activities were measured using Ellman's method, as previously reported (Akkol et al., 2012) with modifications. In this study, 50 µl of 0.1 M sodium phosphate buffer (pH 8.0), 25 µl of AChE (or BuChE) solution, 25 µl of leaf extract and 125 µl of DTNB were added in a 96-well microplate and incubated for 15 min at 25 °C. All extracts were re-suspended in the DMSO followed by dilution in the buffer so that the DMSO does not exceed 1%. The reaction was then initiated with the addition of 25 µl of acetylthiocholine iodide (or butyrylthiocholine chloride). The hydrolysis of acetylthiocholine iodide (or butyrylthiocholine chloride) was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm. The concentration of the extracts which caused 50% inhibition of the AChE (or BuChE) activity (IC_{50}) was calculated by nonlinear regression analysis. The percentage of inhibition was calculated from $(1 - S/E) \times 100$, where E and S were the respective enzyme activities without and with the test sample, respectively. Galanthamine was used as positive control.

2.12. Cytotoxicity evaluation

Cytotoxicity of sample was estimated on human breast cancer cells (MCF-7) as described by Natarajan et al. (2011) with modification. Cells were distributed in 96-well plates at 3×10^4 cells/well in 100 µl, and then 100 µl of culture medium containing sample at various concentrations were added. Cell growth was estimated by The MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. All extracts were re-suspended in the DMSO followed by dilution in the buffer so that the DMSO does not exceed 1%. Doxorubicin was used as positive control.