Proceedings

Punica granatum L. Fruit Parts from Algerian Cultivar Bioactive Compounds and In Vitro Biological Activities: A Comparative Study †

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Abstract: Fruits are a source of antioxidant compounds, such as phenolics, vitamins and carotenoids, which contribute to their chemo preventive potential. The mentioned compounds, which scavenge free radicals, may reduce the level of oxidative stress and prevent the oxidation of biomolecules, that would break the reaction chains of pathogenesis in the deterioration of physiological functions, which could occur in the coronary heart diseases and cancer. Apart from their biological properties, natural antioxidants are also of interest in the cosmetic, pharmaceutical and especially in food industries, since they can be also used as natural antioxidants, Nutraceuticals, prebiotics, dyes.

The present work evaluated phenols, and flavonoids contents (quantity) of organic pomegranate juice, peel and seeds cultivated in the Northeastern part of Algeria, in vitro antioxidant activity, using: CUPRAC, GOR, PHENYL, DPPH, ABTS assays and enzymatic activity: α-glycosidase were also investigated and confirms traditional uses of pomegranate parts. Furthermore, a comparative study of all these essays on different pomegranate’s parts tests will be given. However, further investigations should assessed safety of by products “seeds, arils and peels” at efficient but non toxic doses, if we want to use it as added value in our daily feeding.

Keywords: Punica granatum L.; traditional medicine; bioactive compounds; in vitro biological activities

1. Introduction

Pomegranate fruit parts are naturally bioavailable sources rich in nutrients and bioactive compounds with establish antioxidant activities. Global demand to this fruit has been increasing and pomegranate is now harvested in many parts of the world. [1,2]. Pomegranates are enjoyed for their sweetness, acidic juice, and antioxidant properties and are considered as a super fruit [3]. All parts of the pomegranate tree including the roots, the reddish-brown bark, leaves, flowers, rinds, and seeds, have been used in traditional healing as medicinal food of great importance for treating gastrointestinal ailments [4–6]. The juice from pomegranates is known to be nature’s most powerful antioxidants [7]. Furthermore, Pomegranate phenol compounds are mostly concentrated in the fruit peels [8], but that’s large amounts of by-products composed of peels (78%) and seeds (22%) generated in pomegranate juice processing, has limited use as animal feed [9]. Thus, scientific studies on bioactive constituents and pharmacological properties of pomegranate have increased considerably in the last decade. An important portion of these studies is on the determination of the nutrition value of cultivars from different cultivars.
regions [2], in addition to the valorization of pomegranate peels, focused on their exploitation in the food industry as natural antioxidants [8].

In this context, we report phytochemical and pharmacological investigations on an Algerian cultivar’s *Punica granatum* L. from the region of Constantine for its fruit parts extracts (peel, juice, seed), where total phenols and flavonoids are quantified, in vitro enzymatic and several antioxidant activities, exhibit pomegranate ethanol peel extract as a promising hypoglicemic and antioxidant natural agent. In addition, peel extracts was used as an additive in derma-cosmetic formula as natural dye and antioxidant and also in a dietary vegetal milk to supplement it with functional hypoglicemic metabolites, antioxidant activity, color, and sensory attributes, in order to promote human health, well being as well as increasing its exploitation possibilities for more responsible and sustainable eco-economy.

2. Experiments Material and Methods

2.1. Reagent and Standards

All chemicals and reagents used in this work were of analytical grade and purchased from Sigma-Aldrich Company Ltd. (Germany).

2.2. Plant Material

Local Fruits of *Punica granatum*. L (Saffri variety) were commercially purchased in September 2018 and identified as cultivated in El Hama, Constantine region, Algeria.

Peels: after cleaning the fruit with water, peels are well dried in dark at room temperature, then ground to powder and stored in freeze until use.

Juice: fruit juice was obtained by passing the fruit through a reel; filtered then lyophilized and stored in freeze until use.

Seeds: seeds are well washed then dried and stored in freeze until use.

2.3. Extraction and Phenol Content Determination

Solide-liquide extraction of dried peels and seeds was done using cold maceration which is indicated for the safe assessing and processing of biological products, for this 72,32 g of peels powder and 2,89 g of seeds were pooled into sterile amber jars with ethanol (1:3 *w/v*) as extracting solvent for 15 days at room temperature under stirring conditions. The obtained extracts are then filter on a Büchner funnel, concentrated under reduced pressure in a rotavap and then lyophilized and stored in freeze until use. Total polyphenols were determined by the Folin–Ciocalteau method [10,11]. Results were expressed as mg Gallic acid equivalents (µg GAE)/ mg dry weight (DW) of crud extract. The flavonoids content was determined according to Topçu et al., [12] with few modifications and expressed as µg quercetin equivalents per mg of dry weight (µg QE/ mg DW).

2.4. Enzyme Inhibition and Antioxidant Assays

The following tests were performed on different extracts dilutions using a microplate reader (*Multi-mode EnSpire*® PerkinElmer).

2.4.1. Inhibitory Activity of an Enzyme Related to Metabolic Syndrome

α-Glycosidase Inhibition

The inhibition of α-glycosidase was determined according to Sinéad et al., [13] method, briefly: 100 µL (0.1 U/mL) α-glycosidase enzyme solution (1 mg/9,8 mL of sodium phosphate buffer (100 mM, pH 6,9)) were added to 50 µL of extracts, then the enzyme reaction was started by adding 50 µL of substrate solution p-nitrophenyl-α-D-glucopyranoside (5 mM) (15,06 mg/10 mL of sodium phosphate buffer (100 mM, pH 6,9)),
the reaction mixture was incubated 30 min at 37 °C, the absorbance was measured at 405 nm. Enzyme inhibition was calculated using the following equation:

\[
\% \text{ inhibition} = \frac{(A_0 - A_s)}{A_0} \times 100
\]

where \(A_0\) is the absorbance of the negative control (blank, without extract) and \(A_s\) is the absorbance in presence of the extract, acarbose was used as positive control.

IC50 values denote the \(\mu\)g GAE/mL required to inhibit the enzyme by 50%.

2.4.2. Antioxidant Activity
- ABTS radical scavenging

The assay was carried out as described by Re et al., [14]. ABTS•+ was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate and incubating at room temperature in the dark for 16 h. After that, 160 \(\mu\)L of ABTS•+ solution were added to 40 \(\mu\)L of extracts, the mixture was then incubated at room temperature and the absorbance measured at 734 nm. A blank without the extract was used as negative control. \(\alpha\)-Tocopherol and BHA were used as positive controls.

- DPPH radical scavenging

The DPPH scavenging activity was evaluated according to Blois method [15]. 160 \(\mu\)L of DPPH methanolic solution (6 mg /100 mL) were added to 40 \(\mu\)L of extracts, the mixture was then incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm. A blank of 40 \(\mu\)L of methanol with 5 mL of DPPH solution was used as negative control. \(\alpha\)-tocopherol, BHT and BHA were used as positive controls.

- Galvinoxyl (GOR) radical scavenging

Galvinoxyl free radical scavenging activity was evaluated by using Shi et al., [16] protocol.

To 40 \(\mu\)L of extracts, 160 \(\mu\)L of Galvinoxyl radical 0,1 mM (4 mg in 100 mL MeOH) are added. The mixture solutions are then incubated for 120 min, and the absorbance measured at 428 nm. Methanolic solution of Galvinoxyl was used as a blank. BHT and BHA were used as antioxidant standards.

- Cupric reducing capability (CUPRAC)

Cupric reducing capacity was evaluated according to Apak et al., [17] method. Solution (S1) at PH = 7.0 is prepared by adding 1,927 g of ammonium acetate (ACNH4) to 25 mL of water. Solution (S2): 0,039 g of Neocupronin are dissolved in 25 mL of methanol.

Solution (S3): 0,042625 g of (Cu Cl2, 2H2O) are dissolved in 25 mL of water.

Then 60 \(\mu\)L of (S1), 50 \(\mu\)L of (S2) and 50 \(\mu\)L of (S3), were respectively added to 40 \(\mu\)L of extracts, the all is incubated for 1 h in the dark and the absorbance measured at 450 nm. For the blank replace extracts with methanol. BHA and BHT are used as positives controls.

- Reducing power activity

The reducing power test was undergoing following Oyaizu [18] protocol with slight modifications. To 10 \(\mu\)L of extracts, 40 \(\mu\)L of phosphate buffer pH (6.6) and 50 \(\mu\)L of potassium ferricyanure K3Fe(CN)6 1% (1 g K3Fe(CN)6 in H2O) are added. After 20 min of incubation at 50 °C, 50 \(\mu\)L of trichloroacetic acid (TCA) at 10%, 40 \(\mu\)L of water and 10 \(\mu\)L ferric chloride FeCl3 (0.1% in H2O) are added. The solution absorbance is measured at 700 nm. For the blank replace the extracts with methanol. Ascorbic acid, Tannic acid and \(\alpha\)-Tocopherol are used as standards.

- Phenanthroline activity

Phenanthroline activity was evaluated according to Szydlows Czerniaka et al., [19] method.

50 \(\mu\)L of FeCl3 à 0.2%, 30 \(\mu\)L of Phenanthroline methanol solution (0.5%) and 110 \(\mu\)L of methanol, are respectively added to 10 \(\mu\)L of extracts. The mixture solutions are
incubated at 30 °C for 20 min in the dark; absorbencies are measured at 510 nm. For the blank replace the extracts with methanol. BHT is used as standard for this assay.

2.5. Formulation of Cosmetic and Functional Food Products

The formula of solid soap and vegetal milk almond, pomegranate fruit based were developed. Pomegranate fruit part extract that had the highest TPC, TFC, α-glycosidase inhibition and antioxidant activities was chosen as active ingredient [8]. Physical properties (e.g., texture, Color, Odor, pH of products) were determined.

2.6. Statistical Analysis

Sampling and analyses were performed in triplicate, and the data are presented as mean ± standard deviation (S.D.). Statistical analysis was performed using Microsoft Office Excel 2008 ($p < 0.05$).

3. Results and Discussion

The present work aimed to determine the total polyphenols content of *Punica granatum* L. fruit different parts from an Algerian cultivar and its efficacy against an enzyme involved in hyperglycemia related with metabolic syndrome and species generated oxidative stress.

3.1. Total Phenols and Flavonoids Quantification

Quantification results of total polyphenols compounds and flavonoids is reported and, obtained results exhibit a high phenols content for peels extract (699.29 ± 0.00 μg GAE/mg DW), followed by juice extract (60.27 ± 23.42 μg GAE/mg DW) then seeds extract (28.01 ± 11.53 μg GAE/mg DW). Similar results were found for Flavonoids quantification with peels extract as the highest content (95.41 ± 3.53 μg QE/mg DW), followed by juice extract (12.08 ± 0.58 μg QE/mg DW), then seeds extract (12.84 ± 1.03 μg QE/mg DW), these values are the in range of most tested cultivars worldwide [20–23].

Predominant phenol classes found in pomegranate peels are flavonoids, anthocyanins, and tannins. The main phenol compound present in the pomegranate peel is punicalagin, which accounts for approximately 70% of the hydrolysable ellagitannins and 75–78% of total phenolics, it is responsible of the antioxidant activity of pomegranate [24].

3.2. Enzyme Inhibition

Several studies linked pomegranate with type 2 diabetes prevention and treatment. Fasting blood glucose levels were decreased significantly by punicic acid, methanol seed extract, and pomegranate peel extract [25]. The activity of Pomegranate fruit parts extracts was assessed towards an enzyme associated with metabolic syndrome: α-glycosidase.

α-Glycosidase Inhibition

One therapeutic approach to decrease postprandial hyperglycemia is to suppress the production and/or absorption of glucose from the gastrointestinal tract through inhibition of α-glycosidase enzyme [26]. Hypoglycemic chemical agents used in clinical practice, such as acarbose, competitively inhibit α -glycosidase in the brush border of the small intestine, which consequently delay the hydrolysis of carbohydrates and alleviate postprandial hyperglycemia, with side effects at long administration (diarrhea, abdominal discomfort, flatulence, and hepatotoxicity). Therefore, α-glycosidase novel natural inhibitors are prized. Phenol compounds of *Punica granatum* L. fruit parts may freely interact with enzymes present in the digestive tract modulating their activity [26,27].

The inhibitory activity of pomegranate fruit parts’ ethanol extracts towards α-glycosidase enzyme exhibit a very high activity for peels extract (IC50 = 44.65 ± 5.77 μg/mL) with a relation dose-response presenting better effect than used standard acarbose (IC50
= 275, 43 ± 1.59 μg/mL), followed by juice concentrate (208.45 ± 14.61 μg/mL) which has close activity to acarbose, then seeds extract (IC50 = 402.83 ± 3.29 μg/mL), results are recorded. Several in vivo studies investigated the effect of different pomegranate fractions on fasting blood glucose, which is considered a key variable in the diagnosis of type 2 diabetes [25,28–30]; this mechanism that enables pomegranate to improve postprandial hyperglycemia is mediated by the inhibition of intestinal α-glycosidase activity [31]. A reduction of α-glycosidase activity was also observed in the saliva of healthy humans after the consumption of pomegranate extract [32]. Indeed, many plants, used traditionally to control diabetes or hyperglycemia were reported to exert strong inhibition of α-glycosidase activity [33]. These results provide evidence for the anti-diabetic activity of pomegranate peels by at least inhibiting α-glycosidase enzyme activity and may offer dietary coadjutants (therapeutic complements) to control hyperglycemia in diabetic patients. However, before pomegranate or any of its extracts can be medically recommended for the management of type 2 diabetes, controlled, clinical studies, are recommended.

3.3. Effect of Pomegranate Fruit Parts Extracts on Oxidative Stress

Oxidative stress is associated with several pathologies like cardiovascular, neurodegenerative, cancer and aging. A diet rich in natural antioxidants could be beneficial to human health and a lot of interest is focused on the determination of antioxidant capacity of natural products [34]. The antioxidant capacity of Punica granatum L. fruit parts extracts was determined using five different experimental models, with higher antioxidant potency as expected for peels extract (IC50 = 20.53 ± 0.52 μg/mL, 9.51 ± 0.48 μg/mL, 7.42 ± 0.84 μg/mL) for radical scavenging (DPPH, ABTS, GOR respectively) mostly similar to referential compounds, as well as for reducing activity (A0.50 = 3.44 ± 0.10 μg/mL, 32.20 ± 3.43 μg/mL, 14.04 ± 2.35 μg/mL, for CUPRAC, reducing power, phenantroline respectively) which are also similar or even better than used standards, this highlights the electron and proton donor potent of pomegranate ethanol peels extract and dose-response relation with antioxidant capacity, which is in agreement with obtained total phenols content mostly higher in peels extract. Similar antioxidant activity on ABTS radical, reducing power and DPPH was reported [22,23]. Obtained results exhibit the studied pomegranate as from very good quality and indicated as health promoting natural agent. However, it is necessary to perform cellular assays in order to evaluate the potential antioxidant activity of an extract [34].

3.4. Formulation of Cosmetic and Functional Food Products

3.4.1. Formulation of Solid Soap Product by Cold Saponification

According to bioactive compounds and antioxidant activity results, the pomegranate ethanol peel extract was chosen as active ingredient in solid soap product by adding it in the base formula. With an optimal dose in the range of 0.5–3 mg/100 g. Characteristics of soap product with this additive exhibit a natural pleasant color and smell without any chemical dyes besides neutral Ph value of 7.5 is appropriate and recommended for sensitive skins.

3.4.2. Formulation of Enriched Vegetal Almond Milk with Pomegranate Peels Extract

According to the same criteria in addition to α-glycosidase inhibition, peel extract was also chosen for the enrichment of vegetal almond milk in the base formula with an optimal dose of 4–5 mg/100 g, in order to have a functional food for diabetic persons or simply as dietary food for health caring and prevention with a natural color and very nice taste.

4. Conclusions

The properties of pomegranate depend on cultivars and growing locations. Bioactive components of pomegranate fruit are attractive potent targets for the scientific community
to develop novel food products for treatment/prevention of chronic diseases. This study highlighted, Constantine cultivar (Algeria) among the best cultivars in the world and also pomegranate peels as the richest sources of phenols and glycemic regulator, these by-products can be used to produce several economic and agri-waste management benefits. Therefore, future research should focus on the development of technologies for commercial production of novel food products from fruits, peels, and seeds and conducting in vivo clinical trials to determine optimal dietary regimens to achieve the desired beneficial health effects and strengthen the therapeutic reputation of pomegranate food products.

The functional properties that were demonstrated for the pomegranate could stimulate pomegranate agric-waste especially peel promotion which should be used as alternative source of natural antioxidant and glycolic regulator in food and non-food products.

Author Contributions: M.T.N. and B.C. conceived and designed the experiments and analyzed the data; C.A. and C.L. performed the experiments; Z.K. contributed reagents/materials/analysis tools; M.T.N. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABTS</td>
<td>2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>A0.50</td>
<td>Absorbance at 50% of substrate reduction</td>
</tr>
<tr>
<td>BHA</td>
<td>Butylhydroxyanisol</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylhydroxytoluène</td>
</tr>
<tr>
<td>CUPRAC</td>
<td>Cupric Ion Reducing Antioxidant Capacity</td>
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<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
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<tr>
<td>DW</td>
<td>Dry weight</td>
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<tr>
<td>GAE</td>
<td>Gallic acid equivalent</td>
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<td>GOR</td>
<td>Galvinoxyl Radical</td>
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<tr>
<td>IC50</td>
<td>Concentration at 50% of Inhibition</td>
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<td>QE</td>
<td>Quercetine equivalent</td>
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<td>TFC</td>
<td>Total flavonoids content</td>
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<tr>
<td>TPC</td>
<td>Total polyphenols content</td>
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References


