

Article

The Efficiency of Pomegranate Fruit Punica Granatum L Extract in Curing the Resistance of Some Isolated Bacterial Species from Urinary Tract Infection to Antibiotics

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Abstract: Introduction. The growing prevalence of multidrug-resistant bacterial strains poses a major obstacle to the effective management of urinary system infections, as a result of the spread resistance associated plasmids. **Objectives.** This research investigates the antimicrobial and plasmid-curing potential of watery and alcoholic extracts of P.granatum L. fruits towards six clinically relevant uropathogenic bacteria: Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris, Proteus mirabilis, and Klebsiella pneumoniae. **Materials and Methods.** The antibacterial activity of the tested extracts was rated via the agar well diffusion test, while the minimum inhibitory concentration was determined using the serial dilution method. Sub-minimum inhibitory concentrations of both extracts were used to evaluate their efficacy in curing and removing plasmids. **Results.** The loss of antibiotic resistance in several isolates was confirmed through phenotypic assays and agarose gel electrophoresis, which revealed the partial or complete disappearance of plasmid DNA bands in treated samples. **Conclusions.** This research confirm that the pomegranate plant is a promising therapeutic alternative through two mechanisms: its antimicrobial activity against urinary tract pathogens and its role in helping to curb the growing crisis of bacterial resistance to antibiotics. However, this calls for further laboratory and clinical studies on its most active compounds in order to incorporate them into treatment strategies for urinary tract diseases.

Keywords: UTIs, curing plasmids, pomegranate, Antibacterial resistance, antibiotic.

Introduction

Urinary system bacterial infections are considered the most prevalent types of infections, that affect a large number of people[1][2]. UTIs can take place in different parts of the urinary system including, the kidneys, ureters, bladder, and urethra[3]. Bacteria and fungi are among the most common causes of UTIs, and bacterial virulence factors play an important role in bacterial colonization, disease progression, and damage to urinary tract tissues. In addition, renal dysfunction, anatomical

abnormalities such as urethral or collecting system obstruction diabetes, immunodeficiency, and urinary tract contamination following surgery or the use of urinary catheters are all elements that rise the hazard of urinary systemt infections[4]. The development of multidrug-resistant (MDR) strains has caused decrease to antibiotic efficacy in treating these infections and a shortage of treatment options, which in turn has resulted in increased healthcare costs, morbidity, and mortality rates[5]. Hospitalized patients who are immunocompromised or have chronic diseases are the most vulnerable to pathogenic bacteria. The inappropriate use of antibiotics, has contributed to the widespread prevalence of antibiotic-resistant bacteria worldwide, posing a significant risk to public health as a “silent pandemic”[6]. Given these alarming circumstances, there is an urgent need for new and safe alternatives to antibiotics that possess anti-inflammatory and antibacterial properties. Plants contain numerous compounds with high efficacy against pathogenic microorganisms. These compounds include minerals, vitamins, phenols, tannins, glycosides, alkaloids, volatile oils, and saponins, which exhibit antibacterial and antifungal properties via different mode of actions including halting of bacterial protein and nucleic acid biosynthesis and disruption of microbial cell wall and plasma membrane integrity[7]. Medicinal plants derive their therapeutic value from their naturally occurring bioactive components primary and secondary metabolites which constitute a major source of essential pharmaceutical compounds[8]. The increasing reliance on medical plants as a source of medicines and other products is primarily due to their efficacy, the few or no side effects associated with their use, and their potential as an alternative to expensive drugs. Herbal medicine differs from chemical medicine in the interaction between active ingredients, which enhances the effectiveness of inhibiting microscopic pathogens. These interactions are either antagonistic or synergistic, with interactions occurring between different components, resulting in a pharmacological effect that surpasses that of equivalent doses of antibiotics[9]. The pomegranate (*P. granatium* L.) is a fast-growing plant belonging to the flowering family (Punicaceae)[10]. Most of the curative benefits of pomegranate fruit are due to its primary and secondary bio compounds, like tetraphenols, including flavonoids and anthocyanin's, hydrolysable tannins, fatty acids, and lipids[11]. Pomegranate fruit and peel has numerous uses, and mounting evidence reveals a myriad of medicinal properties, including ant diabetic, anticancer, anti-inflammatory, antimalarial, ant fibrotic, antifungal, and antibacterial properties. It is also used to treat diarrhea, expel worms, strengthen the heart, reduce fever, act as a diuretic, and possess antioxidant properties[12][13]. This study aims to use The pomegranate fruit as therapeutic alternatives that differ from traditional antibiotic regimens, and has the ability to remove the plasmid, eliminate bacterial resistance to multiple drugs, and convert bacteria into antibiotic-sensitive bacteria. and have the ability to removal plasmid and eliminate multidrug-resistant bacteria and convert them into antibiotic-sensitive bacteria.

Materials and Methods

1. Collection and Preparation of Material

Punica granatium L fruits were obtained from local markets in the city of Mosul -Iraq.

Ethanolic extract: "Method[14] was used. Twenty five grams of fresh seeds were weighed and placed in a Soxhlet apparatus. One hundred fifty grams of ethanol were added to the apparatus's glass flask, and the ethanol was heated until it evaporated and condensed inside the apparatus, causing the solvent to fall onto the seeds and extract the active compounds. As the solvent continuously returned to the flask, the extraction process continued for approximately 4 hours. The ethanol was discarded using a rotary evaporator to obtain the concentrated extract, which was stored at 4°C until use.

Aqueous extract: Preparation was performed as per the method by[15], Approximately 25 g of fresh, freeze-dried pomegranate seeds were weighed and added to 150 mL of dist. water. The mix was placed in a homogenizer for one hour to extract the biologically active compounds from the fresh pomegranate seeds. Centrifugation was performed at 11,000 rpm for 5 minutes at 20 m to separate the supernatant from the insoluble precipitate. The supernatant was used in subsequent experiments. The samples were stored at 4 m until use.

2. Bacterial Isolates

Six clinically important uropathogenic strains were included: the Gram-positive *S. aureus* and Gram-negative species such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. vulgaris*, and *P. mirabilis*. All bacterial isolates used in this study were sourced from the laboratories of the Department of Biology, College of Education for Pure Sciences, University of Mosul. Their identification was confirmed via standard microbiological and biochemical characterization. Including Gram staining, catalase, coagulase, citrate utilization, and triple sugar iron agar reactions, following established microbiological protocols.

3. Antibiotic Susceptibility Testing

Sensitivity tests were conducted following the [16] CLSI instructions. The examined antibiotics included: Amoxicillin (AX), Ampicillin (Am), Streptomycin (Sm), Erythromycin (Ery), Gentamicin (GN), Ciprofloxacin (CF), Nalidixic acid (Nal), Refampicin (RA) Trimethoprim (Tr) and Tetracycline (TE). The medium was prepared by adding the appropriate antibiotic to a sterile nutrient agar medium cooled to a temperature of (45–50 °C), at a final concentration of (micrograms/mL). Concentrated antibiotic solutions were prepared according to the [17] by dissolving a specified amount of the antibiotic in the appropriate solvent; all concentrated solutions were then sterilized by filtration (0.22 micrometers) and stored at until use in 20°C[18].

4. Evaluation Antibacterial Activity

Antimicrobial testing of both aqueous and ethanol based extracts was performed utilizing the agar well diffusion technique, in accordance with the method described by[19]. Standardized bacterial, adjusted to a 0.5 McFarland turbidity, were evenly distributed across Mueller Hinton agar plates. Circular wells of 6 mm diameter were aseptically created in the agar medium, into which 100 µL of the plant extract solutions prepared at concentration of 12.5, 25, 50, 100, and 200 mg/mL were dispensed. incubated for a 24 hour period at 37 °C to allow for bacterial growth inhibition. After incubation, the clear inhibition zones surrounding each well were measured in millimeters. Furthermore, spectrophotometer (model CECTL CE 1021,1000S) at 600 nm were used to monitor growth density of bacteria

5. Determination of MIC and Sub-MIC

MIC values were calculated using the agar dilution approach, adapted from the procedure of [20] with relevant experimental modifications. Plates were inoculated with bacterial suspensions and They were grown under standard conditions. Meanwhile, the Sub-MIC is the concentration that allows partial bacterial growth and is used in subsequent plasmid curing assays.

6. Plasmid Curing Assay

To investigate the potential of the plant extracts in eliminating plasmid-mediated antibiotic resistance, "Plasmid elimination procedures were conducted in conformity with the [21]. Bacterial isolates were cultured in nutrient broth supplemented with sub-MIC concentrations of the extracts. After incubation, treated bacteria were recultured on antibiotic-containing media, and changes in resistance phenotype were evaluated.

7. Plasmid DNA Extraction and Electrophoresis

Plasmid DNA was extracted from both untreated and extract-treated bacterial cultures using a commercial miniprep kit (Promega, USA) "As per the kit's protocol (Promega, USA), plasmid DNA was isolated, and its concentration and purity were subsequently determined using a BioDrop spectrophotometer. "DNA samples were resolved using agarose gel electrophoresis, then stained with ethidium bromide and imaged using ultraviolet illumination."

Results and Discussion

Antibiotic Resistance

1. Antibiotic Resistance Patterns Among Uropathogenic Isolates

The results, as shown in Table (1), revealed differences in antibiotic resistance levels among the six bacterial species included in the study (*S. aureus*) exhibited high resistance rates to certain antibiotics, including (Nal), with a 100%, while resistance to the remaining antibiotics ranged from 48% to 86%. As for the results of (*E. coli*) isolates, they also showed variation in antibiotic resistance; they

exhibited very high resistance to certain antibiotics, including oxalate (Ax) and amphotericin B (Am), with resistance rates of 100% for both. Resistance to the remaining antibiotics ranged from 40% to 90%. (*Klebsiella pneumonia*) isolates also showed marked variation in resistance to the antibiotics under study, exhibiting very high resistance (100%) to erythromycin (Ery), (Nal), and (Am), while resistance ranged from 11% to 88% for the remaining antibiotics. (*P. vulgaris*) showed high resistance to both antibiotics (Am and Ax), at 100% for each, and moderate resistance to the antibiotic (Tri), at 50%, while it showed no resistance to the other antibiotics used in the study. Similarly, (*P. mirabilis*) exhibited low resistance (25%) to each of the following antibiotics: Am, Ax, Tri, and Sm. As for the (*Ps. aeruginosa*) strain, it exhibited high resistance rates to certain antibiotics, including (Nal and Ax), with a resistance rate of 100% for each, while resistance to the remaining antibiotics ranged from 27%.

Table 1. The numbers and percentages of antibiotic-resistant bacterial species.

Antibiotics Bacteria	No. Species	AX (%)	Am (%)	Sm (%)	Ery (%)	GN (%)	RA (%)	Cf (%)	TE (%)	Tri (%)	Nal (%)
<i>S. aureus</i>	29	(86) 25	(82) 24	(48) 14	(82) 24	(68) 20	(48) 14	(65) 19	(51) 15	(68) 20	100
<i>E. coli</i>	32	100	100	(53) 17	(90) 29	(40) 13	(46) 15	(65) 21	(87) 28	(84) 27	(90) 29
<i>K. pneumonia</i>	9	(71) 10	(66) 6	(88) 8	100	(11) 1	(11) 1	(88) 8	(66) 6	(88) 8	100
<i>Pr. vulgaris</i>	4	100	100	S	S	S	S	S	S	(50) 2	S
<i>Pr. Mirabilis</i>	4	(25) 1	(25) 1	(25) 1	S	S	S	S	S	(25) 1	S
<i>Ps. aeruginosa</i>	11	100	(90) 10	(72) 8	(81) 9	(45) 5	(27) 3	(27) 3	(63) 7	(90) 10	100

S :sensitive

2. Antibacterial Activity of Pomegranate Extracts

Table 2 shows the response of bacterial species to both the aqueous and ethanolic extracts of the pomegranate. The results depended on the concentration of the extracts, with the 200 mg/mL concentration exhibiting the greatest inhibitory effect on the bacterial isolates. The aqueous extracts exhibited a greater inhibitory effect than the ethanolic extracts. (*S. aureus*) showed the highest response to pomegranate extracts, although there were some cases in which no inhibitory action was noticed, as in the case of *E. coli*. MIC values ranged between 0.125 and 1 mg/mL, with aqueous extracts reaching maximal efficacy at 200 mg/mL, suggesting stronger solubility and bioavailability of phenolic compounds in water.

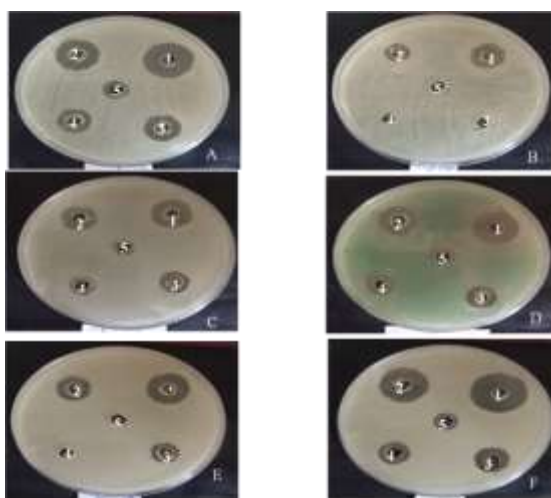
Table 2. Zone of inhibition (mm) at varying concentrations of aqueous and ethanolic pomegranate extracts.

Bacteria	Type of extract	Concentration of extract (mg/ml)					MIC
		200	100	50	25	12.5	
<i>Staphylococcus aureus</i>	Aqueous	29	24	19	14	11	0.125
	Alcoholic	23	16	16	15	13	0.125
<i>Escherichia coli</i>	Aqueous	26	21	11	9	-	0.25
	Alcoholic	21	16	-	-	-	1
<i>Klebsiella pneumonia</i>	Aqueous	24	19	15	14	14	0.125
	Alcoholic	18	13	13	13	12	0.125
<i>Proteus mirabilis</i>	Aqueous	26	23	18	16	13	0.5
	Alcoholic	23	19	18	16	13	0.125
<i>Proteus vulgaris</i>	Aqueous	29	24	19	-	-	0.125
	Alcoholic	18	17	16	14	13	0.125
<i>Pseudomonas aeruginosa</i>	Aqueous	27	23	19	16	11	0.125
	Alcoholic	23	19	18	13	11	0.125

(-) without effect

3. Visualization of Antibacterial Zones

Figures 1 and 2 illustrate the concentration-dependent inhibition zones caused by aqueous and alcoholic extracts. A progressive expansion in the diameter of clear zones was observed with increasing extract concentration, particularly in *S. aureus* and *P. mirabilis*. Figure 1 and 2, Growth inhibition zones at five extract concentrations for each bacterial isolate (aqueous vs ethanolic).

**Figure 1.** The impact of Aqueous extract of *P. granatum* L fruits at different concentrations on isolated bacteria.A. *Sta. aureus* B. *E. coli* C. *K. pneumonia*D. *Ps. aeruginosa* E. *Pr. Vulgaris* F. *pr. mirabilis*Concentrations 1(200 mg/cm³), 2(100 mg/cm³), 3(50 mg/cm³), 4(25 mg/cm³), 5(12.5 mg/cm³).

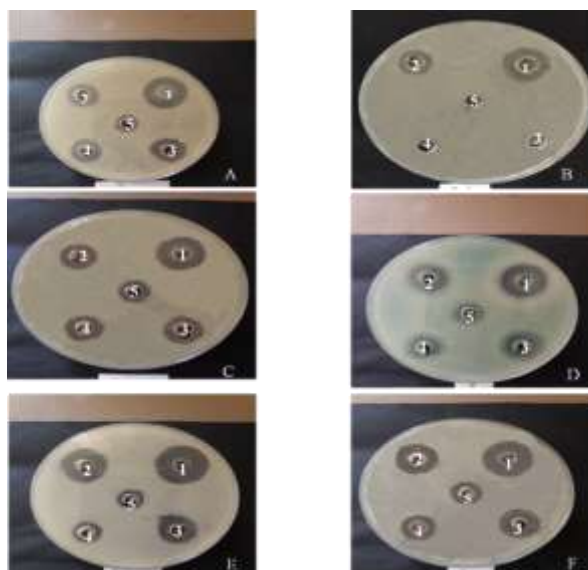


Figure 2. The impact of alcoholic extract of *P. granatum* L fruits at different concentrations on isolated bacteria.

A. *Sta. aureus* B. *E. coli* C. *K. pneumonia*

D. *Ps. aeruginosa* E. *pr. vulgaris* F. *pr. mirabilis*

Concentrations 1(200 mg/cm³), 2(100 mg/cm³), 3(50 mg/cm³), 4(25 mg/cm³), 5(12.5 mg/cm³)

4. Plasmid Curing Activity of Pomegranate Extracts

The plasmid curing potential of *P. granatum* L. extracts is presented in (Table 3). Post treatment results showed a marked loss of antibiotic resistance in most isolates, particularly *S. aureus* and *E. coli*, when treated with aqueous extract. Agarose gel electrophoresis confirmed these findings through the disappearance of plasmid DNA bands in treated samples (Figure 3).

Table 3. Antibiotic resistance pre- and post-treatment and visual confirmation of plasmid band disappearance.

Bacteria	Antibiotic media added in µg/ml									
	Nal 30	Tri 10	TE 15	Cf 25	RA 50	Gn 25	Ery 15	Sm 25	Am 50	AX 50
<i>Staph. aureus</i> / control	R	R	R	R	R	R	R	R	R	R
<i>Staph. aureus</i> /curing by aqueous extract	S	S	S	S	S	S	S	S	S	S
<i>Staph.aureus</i> /curing by alcoholic extract	S	S	S	S	S	S	S	S	S	S
<i>E. coli</i> / control	R	R	R	R	R	R	R	R	R	R
<i>E. coli</i> / curing by aqueous extract	S	S	S	S	S	S	S	S	S	S
<i>E. coli</i> / curing by alcoholic extract	R	S	R	R	S	S	S	S	S	R
<i>Klebsiella pneumonia</i> / control	R	R	R	R	R	R	R	R	R	R
<i>Klebsiella pneumonia</i> /curing by aqueous extract	S	S	S	S	S	S	S	S	S	S
<i>Klebsiella pneumonia</i> / curing by alcoholic extract	R	R	R	R	S	S	S	S	S	W
<i>Proteus mirabilis</i> / control	S	R	S	S	S	S	S	S	R	R
<i>Proteus mirabilis</i> / curing by aqueous extract	---	S	---	---	---	---	---	---	S	S
<i>Proteus mirabilis</i> / curing by alcoholic extract	---	S	---	---	---	---	---	S	S	S
<i>Proteus vulgaris</i> / control	S	R	S	S	S	S	S	R	R	R
<i>Proteus vulgaris</i> / curing by aqueous extract	---	R	---	---	---	---	---	S	S	S

<i>Proteus vulgaris</i> / curing by alcoholic extract	---	S	---	---	---	---	---	S	S	R
<i>Pseudomonas aeruginosa</i> / control	R	R	R	R	R	R	R	R	R	R
<i>Pseudomonas aeruginosa</i> / curing by aqueous extract	S	S	S	S	S	S	S	S	S	S
<i>Pseudomonas aeruginosa</i> / curing by alcoholic extract	w	R	R	S	S	S	S	R	R	R

-----: The test not done: R: Resistance S: sensitive W: Weak

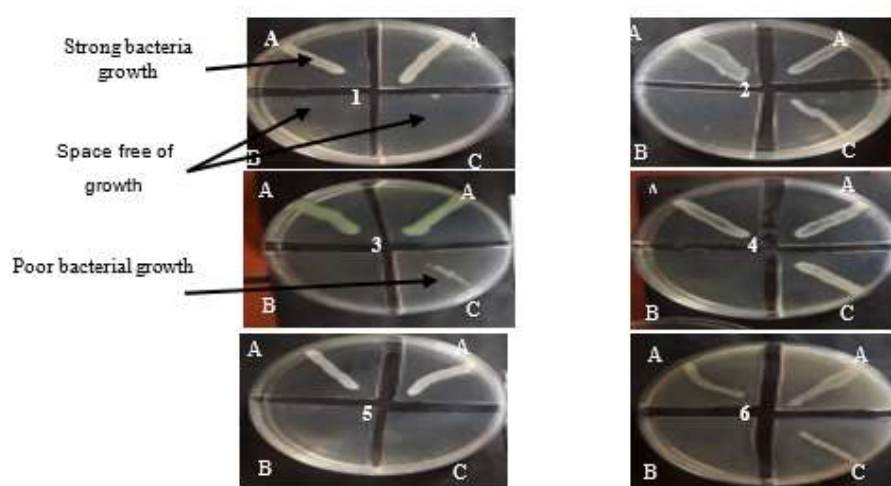


Figure 3. Effectiveness of the alcoholic and aqueous extract of *P. granatum* L. plant on bacteria grown on the antibiotic media under study.

A: Before adding *P. granatum* L. extract **B:** After adding the aqueous extract of *P. granatum* L.

C: After adding the alcoholic extract of *P. granatum* L.

1: *Staph. aureus* on Cf 2: *E. coli* on Cf 3: *Ps. aeruginosa* on Nal

4: *Kleb. pneumonia* on Cf 5: *Staph. aureus* on Tr 6: *Kleb. pneumonia* on Ax

Classification of plasmid DNA content by gel electrophoresis

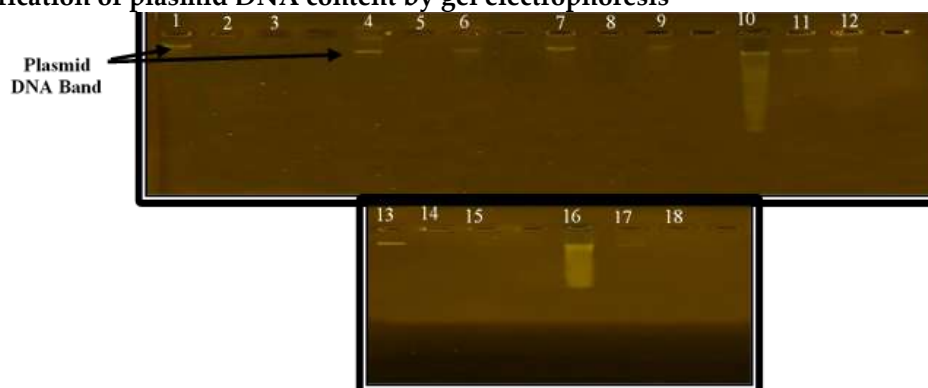


Figure 4. Agarose gel electrophoresis of plasmid DNA extracted from uropathogenic bacteria before and after treatment with aqueous and ethanolic extracts of *P. granatum* L. fruits.

Lane	Sample Description	Lane	Sample Description
1	<i>S. aureus</i> (untreated)	10	<i>P. aeruginosa</i> (untreated)
2	<i>S. aureus</i> + aqueous extract	11	<i>P. aeruginosa</i> + aqueous extract
3	<i>S. aureus</i> + ethanolic extract	12	<i>P. aeruginosa</i> + ethanolic extract
4	<i>E. coli</i> (untreated)	13	<i>Pr. vulgaris</i> (untreated)
5	<i>E. coli</i> + aqueous extract	14	<i>Pr. vulgaris</i> + aqueous extract
6	<i>E. coli</i> + ethanolic extract	15	<i>Pr. vulgaris</i> + ethanolic extract
7	<i>K. pneumoniae</i> (untreated)	16	<i>Pr. mirabilis</i> (untreated)

8	<i>K. pneumoniae</i> + aqueous extract	17	<i>Pr. mirabilis</i> + aqueous extract
9	<i>K. pneumoniae</i> + ethanolic extract	18	<i>Pr. mirabilis</i> + ethanolic extract

Note: The disappearance or fading of plasmid bands was evident in treated samples, especially lanes 2, 5, 8, and 17, indicating successful plasmid curing by aqueous and ethanolic extracts. Visualization of plasmid DNA was accomplished by staining the agarose gels with ethidium bromide, followed by exposure to ultraviolet (UV) light. A 1 kb DNA ladder served as a molecular size marker, although it is not displayed in the figure.

5. Quantification of DNA Concentration and Purity

Figures 5 and 6 present spectrophotometric data indicating a substantial reducing in plasmid DNA concentration and purity after treatment with both extracts.

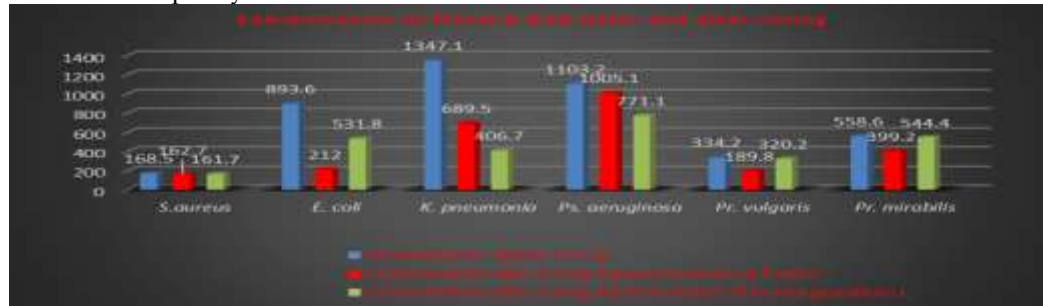


Figure 5. BioDrop measurement of plasmid DNA concentration before and after treatment.

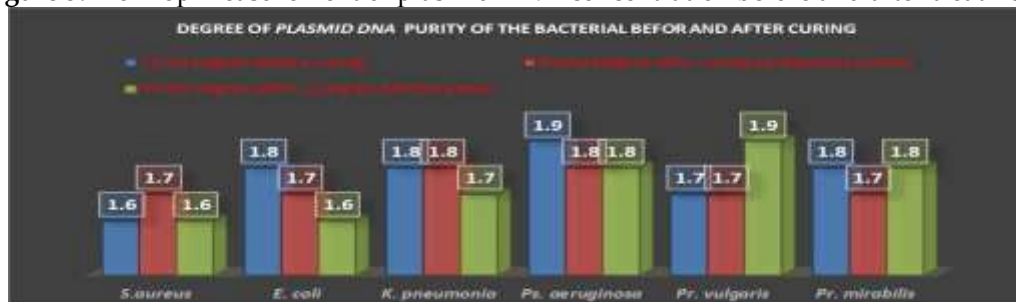


Figure 6. BioDrop measurement of plasmid DNA purity before and after treatment.

6. Spectrophotometric Evaluation of Bacterial Growth

As shown in Figure 7, treatment with both types of extracts at 200 mg/mL led to a reduction in optical density (OD₆₀₀).

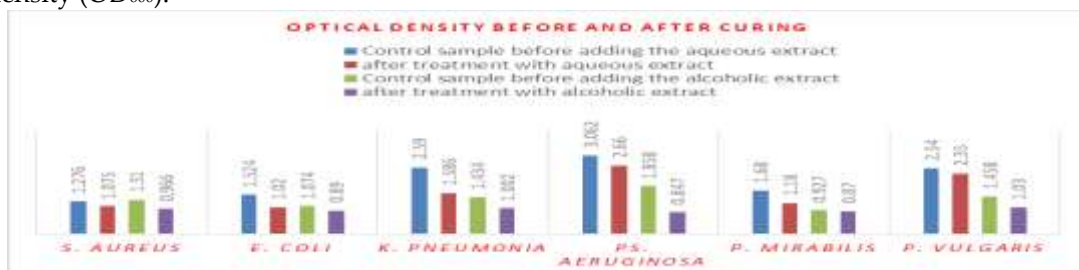


Figure 7. Optical density (OD₆₀₀) of bacterial cultures before and after treatment with pomegranate extracts.

The progressive decline in bacterial turbidity (OD₆₀₀) following exposure to increasing concentrations of *P. granatum* extracts reveals.

Discussion

Resistance to antibiotic is one of the most momentous problem currently menacing public health, and estimates suggest that if no alternative treatments are discovered to eliminate pathogens, there will be no effective antibiotics by 2050, as several factors contribute to the development of this resistance[22]. As shown in Table (1), the high resistance to antibiotics may be attributed to the

overdoing and abuse of antibiotics, In addition to the adaptive capabilities of bacteria, such as horizontal gene transfer and genetic mutations, therefore, lead to the inactivation of antibiotics through enzymatic activity, alteration of the drug's target site, disruption of membrane permeability, and activation of efflux pumps. All of these pathways ultimately result in the alteration or neutralization of the effects of antibiotics. [23]. Abundant production of β -lactamases enzymes that deactivate penicillin type antibiotics by breaking their β -lactam ring, preventing antibiotics from binding to penicillin-proteins rendering them ineffective thus [24]. These findings align with previous reports on MDR pathogens in UTIs, where efflux pump overexpression and enzymatic inactivation contribute to multidrug resistance. Given the worsening problem of antibiotic resistance and the lack of strategic plans to address this issue, researchers have turned to plants and plant derived substances as alternatives to antibiotics or as resistance modifiers [25]. The growth inhibition results (Table 2) and (Fig 1,2) show the effect for both the aqueous and ethanolic extracts of pomegranate (*P. granatum* L). The aqueous pomegranate extract has greater inhibitory activity than its ethanol extract, and that the highest rate of growth inhibition occurred at a concentration of 200 mg/cm³. *Staphylococcus* bacteria showed the highest inhibitory response to both the aqueous and alcoholic pomegranate extracts. Other bacterial species showed a lower response, particularly to the alcoholic extract. The antibacterial activity of the extracts was estimated by determining the minimum inhibitory concentration (MIC), which ranged from 0.125 to 1 millimeter. The primary reason for most of the pomegranate plant's effects may be attributed to its primary and secondary metabolites. The most important of these are adriphenols, including anthocyanins and flavonoids, which have hydrolytic activity, as well as lipids and fatty acids. These compounds are highly effective in inhibiting bacterial growth, as they inhibit cell wall production and alter cell membrane function. Furthermore, they inhibit the biosynthesis of essential proteins in living cells and degrade DNA. The inhibitory effect of pomegranate fruit extracts may be attributed to their antioxidant content. Our results were consistent with a study [26],[27]. on the efficacy of pomegranate extracts, in which an increase in the concentration of pomegranate peel extract led to an increase in the inhibitory effect on bacterial growth, reaching 100% inhibition, and that polyphenols, punicalagins, gallic acid, punicalic acid, and 5-hydroxymethylfurfural (5-HMF) are responsible for the plant's inhibitory activity. These compounds act to disrupt and inactivate the plasma membrane and antibiotic-resistant proteins. Additionally, Gram-positive and Gram-negative bacteria varied in their response depending on the nature of their cell walls, The punicalagin compounds in pomegranate extracts play a role in disrupting and inhibiting the integrity of the cell membranes of *Staphylococcus* bacteria and hindering their growth, in addition to inhibiting fatty acid synthesis enzymes, making them more responsive than Gram-negative bacteria. As shown in Table 2, the MIC values ranged from 0.125 to 1, indicating strong efficacy of pomegranate extracts, with the aqueous extract clearly outperforming the others, as confirmed by a study [28]. *S. aureus* showed the highest response to both extracts, with an MIC value of 0.125 mg/mL. A study [29] demonstrated that pomegranate peel extract had a greater effect on *S. aureus*, with an inhibition value ranging from 15.62–500.00 μ g/mL, compared to Gram-negative bacteria, for which the MIC ranged from 125.00–500.00 μ g/mL. This may be attributed to the punicalagin compounds in pomegranate, which are more influential against Gram-positive bacteria because of their ability to bind to the peptidoglycan wall.

Numerous studies have shown that pomegranate extracts exhibit potent antibacterial activity and have the potential to treat recurrent urinary tract infections.[30] Clinical trials and post-hoc analysis have confirmed the role of pomegranate in reducing the recurrence of infection, especially when combined with antibiotics.[31] Overall, the results highlight the significant role of pomegranate as a safe and sustainable antibacterial agent, making it a promising candidate for the development of new natural antibiotics or for incorporation into existing antibiotics to help address the growing problem of antibiotic resistance building on previous studies and conducting further in-depth research on its most important active components and mechanisms of action against bacteria. Statistical analysis was not performed due to the lack of statistically significant differences; therefore, we relied on electrophoresis experiments, which effectively demonstrated the validity of the study by showing that certain bacteria lost their plasmids after being exposed to pomegranate extracts.

Table and Figure (3) show the results of the inactivation of the studied bacteria using pomegranate extracts. The aqueous extract demonstrated a high ability to eliminate resistance plasmids and render all bacterial species susceptible to antibiotics, while the alcoholic extract showed variable efficacy, which reflects the extracts' efficacy against multidrug resistant (MDR) bacteria a process known as plasmid curing. This is consistent with a study [32] that demonstrated the ability of *Plumbago zeylanica* root to curing antibiotic resistant plasmids.

In a study [33] that used *Nigella sativa* extract against multidrug resistant *Staphylococcus aureus*, analysis of the plasmid profiles on agarose gel revealed that the bacteria underwent plasmid loss and transformed from resistant to antibiotic sensitive bacteria. This provides evidence that plasmids have crucial role in the transfer of resistance genes. In contrast, *P. aeruginosa* and *K. pneumoniae* did not respond to treatment with pomegranate extracts and remained resistant to certain antibiotics; this may be attributed to the fact that the resistance genes are located on the bacterial chromosome, or to the low membrane permeability of these plant extracts.

Our results demonstrated the ability of the aqueous extract of pomegranate fruit to eliminate plasmids; this may be due to a molecular mechanism that targets plasmid stability or replication. The strategy of killing bacteria with antibiotics has begun to backfire as antibiotic resistance increases. Therefore, neutralizing plasmids using plant extracts is a promising solution for advancing to the gene extraction stage, as the active compounds in plant extracts help eliminate plasmids through molecular mechanisms, including the inhibition of plasmid replication initiation (Rep-ori), plasmid division, and the supercoiling of DNA. while cryptolipin, alkaloids, and serpentine molecules act by sliding between DNA bases, causing denaturation and DNA elongation, interfering with the DNA gyrase enzyme, and inhibiting quorum sensing (QS) [34]. There is evidence that pomegranate compounds rich in polyphenols are capable of disrupting the bacterial plasma membrane and inhibiting the action of proteins associated with antibiotic resistance. The pomegranate plant (*P. granatum*) exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria, with significant potential against drug-resistant strains[35].

Figure (4) shows the electrophoresis of agarose gel and the disappearance of plasmid DNA bands following neutralization with pomegranate extracts. This indicates the success of the curing process and the effectiveness of pomegranate extracts in plasmid treatment, which offers promising hope for controlling the problem of antibiotic resistance. Studies have shown, that plasmids may carry multiple resistance genes and are highly stable, which contributes to the persistence of the resistance trait within the bacterial cell and its horizontal transfer to other bacteria [36]. Nevertheless, some bacterial species, including *K. pneumoniae* and *P. aeruginosa*, have retained their resistance after plasmid treatment. This may be due to the presence of resistance genes on the chromosome, the ineffectiveness of plasmid targeting, cell efflux systems, or changes in the permeability barrier. Therefore, the process of identifying the locations of resistance determinants requires molecular studies using PCR to estimate the number of copies of the plasmid and the resistance genes.

The results (Figures 5 and 6) support the hypothesis that *P. granatum* L. induces plasmid curing, potentially by DNA strand breakage, intercalation, or inhibition of DNA replication machinery. The observed decline in DNA yield is consistent with plant-derived alkaloids and phenolics disrupting plasmid stability [37] Figure (7) Optical density (OD₆₀₀) measurements of bacterial cultures before and after treatment with pomegranate extracts showed a marked change in bacterial growth depending on the extract concentration. These results supported the agar diffusion test findings and the plasmid removal results obtained in the present study. A decrease in OD₆₀₀ is an indicator of reduced bacterial growth or biomass and may result from slowed cell division or reduced cell density[38]. These findings are agreed with reports indicating that pomegranates are particularly rich in phenolic compounds, punicalagin, flavonoids, and biologically active chlorogenic acid. These compounds act as antioxidants, anti-inflammatory agents, and anticancer agents.[39] These compounds may contribute to antibacterial activity by affecting the integrity of the cell membrane and disrupting its functions; furthermore, influence the entry of active compounds into the cell and the cell's sensitivity to plant extracts [40][41]. It is possible that certain environmental factors, such as pH and temperature, may have influenced the stability, distribution, and interaction of the active compounds with bacterial cells. This is consistent

with findings from previous studies on the antimicrobial activity of *Psidium guajava* extracts, which showed that changes in temperature and pH can affect the activity of plant extracts as antibacterial agents [42]. Considering the results as a whole, the correlation between minimum inhibitory concentration (MIC) values and changes in light density at 600 nanometers, along with the results of agar diffusion tests, suggests that pomegranate extracts possess a concentration-dependent antibacterial effect. This effect is likely the result of the interaction of several mechanisms, including disruption of cell membrane integrity, effects on enzyme functions and metabolic pathways, and possibly synergistic effects among the active compounds present in the extract[43]. These findings support the potential for studying pomegranate extracts as possible sources of compounds that may help combat urinary pathogens resistant to multiple antibiotics.

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Ethical Considerations

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Declaration of Competing Interest

None.

Conclusions

This study showed that pomegranate fruit extracts, particularly the aqueous extract, possess antibacterial and plasmid inhibiting activity effective against multidrug resistant pathogens in the urinary tract. The efficacy of these extracts was not limited to concentration dependent inhibition of bacterial growth; they also led to the loss of antibiotic resistance through the disruption of plasmids, as confirmed by electrophoresis and in vitro assays. Our results reflect the promising role of pomegranate not merely as an antibacterial agent but as a source of natural compounds that act as a safe and effective plasmide targeting agent to mitigate multidrug resistance. Although our study successfully achieved its objectives, further investigation is needed to identify the most effective compounds in pomegranate that play a role in plasmide modulation. Additionally, further molecular studies targeting resistance genes and virulence factors rather than indiscriminately killing bacteria are required, along with the use of artificial intelligence and nanotechnology to enhance the role that plant extracts can play as future therapeutic alternatives.

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