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Original Research Article

In vitro Antioxidant, anti-Alzheimer and Antibacterial Activities of Ethyl acetate and n-Butanol Fractions of *Punica granatum* Peel from Algeria

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ABSTRACT

Plants constitute a potential source of high-value compounds which can provide a new source of bioactive agents. The present study was carried out to investigate the content of phenolic compounds, the antioxidant, anticholinesterase and antibacterial activities of the peels of pomegranate collected in eastern Algeria. The hydro-ethanolic extract of pomegranate peels was fractionated, then the ethyl acetate and n-butanol fractions were evaluated for their biological activities. DPPH, ABTS, CUPRAC, phenanthroline and reducing power assays were used to evaluate the antioxidant capacity. The enzyme inhibitory activity of the fractions was evaluated against key enzymes in Alzheimer's disease: acetylcholinesterase and butyrylcholinesterase. Moreover, the antibacterial activity of fractions was tested against four bacterial strains: *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 10876), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923). The results demonstrated that the fractions were a good source of phenolics, flavonoids, flavonols and tannins. Furthermore, the extracts showed the highest antioxidant activity than the standards. The extracts exhibited an interesting inhibitory effect against the tested enzymes. The ethyl acetate fraction showed the highest activity against acetylcholinesterase (IC_{50}: 76.51±3.32 µg/mL) while the n-butanol fraction showed the highest activity against butyrylcholinesterase (IC_{50}: 119.15±5.87 µg/mL). Furthermore, the fractions showed promising antibacterial effects against *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa*. The results showed that pomegranate peels could be considered a good source of bioactive compounds with potent scavenging activity and reducing ability. In addition, pomegranate peels showed great potential as antibacterial and anti-neurodegenerative agents.

***Keywords*:** *Punica granatum*, peels, fractions, antioxidant activity, anticholinesterase activity, antibacterial activity

Introduction

Free radicals are naturally present in living organisms,[1] however, high amounts of free radicals can be harmful to biomolecules, including *e.g.* carbohydrates, proteins, lipids and nucleic acids, leading to tissue damage, cell death or degenerative processes.[2]Oxidative stress plays an important pathologic role inAlzheimer's disease (AD), but it is also a possible therapeutic target.[3] Synthetic antioxidants are used to prevent the damage caused by free radicals[4] despite their toxicological side effects,[5] for that reason, many researchers have studied new antioxidants from natural sources such as polyphenols.[6]

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Polyphenols are secondary plant metabolites and are naturally present in almost all plant materials.[7]

Alzheimer's disease is the most common cause of dementia in the elderly population. Clinically, AD is characterized initially by mild memory impairment that declines progressively to the ultimate loss of mental and physical skills.[8] Acetylcholine (ACH) is a signaling molecule in neurotransmission; one of the few therapeutic approaches for AD is cholinesterase inhibitors. By inhibiting the activity of the enzymes acetylcholinesterase and butyrylcholinesterase, which are involved in acetylcholine hydrolysis, ACH levels at synapses can be increased.[9] Plants are estimated to be a potential source of novel cholinesterase inhibitors.[10]

Microorganisms are ubiquitous and can cause huge health problems in humans.[11]*Staphylococcus aureus* is considered one of the most common sources of food borne diseases, while *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa* produce toxins and other metabolites that induce human gastroenteritis diseases.[12]The pathogens can build resistance against antibiotics,[13,14]furthermore, synthetic antibiotic residues have been polluting the environment and ecosystem survival.[15]Plants are non toxic or with low toxicity,[16] they are regarded as a valuable source of naturally antimicrobial agents.[17]

Phytoconstituents like alkaloids, flavonoids, polyphenols, tannins, and others can be used as resistance modifiers and antimicrobials.[18]

Punica granatum L. (family: Punicaceae), commonly known as pomegranate, is a shrub or a small tree native to Asia and is now widely cultivated in the Mediterranean region, particularly in Algeria.[19] The plant is often less expensive and locally available,[20] and it is used in folkloric medicine against various diseases.[21]A recent study on Algerian pomegranate fruit identified more than 50 phenolic compounds, including many substances that were detected for the first time in the pomegranate fruit extract.[22]Akhtar *et al.*[23] mentioned that the properties of pomegranate are not limited to the edible part of the fruit but also to the non-edible part such as peels, even though they are considered waste. According to previous reports, the pomegranate peels are rich in chemical compounds including phenolic acids (*e.g.* gallic acid, hydroxycaffeic acid, vanillic acid), flavonoids (*e.g.* catechin, epicatechin, quercetin 3-O-rhamnoside), hydrolyzable tannin (*e.g.* ellagic acid, punicalagin, granatin B), anthocyanins (*e.g.*delphinidin-3-O-glucoside, cyanidin-3-O-pentoside) and proanthocyanidin (*e.g.* gallocatechinhexoside, procyanidin dimer).[24] These constituents exhibit a broad range of bioactivities, such as antioxidant, antimicrobial, antiproliferative,[25] anti-diabetic,[26] antidiarrheal,[27] anti-inflammatory,[28] hepatoprotective,[29] antiviral,[30] nematocidal,[31] and immunomodulatory effect.[19]

The present study was undertaken to valorize the natural sources and encourage their use in the food and pharmaceutical industries. The biological activities of the Algerian *Punica granatum* L. peel fractions were investigated in terms of phytochemical content, *in vitro* antioxidant capacity, anticholinesterase effect and antibacterial activity.

Materials and Methods

Chemicals, reagents and apparatus

The chemical products and reagents used were: Folin-ciocalteu reagent, tannic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), trolox, ascorbic acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), trichloroacetic acid, neocuproine, potassium ferricyanide, acetylcholinesterase from electric eel (AChE, type-VI-S, EC 3.1.1.7, 827,84 U/mg), butyrlcholinesterase from horse serum (BChE, EC 3.1.1.8, 7,8 U/mg), acetylthiocholine iodide, butyrylthiocholine chloride, 5.5`-dithiobis (2-nitro-benzoic acid) (DTNB), galantamine, were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Steinheim, Germany). Mueller–Hinton broth (MHB), Mueller–Hinton agar (MHA) were obtained from Liofilchem srl (Italy). Cefoxitin (CX 30 µg/disc) and cefazolin (CZ 30 µg/disc) were obtained from HiMedia laboratories (India). Sodium carbonate, aluminum nitrate, iron (III) chloride ($FeCl_3$), copper (II) chloride, potassium persulfate, were obtained from Biochem Chemopharma. All other chemicals and solvents were of analytical grade.

The equipment used were rotavapor (BÜCHI B-490), oven (Mermmet, Germany), visible spectrophotometer (Jenway, UK) and a 96-well microplate reader (PerkinElmer Multimode Plate Reader EnSpire, USA).

Plant collection

Pomegranate fruits were harvested in May 2017 from Constantine (36°16'03"N 6°30'12"E, Algeria). The identification was done by Dr. Nouioua Wafa, Department of Biology and Plant Ecology, Faculty of Natural and Life Sciences, University Ferhat Abbas Setif 1, Setif,voucher specimen was deposited at the herbarium of the faculty under the number PNG/2/2023. Peels were separated manually from the seeds and air-dried in the shade at room temperature. After drying, the plant material was ground to a fine powder using mortar and pestle.

Preparation of fractions

The hydro-ethanolic extract was obtained from pomegranate peels powder (60 g) by maceration in ethanol:water mixture (70:30; v/v) for 24 hours. The operation was repeated twice on the residues. The three successive extracts were mixed together, filtered and then concentrated under reduced pressure at 45°C to get pomegranate peels hydro-ethanolic extract.[32] The fractionation of the crude extract is carried out using a series of solvents of increasing polarity. The crude extract is first mixed with petroleum ether (v/v) to remove lipids. The aqueous phase was further successively fractionated with diethyl ether, ethyl acetate and then n-butanol, all steps were repeated four times to get finally the aqueous fraction.[33] All fractions were evaporated, dried and stored at 4°C for further experiments. Only ethyl acetate (AP) and n-butanol (BP) fractions were studied in this research. The yield percentage was calculated using the following equation (1):

$$\text{Yield\%} = (WF/PP) \times 100 \quad (1)$$

Where WF is the weight of the dried fraction (g) and PP is the weight of the peel powder (g).

Determination of bioactive compounds

Determination of total phenolics content

The Folin–Ciocalteu colorimetric method of Singleton and Rossi[34] was used to determine the total phenolics content in extracts, with slight changes according to Müller *et al*.[35]. A volume of 20 µL of extract was mixed with 100 µL Folin–Ciocalteu reagent (diluted 1/10) and 75 µL sodium carbonate (7.5%). After incubation at room temperature and darkness for 120 mins, the absorbance was measured at 765 nm. The total phenolics content was determined from a standard curve of gallic acid, and the results were expressed as µg gallic acid equivalent (GAE)/mg extract.

Determination of flavonoids content

The method of Topçu *et al*.[36] was used to determine the flavonoids content in extracts, with slight modifications. A volume of 50 µL of extract was mixed with 130 µL of methanol, 10 µL of aluminium nitrate (10%) and 10 µL of potassium acetate (1M). After 40 mins at room temperature, the absorbance was measured at 415 nm. The flavonoids content was determined from a standard curve of quercetin, and the results were expressed as µg quercetin equivalent (QE)/mg extract.

Determination of flavonols content

The method of Miliauskas *et al*.[37] was used to determine the flavonols content in extracts. A volume of 400 of µL extract was mixed with 400 µL of aluminum trichloride (2%) and 1.2 mL of sodium acetate (5%). After 150 mins at 20°C, the absorbance was measured at 440 nm. The flavonols content was determined from a standard curve of quercetin, and the results were expressed as µg quercetin equivalent (QE)/mg extract.

Determination of tannins content

The method of Medpilwar *et al*.[38] was used to determine the tannins content in extracts. A volume of 100 µL of extract was mixed with 20 µL of Folin-Ciocalteu reagent (0.5 N) and left at room temperature for 15 mins. 500 µL of saturated sodium carbonate (20%) was added. After 30 mins at room temperature, the absorbance was measured at 760 nm. The tannins content was determined from a standard curve of tannic acid, and the results were expressed as µg tannic acid equivalent (TAE)/mg extract.

Evaluation of the antioxidant activity

DPPH radical scavenging assay

The method of Blois[39] was based in measuring the DPPH radical scavenging activity. A volume of 40 µL of extract was added to 160 µL of DPPH solution (0.1 mM). Trolox and ascorbic acid were used as antioxidant standards. The absorbance of the resulting solutions was measured at 517 nm after 30 mins of incubation in the dark at room temperature. The antiradical activity was calculated as percentage of inhibition (I%) using the following equation (2):

$$I\% = [(A_{Control} - A_{Extract})/A_{Control}] \times 100 \quad (2)$$

Where $A_{Control}$ is the absorbance of the control without the extract and $A_{Extract}$ is the absorbance in the presence of the extract. The results were expressed as the half inhibitory concentration (IC_{50}) (µg/mL) corresponding to the concentration of 50% inhibition. The lower IC_{50} value indicates higher radical scavenging activity.

ABTS radical scavenging assay
The method of Re *et al*.[40] was followed in the scavenging capability of ABTS radical, with slight modifications. A volume of 160 µL of ABTS solution was added to 40 µL of extract. Trolox and ascorbic acid were used as antioxidant standards. The mixtures were left at ambient temperature for 10 mins and then the absorbance was measured at 734 nm. The percentage of inhibition (I%) of ABTS radical was calculated using the equation (2). The results were expressed as IC_{50} values.
Cupric ion reducing antioxidant capacity assay (CUPRAC)
The method of Apak *et al*.[41] was used in the determination of the cupric-reducing antioxidant capacity, with minor changes as described by Tel *et al*.[42]. A volume of 50 µL of copper (II) chloride (10 mM), 50 µL of neocuproine (7.5 mM) and 60 µL of ammoniumacetate buffer (1 M, pH 7.0) were mixed. A volume of 40 µL of the extract was then added to the mixture. After 60 mins, the absorbance was measured at 450 nm. The results were given as $C_{A0.5}$ corresponding to the concentration (µg/mL) indicating 0.5 absorbance. Trolox and ascorbic acid were used as antioxidant standards.

Phenanthroline assay
The method of Szydlowska-Czerniak *et al*.[43] was followed in phenanthroline assay with minor changes. A volume of 10 µL of extract, 50 µL of $FeCl_3$ (0.2%) and 30 µL of 1,10-phenanthroline (0.5%) were placed. The volume of the reaction mixture was brought to 200 µL by adding ethanol. After 20 mins at 30°C, the absorbance was measured at 510 nm. The results were given as $C_{A0.5}$; trolox and ascorbic acid were used as antioxidant standards.

Reducing power assay
The method of Oyaizu[44] was applied to evaluate the reducing antioxidant power with slight modifications. A volume of 10 µL of extract was mixed with 40 µL of phosphate buffer (0.2 M, pH 6.6) and 50 µL of potassium ferricyanide (1%). After allowing the mixture to incubate for 20 mins at 50°C, 50 µL of trichloroacetic acid (10%), 40 µL of distilled water and 10 µL of ferric chloride (0.1%) were added to the mixture. The absorbance of the resulting solution was measured at 700 nm. The results were given as $C_{A0.5}$; trolox and ascorbic acid were used as antioxidant standards.

Evaluation of the anti-Alzheimer activity
The method of Ellman *et al*.[45] was applied to determine the anti-Alzheimer activity of the extracts, as acetylcholinesterase and butyrylcholinesterase inhibitors. A volume of 150 µL of sodium phosphate buffer (0,1 M, pH 8.0), 10 µL of extract and 20 µL of AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) solution were prepared and incubated for 15 mins at 37 °C. Afterwards, 10 µL of DTNB (0.5 mM) and 10 µL of acetylthiocholine iodide (0.71 mM) or 10 µL of butyrylthiocholine chloride (0.2 mM) were added. The absorbance at 412 nm was recorded at zero-time and after incubation for 15 mins at 37°C. Galantamine was used as reference. The percentage of inhibition (I%) of the AChE or BChE was determined using the following equation (3):

$$I\ \% = [(E-S) / E] \times 100 \quad (3)$$

Where E is the activity of the enzyme without extract or reference, and S is the activity of the enzyme with extract or reference. The results were expressed as IC_{50}.

Evaluation of the antibacterial activity
In order to determine the antibacterial activity of both extracts, various Gram-positive (*Bacillus cereus* (ATCC 10876) and *Staphylococcus aureus* (ATCC 25923)) and Gram-negative bacteria (*Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853)) were used.

Agar diffusion method
The agar disc diffusion method was employed for the determination of antibacterial activities of extracts.[46] The Gram positive bacteria (*B. cereus* and *S. aureus*) and Gram negative bacteria (*E. coli* and *P. aeruginosa*) were cultured in MHB for 24 hours at 37°C and an inoculum of each bacterial strain was prepared. The suspensions were adjusted to the absorbance 0.08 at 625 nm and uniformly spread on MHA medium in Petri plates**.** The dried extracts were dissolved in ethanol 96% or in sterile distilled water to a final concentration of 100 mg/mL. A discs of 5 mm in diameter of sterile filter paper were soaked in solution extracts, allowed to dry completely for 20 mins, and then placed **aseptically** on the surface of previouslyinoculated cultures. The plates were left at room temperature for 30 mins to allow the diffusion of extracts in the media and then incubated for 24 hours at 37 °C. Ethanol 96% and sterile distilled water were used as negative controls, while CX 30 µg/disc and CZ 30 µg/disc were used as positive controls. Antibacterial activities were evaluated by measuring the inhibition zone diameters (in millimetres including disc diameter) produced by the extracts against tested microorganisms.

Determination of the minimal inhibitory concentration and the minimal bactericidal concentration
Minimum inhibitory concentrations (MICs) of the extracts for antibacterial activity were determined using the microdilution bioassay as described by Eloff[47]with minor changes.
The inoculums from the bacterial strains were prepared in MHB and incubated for 24 hours at 37 °C, and the suspensions were diluted to obtain an absorbance of 0.08 at 625 nm. The extracts were dissolved at 100 mg/mL in ethanol 96% or sterile distilled water. 50 µL of extract were serially diluted two-fold with MHB in a 96-well microtiter plate. A volume of 50 µL of each bacterial culture were added to each well. One inoculated well was included to enable control of broth adequacy for organism growth. One non-inoculated well, free of antimicrobial agents, was added to ensure sterility of the culture medium. The extract sterility was also controlled.[48]The plates were then covered and incubated at 37 °C for 24 hours. The bacterial growth was indicated by the presence of a pellet on the well bottom. The MIC was defined as the lowest concentration (mg/mL) of the extract preventing visible *growth*.[12]
The minimal bactericidal concentrations (MBCs) were determined according to Mostafa *et al*.[12] method, with some modifications. Samples were taken from the concentrations of the plant extracts exhibiting invisible growth (from wells of MIC microtiter plates) and were subcultured onto sterile MHA plates. The plates were incubated at 37°C for 24 hours and then examined for bacterial growth in corresponding to plant extracts concentration. MBC was defined as the lowest concentration that did not exhibit any bacterial growth on the inoculated agar plates.

Statistical analysis
The experimental results were expressed as the mean ± standard deviation (SD). The data were subjected to one-way analysis of variance (ANOVA) and the significance of differences between means was calculated by Tukey's multiple comparison test using GraphPad Prism 5 and the significance was accepted at $p<0.05$.

Results and Discussion

In the present work, two fractions obtained from *Punica granatum* peels crude extract were assessed for their total phenolics, flavonoids, flavonols and tannins contents, and their antioxidant activity using five *in vitro* tests: free-radical scavenging (DPPH, ABTS), CUPRAC, phenanthroline and reducing power assays. The anticholinesterase as well as the antibacterial activities were also carried out.

Extraction yield
The extraction yield was calculated for both fractions of *Punica granatum* peels and presented in Table 1. The highest yield was gained in n-butanol fraction (11.83 %) followed by ethyl acetate fraction (8.2 %). In our extraction method, we fractionated the pomegranate peels hydro-ethanolic extract using a series of solvents of increasing polarity, the n-butanol fraction, which was the most polar fraction, gave a high yield. According to the values obtained in this study, the yields of fractions ethyl acetate and n-butanol were higher than those obtained by Šavikin *et al*.[49]The differences in yields could be related to the extraction method performed,[50] climatic and growth locations/conditions, maturity, and other crucial factors.[18]

Bioactive compounds

Polyphenols are ubiquitous molecules in plants. They differ from simple compounds such as phenolic acids to very complicated substances such as tannins.[51] The quantification of total phenolics, flavonoids, flavonols and tannins in both fractions acquired from the hydro-ethanolic extract of the pomegranate peels is presented in Table 1. With regard to the results, polyphenols content of AP showed the highest concentration (753.89±22.07 µg GAE/mg) followed by BP (677.55±27.39 µg GAE/mg) and the highest flavonoids content was recorded in BP (93.48±3.7 µg QE/mg) followed by AP (70.64±2.99 µg QE/mg). Also, the BP was recorded the highest flavonols amount with 21.84±0.00 µg QE/mg followed by AP (8.72±0.22 µg QE/mg). The results showed that both fractions, AP and BP, contained almost the same concentration of tannins (107.66±3.03 and 106.95±0.4 µg TAE/mg, respectively).

Several factors, such as the extraction time, pH,[52] solvent polarity[53] and temperature,[54] can affect the amount of extracted phenolics. In this study, the use of different solvent polarities in the extraction of phytocompounds may explain the variation in phenols, flavonoids, flavonols and tannins quantities between pomegranate peel fractions. Regarding the content of total phenols, our results were higher than those obtained by Belkacem *et al*.[55] and Rosas-burgos *et al*.[56]. In previous studies, Kachkoul *et al*.[57] quantified flavonoid and flavonol contents in the hydro-ethanolic extract of pomegranate peels, and found concentrations of 36.05±2.95 mg QE/g and 7.17±0.49 mg QE/g for flavonoids and flavonols, respectively, also,Amjad and Shafighi[58] found flavonoids values of 258.127±16.19 and 359.6±13.91 mgCA/100 g dw in n-butanol and ethyl acetate extracts of pomegranate flowers, respectively. From the aforementioned results, it appears that the ethyl acetate and n-butanol fractions contained high amounts of tannins. It has revealed that pomegranate peels are a rich source of ellagitannins.[59,60]

Antioxidant activity

The antioxidant activity of pomegranate peel fractions was assessed using various methods based on different principles, because using multiple tests is recommended for displaying the activities of diverse antioxidants.[35] The results are summarized in Table 2, expressed in terms of IC_{50} and $C_{A0.5}$, and demonstrated that the fractions AP and BP contain substances that prevent oxidation in low concentrations and react more efficiently than the antioxidant standards utilized in this work. In DPPH radical scavenging activity, the results revealed that *P. granatum* peel fractions exhibited higher activity compared to the standards, ascorbic acid and trolox (IC_{50}: 4.39±0.01; 5.12±0.21µg/mL, respectively), where the AP (IC_{50}: 3.69±0.11µg/mL) was the most potent, followed by the BP (IC_{50}: 4.07±0.07 µg/mL). There was no significant difference between BP and ascorbic acid (p<0.05). In comparison to the other scavenging assay, the ABTS radical scavenging assay was relatively a sensitive model because of the efficient IC_{50} values obtained. The analysis data of the ABTS assay revealed that the peel fractions were significantly higher than ascorbic acid and trolox (IC_{50}: 3.03±0.04; 3.20±0.05 µg/mL, respectively) (p<0.05), where the AP exhibited the strongest activity with an IC_{50}: 1.25±0.03 µg/mL followed by the BP with an IC_{50}: 1.50±0.08 µg/mL.

In the CUPRAC assay, the results showed that the used standards (ascorbic acid (8.31±0.15 µg/mL) and trolox (8.69±0.14 µg/mL)) were significantly less active than fractions AP and BP ($C_{A0.5}$: 2.59±0.20 and 4.33±0.22 µg/mL, respectively) at p<0.05. Comparable results were obtained from the phenanthroline assay, as seen in Table 2, both fractions reacted better than the standards. The results revealed that the AP was the most efficient with $C_{A0.5}$: 1.78±0.08 µg/mL followed by the BP with $C_{A0.5}$:1.88±0.20 µg/mL, which were significantly higher than those of ascorbic acid and trolox ($C_{A0.5}$: 3.08±0.02; 5.21±0.27 µg/mL, respectively) at p<0.05. From the results of the reducing power assay, the highest antioxidant activity was recorded in AP ($C_{A0.5}$: 3.41±0.08 µg/mL), which was not significantly different from ascorbic acid ($C_{A0.5}$: 3.62±0.28 µg/mL) (p<0.05), followed by BP and then trolox ($C_{A0.5}$: 4.29±0.29; 5.24±0.20 µg/mL, respectively). In previous works on Moroccan pomegranate peel crude extracts, they investigated the antioxidant activities by DPPH and ABTS tests and found values ranging between 42.71±0.04-65.55±0.01 and 62.15±0.01-85.32±0.08 µg/mL, in both assays respectively,[60]in addition, by using the DPPH and the reducing power assays, they found results of 60.87±0.27 and 42.17±7.46 µg/mL, respectively.[57] Besides, Pagliarulo *et al*.[61] proved by RP-HPLC that punicalagin isomers are the most abundant ellagitannins in pomegranate peels, and Aguilar-Zárate *et al*.[62] examined the antioxidant activity of purified punicalagin using DPPH and ABTS radicals. They found IC_{50}: 109.53 µg/mL and 151.50 µg/mL for both assays, respectively, which are weaker than the results found in our study, this can be explained by a synergistic effect between the antioxidants.[63] On the other hand, AP and BP have high cupric and iron ions reducing abilities. Plant phenols play an important role as antioxidant compounds; they possess multifunctional properties and can act as anti-reactive oxygen species, singlet oxygen scavengers, reducing agents, and hydrogen atom donors.[52]

Anti-Alzheimer activity

Cholinesterase inhibitors have been used for the treatment of Alzheimer's disease by increasing acetylcholine levels.[64,65]The AChE and BChE inhibitory activities of pomegranatepeel fractions were tested and compared with galantamine. As shown in Table 3, the AP demonstrated the highest inhibitory effect against AChE (IC_{50}: 76.51±3.32 µg/mL) compared to the BP (IC_{50}: 150.17±5.00 µg/mL).

Table 1: Yield, total phenolics, flavonoids, flavonols and tannins contents of AP and BP fractions of *Punica granatum* peels

Fractions	Yield (%)	Total phenolics (µgGAE/mg)	Flavonoids (µgQE/mg)	Flavonols (µgQE/mg)	Tannins (µgTAE/mg)
AP	8.2	753.89 ± 22.07	70.64 ± 2.99	8.72 ± 0.22	107.66 ± 3.03
BP	11.83	677.55 ± 27.39	93.48 ± 3.7	21.84 ± 0	106.95 ± 0.4

AP: ethyl acetate fraction; BP: n-butanol fraction. Data are presented as mean ± SD.

Table 2: Antioxidant activity of AP and BP fractions of *Punica granatum* peels

Samples	DPPH assay	ABTS assay	CUPRAC assay	Phenanthroline assay	Reducing power
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	$C_{A0.5}$ (µg/mL)	$C_{A0.5}$ (µg/mL)	$C_{A0.5}$ (µg/mL)
AP	3.69 ± 0.11^{a}	1.25 ± 0.03^{a}	2.59 ± 0.20^{a}	1.78 ± 0.08^{a}	3.41 ± 0.08^{a}
BP	4.07 ± 0.07^{b}	1.50 ± 0.08^{b}	4.33 ± 0.22^{b}	1.88 ± 0.20^{a}	4.29 ± 0.29^{b}
Ascorbic acid*	4.39 ± 0.01^{b}	3.03 ± 0.04^{c}	8.31 ± 0.15^{c}	3.08 ± 0.02^{b}	3.62 ± 0.28^{a}
Trolox*	5.12 ± 0.21^{c}	3.20 ± 0.05^{d}	8.69 ± 0.14^{c}	5.21 ± 0.27^{c}	5.24 ± 0.20^{c}

AP: ethyl acetate fraction; BP: n-butanol fraction; *:standard. Data are presented as mean ± SD. Means in the same column followed by different letters (^{a}, ^{b}, ^{c}) differ significantly at p<0.05.

Moreover, both fractions showed an inhibitory effect against BChE, where the BP was the strongest (IC_{50}: 119.15±5.87 µg/mL) followed by the AP (IC_{50}: 143.30±24.18 µg/mL). Statistical analysis revealed significant variation at $p<0.05$ between the fractions and galantamine (IC_{50}: 6.27±1.15 µg/mL and 34.75±1.99 µg/mL against AChE and BChE, respectively). The fractions showed a good effect against AChE and BChE, the obtained results are in disagreement with those of earlier studies; which found that non-polar ethyl acetate and n-butanol extracts of pomegranate peel had lower AChE inhibition. They reported that the inhibition percentages reached maximum activity at 500 µg/mL with 12.63±2.51 and 23.26±4.70% for ethyl acetate and n-butanol extracts, respectively.[49] Also, the non-polar dichloromethane and hexane extracts of pomegranate leaves did not show an effect against AChE and BChE (IC_{50}>250 mg/l).[66] In this study, anticholinesterase activity may be due to the phytochemical composition of the fractions and their antioxidant capacities. It was mentioned by Xia *et al*.[67]that phenolic compounds have beneficial effects on health and have positive impact on the prevention of several degenerative diseases. In addition, due to their antioxidant effect they could modify the body negative mechanism of redox status and prevent organs and tissues from oxidative damage.[67]

Antibacterial activity

The antibacterial activity of fractions of *Punica granatum* peels was investigated against two strains of Gram-positive bacteria (*B. cereus* and *S. aureus*) and two strains of Gram-negative bacteria (*E. coli* and *P. aeruginosa*) using qualitative and quantitative methods. The results are presented in Tables 4 and 5. The fractions showed variable inhibitory activity against all the tested bacteria (Gram positive and Gram negative). At $p<0.05$, the AP and BP showed significant antibacterial activity compared to the references against *S. aureus* with inhibition zone diameters of 25 and 23±1.41 mm, respectively. We also found that *B. cereus* was sensitive to both fractions, but this inhibition was significantly lower than CX30 and CZ30. Furthermore, the assayed extracts indicated no effect against *E. coli* strain. On the other hand, the results showed no activity of AP against *P. aeruginosa*, whereas the BP was significantly active (21.5±0.70 mm zone of inhibition) in comparison with the references ($p<0.05$). The solvents used as negative controls showed no inhibition zone. In this study, the agar diffusion method indicated the highest sensitivity of Gram positive bacteria than Gram negative against the fractions; this result is in accordance with several studies.[56,18] Due to the presence of an outer lipopolysaccharide membrane, Gram negative bacteria are more resistant to antimicrobial agents.[68] The MIC values varied from 0.39 to 12.5 mg/mL and MBC values varied from 25 to 100 mg/mL (Table 5). *B. cereus* was the most susceptible bacterial species to ethyl acetate fraction followed by *S. aureus* with MIC values of 0.39 and 12.5 mg/mL, respectively. Furthermore, *B. cereus* was also the most susceptible bacterial species to n-butanol fraction followed by *P. aeruginosa* and *S. aureus* with MIC values of 1.56, 3.12 and 12.5 mg/mL, respectively. Concerning the bactericidal activity (Table 5), the AP had an effect on *B. cereus* and *S. aureus*, with MBC values of 25 and 50 mg/mL, respectively. Besides, the BP had an effect on *B. cereus*, *S. aureus* and *P. aeruginosa* with MBC values of 50, 100 and 100 mg/mL, respectively. In previous study, Debib *et al*.[69] showed that petroleum ether, ethanolic and acetonic peel extracts inhibited the growth of *E. coli* with inhibition zones ranging from 14 to 28 mm and MIC values ranging from 8 to 256 µg/mL; in contrast, our fractions did not show any effect against this bacteria. The activity of pomegranate peels against microbes can be attributed to its high polyphenolic content, particularly its flavonoids and tannins.[70] One of the anti-infective properties of tannins is causing membrane instability,[56]and for flavonoids is forming complexes with various proteins found inside the bacterial cell walls or extracellular proteins,[68]consequently, causing leakage of the cell membrane and cell lysis, which ultimately leads to microorganism death.[69]The results above indicate the richness of AP and BP in tannins and flavonoids, in addition to the efficient capacities of extracts as antioxidants, which may explain their antibacterial effect (inhibition zone: 13-25 mm, MIC: 0.39-12.5 mg/mL and MBC: 25-100 mg/mL). The good activity of BP against *P. aeruginosa* may be interpreted by the higher content of flavonoids and flavonols compared to the ethyl acetate fraction.

Table 3: Cholinesterase inhibitory effect of AP and BP fractions of *Punica granatum* peels

	Anti-acetylcholinesterase	Anti-butyrylcholinesterase
Samples	**IC_{50} (µg/mL)**	
AP	76.51 ± 3.32^{b}	143.30 ± 24.18^{b}
BP	150.17 ± 5.00^{c}	119.15 ± 5.87^{b}
Galantamine*	6.27 ± 1.15^{a}	34.75 ± 1.99^{a}

AP: ethyl acetate fraction; BP: n-butanol fraction; *: standard. Data are presented as mean ± SD. Means in the same column followed by different letters (^{a}, ^{b}, ^{c}) differ significantly at $p<0.05$.

Table 4: Antibacterial activity of *Punica granatum* peel fractions using agar diffusion method

	Inhibition zone (mm)			
	Bacterial strains			
Samples	***E. coli***	***P. aeruginosa***	***B. cereus***	***S. aureus***
AP	-	-	13.5 ± 0.70^{c}	25^{a}
BP	-	21.5 ± 0.70^{a}	13^{d}	23 ± 1.41^{b}
CX30*	19	21^{b}	20^{b}	21^{c}
CZ30*	19	21^{b}	38^{a}	20^{d}
H_2O^{**}	-	-	-	-
Ethanol 96%**	-	-	-	-

AP: ethyl acetate fraction; BP: n-butanol fraction; CX: Cefoxitin; CZ: Cefazolin; *: positive control; **: negative control. -: no inhibition. Data are presented as mean ± SD. Means in the same column followed by different letters (^{a}, ^{b}, ^{c}, ^{d}) differ significantly at $p<0.05$.

Table 5: Minimal inhibitory concentrations and minimal bactericidal concentrations of *Punica granatum* peels fractions

	MIC (mg/mL)		MBC (mg/mL)	
	Fractions			
Bacterial strains	AP	BP	AP	BP
E. coli	-	-	-	-
P. aeruginosa	-	3.125	-	100
B. cereus	0.39	1.562	25	50
S. aureus	12.5	12.5	50	100

AP: ethyl acetate fraction; BP: n-butanol fraction. -: not tested. MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration

Conclusion

The present findings showed that the ethyl acetate and n-butanol fractions derived from the hydro-ethanolic extract of Algerian pomegranate peel possess promising natural sources as bioactive components with good antibacterial, anticholinesterase activities and highly potent antioxidant properties, acting as free radical scavengers and reducing agents. Further *in vitro* and *in vivo* studies are needed to isolate and identify the bioactive molecules from Algerian pomegranate peel, especially those with anticholinesterase activity, which could be exploited to obtain pharmaceuticals and help combat neurodegenerative disorders. Furthermore, the antibacterial activity of these compounds from pomegranate peel can provide good preservation in the food industry.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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