Potential benefits of phenolics from pomegranate pulp and peel in Alzheimer’s disease: antioxidant activity and inhibition of acetylcholinesterase

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DOI: 10.31665/JFB.2019.5.181

Received: March 01, 2019; Revised received & accepted: March 21, 2019


Abstract

Oxidative stress plays an important role in Alzheimer’s disease. To arrest oxidative stress, this contribution first identified and quantified phenolic bioactives present in the pulp and peel of pomegranate using high-performance liquid chromatography. Punicalagin $\beta$ rendered the greatest antiradical activity as evaluated by on-line HPLC-ABTS method, which was followed by punicalagin $\alpha$, gallic acid, and epicatechin. Furthermore, the scavenging activity against peroxyl and DPPH radicals, as well as the reducing power were investigated. Extracts obtained from the peel showed much higher phenolic contents and antioxidant properties than that of the pulp, hence being selected as potential inhibitor of acetylcholinesterase, a key enzyme involved in the progress of Alzheimer’s disease. Phenolics from pomegranate peel showed inhibition of acetylcholinesterase, which was dependent on the phenolic concentration. Therefore, pomegranate peel may be considered by the industry as a functional food ingredient and possibly for manufacturing of nutraceuticals.

Keywords: Punica granatum L.; Phenolic profile; Antiradical activity; Reducing power; Acetylcholinesterase.

1. Introduction

Pomegranate (\textit{Punica granatum} L.) and its processing by-products are rich sources of phenolic antioxidants (Ambigaipalan et al, 2016, 2017; Yang et al., 2016). In recent years, the interest in this fruit has increased due to scientific evidence supporting its numerous benefits to human health (Akhtar et al., 2015). In fact, pomegranate bioactives have been reported to potentially prevent and/or reduce the risk of development of atherosclerosis, Alzheimer’s disease, and certain types of cancer (Al-Jarallah et al., 2013;
2. Materials and methods

2.1. Material

The pomegranate fruits (species Wonderful) were purchased from CEAGESP, São Paulo, Brazil. The fruit was imported from the United States. The peel was separated from the pulp and seeds. The fruit was imported from the United States. The peel was separated from the pulp and seeds. The samples were stored in laminated polyethylene at 4 °C until use.

2.2. Chemicals

The compounds 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), 2,2’-azobis(2-aminodipropionate) dihydrochloride (AAPH), physostigmine, acetylcholinesterase, acetyltiocholine iodide, 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB), Folin-Ciocalteu, punicaglins α and β, rutin, quercetin, epicatechin, gallic acid, ferulic acid, p-coumaric acid, delphinidin 3-glucoside, cyanidin 3-glucoside, cyanidin-3,5-diglucoside, pelargonidin 3,5-diglucoside and pelargonidin 3-glucoside were purchased from Sigma Aldrich (St. Louis, MO, USA). Tris-HCl was purchased from Merek (Merk, Darmstadt, Germany).

2.3. Phenolic extraction

The extracts were prepared using 80% ethanol [1:10 sample/solvent ratio (m/v)] (Qu et al., 2010). The samples were extracted in shaker table (Model ET-1401, Tecnal, Piracicaba, SP, Brazil) protected from light at room temperature (24 ± 2 °C) for 24 hours. The resulting extract was centrifuged at 15656.2 g (Model NT 825, New Technique, Brazil) and the supernatant was concentrated at 35 °C using a rotary evaporator (Model 801, Fisatom, Brazil). The extracts were stored at −80 °C for further analysis. Chemical analyses were then performed on the pulp and peel extracts.

2.4. Phenolic composition using high-performance liquid chromatography (HPLC)

The identification of major phenolic compounds was performed on a high-performance liquid chromatography (HPLC) system (Shimadzu 20A, Shimadzu Co., Kyoto, Japan), equipped with a pump system (LC-20 AT), autosampler (SIL-20AHT), column oven (CTO-20A), communicator (CBM-20A) and UV/Vis detector (SPD-20A). Phenolic acids and monomeric flavonoids were separated on a Spherisorb ODS-Cl8 column (4.6 × 250 mm × 5 μm, Waters, Milford, MA, USA) at 35 °C. The injection volume was 10 μL. Data acquisition and processing were performed using LC solution software (Kyoto, Japan). The mobile phase consisted of 1% formic acid (A) and methanol (B). The flow rate was adjusted to 0.7 mL min⁻¹. The detection of monomeric flavonoids and phenolic acids was carried out at 280 and 370 nm, respectively, while anthocyanins were detected at 520 nm. The elution gradient used was as follows: 0–60% B for 45 min; 100% B, 50 min; 100% A, 60 min (He et al., 2011). To improve the method performance, punicalagens were separated with the same HPLC system and column but with a different solvent system. The mobile phase consisted of acetonitrile (A) and 1% formic acid (B). The elution gradient used was as follows: 97% B, 0 min; 95% B, 5 min, 85% B, 10 min; 70% B, 16 min; 97% B, 18 min, held at 97% B from 18–30 min. The flow rate was adjusted to 1 mL min⁻¹. The injection volume was 10 μL, the column temperature was 40 °C, and the detector was set at 260 nm. Phenolic compounds were identified by comparing their retention times and UV spectral data with coded and authentic standards under the same conditions as the samples. Calibration curves were used for quantification.

2.5. On-line HPLC-ABTS radical cation scavenging activity

The identification of phenolics was performed on a Shimadzu 20A high-performance liquid chromatography (HPLC) system equipped with a SPD-M10AVP photodiode array detector (DAD) and a SPD-20A UV/vis detector (Shimadzu Co., Kyoto, Japan).
Separations were conducted on an ODS-A C18 column (4.6 × 250 mm × 5 μm). The extracts were filtered using a 0.22 μm filter (Milipore) before the injection (10 μL). The mobile phases consisted of 1% formic acid (A) and methanol (B). The flow rate was adjusted to 0.7 mL min⁻¹ and the elution gradient used was as follows: 0 min, 100% A, 45 min 40% A, 50 min 100% A, which was followed by column equilibration from 50 to 60 min (He et al., 2011). The compounds were identified using UV spectral data and by comparing their retention times with coded and authentic standards. The contribution of individual phenolics to the ABTS radical scavenging activity was evaluated using an HPLC-ABTS radical cation scavenging method (Tiveron et al., 2016; Tremocoldi et al., 2018). A stock solution containing 140 nM potassium persulfate and 7 mM ABTS was prepared and kept at 25 °C in the dark for 16 hours. The stock solution was diluted in methanol to obtain an absorbance of 0.700 ± 0.02 at 734 nm. Each phenolic compound previously separated by HPLC reacted with the ABTS radical cation in the on-line system. The ABTS radical cation flow was adjusted to 0.7 mL min⁻¹, the absorbance was monitored at 734 nm and the scavenging activity was calculated according to their respective negative peaks. The results were expressed as trolox equivalents.

2.6. DPPH radical scavenging activity (DRSA)

The test samples were screened for their ability to scavenge DPPH radicals (Brand-Williams et al., 1995). The phenolic extract (0.50 mL) was added to ethanol (3.0 mL) and a 60 μM DPPH solution (300 μL). After 45 min in the dark, the absorbance of the mixture was read at 517 nm using a UV-visible spectrophotometer (UNICO Instrument Co.) (de Camargo et al., 2012). The radical scavenging activity was calculated using the following equation.

\[
\text{DPPH radical scavenging activity (％) } = \left(\frac{\text{Abs}_{\text{scontrol}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{scontrol}}}\right) \times 100
\]

where Abs kontrol is the absorbance of DPPH radical + ethanol and Abs sample is that of DPPH radical + phenolic extract or trolox. The results were expressed in μM trolox equivalent g⁻¹ of phenolic extract.

2.7. Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed as described by Melo et al. (2015). The extracts were diluted in 75 mM potassium phosphate buffer (pH 7.4). In a microplate, each extract (30 μL) was mixed with 508.25 mM fluorescein (60 μL) and 76 mM AAPH (110 μL) which was followed by incubation at 37 °C in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The absorbance was read every min for 2 h and the excitation and emission wavelength were set to 485 and 528 nm, respectively. The results were expressed as μmol trolox equivalent g⁻¹ of phenolic extract.

2.8. Ferric reducing antioxidant power (FRAP)

The reducing power assay (Benzie and Strain, 1996) was based on the method described by de Camargo et al. (2014). The FRAP reagent, which is a mixture [25 mL of acetate buffer (acetate 300 mM, pH = 3.6) + 2.5 mL of TPTZ] 10 mM TPTZ in 40 mM HCl] + 2.5 mL of FeCl₃ (20 mM in aqueous solution) was prepared at the time of analysis. Phenolic extracts (90 μL) were added to the FRAP reagent (2.7 mL). The absorbance was read at 595 nm using a UV-visible spectrophotometer (UNICO Instrument Co.) after incubation in a water bath at 37 °C for 30 min. The results were expressed in μM iron sulphate g⁻¹ of phenolic extract.

2.9. AChE inhibition assay

The photometric method described by Ellman et al. (1961) was used to determine acetylcholinesterase activity. Physostigmine (1 mg mL⁻¹) was used as the positive control. The reaction rates were calculated using GraphPad Prism software (version 5.0) and the IC₅₀ values were determined.

2.10. Statistical analysis

The experimental design was randomized with four replicates and the results expressed as mean ± standard deviation. The results were submitted to analysis of variance (ANOVA) using SAS software and F test (p < 0.05).

3. Results and discussion

3.1. Identification and quantification of phenolic compounds by high performance liquid chromatography

Pomegranate peel extracts contained a higher amount of total phenolics (128.4 mg g⁻¹) in relation to that of pomegranate pulp.
Table 2. Radical scavenging activity (µmol TE g−1 DW) of pomegranate peel and pulp extracts by HPLC-DAD-ABTS on-line

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compound</th>
<th>µmol TE g−1 DW</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>gallic acid</td>
<td>860.62 ± 20.24c</td>
</tr>
<tr>
<td></td>
<td>epicatechin</td>
<td>545.53 ± 23.17d</td>
</tr>
<tr>
<td></td>
<td>punicalagin α</td>
<td>1,129.73 ± 43.90b</td>
</tr>
<tr>
<td></td>
<td>punicalagin β</td>
<td>3,248.98 ± 49.59a</td>
</tr>
</tbody>
</table>

Data represent mean values for each sample ± standard deviation (n = 3). Means followed by different lower case letters within a row show difference between the samples (p < 0.0001). Abbreviations are: TE, trolox equivalents; and DW, dry weight of phenolic extract.

(17.9 mg g⁻¹) extracts (Table 1), as evaluated by HPLC. Likewise, pomegranate pulp extracts showed a lower content of total flavonoids (17.6 mg g⁻¹) compared to that of the peel extracts (127.2 mg g⁻¹). Various studies have shown that fruit by-products serve as excellent sources of phenolic compounds (Madrigal-Carballo et al., 2009; Mastrodi Salgado et al., 2012). Furthermore, their biological activity has been demonstrated in several model systems in relation to their protective role in prevention of human LDL-cholesterol oxidation and DNA damage, which are biomarkers related to cardiovascular disease and cancer development, respectively.

Gallic acid was the main phenolic acid in both tested samples (Table 1), followed by ferulic acid. The concentration of the latter one was 7.8-fold higher in the peel compared to the pulp. The presence of p-coumaric acid was also noted. Epicatechin and rutin were the monomeric flavonoids present. Structure/activity discussions have been in the spotlight due to their potential in anticipating potential benefits under physiological conditions. While all phenolic acids and epicatechin are simple phenolics (aglycons), rutin is a quercetin molecule linked to a sugar moiety (quercetin-3-O-rutinoside). Several pieces of evidence have demonstrated phenolic aglycons may be more bioaccessible than their conjugated counterparts (Shahidi and Peng, 2018). Likewise, extracts containing high amounts of aglycons also show a higher antiradical activity compared to that of extracts containing their conjugated counterparts (Yoshiara et al., 2018). Amongst the monomeric phenolics, the high content of gallic acid in both samples makes the test materials good candidates as functional foods and/or nutraceutical ingredients. However, considering all phenolics, hydrolysable tannins (punicalagin α and β) made the highest contribution to the phenolic profile of pomegranate peel extract, which is in good agreement with the literature (Ambigaipalan et al., 2016). These bioactive compounds are involved in plant defense against ultraviolet radiation and/or attack by pathogens (Diaz-de-Cerio et al., 2016).

To our knowledge, no genetic study has been reported yet that could explain the differences in the hydrolyzable tannins (HT, i.e., punicalagin) levels between the pulp and the peel pomegranate. However, Ono et al. (Ono et al., 2012) found that the pomegranate hairy root culture system is appropriate for expressing heterologous genes such as yellow fluorescent protein (YFP). In their study, 26 putative UDP-glucosyltransferase (UGT) genes, obtained from a pomegranate fruit peel (a tissue highly abundant in HTs) RNA-Seq library, were expressed and studied in wild type and hairy roots. Additionally, two candidate UGTs for HT biosynthesis were identified based on HPLC and differential gene expression analyses of various pomegranate tissues. In another work, overexpression and single RNAi knockdown pomegranate hairy root lines of two UDP-glucosyltransferase (UGT) genes, UGT84A23 and UGT84A24, did not result in clear changes in punicalagin accumulation, although double knockdown lines of these two UGTs showed reduced levels of punicalagins and bis-hexahydroxydiphenyl glucose isomers. In addition, accumulation of galloyl glucosides (ether-linked gallic acid and glucose) was identified in the double knockdown lines, suggesting that gallic acid was used by an unidentified UGT activity for glucoside synthesis, which gives rise to future prospects for understanding the regulatory control of HT metabolism in plants and its coordination with other biochemical pathways in the metabolic network (Ono et al., 2016).

More recently, Qin et al. (2017) sequenced and assembled the pomegranate genome with 328 Mb anchored into nine pseudo-chromosomes and annotated 29,229 gene models. They discovered that the integument development gene INNER NO OUTER (INO) was under positive selection and potentially contributed to the development of the fleshy outer layer of the seed coat, an edible part of pomegranate fruit. In addition, the genes encoding the enzymes for synthesis and degradation of cellulose, hemicelluloses and lignin were also differentially expressed between soft-and hard-seeded varieties, showing differences in their accumulation in cultivars differing in seed hardness. In parallel, candidate genes for punicalagin biosynthesis were identified and their expression patterns indicated that gallic acid synthesis in tissues could follow different biochemical pathways. As a result of the sequencing of the pomegranate genome, there will be important opportunities for unraveling and understanding the biochemical pathway(s) involved in the punicalagin biosynthesis that will be very valuable for breeding efforts to increase production of this bioactive compound.

3.2. Main phenolics from pomegranate pulp and peel as scavengers of ABTS radical cation

Several studies have reported the scavenging properties of phenolics from pomegranate and their processing by-products towards ABTS radical (Ambigaipalan et al., 2016, 2017). However, the individual contribution of pomegranate phenolics to such activity has not yet been entirely clarified. Furthermore, higher concentrations of particular phenolics do not always translate to higher activities. Therefore, to shed light on this situation and to fill an apparent gap in the literature, pomegranate phenolics from pulp and peel were tested against ABTS radical cation by using an on-line HPLC-ABTS method.

Regardless of the compound, pomegranate peel showed much higher scavenging activity compared to that of pomegranate pulp (Table 2). Amongst pomegranate peel phenolics, punicalagin β rendered the greatest antiradical activity as evaluated by on-line HPLC-ABTS method (1,324.9 ± 49.60 µmol trolox g⁻¹), which was followed by punicalagin α (1,129.73 ± 43.90 µmol trolox g⁻¹). Gallic acid, a simple phenolic compound, rendered the third highest antiradical activity (860.62 ± 20.24 µmol trolox g⁻¹), while the monomeric flavonoid epicatechin (545.53 ± 23.17 µmol trolox g⁻¹) made the lowest contribution. The same trend was found with respect to the antiradical activity of phenolics from pomegranate peel. Therefore, irrespective of the source (peel or pulp), ellagitannins (punicalagin α plus β) serve as the most important antioxid-
dants in pomegranate.

3.3. Phenolics from pomegranate pulp and peel as scavengers of peroxyl radicals and reducing agents

Reactive oxygen species (ROS), including peroxyl radicals, participate in several detrimental processes in biological systems. Likewise, ferric ions also catalyse oxidative reactions. The relatively long half-life of peroxyl radical makes these ROS potentially deleterious to intracellular components and biological fluids (de Camargo et al., 2014). Peroxyl radical is an intermediate of the Fenton reaction. Likewise, both Fe²⁺ and Fe³⁺ participate in the Haber-Weiss cycle, however, the ratio of these two chemical forms is important to the reaction kinetics and the ideal has been reported to be in the range of 1:1 to 7:1 (Fe³⁺/Fe²⁺) (Braughler et al., 1986). A lower concentration of Fe³⁺ may be helpful to stop and/or delay the Fenton reaction, which produces ROS involved in lipid oxidation. Therefore, phenolics from pomegranate pulp and peel were tested against peroxyl radicals (ORAC assay) and for their reducing power (FRAP assay).

In agreement with the results obtained by on-line HPLC-ABTS, phenolics from pomegranate pulp and peel were more effective in scavenging peroxyl radicals and in reducing Fe³⁺ (Table 3). In addition, according to the literature, phenolic extracts from pomegranate by-products also chelated Fe²⁺ and scavenged hydroxyl radicals (Ambigaipalan et al., 2016, 2017). The presence of galloyl moieties in phenolic compounds is important for complex formation with metal ions (Andjelković et al., 2006) and galloyl groups in phenolics from pomegranate juice and their processing by-products have been confirmed by mass spectrometry (Ambigaipalan et al., 2016, 2017; Mena et al., 2012).

3.4. Acetylcholinesterase (AChE) inhibition

Pomegranate peel showed a higher total phenolic content, total flavonoids and higher concentration of all individual phenolics, than that of the pulp. As mentioned earlier, oxidative stress plays an important role in Alzheimer’s disease. Pomegranate peel also displayed a higher antioxidant activity and reducing power than that of the pulp and was, therefore, evaluated for its potential as a source of phenolics with inhibition capacity towards AChE enzyme. The selected extract showed a dose-dependent inhibition towards AChE (IC₅₀ 2.48 mg/mL). The highest inhibition of AChE (58%) was found in extracts at 3 mg/mL, while extracts at 0.3 mg/mL showed 26% of inhibition. Ademosun and Oboh (2012) evaluated the effects of some citrus fruit juices (grapefruit, lemon, orange and tangerine) on AChE activity in vitro. According to these authors, AChE activity was also dose-dependently inhibited by their bioactive compounds. Orange juice, which showed the highest antioxidant activity, also displayed the highest AChE inhibitory activity, thus lending support to the findings of the present study. Therefore, considering data from the literature (Morzelle et al., 2016) and our contribution, dietary inclusion of pomegranate peel extract may prevent Alzheimer’s disease, which may at least partly be attributed to their high content of bioactive compounds, their antioxidant capacity and inhibitory activity against AChE.

4. Conclusion

The present study demonstrated that pomegranate peel had a higher concentration of total phenolics and flavonoids as well as individual phenolics as evaluated by HPLC compared to those of pomegranate pulp. Irrespective of the starting material, punicalagin β rendered the greatest antiradical activity as evaluated by on-line HPLC-ABTS method, which was followed by punicalagin α, gallic acid, and epicatechin. Likewise, pomegranate peel showed higher antioxidant activity and reducing power than that of the pulp. Pomegranate peel proved to serve as a rich source of phenolics with potential inhibitory activity against AChE enzyme. The extracts so obtained showed a dose-dependent inhibition towards AChE. Therefore, due to its antioxidant activity, reducing power and inhibitory effect against AChE, pomegranate peel may be used as multifunctional ingredient and a rich source of nutraceuticals.

Acknowledgments

The first author acknowledges FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), grant #2013/08989-1 for her Ph.D. fellowship. S. M. Alencar is thankful to FAPESP (grant #2013/13190-2). A. C. de Camargo acknowledges CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (process #88887.169471/2018-00) and Fondo Nacional de Desarrollo Científico y Tecnológico-FONDECYT postdoctorado (project 3180432).

Conflict of interest

There are no conflicts to declare.

References


Table 3. Antioxidant activities and reducing power of phenolics of pomegranate pulp and peel

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH (µmol TE g⁻¹ DW)</th>
<th>ORAC (µmol TE g⁻¹ DW)</th>
<th>FRAP (µmol Fe²⁺ g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulp</td>
<td>108.3 ± 5.95⁰</td>
<td>323.8 ± 10.3⁰</td>
<td>2.47 ± 0.12²</td>
</tr>
<tr>
<td>Peel</td>
<td>2,372 ± 30.5⁰</td>
<td>7,423 ± 180</td>
<td>56.5 ± 5.90⁰</td>
</tr>
</tbody>
</table>

Data represent mean values for each sample ± standard deviation (n = 3). Means followed by different lower case letters within a column show difference between the samples (p < 0.0001). Abbreviations are: TE, trolox equivalents; and DW, dry weight of sample.
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