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Pomegranate peel extract is an effective agent against MDR bacteria

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ABSTRACT

Multidrug-resistant (MDR) bacteria are one of the major public health threats facing humanity. Infections with MDR strains are difficult or impossible to treat with standard antibiotics leading to severe illnesses and even deaths. The spread of MDR bacteria has necessitated the search for alternative approaches that tackle MDR pathogens. Natural plants can be utilized as alternative therapeutic agents against the rise of MDR bacteria. In this study, we aimed to assess the antimicrobial activity of pomegranate peel extracts (PPE) against MDR clinical isolates. A total of 9 clinical isolates (8 MDR and 1 non-MDR clinical isolates) were collected and examined for their susceptibility to PPE. Using the zone of inhibition assay, 4 isolates (*S. aureus, three MRSA* isolates*, Vancomycin-resistant Enterococci* (VRE), and *Acinetobacter baumannii)* were sensitive to PPE. In Broth assay, 4 mg/ml PPE significantly reduced the growth (*S. aureus, three MRSA* isolates*, Vancomycin-resistant Enterococci* (VRE), and *Acinetobacter baumannii),* while 40 mg/ml PPE either significantly reduced or completely inhibited the growth of the isolates. The minimum bactericidal concentration (MBC) of PPE against *S. aureus* and MRSA-88 was 10 mg/ml. This study showed the potential of PPE as an alternative compound for treating infections caused by PPE-sensitive MDR bacteria.

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Introduction

Pomegranate peel is a byproduct of the fruit juice processing industry, comprising nearly 30–40% of fruit portions (1). Pomegranate peel has been reported to contain a large number of phytochemicals such as hydrolyzable tannins, phenolic acids, and flavonoids (2, 3). Pomegranate peel is rich in polyphenols (phenolic acids, tannins, and flavonoids particularly anthocyanins) which are known to have diverse biological functions including effectiveness against pathogenic microorganisms (2-6). These activities of pomegranate peel may be exploited as a phytomedicine for humans, to eliminate the use of antibiotics and reduce their cost (1).

It has been reviewed by that pomegranate has a broad spectrum of antimicrobial effects against Gram-negative, Gram-positive bacteria (*B. coagulans, B. cereus, B. subtilis, S. aureus, E. coli, K. pneumoniae, Salmonella, Salmonella enterica, P. aeruginosa, E. aerogenes, S. marcescens, Brucella spp.,* and *R. glutinis*), fungi, and mold (*F. sambucinum, P. digitatum, Saccharomyces cerevisiae, Monilinia laxa, M. fructigena, B. cinerea, and P. expansum* and *F. oxysporum f. sp. lycopersici*) (7). However, different extracts from different parts of pomegranate have various antimicrobial activities. A study by many scholars showed that the antimicrobial activity of pomegranate peel extract was more potent than other parts, and the antimicrobial activity of pomegranate peel extract was related to the total flavonoids and tannins content (1, 5, 7). Also, punicalagin is one of the polyphenols isolated from pomegranate peels. Punicalagin is reported to possess antimicrobial activities against pathogenic Gram-positive, Gram-negative bacteria and yeast strains (*Candida albicans, Pseudomonas aeruginosa, Salmonella enteritidis, Escherichia coli, Staphylococcus epidermidis, Staphylococcus xylosus, Staphylococcus aureus, Bacillus cereus, Lactobacillus sakei ssp. Sakei, Lactobacillus plantarum, Pediococcus acidilactici, Enterococcus faecium, Enterococcus faecalis, Enterococcus mundtii, Enterococcus sulfureus, Enterococcus casseliflavus,* and *Enterococcus columbae*). The high levels of polyphenols, particularly punicalagin and ellagic acid, present in pomegranate peel have been responsible for its antifungal properties (1, 8). In addition to the reported antibacterial and antifungal activities of pomegranate peel extracts, a recent study reported the anti-biofilm potential against biofilms of *B. cereus, B. subtilis,* and *E. faecalis (9)*.

The pathogenic *S. aureus* is one of the leading nosocomial pathogens in hospitals. Particularly, the extraordinary capability of this bacterium to attain antibiotic resistance elements is regarded as a major motive for the elevated nosocomial infections initiated by *S. aureus (10)*.Therefore, infections with MRSA are one of the fundamental sources of morbidity and mortality worldwide (11, 12). Herein,

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this study investigated the antimicrobial activity of pomegranate peel extracts (PPE) against 8 multidrug-resistant (MDR) clinical isolates including MRSA isolates.

Materials and Methods

Preparation of plant extract

The dried powder of *Punica granatum* was procured from Abha city in Saudi Arabia. 50 gm of powdered material was filled in a muslin cloth and exposed to a Soxhlet extractor for hot extraction with absolute ethanol. Subsequently, the ethanolic extract of *P. granatum* was filtered by Whatman-1 filter paper and the filtrate was evaporated at a reduced temperature and pressure by a rotary evaporator. The dried extracts were re-dissolved further in 20% ethanol at the concentration of 1 mg/ml that was used for antibacterial assays.

Bacterial strains and growth medium

Bacterial strains are listed in Table 1. A total of 9 clinical isolates were collected from patients at Asir Central Hospital, Abha city, Saudi Arabia. Specimens were collected from different sources from patients including wounds, blood, sputum, oropharyngeal secretion, and cerebrospinal fluid. All the clinical isolates were identified and their susceptibility to antibiotics was determined using an automated system (Vitek 2®, Biomérieux®) as recommended by the manufacturer at the Clinical Microbiology Laboratory, Asir Central Hospital. Bacteria were routinely cultured aerobically in nutrient media at 37°C with shaking (200 rpm) for 18 h.

Assessment of antimicrobial activity

Zone of inhibition assay

An overnight culture of the tested strain was adjusted to an OD600 of 0.1, and 100 µL of the diluted culture was spread over the surface of a nutrient agar plate. A 10 µL of either pomegranate extract (0.5 mg) or 25% ethanol (negative control) was spotted on the inoculated plates, and the plates were incubated overnight at 37 °C. The plates were inspected by measuring the average diameter of the zone of inhibition.

Broth inhibition assay

An overnight culture of the tested strain was adjusted to an OD600 of 0.1, and 50 µL of the diluted culture was inoculated into glass tubes containing 2 mL nutrient broth (initial inoculum of 10^5–10^6 CFU/mL). A 160 µL of either pomegranate extract (40 mg/ml) or 25% ethanol was added to one tube. A 16 µLof either pomegranate extract (4 mg/ml) or 25% ethanol was added to another tube. After 24 h of incubation at 37 °C, the cultures were serially diluted 10-fold and 10 µL aliquots of each dilution were spotted on a nutrient agar plate. After 24 h of incubation at 37 °C, the number of CFUs produced by each dilution was determined using the following equation: (CFU * dilution factor) *100 = CFU ml^{-1}.

Determination of minimum bactericidal concentrations (MBC)

The MBC of pomegranate extract was determined using a standard broth dilution method as previously described (13). Four milliliters of nutrient broth were added to the first series of glass tubes, and 2 mL of nutrient broth was added to the rest. A 320 µL aliquot of pomegranate extract containing 160 mg was added to the first tube for a final pomegranate extract concentration of 40 mg/ml and serially diluted two-fold to reach a final concentration of 0.31 mg/ml in the final tube. The inoculum was prepared as described above. A 50 µL of the tested strain (5×10^5 CFU/mL) was added to the tubes and the tubes were incubated aerobically at 37 c with shaking (200 rpm). After 24 h of incubation, the cultures were serially diluted 10-fold and 10 µL aliquots of each dilution were spotted on a nutrient agar plate. After 24 h of incubation at 37 °C, the number of CFUs produced by each dilution was determined as described above. The lowest concentration of pomegranate extract that kills 99.9% of the tested strain was considered as the MBC.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 9.2.0) (GraphPad Software; San Diego, CA, USA). Descriptive statistics were also reported for the collected data. A t-test was performed to analyze two independent data groups. A p-value < 0.05 was considered statistically significant**.**

Results

PM shows antibacterial activity against several multidrug-resistant (MDR) clinical isolates

We utilized PM to screen 8 clinical isolates including Gram-positive and –negative bacteria (Table 1). These isolates were obtained from patients with respiratory infection, bacteremia, and wound infection (Table 1). Antibiotic susceptibility testing was conducted using an auto-

Table 1. Strains used in this study.

Strain	Characteristics		Reference
S. aureus	Clinical isolate from an unknown source	(14)	
MRSA-22	Clinical isolate from a wound		This study
MRSA-71	Clinical isolate from a wound		This study
MRSA-88	Clinical isolate from blood		This study
A. bumannii	Clinical isolate from sputum		This study
K. pneumoniae	Clinical isolate from a wound		This study
P. fluorescens	Clinical isolate from an oropharyngeal secretion		This study
Neisseria spp	Clinical isolate from cerebrospinal fluid (CSF)		This study
Vancomycin-resistant Enterococci (VRE)	Clinical isolate from blood		This study

Table 2. Antibiotic resistance profiles of bacterial clinical isolates.

Antibiotics	MRSA-22	MRSA-71	MRSA-88	VRE	*A. bumannii*	*K. pneumoniae*	*P. fluorescens*	*Neisseria spp*
Amikacin	--	--	--	--	--	R	S	--
Amoxicillin/Clavulanate	R	R	R	--	--	R	--	--
Ampicillin	R	R	R	--	--	R	--	--
Azithromycin	S	S	S	--	--	--	--	--
Cefepime	--	--	--	--	R	R	S	S
Cefitriaxone	--	--	--	--	--	--	--	S
Cefotaxime	--	--	--	--	R	R	R	--
Cefoxitin	--	--	--	--	--	--	--	--
Ceftazidime	--	--	--	--	R	R	I	--
Cefuroxime	--	--	--	--	--	--	--	S
Ciprofloxacin	R	R	S	--	R	R	I	--
Clindamycin	S	S	S	--	--	--	--	--
Colistin	--	--	--	--	S	--	S	--
Daptomycin	S	S	S	--	--	--	--	--
Entrapenem	--	--	--	--	--	R	--	--
Erythromycin	S	S	S	R	--	--	--	S
Fosfomycin	S	S	S	--	--	--	--	--
Fusidic Acid	I	I	I	--	--	--	--	--
Gentamicin	S	I	R	--	R	R	S	R
Imipenem	R	R	R	--	R	R	I	S
Levofloxacin	R	R	S	R	--	--	--	--
Linezolid	S	S	S	S	--	--	--	--
Meropenem	--	--	--	--	R	R	I	S
Moxifloxacin	R	R	S	--	--	--	--	--
Mupirocin	S	S	S	--	--	--	--	--
Nitrofurantoin	--	--	--	i	--	R	--	--
Norfloxacin	--	--	--	--	--	R	I	--
Oxacillin	R	R	R	--	--	--	--	--
Penicillin	R	R	R	--	--	--	--	--
Piperacillin And Tazobactam	--	--	--	--	R	R	R	S
Rifampin	S	S	S	--	--	--	--	--
Synercid	S	S	S	--	--	--	--	--
Teicoplanin	S	S	S	R	--	--	--	--
Tetracycline	S	S	R	S	--	--	--	--
Tigecycline	--	--	--	S	S	--	--	--
Trimethoprim / Sulfamethoxazole	S	R	S	--	S	R	R	S
Vancomycin	S	S	S	R	--	--	--	--

mated system. Out of 9 clinical isolates, 8 isolates were considered multidrug-resistant (MDR) bacteria (Table 2). We determined the killing activity of PM against the MDR and non-MDR isolates using the zone of inhibition assay (ZOI). Four clinical isolates; *S. aureus*, three MRSA isolates, Vancomycin-resistant *Enterococci* (VRE), and *Acinetobacter baumannii* were sensitive to 0.5 mg PM (Fig 1). Their zone of inhibition ranged from 12.6 mm to 24.6 mm (Table. 3). In contrast*, K. pneumonia, P. fluorescens, and Neisseria spp* were either partially sensitive or resistant to 0.5 mg PM (Fig 1).

Although the ZOI assay is a convenient method to test whether a compound possesses antibacterial activity, it is not a quantitative method. Thus, we confirmed the results

Table 3. Antibacterial activity of PPE against pathogenic bacterial clinical isolates. Where MBC: minimum bactericidal concentration; NA: not applicable.

Strain	Zone of inhibition Mean ± SD (mm)	MBC (µg/ml)
S. aureus	12.6 ± 0.5	10
MRSA-88	13.3 ± 0.5	10
MRSA-71	16 ± 1	N/A
MRSA-22	14 ± 1	N/A
VRE	24.6 ± 1.5	N/A
A. baumannii	22.6 ± 0.5	N/A

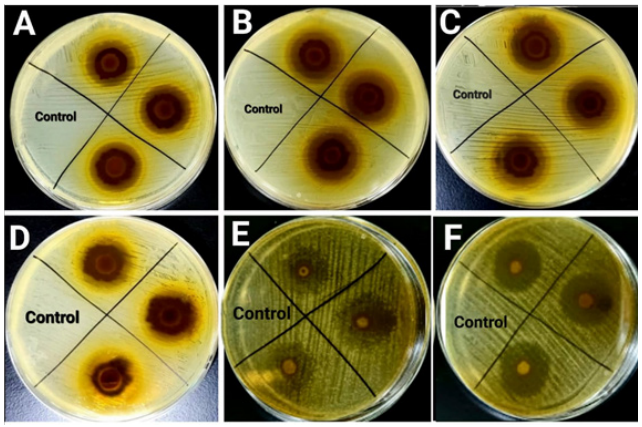

Figure 1. The Zone of inhibition assay of PPE against pathogenic bacterial clinical isolates. A 10 µL of PPE containing 0.5 mg, or ethanol was spotted on a lawn of MRSA-22 (A), MRSA-88 (B), S. aureus (C), MRSA-71 (D), VRE (E), and A. baumannii (F). PPE produced clear zones of inhibition compared to ethanol on the lawn of the tested isolates.

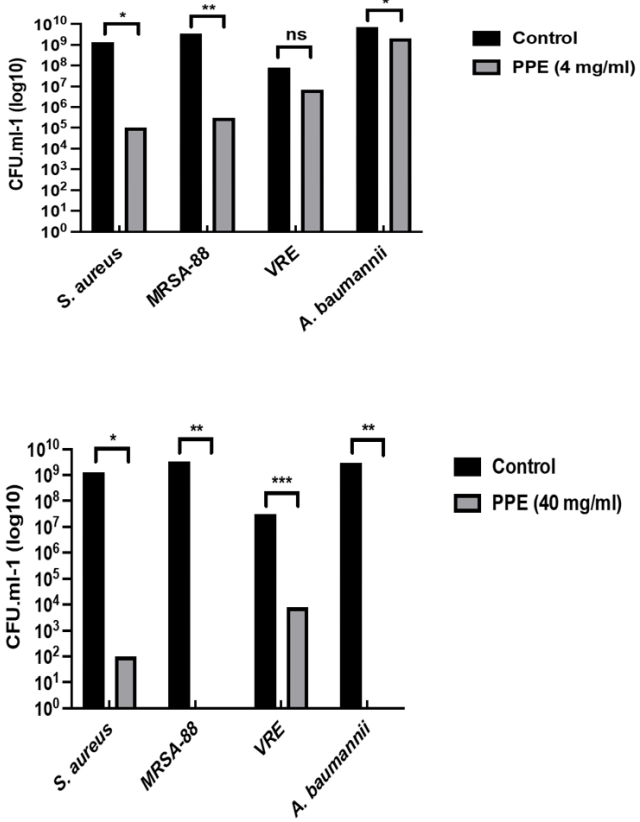

Figure 2. Inhibitory effect of PPE on the growth of pathogenic bacterial clinical isolates. Tubes containing nutrient broth with 4 mg/ml (A) or 40 mg/ml (B) of PPE, or with an equal volume of ethanol (control), were inoculated with 10^5 CFU of the tested isolate. Viable bacteria were quantified by determining the CFU. Statistical significance between treatment and control for each strain was determined by unpaired, two-tailed t-test; *, $P < 0.05$; ****, $P < 0.0001$; ns, not significant.

obtained from the zone of inhibition assay by evaluating the sensitivity of the selected isolates using a broth assay (a quantitative method). We tested two different concentrations of PM; 40 and 4 mg/ml. As shown in Figure 2A, only 4 mg/ml of PM significantly inhibited the growth of *S. aureus* and MRSA-88 by nearly 4 logs. However, the same concentration of PM (4 mg/ml) had a slight effect on the growth of VRE and *A. baumannii* by inhibiting their growth by only ½ to 1 log (Fig 2A). When we increased the concentration of PM to 40 mg/ml, greater inhibition of the tested bacterial growth was reported (Fig 2B). *S. aureus*, MRSA-88, and A. baumannii did not grow in the presence of 40 mg/ml while approximately 3.5 logs reduction of the growth of VRE was reported (Fig 2B).

Furthermore, we determined the MBC of PM for two selected strains, *S. aureus,* and MRSA-88. PM was diluted two-fold from 40 to 0.31 mg/ml and inoculated with either *S. aureus* or MRSA-88. After 24h of incubation, the cultures were serially diluted 10-fold, and the CFUs were determined as explained in the materials and methods. At 10 mg/ml of PM, the growth of either *S. aureus* or MRSA-88 was completely inhibited. Therefore, the MBC value for PM against either *S. aureus* or MRSA-88 is 10 mg/ml.

Discussion

Multidrug resistance (MDR) bacteria are a major public health problem causing nearly 1·27 million deaths worldwide (1). If we did not solve this global health threat, MDR bacteria could kill around 10 million people by 2050 as proposed by The Review on Antimicrobial Resistance, commissioned by the UK Government (2). Today, antibiotics are the conventional treatment for bacterial infections. However, the spread of MDR bacteria necessitates the search for alternative treatments to antibiotics. One approach to tackle MDR bacteria is the search for natural compounds that possess toxic effects against MDR bacteria. Thus, in this present study, we assessed the antibacterial activity of pomegranate peel extract (PPE) against several MRD clinical isolates including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Carbapenem-resistant A. baumannii* (CRA).

Here we used several in vitro assays to determine whether PPE possesses killing activity against MDR clinical isolates. Using qualitative and quantitative methods, we showed that PPE has potent antibacterial activity against S. *aureus* and more importantly MDR clinical isolates including MSRA strains (Fig 2). In agreement with our findings, several studies demonstrated the antibacterial effects of pomegranate extracts against pathogenic bacteria (14, 15). A recent study evaluated the antibacterial efficacy of pomegranate extracts and four polyphenolic constituents; caffeic acid, ellagic acid, epigallocatechin-3-gallate, and quercetin against *M. tuberculosis* and *K. pneumoniae* clinical isolates (14). Among the tested isolates, multidrug-resistant tuberculosis (MDR-TB), extensively drug-resistant tuberculosis (XDR-TB) isolates, and extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* were included in the study (14). The MIC of methanol extract of pomegranate peel against TB isolates ranged from 64–512 ug/ml against TB isolates while ranged from 256 ->1024 ug/mL against *K. pneumoniae* isolates (14). Similarly, the present study demonstrated that PPE inhibited the growth of several clinical isolates; however, the MBC of PPE was nearly 10-fold higher than MIC values reported by Dey's study. The MBC values of drugs are usually higher than that of MIC values (16). It is likely the MIC of PPE would be lower than 10 mg/ml. The reason we did not report the MIC value is that PPE was highly turbid therefore, we could not determine whether the turbidity was a result of the tested bacterial growth or the PPE itself. Thus, we only

reported MBC values. Another study showed the efficacy of pomegranate extract against two bacteria; *S. mutans* and *R. dentocariosa*, which are commonly associated with dental carries (15). At a very low concentration (10-15 ug/ml), pomegranate extract inhibited the growth of *S. mutans* and *R. dentocariosa (15)*. The variation of MIC values among different studies is might due to the tested clinical isolates. Some clinical isolates could be more susceptible than others. For example, Gram-negative bacteria are less accessible to antibacterial agents than Gram-positive bacteria. This is due to the extra layer (outer membrane) that Gram-negative bacteria possess that is missing in Gram-positive bacteria (17).

Although Gram-negative bacteria are difficult to be treated with antibacterial drugs and more frequently show high resistance to antibiotics than Gram-positive bacteria, our data showed that PPE was not only effective against Gram-positive bacteria but also showed potent inhibitory effects against Gram-negative bacteria; *A. baumannii* (Fig 2). Our Finding is in agreement with previous studies that showed a potent effect of pomegranate extracts against Gram-positive and -negative bacteria (18-20). For instance, previously shown that pomegranate extracts inhibited the growth of Gram-negative bacteria including but not limited to *Escherichia coli* O157:H7*,* Salmonella *Typhi, Vibrio cholerae, Shigella spp.,* and *Yersinia enterocolitica*, and Gram-positive bacteria including *Listeria monocytogenes, Staphylococcus aureus, Clostridium spp.,* and *Lactobacillus spp* (21). Thus, pomegranate extract possibly interacts with a common compound or a pathway that is present in both Gram-positive and -negative bacteria to inhibit their growth. The mode of action of pomegranate is not fully understood; however, an attempt by Braga's group investigated the synergistic effects between pomegranate extract and antibiotics and against *S. aureus* clinical isolates including MRSA isolates (22). With respect to the mechanism of action of pomegranate, the study suggested that pomegranate extract significantly affects the function of efflux pumps of *S. aureus* by either inhibiting the efflux pump NorA or enhancing the influx of the drug (22-24). Medicinal plants that have effective extracts against diseases have been introduced in various reports (25-30). However, future studies are needed to investigate the exact mechanism of action of pomegranate.

Observations from this study indicate that PPE has inhibitory effects against certain MDR pathogenic bacteria including MRSA and *A. baumannii* clinical isolates. Thus, PPE has potential therapeutic use for bacterial infections caused by PPE-sensitive MDR strains. However, more studies have to be conducted in this regard. For instance, in vivo studies using animal models are needed to assess the inhibitory effects of PPE. In addition, whether bacteria develop resistance to PPE and the mechanism of resistant bacteria are also should be investigated.

Author contributions

Design the study, conduct the experiments, write the first draft, editing, and reviewing: Dr. Abdulaziz Yahya Alqahtani. Participated in writing the manuscripts and data analyses: Alzhraa Ali Mohamed. Participated in writing the manuscripts and conducting experiments: Dr. Irfan Ahmed. Data analyses and revising: Dr. Mohammed Merae Alshahrani. Reviewing and revising the manuscript: Dr Yasser Alraey. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work. All authors have read and agreed to the published version of the manuscript.

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Institutional review board statement

Before starting data collection, this study had been ethically approved by the local authorities. Authorization had been granted by the Research Ethics Committee at King Khalid University, Saudi Arabia (Ref. No 2022-2609).

Data availability statement

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

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