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Study on the antibacterial and antioxidant activities of *Punica granatum* (pomegranate) peel extracts

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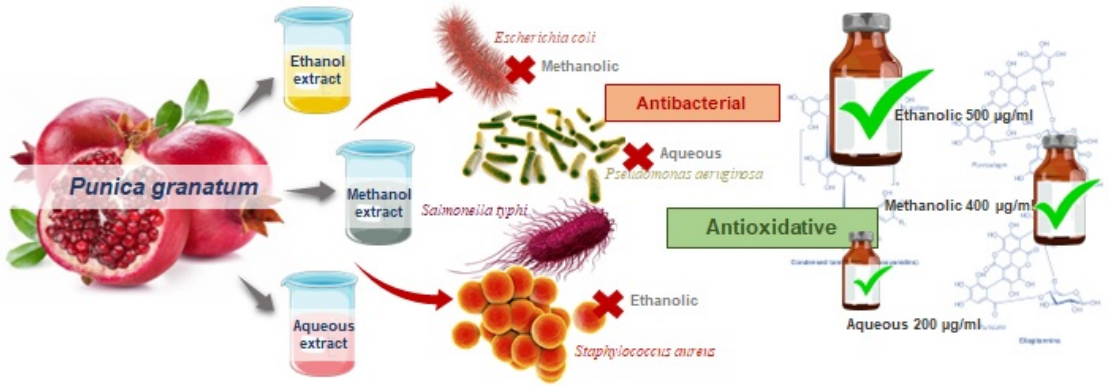
Article

ABSTRACT



This study presents the antimicrobial and antioxidant activities of different extracts from the peel of *Punica granatum* fruit against various standard microorganisms. Three *P. granatum* peel extracts were prepared with ethanol, methanol, and water, using standard protocol, followed by their qualitative phytochemical analysis. Antibacterial and antioxidant activities of *P. granatum* peel extracts against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Staphylococcus aureus* were tested. The phytochemical test of *P. granatum* peel extracts revealed the presence of therapeutic agents such as tannins. The highest antibacterial activity of the ethanolic *P. granatum* extract was against *S. aureus*, the methanolic extract against *E. coli* and aqueous extract against *P. aeruginosa*, while all the three extracts had the lowest antibacterial activity against *S. typhi*. The highest MIC (Minimum Inhibitory Concentration) and MBC (Minimum bactericidal concentration) were attained by the aqueous and ethanolic extracts whereas the lowest was shown by the methanolic extract, against different bacterial strains. Also, the highest antioxidant activity shown by the ethanolic extract was at 500µg/ml, 400 µg/ml in case of methanolic extract, and 200 µg/ml in case of aqueous extract. Despite being a byproduct, *P. granatum* (pomegranate) peel has significant potential as a source of bioactive compounds with important health benefits. The type of solvent used can significantly affect the extract's chemical profile and biological activity, as the relative and absolute concentrations of the extracted compounds play a crucial role in determining their bioactivity.

Keywords: Punica granatum, phytochemical constituents, antibacterial, antioxidants

INTRODUCTION

Antimicrobial resistance (AMR) is becoming a more pressing issue for public health. Multidrug-resistant (MDR) bacteria are emerging, rendering antimicrobial therapies ineffective, and leading to longer hospital stays and higher mortality rates.[1] Plants and herbs have long been used in medicine for their healing properties. Often, these natural treatments are more efficient and cause fewer side effects than man-made medications.[2] Their diverse molecular structures and biological activities are vital for the development of novel pharmaceuticals.[3]

The pomegranate, scientifically known as *Punica granatum*, is a fruit-bearing shrub from the Mediterranean.[4] This shrub produces a fruit that is not only nutritious.[5] but also has recently been recognized for the beneficial qualities of its peel. Studies have shown that the peel possesses properties that can fight bacteria and protect against oxidative damage.[3, 6]

Research has shown that extracts from pomegranate peel can effectively fight various bacteria like *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and several other bacterial strain.[7-10]

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Additionally, these extracts are noted for their antioxidant capacity, which is vital for shielding the body from oxidative stress that leads to chronic diseases such as cancer and diabetes.[11-14] Although these extracts show promise for health benefits, further research is necessary to fully understand their impacts and identify the most effective ways to use them.

The peels of fruits, commonly discarded in food processing, surprisingly contain higher antioxidant levels than the fruit's flesh. These peels are abundant in phenolic substances, including flavonoids and ellagitannins, known for their medicinal value. To extract these compounds, specific methods and solvents are used, typically water or water-alcohol mixtures, due to the compounds' polar nature. The choice of solvent is crucial as it greatly influences the chemical makeup and biological potency of the extracts, where the concentration and combination of the extracted compounds are key to their effectiveness.[15, 16] This study focuses on evaluating the phytochemical content, the antimicrobial efficacy against bacteria like *E. coli*, *P. aeruginosa*, *S. typhi*, and *S. aureus*, and the antioxidant properties of extracts from *P. granatum* peel using three different solvents. This approach also emphasizes the significance of selecting the right solvent for maximum efficacy of the *P. granatum* extract.

MATERIALS AND METHODS

Preparation and processing of plant materials

Punica granatum was collected, and its outer skin was peeled off. The fresh pomegranate peels were left to dry at room temperature for 15 days. These peels were then thoroughly washed with tap water, followed by a final rinse using distilled water, and subsequently dried. After drying, the peels were mechanically ground into a fine powder.

Extraction method

Fresh peels were collected and dried at room temperature. Dried peels were powdered mechanically. Powdered peels were then packed in soxhlet apparatus and extraction was done. Using 15 grams of powdered peel, ethanol, methanol and water were prepared and subjected to soxhlet extraction with 300 mL of ethanol as solvent.[17] Extraction was carried out for 24 hours at 650℉. Itwas observed that all three extracts exhibited brownish color, solid structure, and sticky consistency.

The study focused on four bacterial cultures, each with distinct characteristics and identification numbers from the Microbial Type Culture Collection (MTCC). *Escherichia coli (E. coli)*, a Gram-negative bacterium, was identified with the MTCC number 43. *Staphylococcus aureus (S. aureus)*, which is Gram-positive and was assigned the MTCC number 87. *Pseudomonas aeruginosa (P. aeruginosa)*, also a Gram-negative bacterium, was cataloged under the MTCC number 2488. *Salmonella typhimurium (S typhimurium)*, another Gram-negative bacterium, was included in the study and is associated with the MTCC number 96.

Procedure for Qualitative phytochemical analysis

Detection of Alkaloids

Fifty milligrams of solvent-free extract were mixed with a few milliliters of diluted HCl and then filtered. Numerous alkaloidal reagents were used to properly examine the filtrate. Mayer's test, Wagner's test and Hager's test were performed for the detection of alkaloids. In Mayer's test, an extract is treated with Mayer's reagent, a solution of potassium mercuric iodide; a positive result is indicated by the formation of a cream or white-colored precipitate, suggesting the presence of alkaloids. Wagner's test involves the addition of Wagner's reagent, a solution of iodine in potassium iodide, to the extract, where a brown or reddish-brown precipitate confirms the presence of alkaloids. Hager's test, on the other hand, uses Hager's reagent, a saturated solution of picric acid, which reacts with alkaloids to produce a yellow crystalline precipitate. These tests are essential for the preliminary screening of alkaloids, which are bioactive compounds known for their therapeutic properties.

Test for carbohydrates

Molisch's test, Fehling's test, and Benedict's qualitative test were utilized to identify the presence of carbohydrates. Molisch's test, a sensitive chemical test, was employed to detect the presence of carbohydrates by reacting with alpha-naphthol in the presence of sulfuric acid to produce a violet ring at the interface, indicating positive carbohydrate content. Fehling's test was used as a confirmatory assay, where the reducing sugars present in the extract reacted with Fehling's solution, leading to the formation of a brick-red precipitate of cuprous oxide, confirming the presence of reducing sugars. Lastly, Benedict's qualitative test, which relies on the reduction of copper(II) ions to copper(I) in the presence of reducing sugars, was performed. The development of a red, yellow, or green precipitate upon heating the extract with Benedict's reagent further verified the presence of reducing sugars. These tests collectively provided robust evidence of carbohydrate presence in the extracts, contributing to our understanding of the phytochemical composition of Punica granatum peel.

Proteins and Amino Acids Detection

An extract weighing 100 mg was solubilized in 10 ml of distilled water and filtered using Whatman filter paper. The resulting filtrate underwent tests for proteins and amino acids, including the Ninhydrin test for amino acid detection.

Sterols and triterpenoids Detection

To ascertain the presence of sterols and triterpenoids, both Salkowski's test and the Sulphur powder test were conducted.

Detection of phenol compounds and Tannins

The presence of phenolic compounds was determined through the execution of the Ferric chloride test. In this test, a few drops of ferric chloride solution were added to the extract, leading to the formation of a color complex. The appearance of a deep blue, green, or purple color indicated the presence of phenolic compounds. These compounds are known for their significant antioxidant properties, as they can donate hydrogen atoms to free radicals, thereby neutralizing them and preventing oxidative stress. The positive result of the Ferric chloride test in our study confirmed that the Punica granatum peel extracts are rich in phenolic compounds, which may contribute to their observed antioxidant and antibacterial activities.

Antimicrobial activity test

Preparation of Bacterial Inoculums

A loop full of culture was transferred into sterile nutrient broth and incubated at 37°C overnight to assess antimicrobial activity.

Preparation of Culture Media

Muller Hinton agar (MHA), 38g in 1000ml, was used for antimicrobial testing, sterilized at 121°C for 15-30 minutes.

Concentration of Extract

Stock extract concentrations were prepared at 100mg/ml, 200mg/ml, and 300mg/ml in ethanol, methanol, and water.

Antibiotic Usage

Standard zones of inhibition were determined using Ampicillin, Tetracycline, and Penicillin. Antibiotic discs (3mm) were placed on MHA agar and incubated at 37°C, with observation for zones of inhibition after 24 hours.

Antimicrobial Activity via Well Diffusion Method

Agar well diffusion method was employed. Bacterial strains were cultured in nutrient broth for 24 hours. A 100µl cell suspension was spread on MHA plates. Wells (9mm diameter) were created and filled with 200µl of the respective 100 mg/ml ethanol, 200mg/ml methanol, and 300mg/ml water extracts, which also served as controls. Plates were incubated at 37°C and examined for inhibition zones after 24 hours.

Minimum Inhibition Concentration/Minimum Bactericidal Concentration Determination

The antimicrobial effects of ethanol, methanol, and aqueous extracts of Punica granatum pericarp against *E. coli, S. aureus, S. typhi,* and *P. aeruginosa* were evaluated using the broth dilution method.

Minimum Inhibition Concentration (MIC) Determination

In microtiter plates, 95µl of MHB and 5µl of inoculum were added to each well, except the first. Each column contained a different bacterium. The first and second wells received 100µl of the test sample, which was serially diluted from the second to the eleventh well, with the first and twelfth wells serving as controls. Plates were incubated at 37°C for 16-18 hours, followed by a 2–3-hour incubation with 20µl of resazurin dye.

Minimum Bactericidal Concentration (MBC) Determination

Inoculum from each well of the MIC plate was plated on MHA and incubated for 24 hours at 37°C. The dilution with no growth was identified as the MBC.

Antioxidant activity

The antioxidant activity of the extract was assessed using a DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. This involved mixing the extract at various concentrations (ranging from 50 to 500 µg/ml) with a DPPH solution in methanol. The mixture was incubated for 30 minutes at room temperature in darkness, and then the absorbance was measured using a UV spectrometer.

Standard solutions of ascorbic acid were prepared at the same concentrations as the extract for comparison. Different concentrations of *Punica granatum* peel extract were prepared and mixed with the DPPH solution, following the same procedure as the standard solution. The absorbance of the solutions was measured at 517 nm using a UV spectrophotometer.

RESULTS

Phytochemical screening of peel extract of Punica granatum

Table 1 presents the phytochemical analysis of ethanol, methanol, and aqueous extracts of Punica granatum peel. Tests were conducted for alkaloids, carbohydrates, glycosides, proteins, amino acids, saponins, phenolic compounds, tannins, and steroids. The presence ($^{+}$ve) or absence ($^{-}$ve) of these compounds was recorded for each extract type.

Table 1. Phytochemical analysis of ethanol, methanol and aqueous extract of *Punica granatum* peel sample

Phytochemical Compounds	Name of test	Ethanolic extract	Methanolic Extract	Aqueous extract
Alkaloids	Mayer's Test	-ve	-ve	-ve
Carbohydrates	Wagner's test	–ve	–ve	-ve
Glycosides	Hager's test	+ve	+ve	+ve
Proteins	Fehling's test	-ve	-ve	-ve
Amino Acids	Molisch's test	+ve	+ve	+ve
Saponins	Benedict's test	-ve	+ve	-ve
Phenolic Compounds	Ninhydrin test	+ve	+ve	+ve
Tannins	Foam test	–ve	–ve	–ve
Steroids	Ferric chloride test	+ve	–ve	–ve
Alkaloids	Salkowasky's test	+ve	+ve	+ve
	Sulfur powder test	+ve	+ve	+ve

Antibacterial Properties of Punica granatum peel extracts

The antibacterial activities of ethanol, methanol, and aqueous extracts against *E. coli, P. aeruginosa, S. aureus,* and S. *typhi* are detailed in **Tables 2-4**.

Table 2. Effect of ethanolic extract of *Punica granatum* against different bacteria.

Bacterial culture	Zone of inhibition (diameter in mm)				
	Antibiotic used	Standard zone	Ethanolic extract		
E. coli	Penicillin	13.6±1.52	17.6±2.08	20.6±2.51	22±2
	Tetracycline	15±2.88			
	Ampicillin	16.3±1.52			
P. aeruginosa	Penicillin	12.33±2.51	17.3±2.08	16±1	20±2
	Tetracycline	13.6±2.6			
	Ampicillin	20.33±1.52			
S. aureus	Penicillin	10.6±1.15	22±2	23±4	23±2.64
	Tetracycline	11±1			
	Ampicillin	16.66±2.88			
S. typhi	Penicillin	12±2.64	16.6±1.52	18.3±1.52	19±1
	Tetracycline	11±1			
	Ampicillin	17.66±251			

The zone of inhibition (diameter in mm) was measured for each extract and compared with standard antibiotics like Penicillin, Tetracycline, and Ampicillin. **Figures 1** shows the antibacterial activity of pomegranate peel extracts against four different bacteria. The activity is measured by the zone of inhibition in millimeters. Three types of extracts were tested: ethanol extract, methanol extract, and aqueous extract. The results indicate that the methanol

extract generally exhibited the highest antibacterial activity across all tested bacteria, followed by the ethanol extract, with the aqueous extract showing the least activity. The most significant zones of inhibition were observed against *S. aureus*, especially with the methanol extract.

Table 3. Effect of methanolic extract of *Punica granatum* against different bacteria under study.

Bacterial culture	Zone of inhibition (diameter in mm)				
	Antibiotic used	Standard zone	Methanolic extract		
	Penicillin	11.3±0.57			
E. coli	Tetracycline	11.6±2.08	20±1	21.3±1.52	22.3±1.52
	Ampicillin	15.3±4.16			
	Penicillin	6.6±2.88			
P. aeruginosa	Tetracycline	11±1	17±1	19±1	21±1
	Ampicillin	17±1.73			
	Penicillin	8±2.64			
S. aureus	Tetracycline	12.6±5.5	14.6±1.5	17±2.64	20±1
	Ampicillin	19±1			
	Penicillin	9±1			
S. typhi	Tetracycline	11±1	18.6±1.52	20.3±1.52	21±1.52
	Ampicillin	18±2.64			

Table 4. Effect of aqueous extract of *Punica granatum* against different bacteria under study

Bacterial culture	Zone of inhibition (diameter in mm)				
	Antibiotic used	Standard zone	Aqueous extract		
	Penicillin	7.3±4.04			
E. coli	Tetracycline	9±3.60	16±2.08	20.3±1.52	21.6±2.08
	Ampicillin	18±1			
	Penicillin	11±1			
P. aeruginosa	Tetracycline	13.3±2.88	21±1	20.3±1.52	24±1
	Ampicillin	23±3			
	Penicillin	10.3±1.52			
S. aureus	Tetracycline	12.6±2.08	20.6±1.15	21.6±1.52	21.6±2.08
	Ampicillin	19.6±1.52			
	Penicillin	7.3±2.08			
S. typhi	Tetracycline	10±1	12±1	14±1	16±1
	Ampicillin	16.3±1.52			

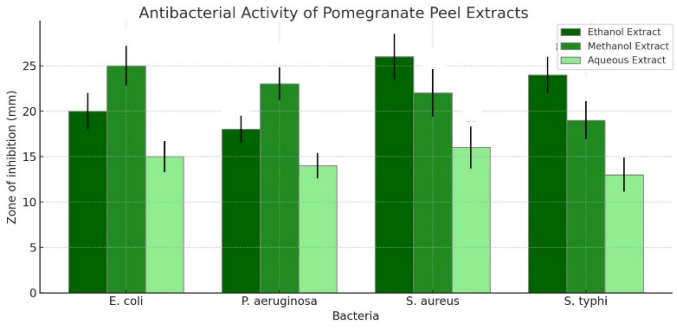

Figure 1. Antibacterial activity of *Punica granatum* peel extracts against various bacteria.

Following the preliminary testing for antibacterial activity using the well diffusion method, the minimum inhibitory concentration (MIC) and maximum inhibitory concentration (MBC) were calculated using the broth dilution method using the aforementioned four distinct bacteria **(Tables 5 and 6)**. For *E. coli*, *P. aeruginosa*, *S. Typhi*, and *S. aureus*, the ethanol extract showed an MIC of 50 mg/ml and an MBC of 75 mg/ml. The methanol extract had an MIC of 25 mg/ml and an MBC of 50 mg/ml. Lastly, the aqueous extract exhibited both an MIC and an MBC of 50 mg/ml and 75 mg/ml respectively (**Figure 2**). These comparisons show that, in all cases, the MBC is higher than comparisons show that, in all cases, the MBC is higher than the MIC, as expected. The MBC value being higher indicates that a higher concentration of the extract is required to kill the bacteria completely compared to just inhibiting their growth.

Antioxidant activities of the extracts of Punica granatum

The antioxidant activity of various extracts and ascorbic acid was assessed using their ability to inhibit DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals. Ascorbic acid, a known antioxidant, was used as a positive control. The results showed that ascorbic acid exhibited high percentage inhibition across different concentrations, indicating its strong antioxidant properties.

For instance, at a concentration of 50 µg/ml, ascorbic acid showed 36.69% inhibition, which increased significantly to 98.19% at 500 µg/ml. Similarly, the ethanol, methanol, and aqueous extracts of *Punica granatum* peels also demonstrated significant antioxidant activities, with the ethanol extract showing a particularly high inhibition percentage, reaching up to 97.55% at 500 µg/ml **(Figure 3)**.

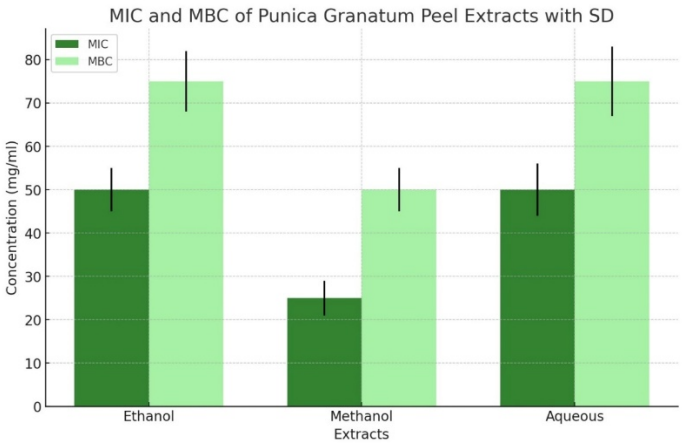

Figure 2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Punica granatum* peel extracts using three different solvents: ethanol, methanol, and aqueous.

DISCUSSIONS

The present investigation advances the understanding of the antimicrobial and antioxidative capabilities of *Punica granatum* (pomegranate) peel extracts, marking a significant contribution to the domains of natural product science and phytotherapeutics. Central to this research is the examination of various extracts (ethanol, methanol, and aqueous) and their effectiveness against

Table 5. Absorbance table of MIC (mg/ml) of *Punica granatum* peel for ethanol, methanol and aqueous extracts.

Name of bacteria	1	2	3	4	5	6	7	8	9	10	11	12
E. coli	C	-	-	-	+	+	+	+	+	+	+	+
Pseudomonas	C	-	-	-	+	+	+	+	+	+	+	+
Salmonella	C	-	-	-	+	+	+	+	+	+	+	+
Staphylococcus	C	-	-	-	+	+	+	+	+	+	+	+

Table 6. Absorbance table of MBC (mg/ml) of *Punica granatum* peel for ethanol, methanol and aqueous extracts

Name of bacteria	1	2	3	4	5	6	7	8	9	10	11	12
E. coli	C	-	+	+	+	+	+	+	+	+	+	+
Pseudomonas	C	-	+	+	+	+	+	+	+	+	+	+
Salmonella	C	-	+	+	+	+	+	+	+	+	+	+
Staphylococcus	C	-	+	+	+	+	+	+	+	+	+	+

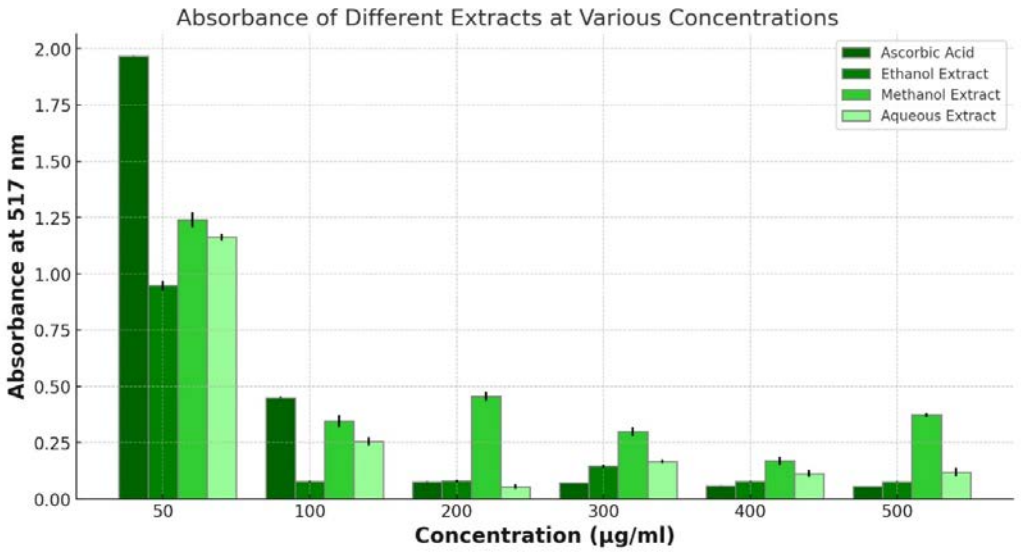

Figure 3: Represents the absorbance data of different peel extract of *Punica granatum* at various concentrations (50, 100, 200, 300, 400, 500 µg/ml). The absorbance values are measured at 517 nm, and the error bars indicate the standard deviation for each measurement. This graph provides a clear comparison of the antioxidant activities of the different extracts at varying concentrations.

a range of bacterial species, including *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. typhi*. A key aspect of this investigation is the detailed chemical analysis of the pomegranate peel extracts. The identification of a wide range of active components, such as alkaloids, phenols, glycosides, flavonoids, terpenoids, steroids, carbohydrates, proteins, and amino acids, highlights the complex makeup of these extracts. These findings are consistent with similar studies, underscoring the potential medicinal uses of pomegranate extracts.[18]

The research demonstrates that the methanol extract from pomegranate peels shows improved effectiveness against bacteria. The choice of ethanol, methanol and water as extraction solvents was primarily guided by the nature of the bioactive compounds targeted for this study, which are predominantly polar. These solvents are known to effectively extract phenolic compounds, flavonoids, and other polar phytochemicals that contribute to the antimicrobial and antioxidant activities of the extracts. Non-polar solvents, such as hexane and dichloromethane, were not employed because they are more suited for extracting non-polar compounds like lipids and certain terpenoids, which were not the primary focus of our investigation. This is indicated by its lower MIC and MBC, which are measures of an antimicrobial's effectiveness. This important discovery supports the potential of pomegranate peel extracts as effective alternatives to conventional antibiotics, consistent with the findings of earlier studies by Missa *et al*. and Naziri *et al.*[16, 19] The observed differences in how various bacteria respond to pomegranate peel extracts can be attributed to the unique structures of their cell walls and their natural sensitivities to the extract's active substances. The primary way these extracts combat bacteria is by compromising the structure of their cell walls, thereby hindering essential enzymatic activities within the bacterial cells.[7] This effect is mainly attributed to polyphenols, a group of compounds known for their ability to bind with and neutralize key bacterial components like adhesins, enzymes, and proteins involved in cell transport. These polyphenols can also interfere with bacterial DNA, thereby hindering their ability to multiply and grow.[20] Additionally, it has been found that the effectiveness of the extracts is influenced by the solvents used for extraction, like methanol and ethanol.[21] These solvents are more efficient in dissolving the active compounds compared to water, highlighting the importance of the extraction method in determining the potency of the extracts. This emphasizes that the choice of solvent is crucial in extracting various beneficial chemicals from plant materials and in determining their

mode of action against bacteria. The higher efficacy of methanol and ethanol extracts seen in the present study could be due to their ability to better solubilize these bioactive compounds compared to water. It is evident that the solvent used for extraction can also play a critical role in the mechanism of action, as it influences the types and quantities of phytochemicals extracted from any plant materials.[22]

The research emphasizes the notable antioxidative capabilities of these extracts, alongside their antimicrobial characteristics. Employing DPPH Radical Scavenging Activity as an indicator for antioxidative potential, the findings indicate that the ethanol extract, more so than other extracts, exhibits robust antioxidative properties. This implies the practicality of harnessing pomegranate peel extracts as organic sources of antioxidants, potentially rivaling ascorbic acid in efficacy. The existence of polar phenolic compounds, recognized for their antioxidative activities, might play a role in this phenomenon.[23, 24]

This research stands out for its thorough examination of the antibacterial and antioxidant effects of pomegranate peel extracts. The results emphasize the significant potential of these extracts in medical treatments, suggesting their usefulness as natural agents in treating various health issues and potentially in creating dietary supplements. The study opens avenues for the development of natural antibiotic and antioxidant agents, setting the stage for future research to understand the processes behind these properties and to assess their efficacy in clinical settings.

CONCLUSION

The present study of *P. granatum* (pomegranate) peel extracts, prepared using ethanol, methanol, and water, has provided valuable confirmations that these extracts, often discarded, exhibit potent antibacterial properties, effective against both Gram-positive and Gram-negative bacteria. Furthermore, these extracts demonstrate considerable antioxidant capabilities, with the ethanol extract showing the highest effectiveness, on par with known antioxidants like ascorbic acid. This outcome was surprising, as it was initially believed that the water extract would be more effective against DPPH radicals. The results underscore the potential of these natural extracts in combating oxidative stress, which is implicated in numerous health conditions.[25, 26]

Investigation into the chemical composition of pomegranate peels uncovered a range of active compounds, including alkaloids, flavonoids, steroids, tannins, cardiac glycosides, and terpenoids. This diversity indicates the rich chemical makeup of pomegranate peels and underscores the importance of the extraction solvent. Different solvents pull out various bioactive compounds, highlighting the need for tailored methods in using these extracts for health benefits.

This study aligns with sustainable development goals (SDGs) by showing how pomegranate peels, often seen as waste, can be transformed into beneficial health products. This approach aids in sustainable consumption and production (Goal 12) by reducing waste and making efficient use of resources. Additionally, the health benefits of these extracts contribute to Goal 3, which aims to ensure health and well-being for all. This method not only offers a sustainable route in healthcare but also encourages exploration into the medicinal value of other typically discarded natural materials.

The research suggests exploring other parts of the pomegranate, like seeds and leaves, for more health benefits. However, detailed biochemical analysis is essential for these natural remedies to gain mainstream acceptance in medicine, especially in determining their long-term safety and compatibility with human health. This research is crucial in maximizing the use of natural resources at a time when sustainable and effective healthcare solutions are increasingly important.

CONFLICT OF INTEREST STATEMENT

Authors declare that there is no financial or academic interest that might have influenced this work.

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