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Evaluation of the antibacterial properties of methanolic pomegranate peel extract against standard strains of *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Escherichia coli*: An in Vitro Study

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Abstract

Background: The increasing prevalence of antimicrobial resistance has intensified the search for effective natural alternatives to synthetic antibiotics. Pomegranate (*Punica granatum*) peel, a widely available agricultural by-product, is known for its rich phytochemical composition. We aimed to quantify the in vitro antibacterial activity of pomegranate peel methanolic extract (PPME) against selected Gram-positive and Gram-negative bacteria and analyze their differential susceptibility.

Methods: Here, PPME was prepared using a Soxhlet apparatus, and its antibacterial efficacy was tested against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa* via the agar well-diffusion method at the concentrations of 50, 100, 200, and 400 mg/mL. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth dilution technique. Data were analyzed employing one-way ANOVA.

Results: The PPME demonstrated notable dose-dependent antibacterial activity ($p < 0.0001$), especially against Gram-negative bacteria. *Escherichia coli* was the most sensitive bacterial strain, with the largest inhibition zone (27 mm) at 400 mg/mL and the lowest MIC (12.5 mg/mL) and MBC (25 mg/mL). Gram-positive bacteria were more resistant to PPME, for which *Bacillus cereus* showed the highest MIC (100 mg/mL) and MBC (200 mg/mL).

Conclusion: Pomegranate peel extract possesses potent antibacterial activity, exhibiting relatively stronger efficacy against Gram-negative pathogens, offering it a promising sustainable candidate for developing novel antibacterial agents, especially against resistant Gram-negative pathogenic strains. Further research is warranted to identify the active compounds of PPME and their mechanisms of action.

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Highlights

What is current knowledge?

Pomegranate peel is known to be rich in phytochemicals and to have general antibacterial properties.

What is new here?

This study provided new evidence that pomegranate peel extract was more effective against Gram-negative vs. Gram-positive bacteria, a reversal of the typical expectation for natural antimicrobials and a key guidance for targeting resistant infections.

Introduction

The use of medicinal plants was one of humanity's earliest innovations for treating diseases, nurturing a close relationship between humans and plants throughout human evolution (1-4). Natural plant derivatives, despite their biodiversity, represent an essential resource for drug discovery. With their broad applications, these compounds serve both as direct therapeutic agents and as building blocks for designing and synthesizing novel drugs (5-7). Plants and their extracts, which have long been used in many areas of traditional medicine, abundantly contain a wide range of structurally diverse secondary metabolites with multiple biological functions (8).

A major healthcare challenge today is the treatment of antimicrobial-resistant infectious diseases, drawing significant attention toward medicinal plants as safer and natural alternatives for synthetic agents. Consequently, global research is steadily expanding to divulge the antibacterial properties of various plant species (9-12). Medicinal plants have gained widespread acceptance in human societies for numerous reasons, including their natural origin, excellent safety, and cost-effectiveness. These natural remedies typically exhibit fewer side effects and, due to their superior compatibility with the human body's genetic and physiological makeup, represent a suitable option for most people (13-17). Research has established that the bioactive compounds of medicinal plants are not limited to their fruits but are also abundant in non-edible parts such as leaves, peels, and seeds. A notable example is pomegranate (*Punica granatum L.*), a member of the *Lythraceae* family native to Central Asia. The peel, seeds, and leaves of pomegranate contain substantial levels of phenolic compounds, contributing to its biological functions as an effective therapeutic agent. Scientific studies have confirmed the potent antioxidant, anti-inflammatory, antifungal, antibacterial, and antimicrobial properties of pomegranate (18,19). Pomegranate extracts prepared via different techniques exhibit antimicrobial activity against various Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus (S. aureus)*, *Bacillus subtilis* (*B. subtilis*), and *Bacillus cereus* (*B. cereus*), as well as against yeasts and molds such as *Candida albicans* (*C.*

albicans) and *Fusarium oxysporum* (*F. oxysporum*) (20). Pomegranate peel methanolic extract (PPME) has shown significant antibacterial effects, particularly against Gram-positive bacteria such as *S. aureus*, which has been attributed to its rich punicalagin and polyphenol content. Also, PPME has been effective against Gram-negative bacteria like *Escherichia coli* (*E. coli*), indicating its capacity to disrupt the cellular integrity of these microorganisms (21). The objective of this study was to evaluate the in vitro antibacterial activity of PPME against four standard pathogenic bacteria, *S. aureus*, *B. cereus*, *Pseudomonas aeruginosa* (*P. aeruginosa*), and *E. coli*.

Methods

This study was conducted in the research laboratory of the Islamic Azad University, Tabriz Medical Sciences Branch. Pomegranate (*Punica granatum*) peels were collected in autumn from the city of Marand. The formal identification of the plant was undertaken by Ms. Fatemeh Chobineh, a botanist at the Islamic Azad University, Ardabil Branch. The methanolic extract was prepared using a Soxhlet apparatus using 500 mL of methanol as the solvent and 300 grams of powdered pomegranate peel at 40°C for 8 hours. The solvent was then removed using a rotary evaporator. Standard bacterial strains, *S. aureus* (ATCC: 25923), *B. cereus* (ATCC: 1247), *P. aeruginosa* (ATCC: 27853), and *E. coli* (ATCC 25922), were obtained from the University of Tehran Microorganism Collection Center. The extracts prepared were stored in dark containers in a refrigerator until testing.

The extracts were concentrated to 50, 100, 200, and 400 mg/mL using 5% dimethyl sulfoxide (DMSO) as the solvent. Bacterial strains were exposed to these concentrations to assess antibacterial activity using the well diffusion assay. The microorganisms were cultured on Mueller-Hinton agar (MHA) (Merck, Germany) one the day prior to the experiment. To prepare the bacterial suspension, several colonies from a fresh young bacterial culture were transferred to MHA. The turbidity of the microbial suspension was adjusted to the 0.5 McFarland standard, equivalent to a concentration of 1.5×10^6 CFU/mL, which was achieved by diluting the bacterial suspension to a 1:100 ratio. Next, the antibacterial activity of PPME was evaluated using the agar well diffusion method. The bacterial suspension with a turbidity equivalent to 1.5×10^6 bacteria/mL was spread over three different directions on MHA plates using a sterile cotton swab. Subsequently, wells with a diameter of 6 mm and a depth of 5 mm were created on the agar, maintaining an appropriate distance of 2.5 cm from each other. Each well was exposed to 100 µL of PPME at different concentrations (50, 100, 200, and 400 mg/mL). The negative control contained only the solvent (5% DMSO), and the positive control included streptomycin antibiotic (10 µg) (Padtan Teb Iran).

The plates were incubated at 37°C for 24 hours, after which the diameters of bacterial growth inhibition zones were measured and recorded in millimeters. To ensure the reliability of the findings, all assays for each extract concentration and each bacterium were performed in triplicate. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using Mueller-Hinton broth (MHB) and MHA (Merck, Germany) and by preparing the serial dilutions of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 mg/mL, where culture media containing bacteria without extract and culture media without bacteria served as positive and negative controls, respectively. The test tubes used for MIC determination were incubated at 37°C for 24 hours, and the lowest extract concentration preventing visible bacterial growth (i.e., no turbidity) was recorded as MIC. To determine MBC, samples were taken from the three tubes preceding the MIC point and cultured on MHA for 24 hours at 37°C, and the concentration showing no growth was considered as the MBC. To minimize experimental errors, each assay was performed in triplicate. SPSS version 26 (SPSS Inc., Chicago, IL, USA) was used to statistically analyze the data using one-way ANOVA.

Results

This study comprehensively investigated the antibacterial activity of PPME against two Gram-positive (*S. aureus* and *B. cereus*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. The findings revealed that PPME possessed significant growth inhibitory effects on the bacteria tested at the 50, 100, 200, and 400 mg/mL concentrations ($P < 0.0001$ for all studied bacteria) (Table 1, Figure 1).

Table 1. Bacterial Growth inhibition zone (mm) of pomegranate peel methanolic extract at various concentrations

Bacterial strains		Extract concentration (mg/mL)						
		50	100	200	400	P-value	Negative control	Positive control
Gram positive	*S. aureus*	0	11.67±0.58	15.33±0.58	20.67±1.53	< 0.0001	-	24
	B. cereus	0	0	9.33±1.53	11.33±1.15	< 0.0001	-	21
Gram negative	*E. coli*	14.33±0.58	19.33±0.58	22.33±1.15	27±1.73	< 0.0001	-	22
	P. aeruginosa	10.67±1.53	15±1.73	19.67±2.08	23±1	< 0.0001	-	20

*The results of one-way ANOVA showed statistically significant differences between various concentrations for all cases ($p < 0.0001$ for all bacteria). The F-values were as follows: *S. aureus*: 285.65, *B. cereus*: 104.87, *E. coli*: 75.23, and *P. aeruginosa*: 31.15.

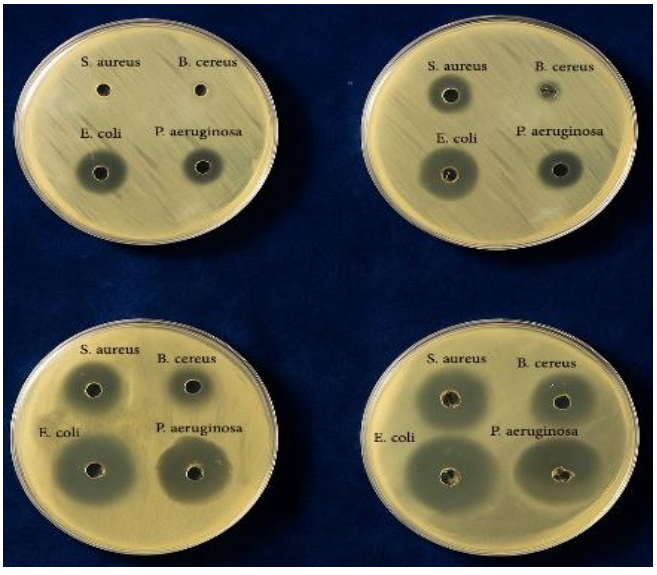

Figure 1. Zone of growth inhibition related to pomegranate peel methanolic extract against *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa* isolates

Regarding the MIC and MBC values calculated, there was a distinct sensitivity pattern among these bacteria. Gram-negative strains (*E. coli* and *P. aeruginosa*) showed remarkably higher sensitivity to PPME, evidenced by much lower MIC values (*E. coli*: 12.5 mg/mL and *P. aeruginosa*: 25 mg/mL) compared to Gram-positive bacteria (*S. aureus*: 50 mg/mL and *B. cereus*: 100 mg/mL) (Figure 2). This pattern was also evident in MBC values, where *E. coli* (With MBC equivalent to 25 mg/mL) was identified as the most sensitive bacteria, and *B. cereus* (With MBC equivalent to 200 mg/mL) was the most resistant microorganism (Table 2).

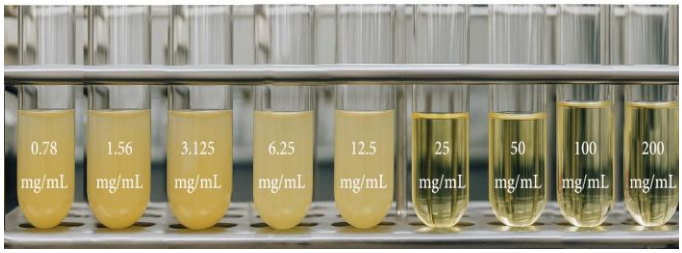

Figure 2. MIC and MBC of pomegranate peel extract against selected bacterial isolates

Table 2. MIC and MBC of pomegranate peel methanolic extract against the bacterial strains tested

Bacterial strains		MIC (mg/mL)	MBC (mg/mL)	P-value
Gram positive	*S. aureus*	50	100	< 0.0001
	B. cereus	100	200	< 0.0001
Gram negative	*E. coli*	12.5	25	< 0.0001
	P. aeruginosa	25	50	< 0.0001

Although the precise underlying mechanism of this selective effect requires further investigations, the low MIC and MBC values for Gram-negative bacteria indicate that PPME active compounds (Such as punicalagin, anthocyanins, and flavonoids) may preferentially interact with specific components residing in the outer membrane of Gram-negative bacteria. Additionally, differences in membrane permeability, variations in efflux systems, or the presence of specific receptors in Gram-negative bacteria could explain this unique pattern.

Discussion

Currently, infectious diseases remain a leading cause of morbidity and mortality around the globe. The discovery of effective treatments for microbial diseases has grabbed increasing attention for disease control, particularly with regard to multidrug-resistant infections. Alkaloids, anthraquinones, saponins, tannins, and polyphenols, which are abundantly present in plants, are rich sources for developing novel antimicrobial agents (22). These natural sources, especially plant-derived bioactive chemicals, have been studied as complements or substitutes for traditional antimicrobial medications in order to address the rising issue of antimicrobial resistance and the pressing need for efficient therapies for bacterial infections (23,24). Accordingly, the present study confirmed the notable performance of PPME as a potential therapeutic agent to fight against pathogenic bacterial strains. In a study by Dahham et al., the antibacterial effects of the methanolic extracts of different parts of pomegranate (Seed, fruit, peel, and juice) were evaluated on some bacteria, including *S. aureus*, reporting that the peel extract of pomegranate had the strongest antimicrobial activity (25). Similarly, we found that PPME exhibited the MIC and MBC values of 50 and 100 mg/mL against *S. aureus*, respectively, with a mean inhibition zone of 23.7 mm. These results are consistent with some previous reports but differ from others. For example, Malviya et al. reported an inhibition zone of 24.5 ± 0.53 mm for pomegranate methanolic extract against *S. aureus* (26), which was close to the value obtained in our study. In another study by Alam Khan et al. in India, the PPME exhibited a minimum inhibition zone of 22 mm against *P. aeruginosa* (27), which was consistent with our findings.

In contrast, Yassin et al. (2021) (28) reported considerably lower MIC and MBC values for the PPME against *S. aureus* (MIC and MBC of 0.125 and 0.250 mg/mL, respectively) compared to our study. Moreover, in the recent report, *E. coli* revealed an MIC of 50 mg/mL and an inhibition zone diameter of 23.7 mm. Likewise, Hamrita et al. reported the MIC and MBC values of 9.37 and 18.75 mg/mL, respectively, for *P. aeruginosa* (29), which differed from our results. In the study conducted by Al-Hassnawi, at the concentrations of 50 and 100 mg/mL, the smallest inhibition zones were related to *P. aeruginosa* (8.33±0.57 mm and 12.16±0.57 mm, respectively), while the largest inhibition zones at these concentrations were related to *S. aureus* (19±1 mm and 21±1 mm, respectively) (30). These results partially aligned with our findings. In another study by Sweidan et al., the highest antimicrobial activity of pomegranate methanolic extract was observed against *P. aeruginosa* (MIC50 = 7.5 mg/mL) (31), which contradicted our findings. Alnees et al. tested the antimicrobial activity of three types of pomegranate extract against *P. aeruginosa*, asserting that the most potent effects belonged to the methanolic extract (32). In another study, the antibacterial activity (Based on growth inhibition zones) of pomegranate peel extract was considerable against *S. aureus* and *P. aeruginosa*, but not against *E. coli* (33). According to Akarca et al., the PPME possessed the highest antimicrobial effects, where *B. cereus* was susceptible to all extracts except aqueous seed extract. In this study, the inhibition zone diameter for PPME was 25.68 ± 0.45 mm against *B. cereus* and 25.14 ± 0.75 mm against *E. coli*, indicating that the greatest antimicrobial effect belonged to *B. cereus* (Mean zone diameter: 21.00 ± 0.05, P > 0.05). In another experiment, the MIC and MBC values of PPME for *B. cereus* were reported as 62.5 and 15.63 µg/mL, respectively (34), showing some deviations from the findings of the present study. Meléndez and Capriles (2006) used the disk diffusion technique to assess the antibacterial activity of a number of tropical plants from Puerto Rico against *S. aureus* and *E. coli*, reporting that pomegranate extract generated growth inhibitory zones of 20 and 11 mm for these bacteria, respectively (35). The antibacterial activity variations observed between our findings and previous reports may be attributed to several factors, including harvest seasons, plant age, geographical origins, extraction methods, drying techniques, and bacterial growth stage.

The most salient finding of this study was the unexpected and significantly higher sensitivity of Gram-negative bacteria, *E. coli* and *P. aeruginosa*, to PPME, as demonstrated by remarkably lower MIC and MBC values compared to Gram-positive strains. These findings suggest that particular bioactive chemicals in the extract, namely punicalagins, ellagitannins, and flavonoids, may target structural weaknesses peculiar to Gram-negative bacteria's envelope, while they cannot penetrate the thick peptidoglycan layer of Gram-positive bacteria. By interacting with and destabilizing the lipopolysaccharides that anchor to structural components, these polyphenols, which are well-known for their metal-chelating and membrane-disrupting capabilities, can efficiently permeabilize the outer membrane. The strong bactericidal effects observed may be the consequence of this first breach, which subsequently allow additional antimicrobial components to enter and enhance damage to the cytoplasmic membrane and the underlying thin peptidoglycan layer. The superior efficacy against Gram-negative organisms can be partly explained by this phenomenon, highlighting the role of outer membrane disruption rather than targeting the peptidoglycan layer, further elaborating on the novelty of our work.

Conclusion

In summary, this study not only confirmed the potent efficacy of the PPME against bacterial pathogens but also revealed a remarkable finding, namely the unique susceptibility patterns of Gram-negative vs. Gram-positive bacteria, as demonstrated by notably lower MIC and MBC values of the former. This discovery opens new avenues for future research into the mechanisms of action and the development of novel antibacterial agents derived from natural compounds. Future studies should include a detailed phytochemical profiling of PPME using techniques such as GC-MS or HPLC to identify and quantify the specific bioactive compounds responsible for the observed antibacterial effects. Furthermore, our findings establish a foundation for designing innovative antimicrobial formulations to cope with infections caused by Gram-negative pathogens and manage antimicrobial resistance.

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Ethical statement

Not applicable.

Conflicts of interest

Authors declare no conflict of interests.

Author contributions

Concept/Design: A.J.S, M.P; Data acquisition: A.J.S; Data analysis and Interpretation: A.G, Z.G; Drafting the manuscript: A.G, Z.G; Critical revision of the manuscript: AJS; Final approval and Accountability: MP; Technical or material support: A.J.S, M.P; Supervision: MP; Securing funding (If available): N/A.

Data availability statement

The authors confirm that the data supporting the findings of this study are included in the article.

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