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POTENTIAL ASSESSMENT OF POMEGRANATE (*PUNICA GRANATUM* L.) FRUIT PEELS AS A SOURCE OF NATURAL ANTIOXIDANTS

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ABSTRACT

Pomegranate (*Punica granatum* L.) belonging to the *Lythraceae* family is commonly known as Rommane (*Arabe*). It is an edible fruit, cultivated in Algeria country for its interests as a nutritional and medicinal product useful for treating a wide range of health maladies. The present work focuses to evaluate the phenolic composition and antioxidant activity of hydroethanolic extract of peel part of this fruit. The phenolic extract of the dried and powdered pomegranate peel was obtained by conventional maceration using 70% ethanol in water as solvent. The extraction yield was 39.72%. Quantitative determination of total phenolic, flavonoid and condensed tannin contents was carried out using colorimetric methods, its amounts were found to be 158.18± 0.66 mg GAE/ g DW, 85.74± 9.77 mg RE/ g DW and 08.98± 0.90 mg CE/ g DW, respectively. The evaluation of antioxidant capacity using phosphomolybdate (PM), 2.2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tests showed that the pomegranate peel extract had a good overall antioxidant potential of 23.53± 1.49 mg AAE/ g DW and showed a concentration-dependent in scavenging activity against DPPH and ABTS free radicals, with an efficient EC_{50} values of 4.64± 0.35 and 3.63± 0.07 µg/ml, respectively. The present findings thus give authority to the fact that pomegranate peel powder could be efficiently used as a potential source of natural antioxidants to prevent damage associated with free radicals.

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Introduction

Oxygen is an indispensable element for life of aerobic organisms, but the very reactivity that underpins its effectiveness as the final electron acceptor in the respiratory chain also renders it a potentially toxic agent, due to the formation of reactive chemical species termed as reactive oxygen/ nitrogen species (ROS/RNS) or free radicals [1]. These reactive species play a dual role in human as both beneficial and toxic compounds, at physiological levels, ROS can serve as immune system modulation and can activate various signal transduction pathways [2]. It is also known that increased levels of these reactive molecules can cause detrimental effects by causing oxidative damage to biological macromolecules and disrupting the cellular redox balance [3, 4], which leads to the generation of oxidative stress-related diseases like arteriosclerosis, diabetes, cancers, and neurodegeneration [4, 5]. In order to scavenge excessive free radicals and reestablish or maintain the balance of homeostasis in human body, thus realizing the prevention and treatment of diseases, the consumption of antioxidants is necessary [6]. Antioxidants are categorized in two groups of synthetic and natural. However, synthetic ones have toxic and carcinogenic effects [7, 8]. Hence, extensive interest has been directed towards the identification of natural antioxidants from various plant sources, such as peels of various fruits. Interestingly, the peel and seed parts of some fruits have higher antioxidant activity than the pulp fractions [9]. For example, pomegranate peel has a higher antioxidant activity than its pulp [10].

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The pomegranate plant (*Punica granatum* L., *Lythraceae* family) is one of the oldest edible fruits and is widely cultivated in parts of Asia, North Africa, the Mediterranean and the Middle East [11]. The pomegranate fruit, referred to as " Rommane " in Arabic, "Grenadier" in French and "Pomegranate" in English, is one of the most frequently used popular plants in Algeria to treat gastrointestinal system diseases (diarrhea, constipation, and stomach bloating), anemia, gum and teeth [12], as well as diabetes [13]. The different parts of this plant such as flowers, seeds and bark have been employed against inflammatory and infectious pathologies [14]. It is also known as the "Super Fruit" due to its excellent properties such as nutritional value, antioxidant capacity. Pomegranate consumed as fresh fruit and functional foods such as juice, beverages, jams and others [15]. Pomegranate peel is characterized by an interior network of membrane comprising almost 26% - 30% of total fruit weight and is an important source of polyphenolic compounds, including flavonoids (anthocyanins, catechins, and other complex flavonoids) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallic and ellagic acid). These compounds are concentrated in pomegranate peel and juice, which depicts 92% of the antioxidant activity allied with the fruit [16, 17].

This research paper aimed to evaluate the phenolic content and antioxidant activity of the ethanolic extract of the fruit peel of *P. granatum* L. applying three different methods, phosphomolybdate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS).

Material and Methods

Collection of fruits

Fresh mature pomegranate fruits were purchased during the harvesting season in the months of October 2016 from a local market at Touggourt city (Wilaya of Ouargla), in southeastern of Algeria. The peels were manually removed from the fruits, dried in the dark at room temperature for three weeks, then crushed into a mortar and stored until further use.

Extraction of phenolic compound

The peel powder (20 g) was extracted by maceration with 100 mL of ethanol-water 70% (v/v) at room temperature for 24 h. The mixture was filtered through filter paper Wattman n°1 to remove fine particles. The residue on the filter paper was re-extracted again twice with the same volume of alcoholic solvent (2× 100 ml). The filtrates were pooled and concentrated in a rotary evaporator under vacuum at 40°C until dryness. The dry residue was re-dissolved in methanol for further analysis in a ratio of 1 mg/ml.

Determination of extract yield

The percentage yield of evaporated dried extract based on dry weight basis was calculated from the following formula:

Yield (g/100 g of dry plant material) = (W1 × 100) / W2 (Eq. 1)

Where W1 was the weight of the extract after the solvent evaporation and W2 was the weight of the dry plant material.

Total phenolic content

Total phenolic content was determined according to Lister and Wilson [18] by using Folin-Ciocalteu reagent. 100 µl of extract were combined with 500 µl of the Folin–Ciocalteu reagent (1/10 dilution) and 1000 µl of distilled water. After incubation 1 min at room temperature, 1500 µl of a 20% sodium carbonate (Na_2CO_3) solution were added. After incubation 2 h at room temperature in obscurity, the absorbance was measured using a UV mini- 1240 UV–Vis spectrophotometer SHIMADZU at a wavelength of 765 nm and total phenolic content was determined on the basis of a standard calibration curve of gallic acid. The results were expressed as mg gallic acid equivalent per gram of dry weight of plant (mg GAE/g DW).

Total flavonoid content

The amount of total flavonoids in the extract was measured spectrophotometrically following the method reported by [19]. 1 ml of ethanolic extract was mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 10 min, the absorbance of the reaction mixture was measured using a UV/Visible spectrophotometry at 430 nm.. The flavonoid content was quantified on the basis of a standard calibration curve of rutin. The results were expressed as mg rutin equivalent per gram of dry weight of plant (mg RE/g DW).

Total condensed tannin content

The amount of condensed tannins or proanthocyanidin in pomegranate peel extract was determined following the method developed by [20]. A volume of 1 ml of the sample was added to 1.5 ml of 4% vanillin solution in methanol. Then, 750 µl of HCl solution (12 M) were added. The tubes capped with a glass marble were shaken by a vortex mixer and heated to 30 °C in a water bath for 20 min. After cooling the tubes, the absorbance was read at 500 nm using a UV/Visible spectrophotometry and condensed tannin content was determined on the basis of a standard calibration curve of catechin. The results were expressed as mg catechin equivalent per gram of dry weight of plant (mg CE/g DW).

Total antioxidant activity

The total antioxidant capacity assay of extract was carried out by the phosphomolybdenum test (PM) according to the procedure described by [21]. An aliquot (300 µl) of hydroalcoholic extract were added to the test tube containing 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were

incubated in a thermal block at 95 °C for 90 min. Once the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm using a UV/Visible spectrophotometry and total antioxidant activity was determined on the basis of a standard calibration curve of ascorbic acid. The results were expressed as mg ascorbic acid equivalent per gram of dry weight of plant (mg AAE/g DW).

DPPH radical scavenging activity

The antioxidant scavenging activity of sample extract was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as described by [22]. The extract was previously dissolved at different concentrations (01 to 08 µg/ml) in methanol. 100 µl of antioxidant solution (sample or control) were added into the test tube individually containing 2900 µl of DPPH° solution (60 µM) freshly prepared in methanol. The mixture was vigorously stirred and incubated 30 min in the dark at room temperature. The absorbance of the resulting solution was measured at 515 nm using a UV/Visible spectrophotometry. The percentage inhibition of free radical DPPH was calculated according to the equation:

$$\% \text{ inhibition} = [1 - (Abs_{sample}/Abs_{blank})] \times 100. \quad (Eq. 2)$$

Where Abs_{sample} and Abs_{blank} are the absorbance of the test sample and the blank control (containing all reagents except the extract solution), successively.

Effective concentration at 50% (EC_{50}) values are widely used parameter to measure the free radical scavenging activity and is defined as the concentration of sample required to decrease by 50% the initial quantity of DPPH. A lower EC50 value indicates a higher antioxidant activity [23]. The IC50 values were calculated from the regression equation for the concentration of extract and percentage inhibition. Trolox was used as a positive control.

ABTS radical scavenging activity

The 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assay done according to the method of [24]. The stock solutions included 7 mM ABTS and 2.45 mM potassium persulfate. The ABTS radical cation ($ABTS^{\bullet+}$) was generated using 5 ml of the ABTS stock solution and 88 µl of the potassium persulfate, in the dark, at room temperature for 12-16 h before use. The solution was then diluted in order to obtain an absorbance of 0.7± 0.02 at 734 nm. Fresh diluted $ABTS^{\bullet+}$ solution was prepared every day. 100 µl of antioxidant solution (sample or control) were mixed with 2900 µl of diluted $ABTS^{\bullet+}$ solution and incubated for 5 min in a dark condition. The absorbance of the resulting solution was measured at 734 nm using a UV/Visible spectrophotometry. The percentage of inhibition of $ABTS^{\bullet+}$ was calculated using above formula (Eq. 2). The anti-radical activity of extract was expressed by EC50 values calculated denote the concentration required to scavenge 50% of ABTS radicals. The EC50 values were determined using the same previously described equation for the DPPH test. Trolox was used as a positive control.

Results and discussion

Extraction yield

The main purpose of extraction is to get high extraction yield and effective at lower concentration suitable for any type of plant material [25]. In the present study, extraction of *P. granatum* peels with 70 % ethanol gave a deep brown color extract with percent yield of dried weight of sample 39.72% (Table 1). An approximate value had been reported by Marchi *et al.* [26] through extraction with aqueous ethanolic solution 80% at the 1/20 ratio and for a period of 48 hours, with above 40% of extraction yield. However, [27] used an extraction process with aqueous ethanol 50% at the 1/5 ratio for an interval of two days, repeated twice, and found value of 22.1% extract yield. These differences in phenolic yield may be attributed to the changes in proportion of water in the solvent system. [28] found that it is necessary to add a certain amount of water (30–40%) to the extraction solvent in order to improve the extraction of phenolic compounds. Water plays an important role in the swelling of the plant material, while ethanol is liable to disrupt the bonding between the solute and the plant matrix and also allow better mass transfer of compounds. Therefore, the binary solvent system (ethanol/ water) shows a synergistic effect which facilitates the phenol extraction [29]. In addition, using a 70% ethanol minimum as solvent is required to inactivate the polyphenol oxidase, enzymes involved in the oxidation of phenolic substances [30].

Total phenolic, flavonoid and condensed tannin contents

Medicinal plants possess a wide range of bioactive compounds, phenolic compounds are the widest occurring one and have been touted as accounting for most of the antioxidant activity of plants or plant products [31]. The quantitative determination of phytochemical constituents of hydroethanolic extract of *P. granatum* peels is summarized in Table 1. The total polyphenolic content in hydroethanolic extract of pomegranate peels was recorded as 170.50± 10.49 mg GAE/ g DW. This finding is higher than the value reported in the study of [32], in which the aqueous ethanol 70% extract of pomegranate peels for the same cultivar (extracted by reflux for 2 h) had 158.18± 0.66 mg GAE/g of dry extract. On the contrary, [33] demonstrated that 70% hyrdoethanolic extract of pomegranate peels from the Thailand cultivar (prepared using a Soxhlet extractor for 24 h) showed higher phenolic content (449.60± 4.40 mg GAE/g of dry extract).

In the current study, a quantitative estimate of the total flavonoids content of the tested cultivar in Table 1 revealed that the peels extract exhibited a high amount of this component 85.74± 9.77 mg RE/g DW, representing 50.29% of total phenolic compounds. Different from our results, some authors studied various cultivars of *P. granatum* from Tunisia, Thialande and Algeria. All authors reported lower levels of total flavonoids in the pomegranate peels extract with 51.52 ± 8.14 mg RE/g

DW [34], 38.44±1.44 mg of catechin equivalents (CAE)/ g DW [33] and 12.8 ± 2.2 mg of catechin equivalents (CEQ)/g DW [32].

The results of the vanillin essay are presented in Table 1, it was observed that the condensed tannins in the aqueous ethanol extract was 08.98± 0.90 mg CE/ g DW. This is lower than that found by [35] for the methanolic extract from the same cultivar (47.78± 3.27 mg CE/g). [36] have shown that the 80% hydromethanolic extract of peels from four Tunisian cultivars (Mekki, Chelfi, Gabsi and Jbeli) contains less condensed tanin contents, ranging from 1.5 mg CE/g DW for Chelfi cultivar to 7.7 mg CE/g DW for Jbeli cultivar.

It is important to note that the condensed tannins accounted for a small amount of total phenolics in the pomegranate peels extract, where their amount represents 05.27 % of the total phenolic content and around ten fold lower than flavonoids concentration. Based on the data presented here, we conclude that the flavonoid compounds are the dominating phenolic group present in tested cultivar.

The variation in total phenolics, flavonoids and condensed tannin contents among studies might be attributed to the differences in cultivars, season and period of harvesting, agro-climatic conditions, as well as methods and time of extraction and extraction solvent [37].

Total antioxidant activity

The hydroethanolic extract of pomegranate peels was screened for it antioxidant capacity using three *in vitro* methods such as PM, DPPH and ABTS. These methods are distinguished by their mechanisms of action and would be complementary to the study of the antioxidant potential of plant material. The reduction of molybdenum (VI) was often used to study the capacity of an antioxidant to transfer electrons. Whereas the trapping of DPPH and ABTS radicals by an antioxidant substances was related to their electron or proton donating ability.

The total antioxidant potential of hydroethanolic extract was measured by their ability to reduce molybdenum (VI) to molybdenum (V) and resulting in formation of green phosphate/ Mo (V) complex with maximum absorption at 695 nm. The experimental data expressed as the number of milligram equivalents of ascorbic acid per gram of extract was found to be 23.53± 1.49 mg AAE/g DW. Only one study done on the total antioxidant activity of pomegranate peels by phosphomolybdenum assay [17]. In that study, the sequential fractionation of pomegranate peels in various solvents revealed that the methanol fraction exhibited the highest antioxidant activity (5067.7 ± 34.6 µmol EAC/ g of dry extract) followed by ethanol (3323.0 ± 42.0 µmol EAC/ g of dry extract), acetone (2481.6 ± 17.6 µmol EAC/ g of dry extract) and ethyl acetate (862.8 ± 2.4 µmol EAC/ g of dry extract) fractions, at the tested concentration of 80 µg/ml. All of which were higher than the value found for the ethanol extract in the present study.

Free radical scavenging activity

The DPPH and ABTS frre radicals scavenging activities were determined by the decrease in its absorbance induced by plant antioxidants. Fig. 1 illustrates the DPPH radical trapping capacity of *P. granatum* peels extract and that of trolox used as reference. The scavenging activity of *P. granatum* peels extract on DPPH radical increased in a dose-dependent manner within the concentration range tested. It effective concentration at 50% (EC50) was 4.64± 0.35 µg/ml, which appeared lower than 26.94 % of the standard trolox (3.39± 1.70 µg/ml). Our results agreed with that reported by Belkacem *et al.* [32], they showed that hydromethanolic extract of pomegranate peels had a relative lower antioxidant activity (EC50= 5.59 µg/ml) to quench the DPPH free radical compared with ascorbic acid (1.09 µg/ml) and butylated hydroxyanisol (3.74 µg/mL). However, [38] reported that the ethanolic extract of pomegranate peel had the highest DPPH radical scavenging activity (EC50 of 3 µg/ml) among the eight fruit peel extracts.

In the present investigation, the ABTS method was used to confirm the results from the DPPH test since it is based on a similar antioxidant mechanism. As observed in Fig. 2, the pomegranate peels extract scavenged the ABTS radical in a similar fashion to that observed against DPPH radical, where their inhibitory ability increased proportionally as the concentration of sample increased for concentrations ranging from 01- 08 µg/ml, with an excellent EC50 value of 3.63± 0.07 µg/ml, which was comparable to that obtained for trolox (3.22± 0.07 µg/ml). In contradiction with our results, [25] extracted antioxidants from the peel of this fruit with the use of ethanol and found an EC50 value of 587.36 µg/mL. This is lower than the value obtained (3.63 µg/ ml).

The observed antioxidant potential for of the investigated pomegranate extract might be due to the presence of phenolic bioactive compounds, which probably act synergistically in crude extract against free radicals. This remark supported by our earlier studies, which have suggested that the polyphenols, flavonoids and condensed tannins, either alone or in combination, represent one of the main classes of phenolic compounents responsible for the antioxidant properties of medicinal plants [39]. These phytochemicals compounds consisting of one or more aromatic rings each having one or more hydroxyl groups [40], an ideal chemical structure that determine the antioxidant activity in terms of their reducing properties as hydrogen or electron donating agents, which could be able to delocalize the charge and stabilize the free radicals [41].

Conclusion

On the basis of this data, it is found that the P. granatum peel ethanolic extract is a rich source of phenolic compounds and powerful antioxidant activities, suggest that this waste is an economically viable source of a natural and potent antioxidants. However, further research is required to isolate and characterization of individual phenolic compounds responsible for this activity and also to understand their mode of action, which could provide health benefits to humans and may be exploited in pharmaceutical and cosmetic purposes.

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Tables

Table 1. Extraction yield, total phenolic, flavonoid and condensed tannin contents of hydroethanolic extract of pomegranate fruit peels.

	Extract yields (g/100 g DW)	**Total phenolic** (mg GAE/ g DW)	**Flavonoids** (mg RE/ g DW)	**Condensed tannins** (mg CE/ g DW)
hydroethanolic extract	39.72	170.50± 10.49	85.74± 9.77	8.98± 0.90

Means of three replicates ± SD (standard deviation)

Figures

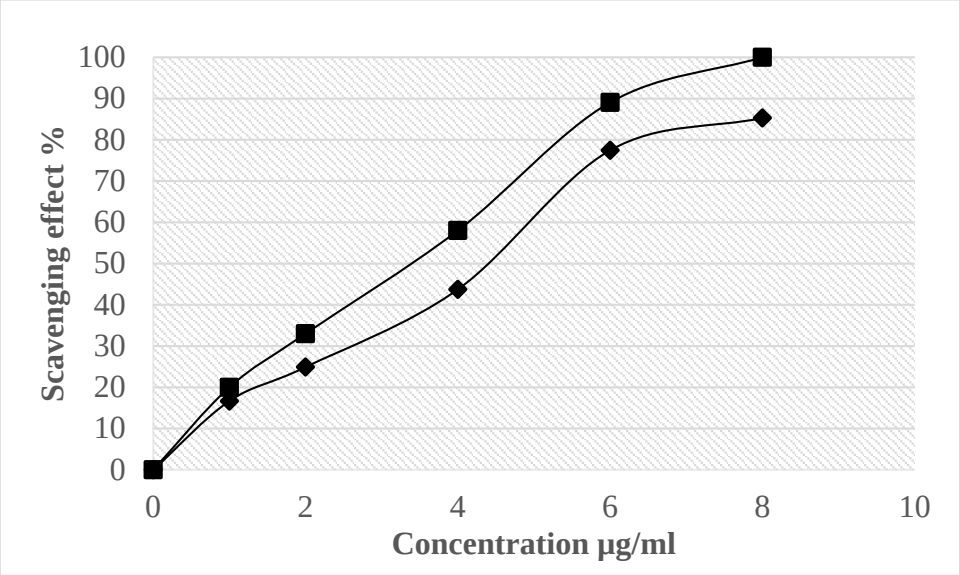

Figure 1 . DPPH radical scavenging activity (%) of pomegranate peel extract (♦) and trolox (■).

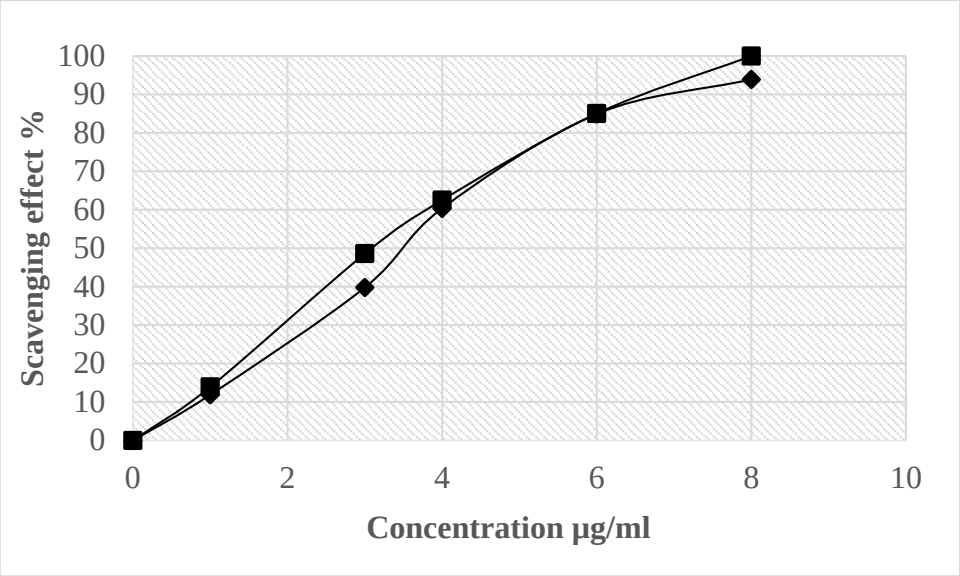

Figure 2 .ABTS radical scavenging activity (%) of pomegranate peel extract (♦) and trolox (■).