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Antiurobacterial Activity of *Punica granatum* L. Seed Extract

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Authors' contributions

This work was carried out in collaboration between all authors. Author SD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SP and PP managed the analyses of the study. Authors SD and SP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The natural products derived from medicinal plants are proven to be the abundant source of biologically active compounds, which have been the basis of development of new pharmaceuticals. The present study was carried out to understand the antibacterial activity of methanolic extract of *Punica granatum* L. seed (MPG) against the clinically isolated urinary tract infections (UTI) causing bacteria i.e. *Escherichia coli* and *Klebsiella pneumoniae* using different methods i.e. disc diffusion, agar well diffusion, modified agar well diffusion. Colony forming units per ml (CFU/ml) were also determined for wild and MPG treated bacteria by spread plate and absorbance method. Phytochemical analysis of MPG and biochemical characterization of *K. pneumoniae* and *E. coli* was carried out using a standard protocol. MPG was a dark red colour sticky mass with a yield of 26.87% (w/w). Phytochemical analysis revealed the presence of steroids, tannins, coumarins, glycosides, flavonoids and phenolic compounds. The present investigation showed that MPG was effective against *K. pneumoniae* and *E. coli*. *K. pneumoniae* was a multi-drug resistant strain, found to be more sensitive to MPG in comparison to *E. coli*. The CFU/ml was found to be reduced in a

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dose-dependent manner in MPG treated urobacteria, which suggests that *P. granatum* seeds can be used as a remedy for UTI and also can be taken regularly to prevent the recurrence of UTI.

Keywords: Escherichia coli; Klebsiella pneumoniae; Punica granatum L.; urinary tract infections.

1. INTRODUCTION

Bacteria are the primary organisms that cause urinary tract infections (UTI). Most common bacteria that cause UTI include *Escherichia coli* and *Staphylococcus saprophyticus*. About 80-90% of UTI are caused by *E. coli*. This bacterium is found in digestive tract of human and is present around the skin in the rectal area. About 10-20% of UTI is caused by *S. saprophyticus* and has a seasonal variation with a higher incidence in summer. And about 5% or less of UTI is caused by other bacteria like *Proteus*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Pseudomonas* and *Enterococcus faecalis*. Microorganisms such as Chlamydia and Mycoplasma may also cause UTI in both male and female. Unlike *E. coli*, Chlamydia and Mycoplasma may be sexually transmitted and infections require treatment of both partners [1].

E. coli is a facultative anaerobe and can grow on ordinary culture medium at an optimum temperature of 37°C (temperature range 10-40°C) in 18-24 hr. Colonies of some strains show beta haemolysis on blood agar. On MacConkey agar medium, colonies look pink due to lactose fermentation. The genus *Klebsiella* consists of gram-negative, capsulated, nonsporing, non-motile bacilli that grow well on ordinary media, produce pink mucoid colonies on MacConkey agar. They are widely distributed in nature and present as commensals in human and animal intestine and also as a saprophyte in the soil. In general, *E. coli* colonies are circular, moist and smooth with an entire margin and nonmucoid unlike colonies of *Klebsiella*, which are mucoid. *K. pneumoniae* subspp. *Pneumonia* is the second most populous member next to *E. coli* of the aerobic bacterial flora of human intestine. It is responsible for severe bronchopneumonia, UTI, nosocomial infections, wound infection, septicaemia, meningitis and rarely diarrhea [2].

Many antibiotics are effective against these bacteria but due to high mutational ability, bacteria are becoming resistant to most of the antibiotics. So, there is a need to choose an alternative form of medicine. Nature has provided us with the best gift in the form of plant and their products. The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, which have now become a curse to the actively growing bacteria.

Pomegranate (*Punica granatum* L.) is a fruit-bearing deciduous shrub or small tree belonging to family Lythraceae under order Myrtales. It is an important fruit of tropical and subtropical region, which originated in the Middle East and India; it has been used for centuries in ancient culture for its medicinal values [3]. Pomegranate has gained popularity in recent years due to its multifunctionality and nutritional benefit in the human diet. The fruit rich in tannin and other biochemical, particularly phenolics, reduce disease risk. But the peel of pomegranate contains high amount of polyphenols than fruit. Besides, antioxidant activity, it has other biological significance like anticancer activity, antimicrobial activity, anti-inflammatory and anti-diabetic activity [4].

This study was undertaken to understand the antibacterial activity of *P. granatum* seed extract against *E. coli* and *K. pneumoniae*, the major causative agents of UTI, frequently isolated from the symptomatic patients visiting MKCG Medical College and Hospital for UTI. These microorganisms cause UTI in human by a variety of ways and the actual mechanism is not clearly understood. There are a few records on antibacterial activity of *P. granatum*. But, the present investigation was carried out to determine the potentiality of *P. granatum* seed extract against UTI causing bacteria by using different methods. In order to understand the rationale behind the bioactivity of any traditional medicine, it is important to know the phytochemicals present in it. So, qualitative and quantitative analysis of the extract was also carried out.

2. MATERIALS AND METHODS

2.1 Plant Part Collection and Extract Preparation

The dried seeds of *P. granatum* were collected from Bhapur bazar, Berhampur, Orissa in the month of August- September 2016 and

authenticated by the botanists of Berhampur University. The shade dried seeds were powdered. The powdered seeds were subjected to exhaustive soxhlet extraction in methanol (300 ml) for 72 hour at 70°C. The filtered extract was concentrated in a rotary evaporator. The crude extract was stored at 4°C in a desiccator for future use and mixed with lukewarm distilled water to prepare the required working concentration depending on the type of study.

2.2 Phytochemical Analysis

Qualitative analysis was carried out for the presence of steroid, tannin, saponin, anthocyanin, coumarin, glycosides and flavonoids using standard protocol [5].

2.2.1 Quantitative analysis for total phenolic compound (TPC)

The total phenolic content of the extract was determined by the folin- ciocalteu (FC) method. 200 µl of crude extract (1 mg/ml), were added to 3.16 ml of distilled water, mixed thoroughly with 0.2 ml of FC reagent for 8 min, and followed by the addition of 0.6ml of 10% Na_2CO_3. The mixture was allowed to stand for 60min in the dark and absorbance was measured at 765nm. The TPC was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent to g dry weight. Statistical analysis was carried out with MS excel 2010 software and results are expressed as mean ± standard deviation.

2.3 Bacterial Strains

Clinical isolates of *E. coli* and *K. pneumoniae* were procured from M.K.C.G Medical College, Berhampur. For routine use, the cultures were maintained on Mueller Hinton agar (MHA) plates. For long-term storage, glycerol stock was prepared by inoculating a single colony into Nutrient broth (NB) and incubated at 37°C for 16 hr. To 0.8 ml of this culture, 0.2 ml of 50% sterile glycerol was added, mixed thoroughly and stored at 4°C for 1 hr and then stored at -20°C until used.

2.4 Biochemical Characterization and Antibiotic Sensitivity Test

Biochemical characterization and antibiotic sensitivity test were carried out according to standard laboratory procedure followed by the Department of Microbiology, MKCG medical college, Berhampur. Briefly, one isolated colony was inoculated into sterile saline or peptone water. A sterile cotton swab was dipped into the dilute culture medium. Excess saline was squeezed and swabbed completely on an agar plate. Different antibiotic discs (Himedia, Mumbai) were aseptically taken and placed properly on agar plate leaving an appreciable gap between two discs. Plates were incubated at 37°C for 18 hr. The clear zone formed around the disc was a measure of the susceptibility of the organism to the antibiotic at a specific concentration.

2.5 Antibacterial Test

The effect of the methanolic extract of *P. granatum* seed (MPG) against the clinically isolated UTI bacteria (*E. coli, K. pneumoniae*) was determined by disc diffusion, agar well diffusion, modified agar well diffusion method. The CFU/ml was determined in wild and drug-treated bacteria by two methods.

- Bacterial colony counting by spread plate method.
- Bacteria counting by absorbance method (OD_{600nm}).

2.5.1 Disc diffusion method

Discs (4 mm diameter) were prepared using whatman no -1 filter paper. Different doses (6.25, 5.5, 4.25, 4 mg/disc) of MPG were added on it and left in hot air oven/ incubator at 37°C for 30 min for complete drying. MHA plates were prepared and swabbed with sterilized cotton bud containing bacterial culture. The MPG treated discs were aseptically placed on them and the plates were incubated overnight. The clear zones of inhibition (ZOI) formed around the discs were measured.

2.5.2 Agar well diffusion method

A single colony was suspended in 1 ml of sterilized saline or peptone water, mixed properly by vortex and incubated at 45°C for 15 min. MHA plates were prepared. 0.1 ml of the bacterial broth was poured onto the plate and swabbed with sterilized cotton bud. The excess broth from the plate was discarded and kept in the incubator for 15 min.

Then wells were dug in it and different doses of the MPG (25, 22.5, 20, 17.5 mg/well) was loaded

into the well. The plates were left at room temperature for 1hr for drug diffusion into media and then incubated overnight at 37°C. The ZOI formed around the wells were measured.

2.5.3 Modified agar well diffusion method

In order to find the sensitivity of different bacteria (*E. coli, K. pneumoniae*), on a single plate, this method was used. A MHA plate was prepared. After solidification, a well was dug in the center. Different organisms were streaked on the plate from the periphery towards the center in a zigzag manner. Then a particular dose of the MPG (25 mg/well) was loaded into the well. Then the plate was left at room temperature for 1hr for drug diffusion and then incubated overnight at 37°C. The distance between the well and the bacterial growth start point was measured.

2.5.4 Cfu/ml determination in control and MPG treated bacteria

2.5.4.1 Bacterial colony count by spread plate method at log phase

The stock solution of the MPG (500mg/ml) was prepared. 3ml of nutrient broth was taken in a different test tube and different doses of extract (1 mg/ml, 2 mg/ml, 4 mg/ml and 8 mg/ml) were added to each test tube, 100 µl of fresh bacterial culture was added to the test tube. A control tube was also prepared by inoculating only bacteria without extract and incubated for 4hr. Aliquots of the culture were taken in Eppendorf tube, contents of the Eppendorf were diluted by adding distilled water (10 µl of culture + 990 µl of distilled water). MHA plates were prepared and 100 µl of the diluted content of the Eppendorf tubes were added to the plate, evenly spread on the plate using a sterile "L" rod. The plates were incubated overnight at 37°C and colonies were counted.

2.5.4.2 Bacterial counting by absorbance method (OD_{600nm}) at stationary phase

The stock solution of MPG (500 mg/ml) was prepared. 3ml of NB was taken in a different test tube and different doses of extract (4mg/ml and 16 mg/ml) were added to the test tubes and to each test tube 100 µl of fresh bacterial culture was added. A control tube was also prepared by inoculating only bacteria without extract. Then the tubes were incubated for 18hr and OD value was measured at 600 nm. Individual sets of blanks were prepared with only media or media with different MPG concentration. These blanks were used while taking OD in order to measure the turbidity resulted by control/wild or MPG treated bacterial growth.

2.6 Salt Agglutination Test

Different concentration of $(NH_4)_2SO_4$ solution were prepared (1M, 1.5M, 2M, 2.5M, 3M, 3.5M, 4M). On a clean slide one drop of control culture or MPG treated culture was taken and added with a different concentration of $(NH_4)_2SO_4$ and observed for agglutination (if any).

3. RESULTS

3.1 Pomegranate Seed Extract

When 80 g of powdered shade dried seed was subjected to exhaustive soxhlet extraction followed by extract concentration in a rotary evaporator, 21.5 g of a dark red colour sticky mass was obtained with a yield of 26.87% (w/w).

3.2 Phytochemical Analysis

Presence of steroid, tannin, coumarin, glycoside and flavonoid was confirmed in MPG. The total phenolic content of the MPG, calculated from the calibration curve (R^2=0.9842) was 70±0.105 mg gallic acid equivalent per gram of extract.

3.3 Antibiotic Sensitivity Test

E. coli strain used in this study was found to be comparatively sensitive showing resistance to only cefuroxime (CXM), cephotaxime (CTX) and sensitive to many drugs i.e. amikacin (Ak), levofloxacin (LE), ceflxime (CFM), norfloxacin (NX), amoxyclav (AMC), nitrofurantoin (NIT), gentamycin (GEN), cotrimoxazole (COT) (Plate 1A).

K. pneumoniae was found to be sensitive to NIT, NX, COT, LE and it was resistant to many drugs i.e. AMC, CTX, GEN, AK, CFM, CXM (Plate 1B).

3.4 Antibacterial Activity of MPG

3.4.1 Disc diffusion method

In disc diffusion method, the MPG was found to be effective against both clinically isolated *E. coli* and *K. pneumoniae* at doses of 6.25, 5.5, 4.75, 4 mg/disc. *K. pneumoniae* (Plate 2B) was found to be comparatively sensitive than *E. coli* (Plate 2A). Table 1 represents the results of disc diffusion method.

Table 1. Effect of MPG on *E. coli* and *K. pneumoniae* in disc diffusion method

MPG in mg/disc	ZOI of *E. coli* in cm	ZOI of *K. pneumoniae* in cm
6.25	1.0	1.5
5.5	0.9	1.4
4.75	0.8	1.4
4	0.7	1.3

**results are expressed as the mean value of triplicate set of experiments*

3.4.2 Agar well diffusion method

Different doses of MPG (25, 22.5, 20, 17.5 mg/ml) were used to determine the antibacterial activity of MPG against *E. coli* and *K. pneumoniae*. The extract was found to be effective against both the strains. *K. pneumoniae* (Plate 3B) was found to be comparatively sensitive than *E. coli* (Plate 3A). Table 2 presents the results of agar well diffusion method.

Table 2. Antibacterial activity of MPG against *E. coli* and *K. pneumoniae* in agar well diffusion method

MPG in mg/well	ZOI of *E. coli* in cm	ZOI of *K. pneumoniae* in cm
25	1.9	2.0
22.5	1.7	1.8
20	1.7	1.7
17.5	1.5	1.6

**results are expressed as the mean value of triplicate set of experiments*

3.4.3 Modified agar well diffusion method

A particular dose of MPG (25mg/ml) was used to determine its antibacterial activity against *E. coli* and *K. pneumoniae*. *E. coli* was found to be comparatively sensitive than *K. pneumoniae* (Plate 4). This method is useful to observe the effect of the extract against different types of bacteria on a single plate. The results were presented in Table 3.

3.4.4 Cfu/ml determination in control and drug-treated bacteria at log and stationary phase

3.4.4.1 Spread plate method

The CFU/ml for the wild *E. coli* was found to be $5.15x10^7$ (Plate 5A) but it was found to be $3.21x10^7$ (1 mg/ml) (Plate 5B), $1.79x10^7$ (2 mg/ml) (Plate 5C), $0.97x10^7$ (4mg/ml) (Plate 5D) and 0.54x107 (8 mg/ml) (Plate 5E) for MPG treated *E. coli* at log phase. The CFU/ml for the wild *K. pneumoniae* was observed to be $2.56x10^7$ and it was $1.8x10^7$ (1 mg/ml), $1.02x10^7$ (2 mg/ml), $0.86x10^6$ (4 mg/ml) and $0.31x10^6$ (8 mg/ml) in MPG treated *K. pneumoniae* at log phase. The results were graphically represented in Fig. 1.

Table 3. Activity of MPG against different bacterial strains in modified agar well diffusion method

Type of organism	ZOI for 25 mg/well of MPG
E. coli	1.5
K. pneumoniae	1.2

**results are expressed as the mean value of triplicate set of experiments*

3.4.4.2 Absorbance method

We know that OD_{600}= $1x10^9$ bacteria. In absorbance method, the CFU/ml for the wild *E. coli* was determined as $1.21x10^9$ and it was $0.95x10^9$ (4 mg/ml) and $0.72x10^8$ (16 mg/ml) for MPG treated bacteria at stationary phase. The cfu/ml of wild *K. pneumoniae* was $0.76x10^9$ and it was $0.24x10^9$ (4 mg/ml) and $0.15x10^8$ (16 mg/ml) for MPG treated *K. pneumoniae* at stationary phase. The results were graphically presented in Fig. 2.

3.5 Salt Agglutination Assay

The wild bacteria agglutinated at 2.5M of $(NH_4)_2SO_4$ or above whereas in the MPG treated bacteria agglutinated at 3M of $(NH_4)_2SO_4$ or above.

4. DISCUSSION

Successive petroleum ether, chloroform, methanol and water extract of *P. granatum* were tested (*in vitro*) for their antibacterial activity. But, the methanolic extract was found to be most effective against all tested microorganisms [6]. Therefore, we used this solvent system for extract preparation and methanol is also known to be an excellent medium for extraction of maximum phytoconstituents. Chemical investigation of methanolic extract of pomegranate fruit following antibacterial activity led to the isolation of pelargonidine 3 galactose, cyanidine 3 glucose, gallic acid, quercetin and myricetin. All these compounds exhibited significant activity against spp. of

Corynebacteria, Staphylococci, Streptococci, Bacillus subtilis, Shigella, Salmonella, Vibrio cholera and Escherichia coli. However, these compounds were more active against gram-positive bacteria [7].

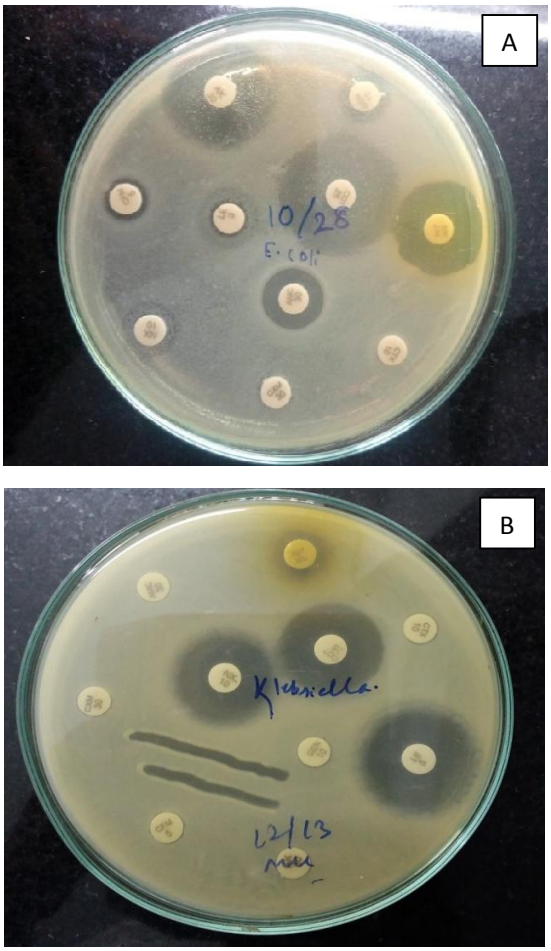


Plate 1. Antibiotic sensitivity test (A) *E. coli* (B) *K. pneumoniae*

In this study, *P. granatum* seed extract was found to have an effective enterobacteriostatic effect, which was evident from disc diffusion, agar well diffusion and modified agar well diffusion method. In disc diffusion and agar well diffusion, *K. pneumoniae* was found to be more sensitive than *E. coli* (Plate 2 and 3), whereas in modified agar well diffusion *E. coli* was found to be more sensitive as compared to *K. pneumoniae* (Plate 4). Plant extract has great potential as antimicrobial compounds against microorganisms. Thus they can be used in the treatment of infectious diseases caused by resistant microbes. In this present study, we found in both disc diffusion and agar well diffusion method *K. pneumoniae*, which was a multidrug-resistant (MDR) strain was found to be more sensitive to MPG in comparison to *E. coli* strain, which was a comparatively sensitive strain as evident from the results of antibiotic sensitivity test.

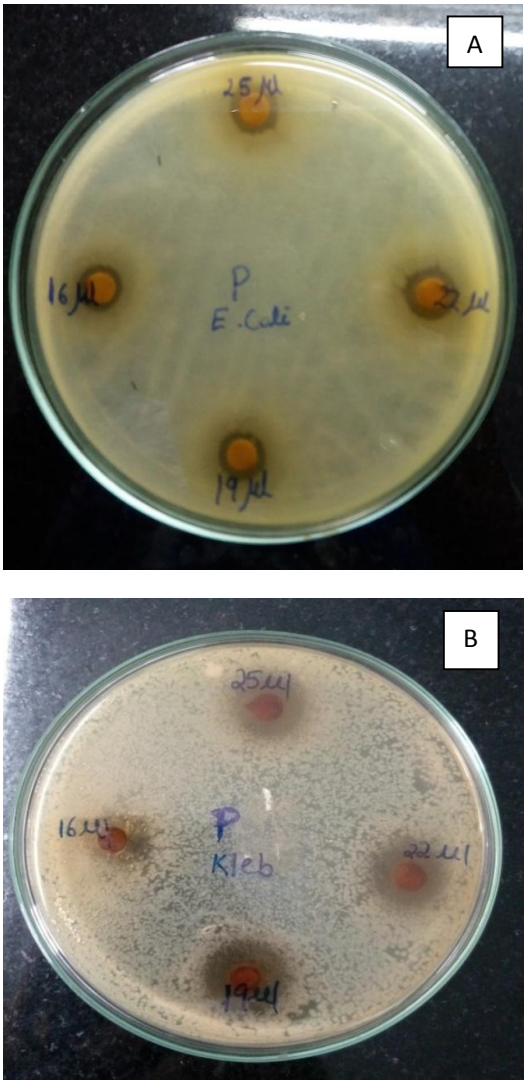
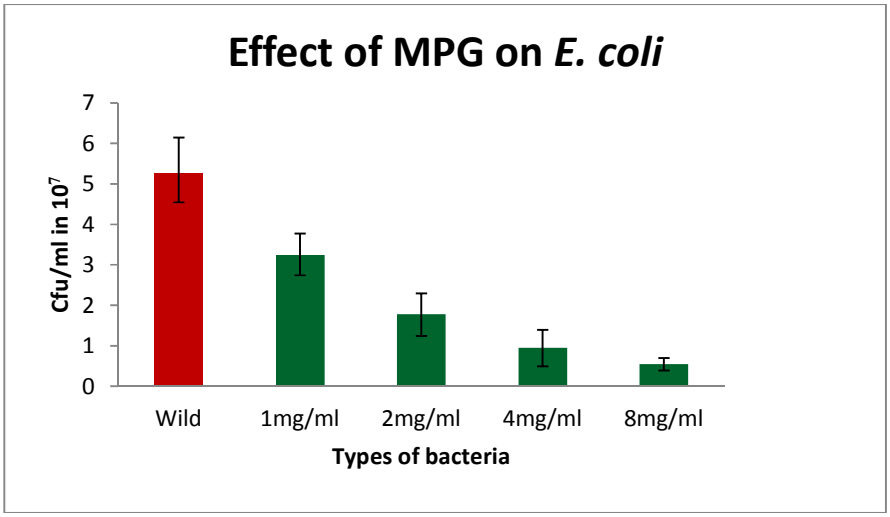


Plate 2. Antibacterial activity of MPG by disc diffusion method (A) *E. coli* (B) *K. pneumoniae*

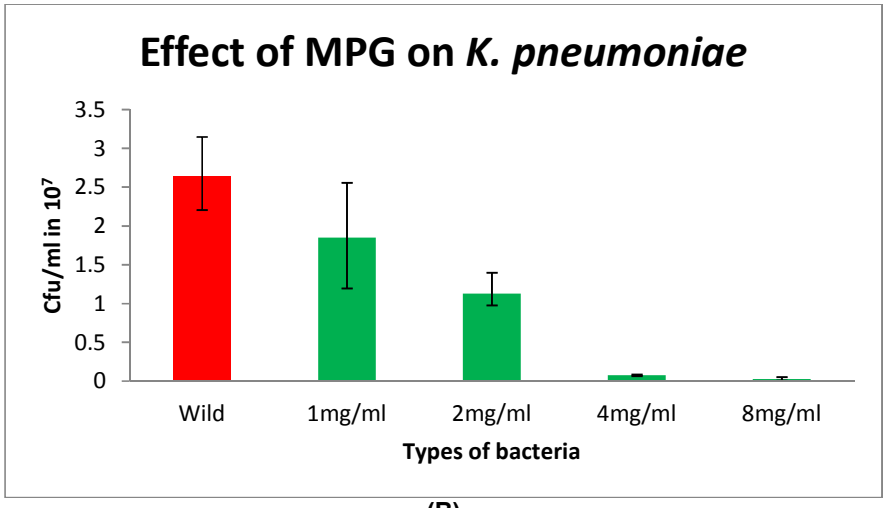
There can be result variation in between different plates. So modified agar well method is used to test the sensitivity of a number of organisms on a single plate. In this present study, we found *E. coli* is more sensitive than *K. pneumonia* (Plate 4) in the modified agar well method, which might be due to the more inoculant concentration of *K. pneumoniae*. Bactericidal or bacteriostatic effect

of any natural product or antibiotic is determined by the kind of target organism as well as its concentration. If the organism is resistant or if it is present in great numbers then we have to take an antibiotic in a higher dose and for a longer duration. So in modified agar well there might be abundant resistant organisms present in comparison to the number of *E. coli,* so we found an opposite result in modified agar well in comparison to disc diffusion and agar well diffusion method.

The synergistic effect of the association of antibiotic with plant extract against resistant bacteria leads to a new choice for the treatment of infectious diseases. The effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment [8]. The crude ethanolic extract from *P. granatum* L. produces inhibition zone against *E. coli* 0157:H7, other enterohemarrhagic *E. coli* and the reference strain. The antibacterial activity of the crude ethanolic extract (2.5 mg) resulted in clear inhibition zone of at least 10 mm for all the strain tested [9]. The present study indicated that methanol extract of *P. granatum* seed is effective against the clinically isolated *E. coli* and in disc diffusion method (6.25 mg/disc), it showed an inhibition zone of 10 mm (Plate 2A).



(A)



(B)

Fig. 1. Antibacterial activity of MPG by spread plate method at log phase (4 hr culture): (A) *E. coli* (B) *K. pneumoniae*

**results are expressed as the mean±SD value of triplicate set of experiments*

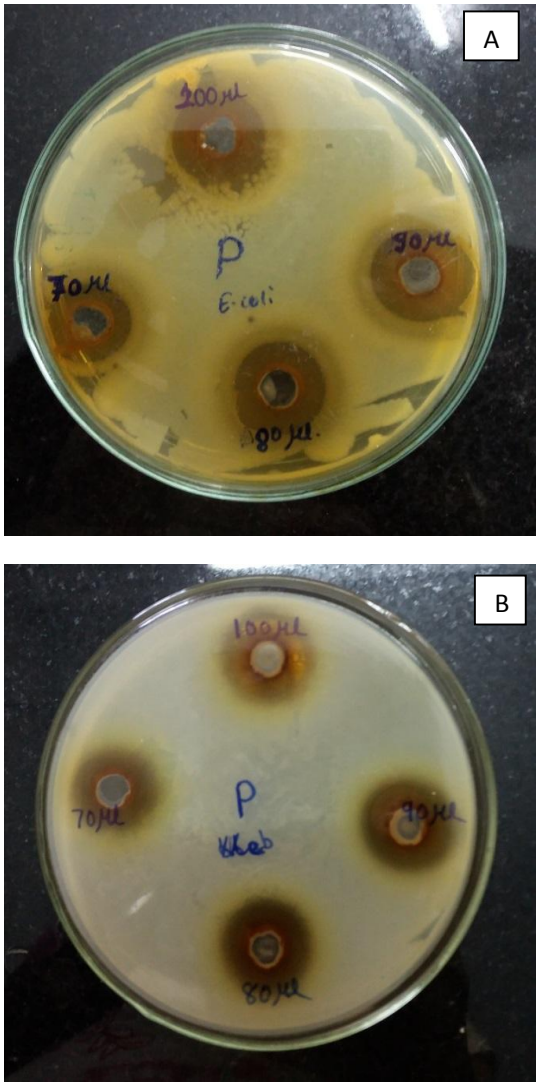


Plate 3. Antibacterial activity of MPG by agar well diffusion method (A) *E. coli* (B) *K. pneumoniae*

The bacteriocidic or bacteriostatic effect can be measured not only through solid plate medium but also in liquid broth culture. In contrast to solid medium in liquid broth culture medium, the bacteria are exposed uniformly to the antimicrobial agents, in this case, pomegranate seed extract and the sensitivity test is better executed in liquid media. So, we used two techniques to find CFU/ml in control and drug-treated bacteria. In the first case, we exposed the bacteria to different doses of the drug for 4 hr (exponential phase), followed by inoculation through spread plate method and directly counting the colonies on MHA plates and compare the number of colonies present in control/wild bacteria and drug-treated bacteria. This method counts the number of live bacteria capable of forming colonies (Fig. 1). In the second method, we exposed the bacteria to different doses of the drug for 18 hr (stationary phase) and used absorbance method (OD_{600}) to measure the bacterial turbidity, which measures the number of living as well as dead bacteria. The CFU/ml was comparatively less in live spread plate method in comparison to absorbance method as it measures both lives as well as dead bacteria and in first case 4 hr culture was taken and in the second case 18 hr culture was used for the study (Fig. 2).

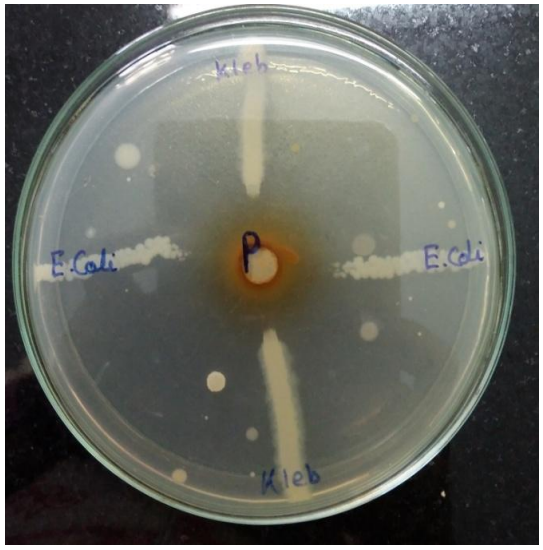
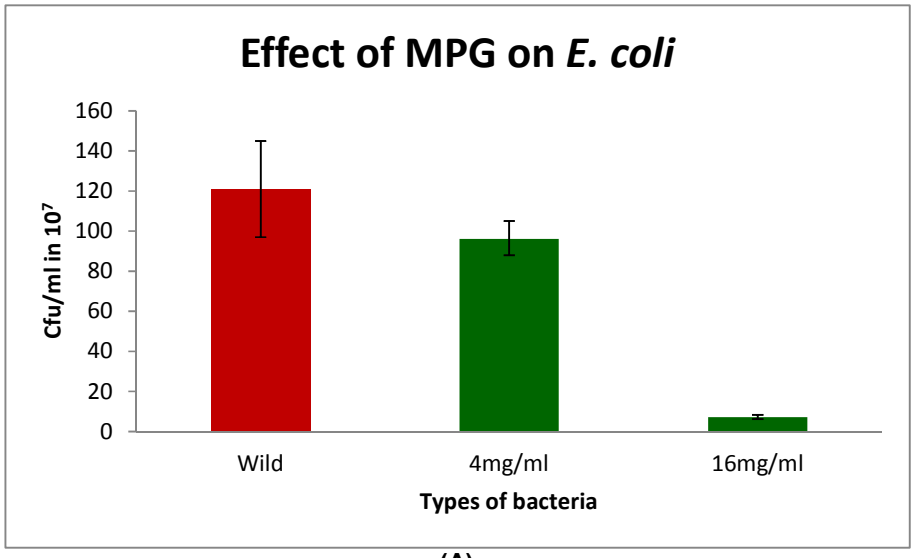
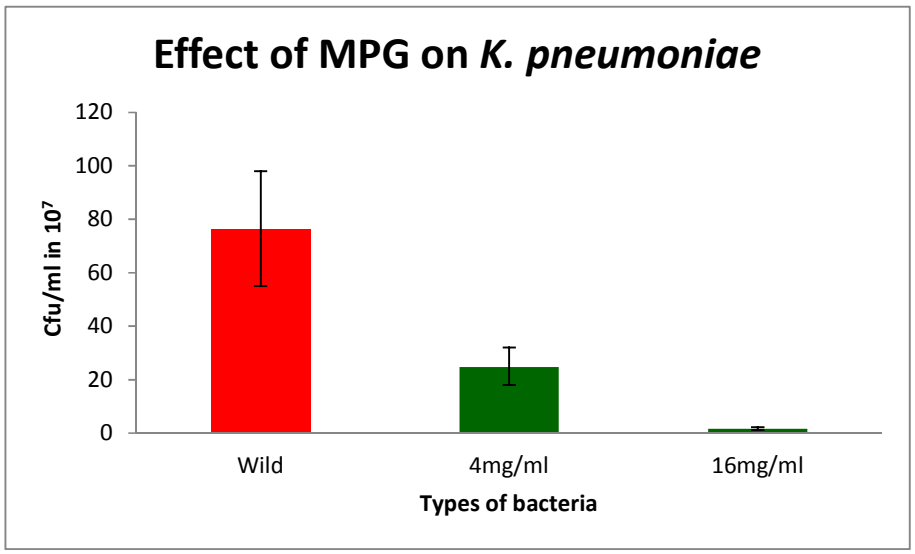


Plate 4. Antibacterial activity of MPG against *E. coli* and *K. pneumoniae* by modified agar well diffusion method

Cell surface hydrophobicity is a measure of virulence in pathogenic bacteria and loss of surface hydrophobicity can play important role in making the bacteria defective for adherence and it can be easily flushed out of the body. Salt agglutination assay measures the surface hydrophobicity. In this study, the wild bacteria agglutinated at 2.5M of $(NH_4)_2SO_4$ or above whereas in the MPG treated bacteria, we found the loss of surface hydrophobicity as the MPG treated bacteria agglutinated at 3M of $(NH_4)_2SO_4$ or above. Our findings suggest that the MPG treated bacteria might be defective to bind to host cell surface, which can reduce their virulence ability. However, further study is needed to confirm these results.



(A)



(B)

Fig. 2. Antibacterial activity of MPG by absorbance method at stationary phase (18 hr culture): (A) *E. coli* (B) *K. pneumoniae*

**results are expressed as the mean±SD value of triplicate set of experiments*

The components of pomegranate contain compounds offering some impressive therapeutic applications [10]. Pomegranate fruit, fruit juice, peel extracts, seed oil and seed extracts have been found to possess a potent antioxidant activity. Antioxidant potential of pomegranate juice and extracts is attributed to their high polyphenolics content including ellagic acid and ellagitannins. Other researchers have shown that pomegranate peel extract has markedly higher antioxidant capacity than the pulp extract. Among methanol, ethyl acetate and n-hexane, used for extraction of antioxidants from pomegranate peel and seed, ethyl acetate extract has maximum antioxidant activity [11].

Pomegranate juice is a polyphenol-rich with high antioxidant capacity shown to exert significant antiatherogenic, antioxidant, antihypertensive and anti-inflammatory effects [12]. Polyphenols are plant-derived natural products, classified as flavonoids and non-flavonoids with well-documented health benefits to human beings, such as antioxidant, anticancerous, antidiabetic

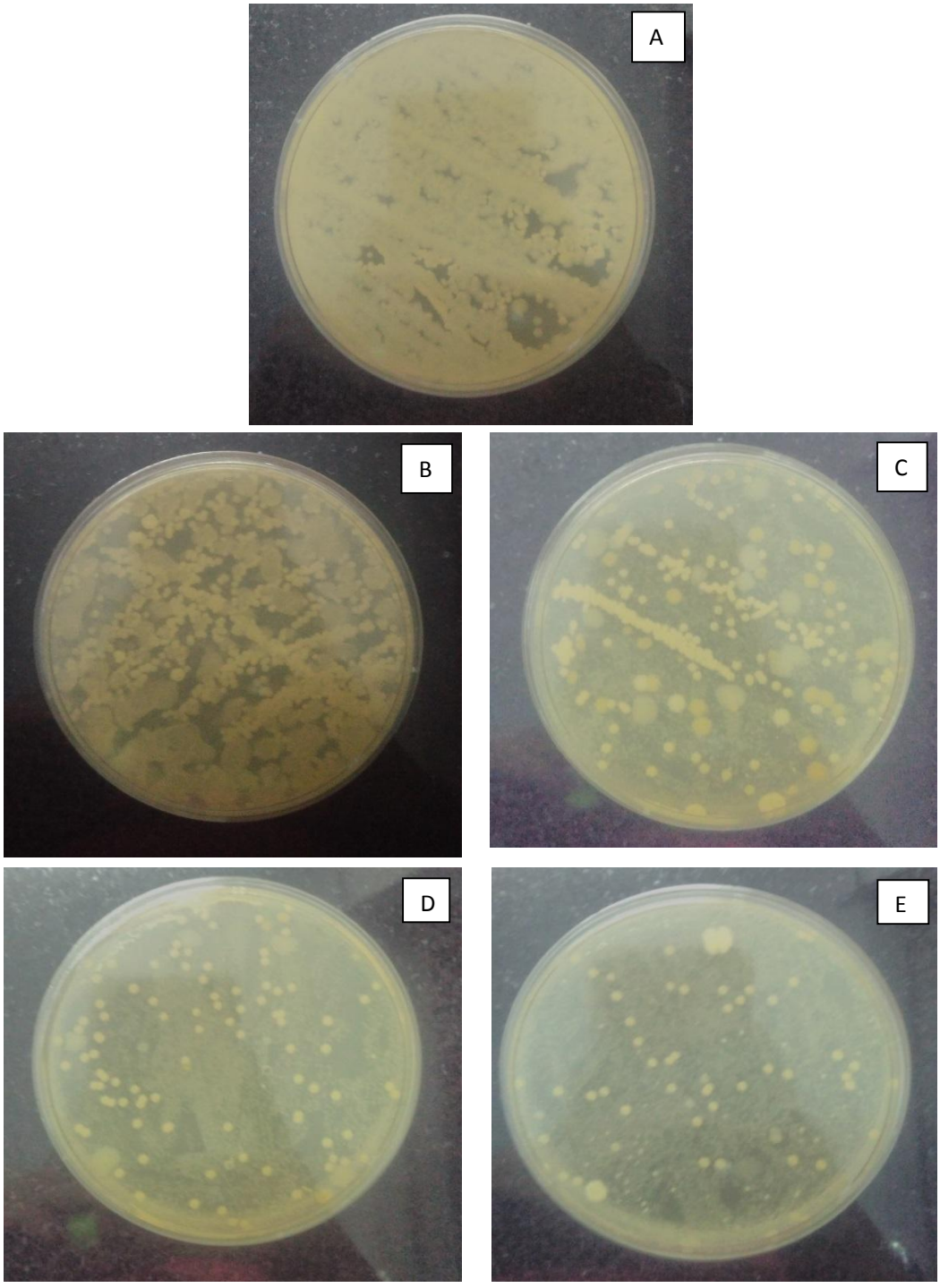


Plate 5. Antibacterial activity of MPG by spread plate method against *E. coli* (A) control/wild (B) 1 mg/ml (C) 2 mg/ml (D) 4 mg/ml (E) 8 mg/ml MPG treated bacteria

along with antibacterial activities. Among these bioactivities, the antibacterial activities were considered to be most important due to their potential use against the drug-resistant bacteria that are insensitive to conventional antibiotics [13]. Polyphenols, especially flavonoids, have

been proposed to exert their antibacterial effects in three different ways i.e. by direct killing of bacteria, synergistic activation of antibiotics, and attenuation of bacterial pathogenicity [14].

The phytochemical screening revealed the presence of triterpenoid, steroids, glycosides, flavonoids, tannins, carbohydrates and vitamin C in peel extract; triterpenoids, steroids glycosides, saponins, alkaloids, flavonoids, tannins and vitamin C in whole fruit extract and triterpenoids, steroids, glycosides, saponins, alkaloids, tannins, carbohydrate and vitamin C in seed extract [15]. This was also confirmed by our phytochemical analysis.

5. CONCLUSION

Our results indicate that the antiuropathogenic activity of pomegranate seed extract might be due to the presence of phenolic and nonphenolic bioactive compounds, which probably act synergistically against MDR strains of UTI causing bacteria. However, further study is going on to isolate and characterize the bioactive compounds and also to understand the mode of action of these phytoconstituents against the UTI causing pathogens at a molecular level.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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