

Original Research

In Vitro Antioxidant and Phytochemical Evaluation of Methanolic Extracts from *Punica granatum* Peels in Northwestern Algeria

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Abstract

Natural antioxidants are becoming increasingly popular because of their importance in preventing and treating many diseases. This study investigates the antioxidant properties and chemical composition of methanolic extracts derived from the fruit peels of *Punica granatum* collected in Mostaganem, Algeria. Maceration was used for methanolic extraction of the dry peel powder, followed by both quantitative and qualitative chemical analyses. The antioxidant capacity of the extract was evaluated using three distinct assays: DPPH, ABTS, and FRAP. The results revealed a rich profile of bioactive compounds, including total phenolic content (187.13 ± 1.92 mg GAE/g dry extract), total flavonoid content (79.51 ± 0.83 mg QE/g dry extract), and total tannin content (48.09 ± 0.63 mg CE/g dry extract). Furthermore, the extract exhibited significant antioxidant activity, with IC₅₀ values of 42.52 µg/mL for the DPPH assay, 47.49 µg/mL for ABTS, and 59.5 µg/mL for FRAP. Thus, very high antioxidant activity ratios were recorded, which were also statistically consistent with vitamin C and trolox at the highest concentration (200 µg/mL). These findings highlight the potential of *Punica granatum* fruit peels as a valuable source of natural antioxidants, contributing to the prevention of oxidative stress-related diseases. Given their notable biological activity, pomegranate peels warrant careful consideration for incorporation into functional foods and dietary supplements, offering promising avenues for enhancing health.

Keywords

antioxidant activity, *Punica granatum*, phytochemical screening, methanolic extract, IC₅₀, fruit peels, and phenolic compounds.

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Vrednotenje antioksidativne aktivnosti in fitokemični pregled metanolnih izvlečkov iz lupin sadežev vrste *Punica granatum*, nabranih v severozahodni Alžiriji

Izvleček

Naravni antioksidanti postajajo vse bolj priljubljeni zaradi svojega pomena pri preprečevanju in zdravljenju številnih bolezni. Ta raziskava proučuje antioksidativne lastnosti in kemično sestavo metanolnih izvlečkov, pridobljenih iz lupin sadežev vrste *Punica granatum*, zbranih v regiji Mostaganem v Alžiriji. Metanolna ekstrakcija suhih lupin v prahu je potekala preko maceracije, temu pa so sledile kvantitativne in kvalitativne kemijske analize. S tremi različnimi analizami, DPPH, ABTS in FRAP, je bila ocenjena antioksidativna zmogljivost izvlečka. Rezultati so razkrili bogat profil bioaktivnih spojin, vključno s skupno vsebnostjo fenolov ($187,13 \pm 1,92$ mg GAE/g suhega ekstrakta), skupno vsebnostjo flavonoidov ($79,51 \pm 0,83$ mg QE/g suhega ekstrakta) in skupno vsebnostjo taninov ($48,09 \pm 0,63$ mg CE/g suhega ekstrakta). Poleg tega je izvleček pokazal pomembno antioksidativno aktivnost z vrednostmi IC₅₀ 42,52 µg/ml za analizo DPPH, 47,49 µg/ml za ABTS in 59,5 µg/ml za FRAP. Pri najvišji koncentraciji (200 µg/mL) so bila zabeležena zelo visoka razmerja antioksidativne aktivnosti, ki so bila statistično skladna z vitaminom C in troloxom. Te ugotovitve poudarjajo potencial lupin sadežev vrste *Punica granatum* kot dragocenega vira naravnih antioksidantov, ki prispevajo k preprečevanju bolezni, povezanih z oksidativnim stresom. Glede na njihovo pomembno biološko aktivnost bi bilo potrebno lupine granatnega jabolka skrbno proučiti glede vključitve med funkcionalna živila in prehranska dopolnila, saj ponujajo obetavne možnosti krepitev zdravja.

Ključne besede

antioksidativna aktivnost, *Punica granatum*, fitokemični pregled, metanolni ekstrakt, IC₅₀, lupine plodov, fenolne spojine

Introduction

Punica granatum (family *Lythraceae*), commonly known as pomegranate, is a deciduous shrub or small tree that can attain heights between 5 and 10 meters (Khan et al., 2020; Mendonca, 2020). This species thrives in subtropical and tropical climates, is prevalent in the Mediterranean region, and has been cultivated for over 4,000 years (Malik et al., 2020; Pfadenhauer et al., 2020; Nair et al., 2021). The pomegranate holds significant cultural and economic value, serving as a staple in various culinary traditions while symbolizing fertility and abundance across numerous cultures (Giesecke, 2023; Ramsay et al., 2023). The fruit is characterized by its distinctive red hue and sweet-tart flavour, comprising numerous edible seeds (arils) that are enclosed within a hard, thick rind (Mandal et al., 2021). Phytochemically, pomegranate is rich in polyphenols, particularly flavonoids and tannins, which are concentrated in both seeds and peels (Yassin et al., 2021; Yisimayili and Chao, 2022). Recent research has indicated that peels contain higher levels of certain bioactive compounds compared to the arils, contributing to their potential health benefits.

These compounds exhibit a range of biological activities, including anti-inflammatory, antimicrobial, and anticancer properties (Kahlaoui, 2021). The utilization of pomegranate peels, often discarded as waste, presents an opportunity for valorization in the food, cosmetic, and pharmaceutical industries (Jimenez-Lopez et al., 2020; Nirmal et al., 2023).

Antioxidants play a crucial role in preserving cellular integrity by counteracting reactive oxygen species (ROS) and mitigating oxidative damage (Dumanović et al., 2021; Houldsworth, 2024). Oxidative stress occurs when there is excessive production of ROS that exceeds the body's antioxidant defences. This discord can result in cellular injury, which can be seen in the development of a variety of conditions, including cancer, cardiovascular diseases, diabetes, and neurodegenerative disorders, such as Alzheimer's disease (Demirci-Cekic et al., 2022). Natural antioxidants, especially those sourced from fruits and vegetables, have garnered attention due to their potential health benefits and safety profiles compared to synthetic alternatives (Gulcin, 2020; Wu et al., 2024). Compounds, such as flavonoids, phenolic acids, and vitamins (vitamins C and E), have demonstrated the ability to scavenge free radicals and

chelate metal ions, thereby preventing oxidative damage (Gulcin, 2020). Due to the growing consumer demand for natural products as a result of the expanding culture of bio-food consumption, exploring the antioxidant properties of plant extracts like those from *Punica granatum* fruit peels could lead to innovative applications in functional foods and dietary supplements (Benchagra et al., 2021).

The objective of this study is to investigate the chemical composition and antioxidant activity of methanolic extracts from *Punica granatum* fruit peels harvested from the Mostaganem region of northwestern Algeria. It includes qualitative identification of bioactive compounds; quantitative analysis of major phytochemicals, total phenolic content, flavonoids, and tannins using standard spectrophotometric methods; and evaluation of antioxidant potential through assays, such as DPPH radical scavenging, ABTS, and FRAP. By detailing the chemical profiles and biological properties of the *Punica granatum* peel extracts, this research seeks to enhance the understanding of their health benefits and promote their utilization, thereby increasing the value of this often overlooked agricultural by-product.

Materials and Methods

Sample Collection

P. granatum peels were collected from fully ripened fruits of pomegranate sourced from the Mostaganem region in northwestern Algeria (the coordinates are approximately 35.8° N latitude and 0.1° E longitude) (Fig. 1), which is characterized by a mild Mediterranean climate with moderate humidity, temperature and radiation, as well as soil fertility that is an essential source for nutrition, plant growth and the production of natural bio-active compounds.

The samples were harvested during the peak harvest season, in the second half of October 2023, to ensure optimal phytochemical composition. After collection, peels were carefully separated from the fruit (Fig. 2), washed thoroughly with distilled water to remove any contaminants, and air-dried in a dark, well-ventilated room, with a temperature not exceeding 40°C to prevent degradation of sensitive compounds. The dried husks were then ground into a fine powder using an electronic grinder and stored in

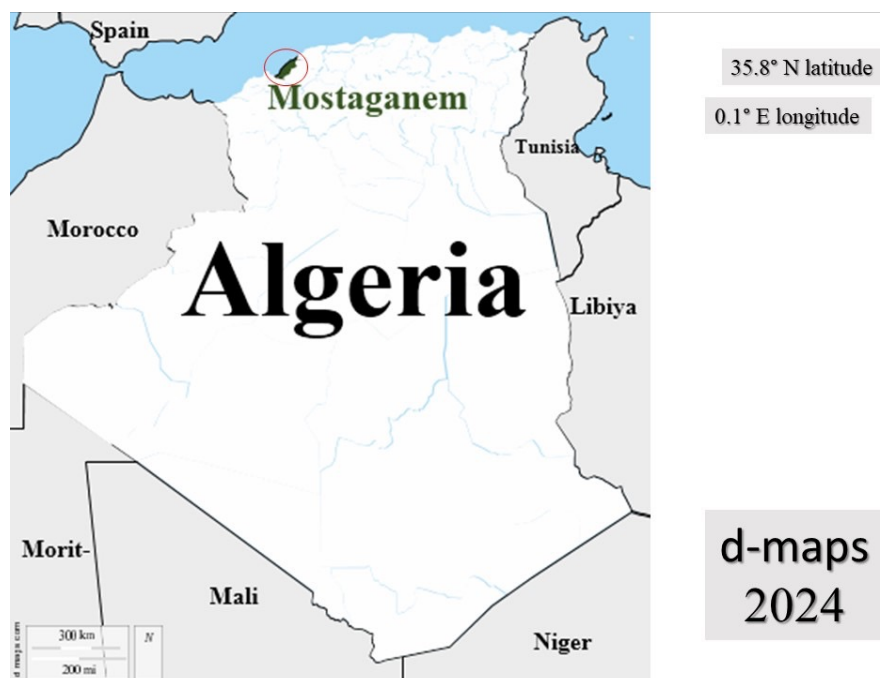


Figure 1. Geolocation of the pomegranate fruit collection area (Mostaganem, Algeria).

Slika 1. Geolokacija območja zbiranja plodov granatnega jabolka (Mostaganem, Alžirija).

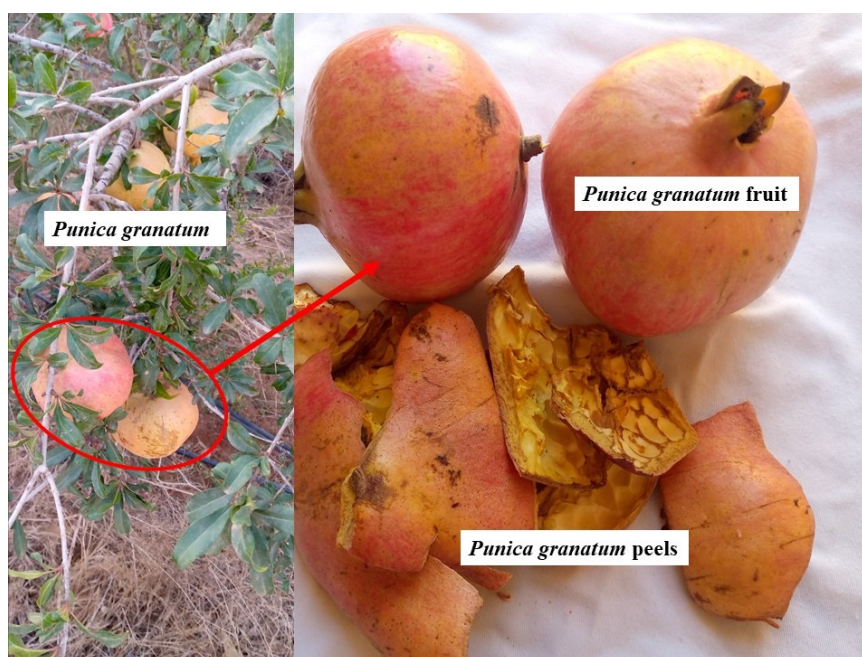


Figure 2. The *Punica granatum* fruit peels.

Slika 2. Lupine plodov *Punica granatum*.

dark, airtight glass containers at room temperature until further analysis. All sample handling is conducted by following standard laboratory protocols to minimize contamination and preserve the integrity of the extracts (Nida et al., 2021; Banza and Rutto, 2022).

Preparation of Methanolic Extract

The methanolic extracts of *P. granatum* fruit peels were prepared using the maceration method. 50 g of the dried and powdered fruit peels were placed in a clean, dry glass container and mixed with 250 mL of analytical-grade methanol. The mixture was allowed to macerate at room temperature for 72 hours, with periodic agitation every 12 hours to enhance the extraction efficiency. After the maceration period, the mixture was filtered through Whatman (N° 1) filter paper to separate the liquid extract from the solid residues. The resulting filtrate was then concentrated using a rotary evaporator at a temperature not exceeding 40°C to eliminate the solvent. The concentrated extract was subsequently transferred to pre-weighed vials and stored

at -20°C until further analysis (Samsuri et al., 2020; Yassin et al., 2021). In addition to minimizing thermal degradation, the maceration method was also chosen to maximize the extraction of bioactive compounds since the methanol molecule has the smallest molecular mass among alcohols, and it is richer in hydroxyls and more connected to the metabolite (Mali and Kumar, 2023; PM et al., 2024).

To determine the methanolic extract yield from *P. granatum* fruit peels, the following formula was followed:

Yield (% w/w) = $(W_{\text{initial}} / W_{\text{extract}}) * 100$, where w indicates weight with the same unit (g) (Samsuri et al., 2020; Yassin et al., 2021).

Phytochemical Screening

The qualitative chemical analysis of the methanolic extract of *P. granatum* fruit peels was performed through a series of standardized phytochemical screening tests to identify the presence of various bioactive compounds. The following classes of phytochemicals were examined (Sutoyo et al., 2021; Maigoda et al., 2022):

Alkaloids: A total of 4 mg of ethanolic extract was dissolved in 3 mL of methanol and 5 mL of ammonia, adjusting the pH to 8-9. The resulting mixture was then filtered. Following filtration, 2 mL of a 2 M HCl solution was added to the filtrate. After thorough shaking, five drops of the upper layer were distributed into four test tubes. The solution in tube 1 served as a blank, while solutions in tubes 2, 3, and 4 received one drop each of Mayer, Wagner, and Dragendorff reagents, respectively.

Flavonoids: Flavonoid content was assessed through the Shinoda test. An aliquot of the extract was treated with several drops of concentrated hydrochloric acid (HCl) and magnesium turnings (Mg). The appearance of a pink or red hue indicated the presence of flavonoids.

Tannins: Approximately 1 mg of ethanolic extract was positioned on a drip plate, to which five drops of methanol were added and mixed using a spatula until complete dissolution occurred. Subsequently, 2-3 drops of a 1% FeCl_3 solution were incorporated. The emergence of a blue, green, or brownish-green colour signified the presence of tannins.

Saponins: Approximately 1 mg of ethanolic extract was placed in a test tube, followed by the addition of 5 mL of distilled water. The mixture was then agitated for 1 minute. In the presence of foam, two drops of a 1 M HCl solution were introduced. If the foam persisted at a height of 1-3 cm for a duration of 10 minutes, it was concluded that the sample contained saponins.

Terpenoids: The detection of terpenoids was carried out using the Liebermann-Burchard test. A small quantity of the extract was combined with chloroform (CHCl_3) and concentrated sulfuric acid (H_2SO_4). The emergence of a red or purple colour signified the presence of terpenoids.

Steroids: Steroids were identified via the Salkowski test. An aliquot of the extract was treated with chloroform (CHCl_3) and concentrated sulfuric acid (H_2SO_4). The appearance of red colour in the chloroform layer indicated the presence of steroids.

Glycosides: The presence of glycosides was assessed using the Keller-Kiliani test. A sample of the extract was treated with a few drops of glacial acetic acid (CH_3COOH), followed by concentrated sulfuric acid (H_2SO_4). The appear-

ance of a reddish-brown ring at the interface indicated the presence of glycosides.

The qualitative screening provides a comprehensive overview of the phytochemical profile of *P. granatum* fruit peels and reveals the presence of bioactive compounds that may contribute to their antioxidant and therapeutic properties, forming a basis for further quantitative analysis and exploration of the biological activities associated with these phytochemicals.

Quantitative Chemical Analysis

The quantitative chemical analysis of the methanolic extract obtained from the fruit peels of *P. granatum* was conducted to ascertain the total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC). The initial concentration of 1 mg/mL of the methanolic extract in PBS (phosphate-buffered saline) was used as the base sample in the determination of the doses of metabolites in the extract (Hayat et al., 2020).

Total Phenolic Content (TPC)

Total phenolic content (TPC) was measured using the Folin-Ciocalteu method. An aliquot of the extract (0.5 mL) was mixed with 2.5 mL of the Folin-Ciocalteu reagent and allowed to react for 5 minutes. Following this, 2 mL of a 20% sodium carbonate solution (Na_2CO_3) was added, and the mixture was incubated in the dark for 30 minutes at room temperature. The absorbance was then measured at 765 nm using a UV-VIS spectrophotometer. TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight of the extract, using the same experimental conditions with a series of reductive concentrations of gallic acid (500, 250, 125, 62.5 and 31.25 $\mu\text{g/mL}$) (Barbosa and Minguillan, 2021; Molole et al., 2022).

Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined using a colourimetric assay. 1 mL of the extract was mixed with 3 mL of distilled water, followed by the addition of 0.3 mL of a 5% sodium nitrite solution (NaNO_2). After 5 minutes, 0.3 mL of a 10% aluminium chloride solution (AlCl_3) was added, and the mixture was allowed to stand for another 6 minutes. Finally, 2 mL of 1 M sodium hydroxide (NaOH) was added, and the absorbance was measured at 510 nm. The results were expressed as milligrams of quercetin equiva-

lents (QE) per gram of dry weight of the extract, using the same experimental conditions with a series of quercetin reductive concentrations (500, 250, 125, 62.5 and 31.25 µg/mL) (Shraim et al., 2021; Sari et al., 2023).

Total Tannin Content (TTC)

Total tannin content (TTC) was assessed using the vanillin method. 0.5 mL of the extract was mixed with 2.5 mL of vanillin-HCl reagent. After incubation for 20 minutes at room temperature, the absorbance was recorded at 500 nm. TTC was expressed as milligrams of catechin equivalents (CE) per gram of dry weight of the extract, using the same experimental conditions with a series of catechin reductive concentrations (500, 250, 125, 62.5, and 31.25 µg/mL) (Padumanonda and Phontree, 2021; Maobe et al., 2022).

Antioxidant Activity

The antioxidant activity of methanolic extracts from *P. granatum* fruit peels was evaluated using three different assays: DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and FRAP (ferric reducing antioxidant power). The extracts were tested at concentrations of 10, 20, 50, 100, 150, and 200 µg/mL to determine their capacity to scavenge free radicals and reduce oxidized substrates. Each assay utilized appropriate controls to ensure the reliability of the results.

DPPH Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed to evaluate the free radical scavenging activity of the extract. A 0.1 mM DPPH solution was prepared in methanol (CH₃OH) and kept in the dark to avoid degradation. 50 µL of each concentration of the extract (10, 20, 50, 100, 150, and 200 µg/mL) was combined with 150 µL of the DPPH solution in a 96-well plate. The reaction was allowed to proceed for 30 minutes in darkness, after which the absorbance was recorded at 517 nm. Ascorbic acid (vitamin C) was included as a positive control in this assay, facilitating a comparative assessment of the antioxidant capacity of the extracts. The formula used to calculate the antioxidant activity in the DPPH assay is typically expressed in terms of the percentage of the DPPH radical scavenging activity (RSA). The basic formula is: $RSA (\%) = [(A_0 - A_s) / A_0] * 100$, where A_0 is the absorbance of the DPPH solution without the sample and A_s is the absorbance of the DPPH solution

in the presence of the sample (Chen et al., 2020; Bashkin et al., 2021).

ABTS Assay

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay was conducted to assess the scavenging capacity of the extracts against the ABTS radical cation. An ABTS solution was prepared by combining seven mM ABTS with 2.45 mM potassium persulfate, allowing it to stand in the dark for a duration of 14 hours prior to use. This ABTS solution was subsequently diluted to achieve an absorbance of 0.700 ± 0.03 at 734 nm. A total of 50 µL of each extract concentration (10, 20, 50, 100, 150, and 200 µg/mL) was combined with 150 µL of the diluted ABTS solution within a 96-well plate and incubated for 6 minutes at room temperature. The absorbance was recorded at 734 nm. Ascorbic acid was used as a control for antioxidant activity benchmarking using the same experimental conditions. The antioxidant activity in the ABTS assay was determined by calculating the percentage of ABTS radical scavenging activity (RSA) with the following formula: $RSA (\%) = [(A_0 - A_s) / A_0] * 100$, where A_0 is the absorbance of the ABTS solution without the sample and A_s is the absorbance of the ABTS solution in the presence of the sample (Gaber et al., 2021; Rumpf et al., 2023).

FRAP Assay

The ferric-reducing antioxidant power (FRAP) assay was employed to assess the reducing capacity of the extract. A freshly prepared FRAP reagent was formulated by mixing 10 mL of 300 mM sodium acetate buffer (NaCH₃COO at pH 3.6), 1 mL of 10 mM TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) dissolved in 40 mM hydrochloric acid (HCl), and 1 mL of 20 mM ferric chloride hexahydrate (FeCl₃·6H₂O). Extract concentrations were prepared in methanol at 10, 20, 50, 100, 150, and 200 µg/mL. 50 µL of each extract was combined with 150 µL of the FRAP reagent in a 96-well plate. The mixtures were incubated at 37°C for 30 minutes, after which the absorbance was measured at 593 nm. A control sample containing Trolox served as a comparison. The antioxidant activity in the FRAP assay was determined by calculating the percentage of inhibited iron ions (I_{Fe}) with the following formula: $I_{Fe} (\%) = [(A_0 - A_s) / A_0] * 100$, where A_0 is the absorbance of the ABTS solution without the sample and A_s is the absorbance of the ABTS solution in the presence of the sample (Mwamatope et al., 2020; Rumpf et al., 2023).

Statistical Analysis

All experiments were performed in triplicate. All data are presented as means $\pm$ standard deviation (SD). Total polyphenols, flavonoids, tannins, and antioxidant activity were examined for statistical significance using analysis of variance (Excel test, two-factor ANOVA test with replication and post hoc test, which was ensured by IBM SPSS version 25). A p-value of 0.05 was considered a threshold to declare a statistically significant difference. This rigorous statistical methodology reinforced the robustness and scientific validity of the study's conclusions.

For the graphical extrapolation of IC₅₀ values for the antioxidant power of *P. granatum* fruit peels, the range between 10 and 200 was chosen for the x values, unlike previous studies, to obtain a more accurate curve and correlation values.

Results and Discussion

Extraction Yield

The yield of methanolic extract by the maceration method from *P. granatum* fruit peels was determined to be 41.13 ± 0.28 (w/w), indicating a high efficiency in the recovery of bioactive compounds. This yield exceeds those reported in previous studies using different solvents, including ethanol and water, while it was largely consistent with studies using methanol as the solvent. In Table 1, these comparisons are shown.

Table 1. Yield of methanolic extract from *Punica granatum* fruit peels by the maceration method.

Tabela 1. Pridobivanje metanolnega ekstrakta iz lupin sadežev *Punica granatum* po metodi maceracije.

Extraction Solvent	Yield (% w/w)	Reference
Methanol	41.13 ± 0.28	Current Study
80% Methanol/ Water	45.4 ± 5.3	Mutahar et al., 2012
Methanol	28.9 ± 0.5	Konsoula, 2016
Water	27.4	Mutahar et al., 2012
Ethanol	21.9 ± 0.6	Konsoula, 2016
Methanol	9.82	Yassin et al., 2021
Acetone	6.51	Yassin et al., 2021
Hexane	4.98	Yassin et al., 2021

Chemical Composition

Qualitative Analysis

Qualitative analysis of methanolic extracts from *P. granatum* fruit peels revealed the presence of several bioactive compounds known for their antioxidant and therapeutic properties. The analysis confirmed the presence of the following compounds: alkaloids, flavonoids, tannins, saponins, terpenoids, steroids and glycosides (Table 2). These results indicate that the peel of the *P. granatum* fruit is rich in diverse phytochemicals, which likely contributes to its antioxidant properties and potential health benefits (Gosset-Erard et al., 2021; Parisi et al., 2022).

Table 2. Qualitative analysis of methanolic extracts from the *Punica granatum* fruit peels, where + indicates presence detected and ++ indicates significant presence detected.

Tabela 2. Kvalitativna analiza metanolnih ekstraktov iz lupin sadežev *Punica granatum*, pri čemer + označuje ugotovljeno prisotnost, ++ pa znatno ugotovljeno prisotnost.

Compound	Presence
Alkaloids	+
Flavonoids	++
Tannins	++
Saponins	+
Terpenoids	+
Steroids	+
Glycosides	+

Quantitative Analysis

Quantitative analysis of the methanolic extracts from *P. granatum* fruit peels was performed to determine the concentration of major bioactive compounds, specifically total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC). The results are summarized below.

Total phenolic content (TPC) was measured using the Folin-Ciocalteu method. A calibration curve was generated using gallic acid as a standard (Fig 3.a). The concentration of phenolic compounds in the methanolic extract was found to be 187.13 ± 1.92 mg GAE/g DE (milligrams of gallic acid equivalents per gram of dry extract), as shown in Fig. 4. This indicates a significant concentration of phenolic compounds known for their antioxidant properties.

A study reported that the total phenolic content in water and acetone extracts of *P. granatum* flowers was approximately 230.8 ± 9.5 and 183.6 ± 15.5 mg GAE/g of extract (Petrova et al., 2020). According to a study by Tunisian scientists, total polyphenols in the pomegranate peel amounted to 85.60 ± 4.87 mg GAE/g (Walid et al., 2012). Another *Punica granatum* fruit peel study from the USA showed the highest total phenols (202.4 mg GAE/g DW) (Wang et al., 2011). *P. granatum* peel methanolic extract had the highest polyphenolic content (190 mg/g) compared to seeds (70 mg/g) and juice (34 mg/g) (Konsoula, 2016).

Total flavonoid content (TFC) was determined using the colourimetric aluminium chloride method using quercetin as a standard (Fig. 3.b). The quercetin equivalent in the methanolic extract was measured at 79.51 ± 0.83 mg QE/g DE (milligrams of quercetin equivalent per gram

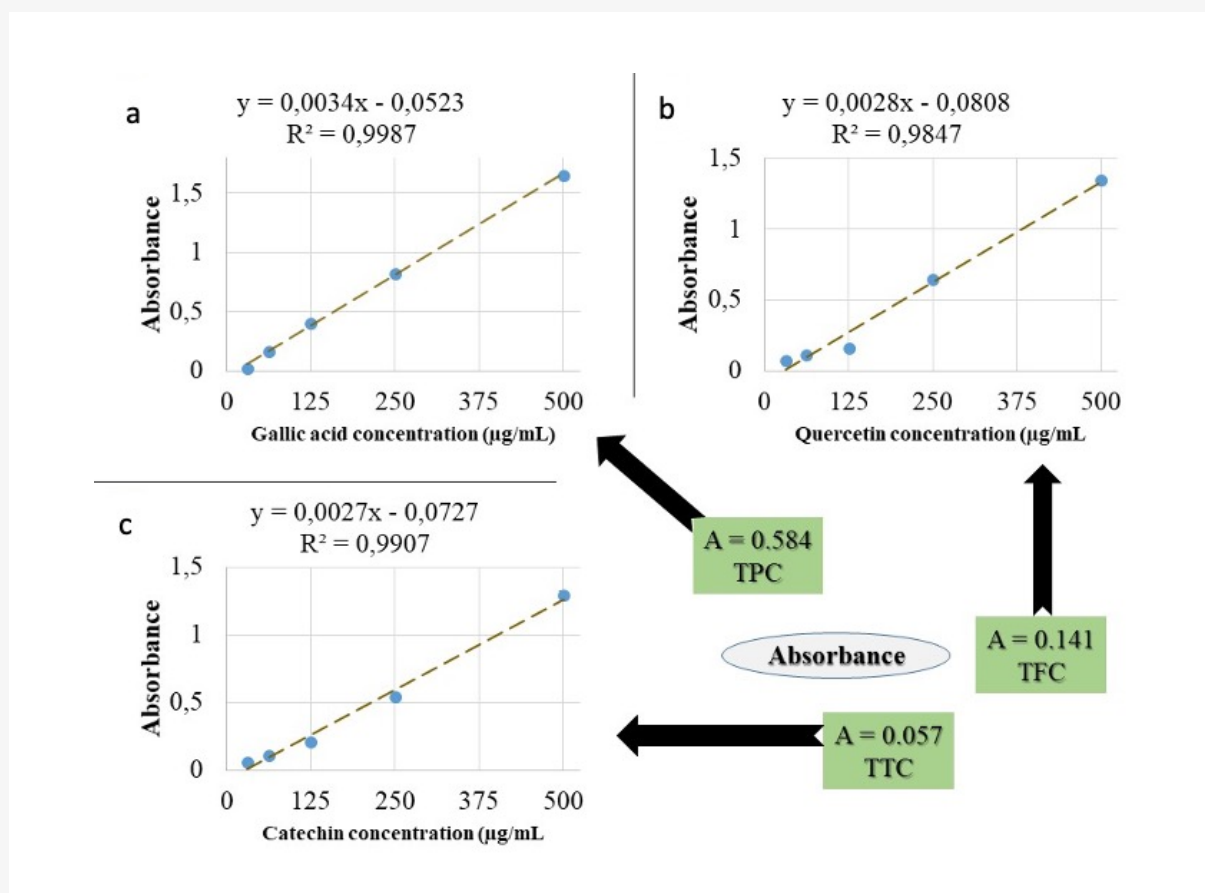


Figure 3. Linear representation of the reference reagents to determine the equivalent values of TPC, TFC, and TTC. a: gallic acid at 765 nm, b: quercetin at 510 nm, and c: catechin at 500 nm. TPC: Total phenolic content, TFC: Total flavonoid content, and TTC: Total tannin content.

Slika 3. Linearni prikaz referenčnih reagentov za določitev ekvivalentnih vrednosti TPC, TFC in TTC. a: galna kislina pri 765 nm, b: kvercetin pri 510 nm in c: katehin pri 500 nm. TPC: skupna vsebnost fenolov, TFC: skupna vsebnost flavonoidov in TTC: skupna vsebnost taninov.

of dry extract), a result shown in Fig. 4. This high level of flavonoids contributes to the overall antioxidant capacity of the extract. A study found that the extracts of *P. granatum* peel contained about 51.52 ± 8.14 mg quercetin equivalents (QE)/g of extract (Walid et al., 2012). Research indicated that the whole fruit peel extract in ethyl acetate contained about 39.2 mg QE/g of flavonoids (Wang et al., 2011). *P. granatum* peel methanolic extract had the highest polyphenolic content – the highest amount of flavonoids (21 mg/g), compared to seeds (9 mg/g) and juice (4 mg/g) (Konsoula, 2016).

Total tannin content (TTC) was assessed using the vanillin assay, which is based on the reaction of tannins with vanillin in the presence of hydrochloric acid, using the standard linear trace (Fig. 3.c). Total tannin content was estimated to be 48.09 ± 0.63 mg CE/g DE (milligrams of catechin equivalents per gram of dry extract), as shown in Fig. 4. This indicates the presence of a significant amount of tannins, which are known for their health-promoting properties. A study found that the extracts of pomegranate peel had a total tannin content of approximately 139.63 ± 4.25 mg tannic acid equivalents per g dry weight (mg

TAE/g DW) (Walid et al., 2012). The authors of a Spanish study found that hydrolyzed tannin content in the extracts of pomegranate peel was approximately 21.25 mg tannic acid equivalents (TAE)/g (Viuda-Martos et al., 2013).

These quantitative results confirm that the methanolic extract of *P. granatum* fruit peels is rich in bioactive compounds, supporting its potential use in functional foods and food applications. The high concentrations of TPC, TFC, and TTC contribute to the overall antioxidant activity observed in the extract. There is a statistically significant difference between the amounts of metabolites studied, with 0.05 being the threshold for statistical significance.

Antioxidant Activity Results

The antioxidant activity of the methanolic extracts of *P. granatum* peels was evaluated using three assays: DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power), and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). The results are presented in Figs. 5, 6, and 7.

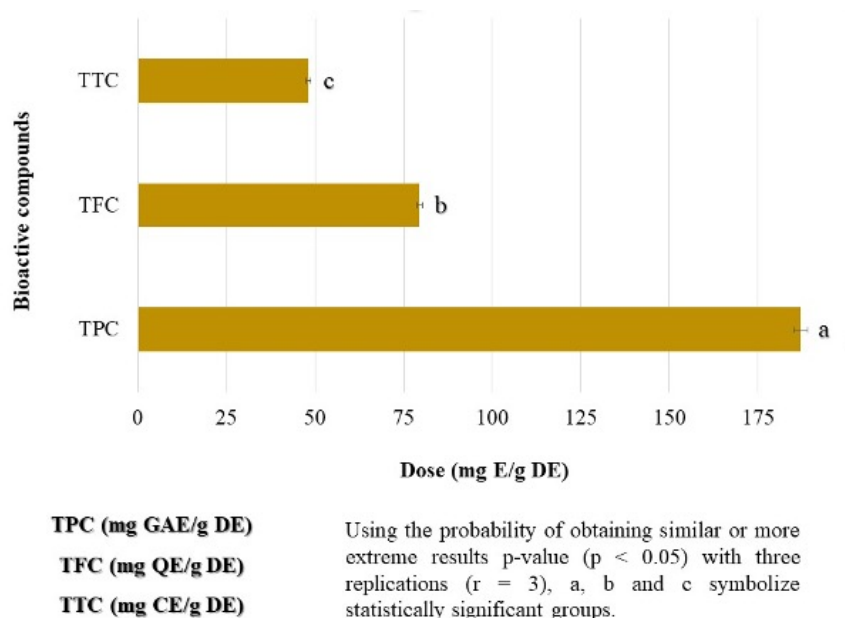


Figure 4. Quantitative analysis of bioactive compounds in the methanolic extracts of *Punica granatum* fruit peels. TPC: Total phenolic content, TFC: Total flavonoid content, and TTC: Total tannin content.

Slika 4. Kvantitativna analiza bioaktivnih spojin v metanolnih izvlečkih lupin sadežev *Punica granatum*. TPC: skupna vsebnost fenolov, TFC: skupna vsebnost flavonoidov in TTC: skupna vsebnost taninov.

DPPH Assay

The DPPH assay conducted on the methanolic extract of *P. granatum* fruit peels demonstrated a significant radical scavenging activity across various concentrations. The results indicated a gradual increase in scavenging activity, with values reaching 94.39 ± 0.15 % at 200 $\mu\text{g/mL}$. The calculated IC₅₀ value of the extract was 14.23 $\mu\text{g/mL}$, suggesting moderate antioxidant potential compared to ascorbic acid, which exhibited a lower IC₅₀ of 42.52 $\mu\text{g/mL}$, affirming its status as a potent antioxidant (Fig. 5).

These findings align with previous research indicating the antioxidant properties of the *P. granatum* extracts. For instance, Benchagra et al. (2021) reported an IC₅₀ value of 21.58 $\mu\text{g/mL}$ for the *P. granatum* juice, highlighting its comparable effectiveness in scavenging free radicals. Similarly, Konsoula (2016) noted an IC₅₀ value of 0.06 to 0.08 mg/mL for the methanolic extracts of *P. granatum* fruit peels, further supporting the notion that pomegranate-derived

compounds possess substantial antioxidant capabilities.

The increasing scavenging activity with higher concentrations of the extract underscores the potential of *P. granatum* fruit peels as a source of natural antioxidants. The observed moderate activity of the methanolic extract can be attributed to the rich phytochemical profile identified in the qualitative analysis, including flavonoids and tannins, which are known for their radical-scavenging abilities (Khan et al., 2020).

Tannins, which are distinguished by their elevated molecular weight and astringent characteristics, act as antioxidants by creating complexes with proteins and other macromolecules. This interaction impedes oxidative enzymes, including lipoxygenase and cyclooxygenase, thereby diminishing the generation of pro-oxidant substances (Molino et al., 2023; Zeng et al., 2023). Additionally, tannins may stimulate endogenous antioxidant enzymes, thereby augmenting the body's inherent defence

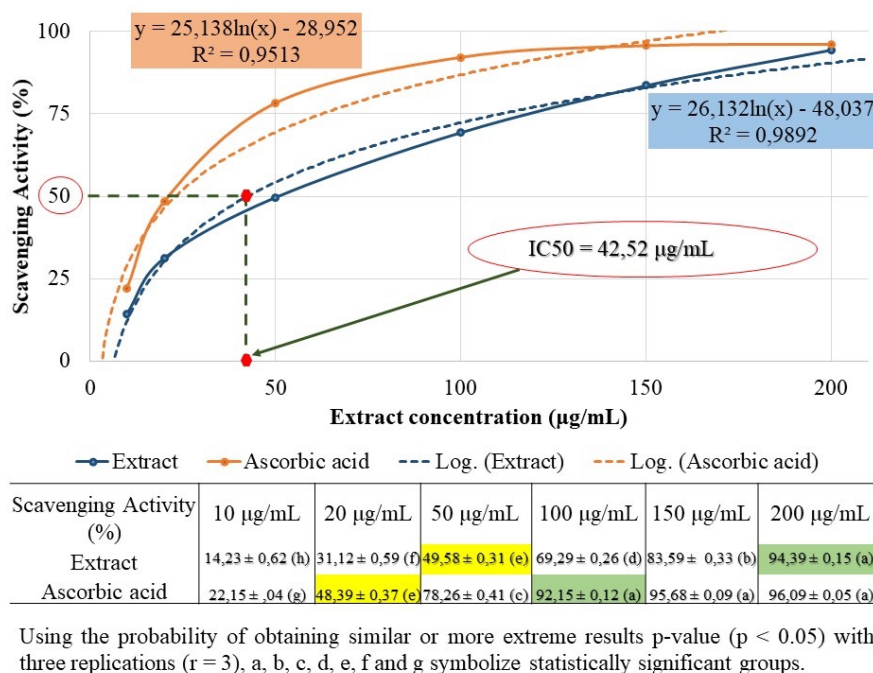


Figure 5. Antioxidant activity of the methanolic extract of *Punica granatum* fruit peels in the DPPH assay. Yellow color: Compatibility in antioxidant efficacy between the minimum concentration of the extract compared to vitamin C. Green color: Compatibility in antioxidant efficacy between the maximum concentration of the extract compared to vitamin C.

Slika 5. Antioksidativna aktivnost metanolnega izvlečka lupin sadežev *Punica granatum* v preskusu DPPH. Rumena barva: združljivost antioksidativne učinkovitosti med najmanjšo koncentracijo izvlečka v primerjavi z vitaminom C. Zelena barva: združljivost antioksidativne učinkovitosti med največjo koncentracijo izvlečka v primerjavi z vitaminom C.

mechanisms against oxidative stress. Both flavonoids and tannins influence signalling pathways associated with oxidative stress, notably the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which results in the upregulation of antioxidant genes. This synergistic interaction of polyphenols highlights their promising therapeutic potential in the prevention of diseases linked to oxidative stress, including cardiovascular diseases, cancer, and neurodegenerative disorders, thereby enhancing overall health and well-being (Ciampi et al., 2020; Soldado et al., 2021). The methanolic extract of *P. granatum* fruit peels demonstrates promising antioxidant activity, reinforcing the fruit's status as a valuable source of bioactive compounds with potential health benefits. Future studies should focus on isolating specific phytochemicals responsible for this activity and exploring their mechanisms of action in oxidative stress-related conditions.

Statistically, with a p-value threshold of 0.05, there is a significant difference between all concentrations of the extract used in our study, while only the lowest concentra-

tions between 10 and 100 µg/ml were found to be significant for vitamin C. On the other hand, we did not observe a significant difference between the 50 µg/mL concentration of the extract and the 20 µg/mL concentration of vitamin C. Statistically, the 200 µg/mL concentration of the pomegranate fruit peel methanolic extract is considered to be compatible with the 100 µg/mL concentration of vitamin C in terms of DPPH root scavenging power.

ABTS Assay

The ABTS test results indicate that the antioxidant activity of the *P. granatum* fruit peel extract increases with increasing concentration, with an IC₅₀ value of 47.49 µg/mL (Fig. 6). This means that at this concentration, the extract exhibits 50% inhibition of the ABTS radical. In comparison, ascorbic acid, a known chemical antioxidant, shows a much lower IC₅₀ value of 3.3 µg/mL, indicating stronger antioxidant activity per unit concentration.

The percentage inhibition data reveals a clear dose-response relationship for both pomegranate extract and

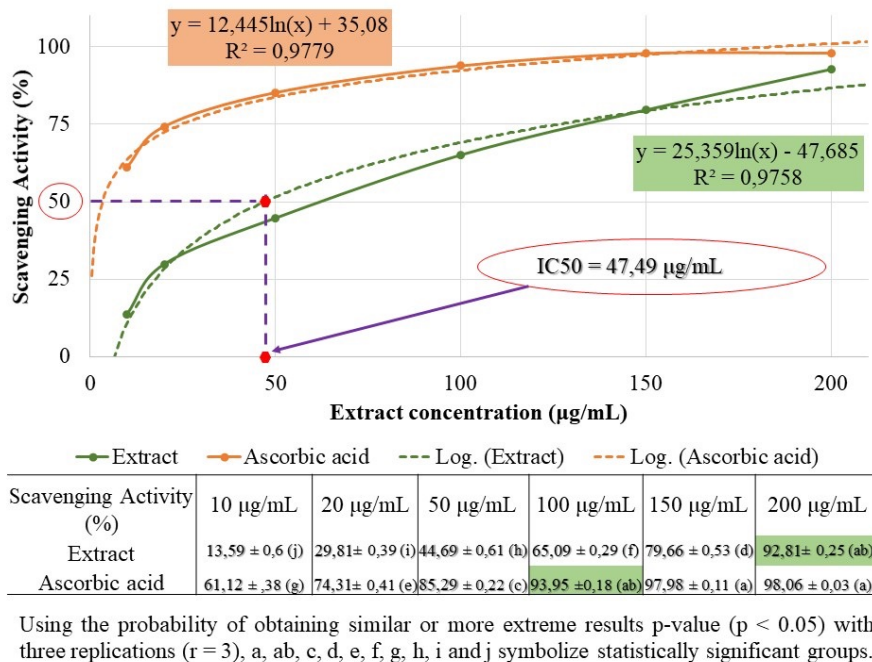


Figure 6. Antioxidant activity of the methanolic extract of *Punica granatum* fruit peels in the ABTS assay. Green color indicates the compatibility in antioxidant efficacy between the concentration of the extract compared to vitamin C.

Slika 6. Antioksidativna aktivnost metanolnega izvlečka olupkov sadežev *Punica granatum* v testu ABTS. Zelena barva označuje združljivost antioksidativne učinkovitosti med koncentracijo izvlečka v primerjavi z vitaminom C.

ascorbic acid. At the highest concentration tested (200 µg/mL), the extract achieves $92.81 \pm 0.25\%$ inhibition, while ascorbic acid reaches $98.06 \pm 0.03\%$ (Fig. 6). This indicates that although the pomegranate extract is highly effective, it is less effective than ascorbic acid in terms of its ability to scavenge ABTS radicals.

The antioxidant activity of the *P. granatum* fruit peels can be attributed to their rich phytochemical profile, which includes metabolites. Polyphenols, encompassing flavonoids and tannins, are essential in countering oxidative stress through their diverse antioxidant mechanisms. Flavonoids, including quercetin and kaempferol, display notable free radical scavenging capabilities by transferring electrons to reactive oxygen species (ROS). This action effectively neutralizes ROS, thereby preventing cellular damage. The donation of electrons stabilizes the radical, diminishing its reactivity and lessening oxidative damage to lipids, proteins, and DNA. Furthermore, flavonoids possess the ability to chelate metal ions, such as iron and copper, which promote the formation of free radicals, consequently reducing oxidative reactions. Studies have shown that compounds, such as ellagitannins and anthocyanins, present in pomegranate contribute significantly to its antioxidant capacity (Benchagra et al., 2021). These compounds can donate electrons or hydrogen atoms to free radicals, effectively neutralizing them.

The IC₅₀ values observed in this study for *P. granatum* are consistent with previous findings. For instance, a study by Sabraoui et al. (2020) reported IC₅₀ values between 42.71 and 65.55 µg/mL for pomegranate peel extracts, which aligns well with our findings. Comparatively, Other plant extracts, such as those from *Satureja khoozistanica*, *Withania somnifera* leaves, and *Cannabis sativa* flowers, have shown similar IC₅₀ values, often in the range of 56-60 µg/mL (Venkatachalapathy et al., 2021; Al Khoury et al., 2022). This positions *P. granatum* fruit peels as a promising natural source of antioxidants, particularly when considering their availability and potential for incorporation into functional foods and supplements. The antioxidant activity of the *P. granatum* fruit peel extract, as evidenced by the ABTS assay, substantiates its potential as a significant source of natural antioxidants. Although it demonstrates considerable activity, it does not match the potency of ascorbic acid. Future investigations should prioritize the isolation and characterization of the active components responsible for the noted antioxidant activity, in addition to examining their synergistic effects with other dietary antioxidants.

Statistically, there is an overall significant difference between all extract concentrations and vitamin C concentrations below 200 µg/mL, with 0.05 being the significance threshold. On the other hand, statistical agreement was recorded between the highest studied concentration of the extract (200 µg/mL) and the 100 µg/mL concentration of vitamin C.

FRAP Assay

The FRAP assay was performed using a methanolic extract of *P. granatum* fruit peels. The absorbance measurements obtained from the assay allowed the calculation of the reducing power expressed in percentage compared with trolox as a potent inhibitor against metal ions that acts directly in the oxidation process and catalyzes the liberation of radicals. The results are summarized in Fig. 7. The evaluation reveals a promising profile of antioxidant efficacy. The extract demonstrated a clear dose-dependent increase in FRAP values, reaching $75.29 \pm 0.14\%$ at 200 µg/mL, which is comparable to the FRAP value of Trolox at the same concentration ($75.39 \pm 0.07\%$). Notably, the IC₅₀ values indicate that the pomegranate peel extract (59.5 µg/mL) requires a higher concentration than trolox (36.8 µg/mL) to achieve a 50% inhibition of oxidative stress. This observation suggests that while *P. granatum* fruit peels possess significant antioxidant properties. Their potency may be slightly lower than that of synthetic antioxidants like trolox, which is consistent with findings of other studies that report varying levels of efficacy among natural extracts (Sihag et al., 2022; Saporbekova et al., 2023).

When comparing the antioxidant activities of *P. granatum* fruit peels with other plant extracts, the studied extract showed similar or even superior abilities to many fruits. The antioxidant activities of these extracts can be largely attributed to the diversity of their phytochemical properties, which are known for their strong free radical scavenging effect, as well as the inhibition of oxidation induced by metals, such as iron and copper. This comparative analysis highlights the competitive nature of the *P. granatum* fruit peels within the broader category of fruit extracts, making them a valuable source of natural antioxidants (Tiji et al., 2021; Nwoso, 2023; Radulescu et al., 2024).

Future research should focus on isolating specific bioactive compounds in the *P. granatum* peels and exploring their mechanisms of action. Furthermore, studies examining the synergistic effects of the *P. granatum* peel extracts with other plant sources may enhance antioxidant activity

and expand their use in functional foods (Sihag et al., 2022; Radan et al., 2024). Such studies will contribute to a more comprehensive understanding of how these natural products can be effectively harnessed to promote health.

With a statistical threshold of 0.05, there is no significant difference between the concentrations above 150 µg/mL, where there is an agreement between the inhibitory activity of the methanolic extract and trolox. All concentrations equal to or less than 100 µg/mL showed a significant difference. There is a statistical agreement between the 20 µg/mL concentration of the extract and the 10 µg/mL concentration of Trolox.

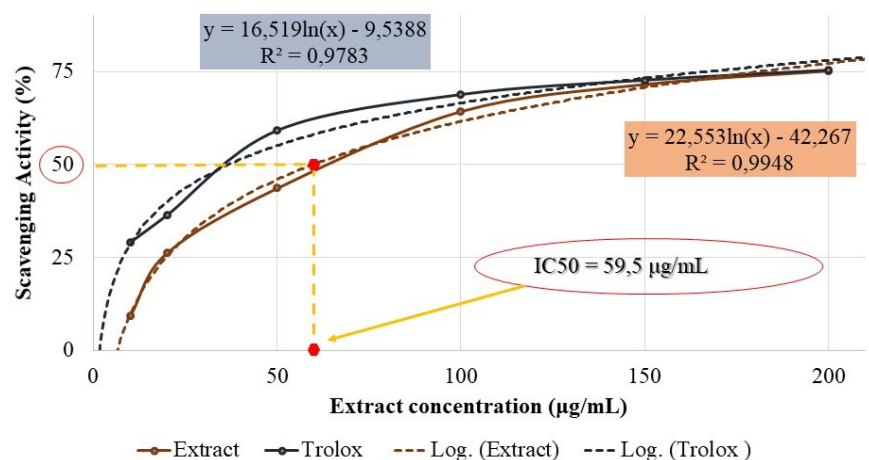
Implications of Findings

The findings of this study have several implications. Firstly, the high antioxidant potential of *P. granatum* fruit peels suggests that they could be utilized as natural additives in

food products to enhance shelf life and nutritional value by reducing oxidative damage. The presence of diverse bioactive compounds also points to potential health benefits, including anti-inflammatory, anticancer, and cardioprotective effects.

Moreover, the results indicate that *P. granatum* peels could serve as an economically viable source of natural antioxidants, particularly in the regions where pomegranate cultivation is prevalent. This could promote the valorization of agricultural waste, contributing to sustainable practices in the food industry.

Future research could explore the specific mechanisms through which these compounds exert their effects, as well as their bioavailability and pharmacokinetics in human health. Additionally, investigations into the synergistic effects of the various phytochemicals present in *P. granatum* peels could provide insights into their potential therapeutic applications.



Scavenging Activity (%)	10 µg/mL	20 µg/mL	50 µg/mL	100 µg/mL	150 µg/mL	200 µg/mL
Extract	9,29 ± 0,21 (h)	26,22 ± 0,46 (g)	43,66 ± 4 (e)	64,36 ± 0,22 (c)	71,66 ± 0,28 (a)	75,29 ± 0,14 (a)
Trolox	28,94 ± 1,13 (g)	36,31 ± 0,26 (f)	59,14 ± 0,71 (d)	68,81 ± 0,15 (b)	72,68 ± 0,11 (a)	75,39 ± 0,07 (a)

Using the probability of obtaining similar or more extreme results p-value ($p < 0.05$) with three replications ($r = 3$), a, b, c, d, e, f, g and h symbolize statistically significant groups.

Figure 7. Antioxidant activity of the methanolic extract of *Punica granatum* fruit peels in the FRAP assay. Yellow color: Compatibility in antioxidant efficacy between the minimum concentration of the extract compared to Trolox. Green color: Compatibility in antioxidant efficacy between the maximum concentration of the extract compared to Trolox.

Slika 7. Antioksidativna aktivnost metanalnega izvlečka lupin sadežev *Punica granatum* v testu FRAP. Rumena barva: Združljivost antioksidativne učinkovitosti med najmanjšo koncentracijo izvlečka v primerjavi s troloxom. Zelena barva: združljivost antioksidativne učinkovitosti med največjo koncentracijo izvlečka v primerjavi s Trolox.

Limitations of the Study

Despite the encouraging findings, this study presents several limitations. The antioxidant assays utilized were conducted *in vitro*, which may not completely mimic the complexities of biological systems *in vivo*. Consequently, while the findings suggest notable antioxidant activity, additional research is required to assess the bioavailability and metabolic pathways of these compounds in human subjects. Lastly, the study did not explore the potential toxicity or adverse effects associated with the consumption of *P. granatum* peel extracts. Investigating these factors is crucial for assessing their safety and efficacy for human use. This study establishes the antioxidant potential of *P. granatum* peels and their rich phytochemical profile. Further research is warranted to explore their application in functional foods and therapeutic settings.

Conclusion

At a time when the use of chemicals in the health and food industry has dominated, many researchers are seeking to reintegrate natural compounds as a healthier alternative, as research into antioxidants derived from natural substances has increased. The methanolic extract of *Punica granatum* fruit peels demonstrates significant antioxidant activity, as evidenced by robust results across three *in vitro* experimental methods: DPPH, ABTS, and FRAP. These findings highlight the potential of the methanolic extract of *P. granatum* fruit peels as a rich source of antioxidant compounds, which could play a crucial role in combating oxidative stress and associated diseases. This is because the metabolites in the peels can scavenge free radicals and inhibit oxidation with metal ions, such as iron. This was confirmed by this study.

Given the rising interest in natural dietary supplements, the incorporation of this extract into functional foods or as a standalone supplement presents an exciting opportunity for enhancing health and wellness. Future research should focus on optimizing the extraction processes, elucidating the specific antioxidant compounds responsible for these effects, and conducting clinical trials to validate the health benefits of *P. granatum* fruit peel extracts in the human population. By advancing our understanding of these compounds, we can pave the way for innovative dietary solutions that harness the full potential of the *Punica granatum* fruit peels.

Author Contributions

Conceptualization, B. B. and B. K.; Methodology, B. B., B. K., M. B., B. A.; Software, B. B., Verification, B. B., B. K., B. A. and M. B. Formal analysis, B. A. ; formal analysis, B. K.; Investigation, B. B. and B. A.; Sources, Scientific Research Laboratory of the Universities of Mostaganem, Mascara and Relizane, Algeria; Data organization, all authors. Preparation of the original draft, all authors; revision and editing of the manuscript, all authors; Conception, M. B.; supervision, B. B.; Project management, B. B. All authors have read and approved the published version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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