Phytochemical analysis and antibacterial activity of *Punica granatum* L. seed extract against clinically isolated uropathogens

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Abstract
The discovery of plant based drugs and dietary supplements derived from plants has accelerated in recent years. Punica granatum L. is one such plant whose products are useful in the treatment of many diseases including urinary tract infections (UTI), since it is rich in various antioxidants like vitamin C, iron and phenolic compounds. Antioxidant rich fluids are known to prevent bacteria from adhering to the walls of the bladder and urethra; as a result UTI is cured or prevented. The present investigation aims to explore the urobactericidal activity of different solvent extracts of the seeds of P. granatum. In this study, P. granatum dried seed powder was extracted exhaustively with different solvents i.e. hexane, chloroform, acetone and methanol (HPG, CPG, APG and MPG). The presence of different phytochemicals in these extracts was assessed both qualitatively and quantitatively before evaluating their urobactericidal and uroprotective potency.

It was found that seeds of pomegranate have many bioactive phytochemicals like terpenoids, steroids, tannins, coumarins, glycosides, flavonoids and phenolic compounds that contribute to its significant antibacterial activity and this was proved by various methods and the zone or degree of growth inhibition was measured against different uropathogens. It was observed that APG and MPG, which are rich in hydrophilic phytoconstituents have significant bactericidal activity in comparison to HPG and CPG, which are rich in hydrophobic compounds. Since, the active phytoconstituents present in P. granatum seed have potent urobactericidal activity, it can be further explored for its tremendous bioactive potentials and also attempts should be made to safeguard and cultivate this miraculous plant which would be beneficial to the society.

Keywords: *E. coli, P. aeruginosa, S. aureus, P. granatum* seed, HPG, CPG, APG, MPG.

Introduction

Medicinal plants have been explored extensively for their therapeutic values which are often contributed by their bioactive secondary metabolites. Herbal medicines are considered to be safe in contrast to the synthetic medicines that are considered as deleterious to human as well as hazardous to the environment. Over three-fourth of the total world population depends mostly on plants and their products for their major health concerns. Since ancient times such plants play an important role in the maintenance of human health. Phytoconstituents such as vitamins (A, C, E and K), carotenoids, pigments, minerals and enzymes have various pharmacological activities[21].

Natural therapeutics may comprise one or more plants or their products and mostly they are complex in nature. Certainly, there are many significant botanicals (plants/plant products) with vital therapeutic values such as *Withania somnifera, Nerium oleander, Lantana camara, Ficus sycomorus, Eucalyptus camaldulensis, Artemisia herba-alba, Allium sativum* etc. Common urotonic plants can be *Punica granatum, Hemidesmus indicus, Phyllanthus amarus, Tribulus terrestris, Mimosa hamata* etc. Many of the herbs and spices like *Foeniculum vulgare, Cucumis sativus, Ammi majus, Allium ascolinicum, Cichorium intybus, Rumex vesicarius* contain useful medicinal compounds, which can be used as an alternative to antibiotics against common bacterial infections[1] and that primes unlimited opportunities for new drug discoveries.

Pomegranate (*Punica granatum* L.) belonging to family Lythraceae is an important medicinal plant as it possess many important compounds like alkaloids, glycosides, terpenoids, flavonoids, tannins and polyphenols[16]. The ripen Pomegranate fruit has a deep red, leathery skin. The fruit has many seeds (Arils) and they are enclosed by small amounts of tangy, red juice which exhibit excellent kinds of antioxidants and other important phytoconstituents[24].

Phytochemistry and ethnopharmacological activity of *P. granatum:* The uroprotective efficacy of pomegranate was verified *in vivo* and *in vitro* studies and also in major biological processes like oxidative stress, hypoxia and inflammation. Chemopreventive, Ellagitannins, different kind of hydrolysable tannins, which are the main class of polyphenolics, have also been found in pomegranate husk, juice, seeds, bark, pericarp and flowers[12,31,32].

Ellagic acid, derived from pomegranate juice and seed oils has anticancer activity against different types of cancer i.e. skin, oral, esophagus, liver, breast, pancreas, bladder, prostate, intestine, colon, neuroblastoma and leukemia[11].

Pomegranate peel is found to be rich in a condensed tannin, prodelphinidins (formed by polymerization of gallocatechin)[22]. Pomegranate fruit is rich in different flavonoids like catechin and gallocatechin having flavon-3-ol among others that have significant pharmacokinetic and antitoxic effect[10]. *P. granatum* also with less amount of different hydrolysable tannins like gallic acid, gallagic acid, gallagyldilactone and pedunculagin exhibits therapeutic applications.

Glances of Antimicrobial Property of *Punica granatum:* Natural dye powders obtained from *P. granatum* have antimicrobial properties against *S. aureus, Shigella sonnei, E. coli, B. megaterium, B. subtilis, B. cereus, Streptococcus epidermidis* and *P. aeruginosa*[6]. Methanolic extract of *P. granatum* seed was reported to have broad-spectrum activity against 159 MDR strains of clinically isolated urobacteria[13]. Ethanolic extract of *P. granatum* seed exhibited strong antiurobacterial activity against clinically isolated *E. coli*[26]. Pomegranate juice was reported to have commendable efficacy in comparison to most well-known antibiotics (streptomycin, ampicillin, bifonazole and ketoconazole). Over 30 non-anthocyanidinic and more than 20 anthocyanidinic compounds were present in pomegranate juice[18].

Etiology and Pathophysiology of UTI: The resident *E. coli* flora present in the large intestine of human is the main causative agent of UTI as abundantly present around the rectal area and contribute to more than 80% of UTI. *S. saprophyticus* mediated UTI is more common during summer responsible for over 10% of UTI cases. Other bacteria like *Enterococcus faecalis, Enterobacter, Citrobacter, Klebsiella, Proteus* and *Pseudomonas* also cause UTI. *Mycoplasma* and *Chlamydia* may cause sexually transmitted UTI that lasts for a longer duration in both male and female and necessitates the treatment of both partners[29].

Women are more prone to have UTI in comparison to men. UTI is more prevalent in sexually active and reproductive age group women. The length of urethra is about 1.5 and 8 inches in women and men respectively. Because of their short urethra, the bacterium reaches the bladder easily in women. Generally, the fecal bacteria colonize the urethra (urethritis) and spread up the urinary tract to the bladder (cystitis) as well as to the kidneys (causing pyelonephritis) or the prostate (prostatitis) in males. Due to the presence of shorter urethra, women are 14 times (50%-60%) more likely to have ascending UTI and it is 25% in persons of >70 years of age and still higher in elderly patients[4]. In this present study, the bactericidal activity of different solvent extracts of *P. granatum* seed powder was explored against different uropathogens isolated from the urine sample of infected patients

Material and Methods

Pomegranate seed extract preparation: The pomegranate seeds were purchased from the local herbal market of Berhampur, Ganjam district of Odisha State, India in the month of August-September, 2018 and identified by the botanists. The phytoconstituents from the powdered seeds were extracted in different solvents like hexane (300ml), chloroform (300ml), acetone (300ml) and methanol (300ml) for 72 hours at 60-70°C using Soxhlet apparatus. The filtered extracts were concentrated, dried and stored in a desiccator at 4ºC until used. The hexane and chloroform extracts were mixed with sunflower oil while the acetone and methanol extracts were dissolved in hot distilled water to formulate the desired working concentration and used in different types of antibacterial study. Graphical representation of extract preparation is illustrated in flowchart 1.

Phytochemical analysis: Standard lab protocols were followed to check the presence of active compounds and also to quantify different phytochemicals.

Qualitative analysis: Standard procedures were carried out to verify the presence of terpenoids, steroids, alkaloids, phenol/tannin, proteins, saponins, coumarin, reducing sugar, glycosides etc[14,28,30].

Quantitative analysis

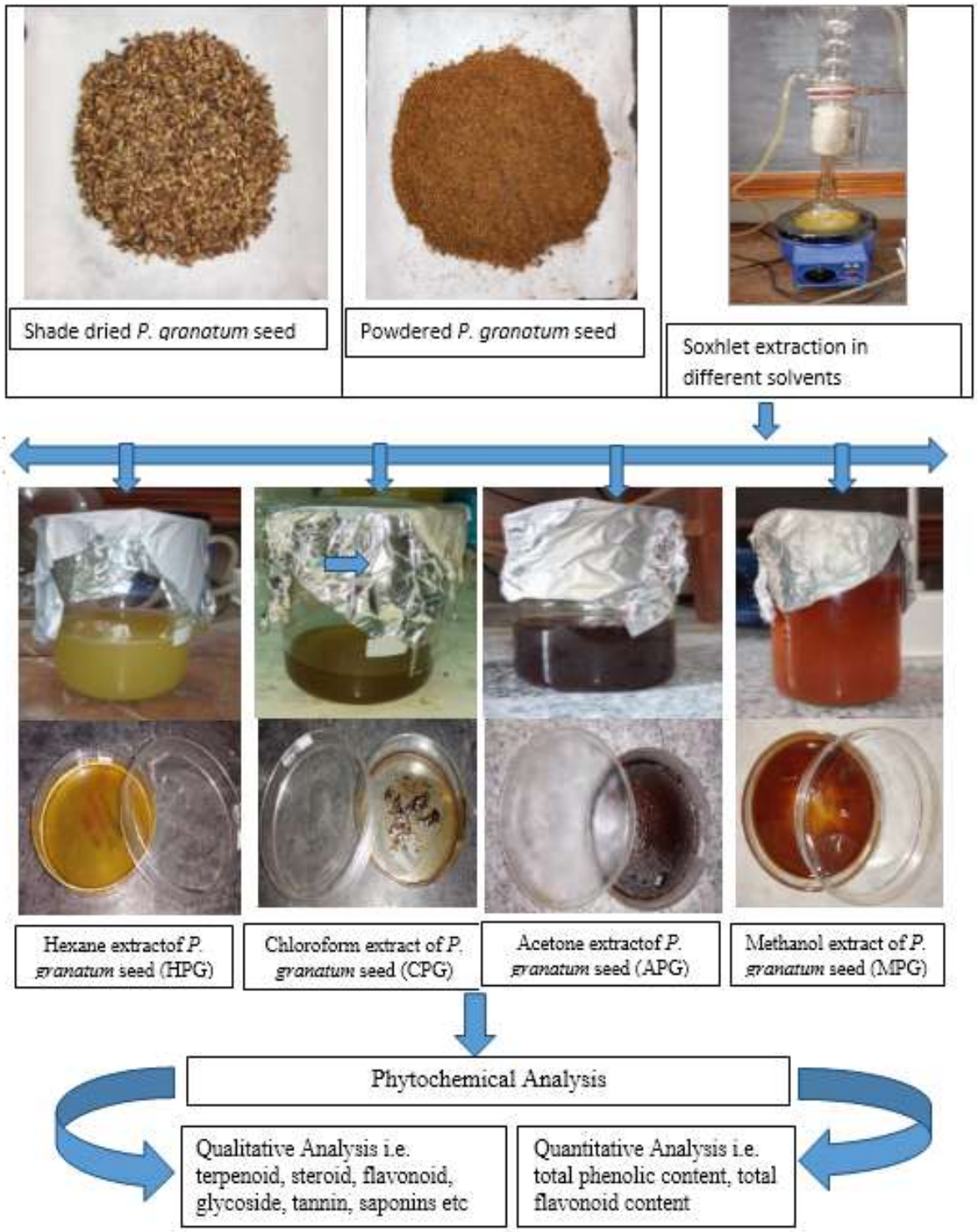
Total phenolic content (TPC): The amount of TPC present in different solvent extracts of *P. granatum* seed was determined by the folin- ciocalteu (FC) method[27]. Briefly, 200 µl of crude extract (1mg/ml) was added to 3ml of distilled water followed by 0.2ml of FC-reagent and mixed thoroughly for 8min, then 0.6ml of 10% Na_2CO_3 was added and incubated in dark for 1hr and absorbance was measured at 765nm. Triplicate sets of test and standard were prepared. The TPC was calculated from the gallic acid standard curve and expressed as mg of gallic acid equivalent per g dry weight of different extracts.

Total flavonoid content (TFC): The TFC was estimated by spectrophotometric method[17,25]. Briefly, different extracts were dissolved in methanol (1mg/ml). To 1ml of this solution, 1ml of 2% $AlCl_3$ dissolved in methanol was added and incubated for an hour at room temperature. Triplicate sets of test and standard were prepared. The absorbance was measured at 415nm. The TFC was calculated from the Rutin standard curve and the test results were expressed as mg of Rutin equivalent per g dry weight of different extracts.

Statistical analysis: MS excel 2013 software was used to carry out the statistical analysis and results were expressed as mean ± standard deviation.

Bacterial strain, maintenance and storage: The test strains of *E. coli*, *P. aeruginosa* and *S. aureus* were isolated from the urine samples of UTI patients in MKCG Medical College and Hospital, Berhampur, Odisha. The bacterial strains were regularly maintained on Mueller Hinton agar (MHA) plates/slants. For long term storage, glycerol stocks were prepared by following standard protocol[8].

Bacterial identification and biochemical characterization: Identification of bacteria was carried out according to standard laboratory protocol that was routinely followed by the Department of Microbiology, MKCG medical college, Berhampur. Briefly, the mid urine samples of the UTI patients were collected and cultured in cystine lactose electrolyte deficient (CLED) agar and incubated over night for bacterial growth. Then the bacterial strains were allowed to grow on specific agar medium such as CLED agar, MacConkey agar, Blood agar etc. for identification of lactose fermenter, non-lactose fermenter, haemolytic or non-haemolytic bacteria. The bacterial strains were then subjected to gram staining. Biochemical characterizations i.e. indole test (I), methyl red (MR), voges proskauer (VP) and citrate test (C) were carried out for confirmation of gram negative bacteria.



Flow Chart 1: Extract preparation *P. granatum* seed

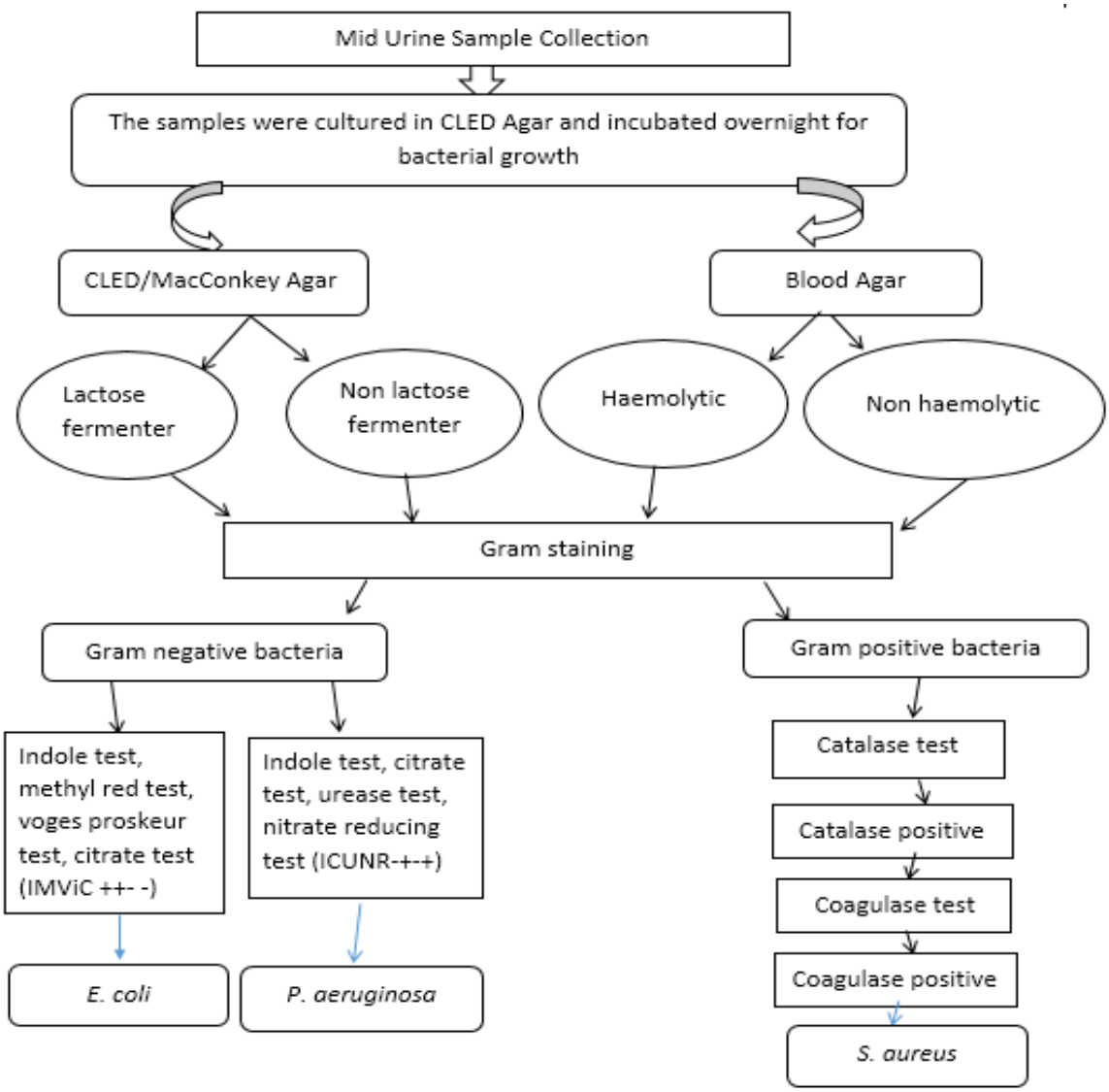
IMViC (++--) confirmed the presence of *E. coli*. Indole test (I), citrate test (C), urease test (U), nitrate reducing test (NR) were basically carried out for *P. aeruginosa* and ICUNR (-+-+) confirmed *P. aeruginosa*. Catalase and coagulase tests were carried out for confirmation of gram positive bacteria. Catalase positive and coagulase positive reaction tests confirmed *S. aureus*. The identification and characterization methods for uropathogenic bacteria are represented in flowchart 2.

Antibiotic sensitivity test (AST): The standard protocol was followed. Briefly, a sterile cotton swab was dipped in a dilute bacterial suspension, squeezed gently by pressing it against the wall of the test tube and swabbed on the sterile MHA plates. Antibiotic discs (Himedia, Mumbai) were picked aseptically and placed gently on MHA plates keeping sufficient gap in between the discs.

Plates were incubated overnight at 37ºC and the zone of inhibition (ZOI), around the discs was measured as a degree of susceptibility of the bacteria to a specific antibiotic at a given dose[8].

Antibacterial test: The efficacy of the different extracts i.e. hexane, chloroform, acetone and methanol extract of *P. granatum* seed abbreviated as HPG, CPG, APG and MPG was determined by different antibacterial techniques (disc diffusion, agar well diffusion and modified agar well diffusion)[8] against the clinically isolated urobacteria (*E. coli*, *P. aeruginosa* and *S. aureus*). The number of live bacteria in control and extract treated culture was also determined by serial dilution followed by spread plate method.

Disc diffusion method: Whatmann no. 1 filter paper was used to prepare discs. *P. granatum* seed extracts (HPG, CPG, APG and MPG) at a dose of 1mg/disc were loaded on it and allowed to dry. MHA plates were prepared and swabbed with sterilized cotton swab containing bacterial culture as mentioned in AST. The extract treated discs were aseptically placed on them. The plates were incubated overnight at 37°C and ZOI was measured.



Flow Chart 2: Identification of Bacterial strains

Agar well diffusion method: An isolated bacterial colony was inoculated into sterile saline/peptone water mixed thoroughly by vortex and activated at 45°C for 15 min. This activated bacterial culture was swabbed on MHA plates and incubated at 37°C for 15 min. Four wells were made on it with the help of sterile micropipette tips and different extracts (HPG, CPG, APG, MPG– 2mg/well) were added into the wells. The plates were kept in RT for 1hr to allow drug diffusion and then incubated overnight at 37°C and the ZOI was measured.

Modified agar well diffusion method: This technique avoids interplate variation of antibacterial testing and it is very useful to find out the sensitivity of different bacteria (*E. coli*, *P. aeruginosa*, *S. aureus*) on one plate. A well was made centrally on a fresh MHA plate and different bacterial strains were streaked on it in a zigzag manner. Then a fixed dose of the HPG, CPG, APG and MPG (2mg/well) was added to the well. Then the plates were incubated first at RT for 1hr and then incubated overnight at 37°C. The ZOI was measured.

Determination of cfu/ml in control and extract treated culture: Different extract solutions (HPG, CPG, APG and MPG – 100mg/ml) were prepared either in sunflower oil or hot water. 300µl of above-mentioned extracts were added to 3 ml of nutrient broth containing test tubes followed by 100µl of activated bacterial culture. A test tube (without extract and with bacteria) served as positive control.

All the test tubes were incubated overnight at 37°C (stationary phase). Aliquots from each culture were serially diluted by adding sterilized distilled water {10 µl of culture + 990 µl of distilled water and repeated twice (dilution factor = 10^{-6})}. 50 µl of this dilute culture was added to MHA plates, uniformly spread by "L" rod and incubated overnight at 37°C. The colony forming units per ml (cfu/ml) were calculated by counting the number of colonies grown on the plates and multiplying it with the dilution factor.

Results

***Punica granatum* seed extract preparation:** When 60g of powdered seeds were extracted in different solvents (hexane, chloroform, acetone and methanol) followed by extract concentration, sticky masses with mean yield of 18.1±0.11%, 1.03±0.09%, 2.14±0.14 % and 5.83±0.13%, respectively were obtained from triplicate sets of extractions, which were used in the following tests. HPG was straw yellow, CPG was brown, APG and MPG were dark brown and reddish brown in color respectively (flowchart 1).

Phytochemical analysis of *P. granatum* seed extract
Qualitative analysis: The results are presented in table 1.

Quantitative analysis
Total phenolic content (TPC): The TPC values of the HPG, CPG, APG and MPG were found to be 9.77±0.06, 20.50±0.10, 66.7±0.42 and 82.2±0.09 mg Gallic acid equivalents/g respectively as determined from the calibration curve (R^2=0.9267).

Total flavonoid content (TFC): The TFC values of the HPG, CPG, APG and MPG were 42.17±0.09, 72.77±0.17, 111.18±0.10 and 147.27±0.11mg Rutin equivalents/g respectively as calculated from the calibration curve (R^2=0.9604).

Antibiotic sensitivity test of different test strains: *E. coli* strain was found to be a MDR strain showing resistance to antibiotics like norfloxacin (NX), nitrofurantoin (NIT), cefixime (CFM), cefuroxime (CXM), gentamycin (GEN), less sensitive to amoxyclav (AMC), levofloxacin (LE), moderately sensitive to amikacin (AK) and highly sensitive to cephotaxime (CEC) and cotrimoxazole (COT). *P. aeruginosa* was found to be a sensitive strain and showed high sensitivity to antibiotics like tobramycin (TOB), imipenem (IC), moderately sensitive to amikacin (AK), levofloxacin (LE), carbenicillin (CB), moxifloxacin (MO), cephotaxime (CEC), piperacillin (PIT) and less sensitive to drugs like colostin (CL), cefepime (CPM).

Table 1
Phytochemical analysis of *P. granatum* seed extract

PHYTOCHEMICALS	HPG	CPG	APG	MPG
Alkaloid	+	+	+	+
Terpenoids	+	+	+	+
Phenol and Tannin	-	-	+	+
Reducing sugar	-	+	-	-
Saponin	+	+	-	-
Protein	+	+	+	+
Steroids	+	+	+	+
Anthocyanin	-	-	-	-
Coumarin	+	+	+	+
Leucoanthocyanin	-	-	-	-
Glycosides	-	+	+	+

Note: + indicates presence and – indicates absence

S. aureus was a MDR strain having resistance to antibiotics like azithromycin (AZM), cefepime (CPM), cefoxitine (CX), less sensitive to ampicillin (A/S), cephotaxime (CEC), moderately sensitive to amikacin (AK), amoxyclav (AMC), vancomycin (VA), levofloxacin (LE) and found to be highly sensitive to only linezolid (LZ). The results are depicted in plate 1 and table 2.

Anti-urobacterial activity of *P. granatum* seed extract

Disc diffusion: APG and MPG showed better efficacy against clinically isolated *E. coli*, *P. aeruginosa*, *S. aureus* than HPG and CPG. *S. aureus* was found to be relatively more sensitive than *E. coli* and *P. aeruginosa.* The results are presented in plate 2 and table 3.

Agar well diffusion: *S. aureus* was found to be more sensitive to APG and MPG fractions of *P. granatum* seed extracts in comparison to *E. coli* and *P. aeruginosa*. The results are depicted in plate 3 and table 4.

Modified agar well diffusion: Both APG and MPG were found to be more effective against all the tested organisms in comparison to other extracts and *S. aureus* was comparatively more sensitive to all the solvent extracts of *P. granatum* seed in comparison to *E. coli* and *P. aeruginosa.* The results are depicted in plate 4 and table 5.

Determination of cfu/ml in control and extract treated culture: At stationary phase the cfu/ml was determined as 8.56×10^8 for the wild *E. coli* and 8.3×10^8, 8.1×10^8, 3×10^8, 2.76×10^8 for HPG, CPG, APG and MPG treated *E. coli* respectively.

Table 2
Antibiotic sensitivity of different bacteria used in this study

Antibiotics	Strains		
	E. coli	***P. aeruginosa***	***S. aureus***
AMC	+	-	++
AK	++	++	++
A/S	-	-	+
AZM	-	-	R
CB	-	++	-
CEC	+++	++	+
CFM	R	+	-
CL	-	-	-
COT	+++	-	-
CPM	-	+	R
CX	-	-	R
CXM	R	-	-
GEN	R	-	-
IC	-	+++	-
LE	+	++	++
LZ	-	-	+++
MO	-	++	-
NIT	R	-	-
NX	R	-	-
PIT	-	++	-
TOB	-	+++	-
VA	-	-	++

Note: R= Resistant, + = Less sensitive, ++ = moderately sensitive, +++ = highly sensitive

Table 3
Effect of *P. granatum* seed extract against different urobacterial strains by disc diffusion method

Concentration of different extracts (1 mg/disc)	ZOI of *E. coli* in cm	ZOI of *P. aeruginosa* in cm	ZOI of *S. aureus* in cm
HPG	0	0	0
CPG	0	0	0
APG	1.8±0.23	1.6±0.11	2.1±0.27
MPG	2.0±0.16	1.7±0.21	2.4±0.19

Results express the mean ± SD of triplicate sets of experiments.

For wild *P. aeruginosa*, cfu/ml was found to be 7.0×10^8 and 2.9×10^8, 2.5×10^8, 6.6×10^7, 4.6×10^7 for HPG, CPG, APG and MPG treated *P.aeruginosa* respectively. For wild *S. aureus*, cfu/ml was found to be 1.13×10^9 and 7.5×10^8, 5.26×10^8, 3.3×10^8, 7.0×10^7 for HPG, CPG, APG and MPG treated *S. aureus* (figure 1, table 6, plate 5, plate 6 and plate 7).

Discussion

Infectious diseases are the second most common cause of mortality and morbidity, often preceded by cardiovascular diseases and succeeded by deaths due to cancer. The condition is still worse in developing and under developed countries. UTI and urinary disorders are the second most common infectious diseases after respiratory infections occurring worldwide and UTI is a very common nuisance and most discomforting disease in women. Many women experience more than one infection during their life time and a single infection increases the chances of its recurrences up to 80 % in the next six months. Therapy for this infection usually begun before the results of microbiological tests are known. The therapy is based on predictable spectrum of etiological agents that cause UTI and their expected antibiotic sensitivity range.

Before choosing the test organisms, it is necessary to do biochemical characterization. It also enables us to distinguish different strains of the same species. In this study, bacterial strains i.e. *E. coli, P. aeruginosa* and *S. aureus* were identified by carrying out standard biochemical characterization tests and used as test organisms to find out the potency of different extracts of *P. granatum* seed (HPG, CPG, APG and MPG) against these common uropathogens. Antibiotic susceptibility can differ among the different strains of a species (some strains may be more resistant than others). Therefore, antibiotic susceptibility test is usually conducted to find out the more suitable antibiotic against a bacterial infection and to treat a disease successfully *in vivo*.

Table 4
Growth inhibitory effect of *P. granatum* seed extract against different urobacterial strains by agar well diffusion method

Concentration of differen extracts (2mg/well)	ZOI of *E. coli* in cm	ZOI of *P. aeruginosa* in cm	ZOI of *S. aureus*in cm
HPG	0	0	0
CPG	0	0	0
APG	1.8±0.12	1.7±0.11	2.2±0.1
MPG	2.1±0.13	1.9±0.7	2.5±0.09

Results express the mean ± SD of triplicate sets of experiments

Table 5
Inhibitory effect of *P. granatum* seed extract against different urobacterial strains by modified agar well diffusion method

Concentration of different fractions (2mg/well)	ZOI of *E. coli* in cm	ZOI of *P. aeruginosa* in cm	ZOI of *S. aureus* in cm
HPG	0.8±0.09	0.6±0.07	2.6±0.12
CPG	1.2±0.05	1.1±0.1	1.2±0.13
APG	1.2±0.08	1.8±0.12	3.2±0.23
MPG	1.4±0.18	1.6±0.13	2.4±0.2

Results express the mean ± SD of triplicate sets of experiments

Table 6
Determination of cfu/ml in control and extract treated culture at stationary phase

Type of organism	cfu/ml for *E. coli*	cfu/ml for *P. aeruginosa*	cfu/ml for *S. aureus*
Wild	8.56×10^8	7×10^8	1.13×10^9
HPG treated	8.3×10^8	2.9×10^8	7.5×10^8
CPG treated	8.1×10^8	2.5×10^8	5.26×10^8
APG treated	3×10^8	6.6×10^8	3.3×10^8
MPG treated	2.76×10^8	4.6×10^8	7×10^7

Results express the mean ± SD of triplicate sets of experiments.

In this study, *E. coli* was found to be a MDR strain showing resistance to 5 antibiotics {cefixime (CFM), cefuroxime (CXM), gentamycin (GEN), norfloxacin (NX)} and nitrofurantoin (NIT)} and less sensitive to some advanced antibiotics like amoxyclav (AMC) and levofloxacin (LE) (plate 1a) while *S. aureus* was also a MDR strain and found to be resistant to 3 of the tested antibiotics {cefepime (CPM), cefoxitine (CX) and azithromycin (AZM)} and less sensitive to ampicillin (A/S) and cephotaxime (CEC) (plate 1c). Yet, these strains were found to be relatively sensitive to different extracts of *P. granatum* seed (especially APG and MPG) than *P. aeruginosa* which was a sensitive or nonresistant strain (plate 1b).

Pomegranate fruit has impressive antioxidant and chemoprotective potential. It was reported to be rich in striking therapeutic compounds[2]. Each part of pomegranate i.e. entire fruit, peel extracts, fruit juice, seed extracts and seed oil are reported to have strong antioxidant activity due to their rich polyphenolics content including ellagic acid and ellagitannins. It is suggested that pomegranate flowers also have significant antioxidant potentiality. Pomegranate peel extract has better antioxidant potency than the pulp extract.

When pomegranate peel and seed were extracted with different solvents like n-hexane, ethyl acetate and methanol, the ethyl acetate extract exhibited maximum antioxidant activity. Pomegranate peel and fruit extracts had better antiperoxidative activity than pomegranate flower extract and they were reported to inhibit lipid peroxidation[15].

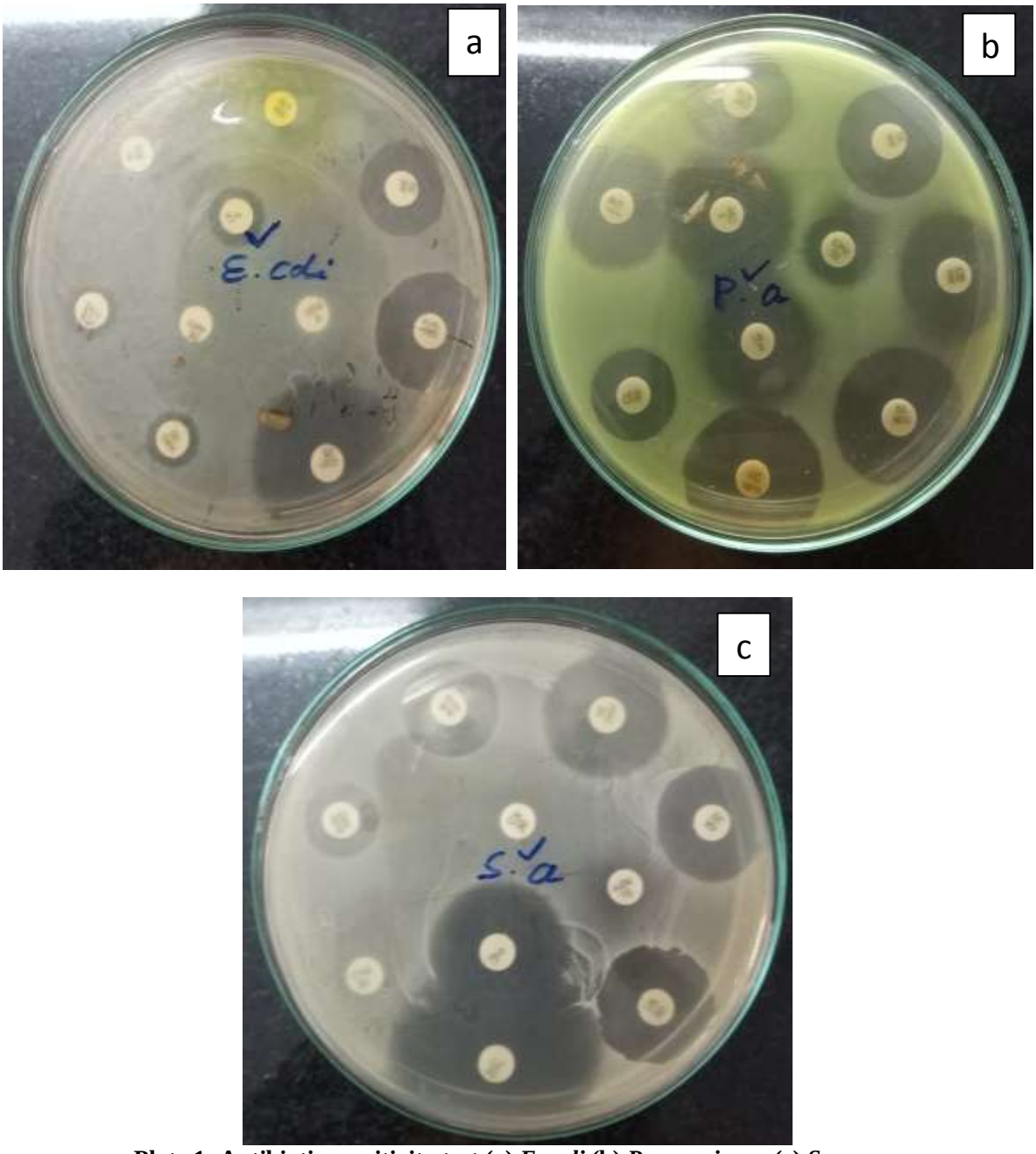


Plate 1: Antibiotic sensitivity test (a) *E. coli* (b) *P. aeruginosa* (c) *S. aureus*

Pomegranate juice is rich in polyphenolic compounds and reported by several authors to have tremendous antioxidant capacity. It is reported to have significant antioxidant, anti-inflammatory, antihypertensive and antiatherogenic effects in human and murine model. It considerably condensed the atherosclerotic lesion areas in immune-deficient mice and thickness of tunica media in cardiac patients. It reduced lipid peroxidation in type 2 diabetes patients and was also found to be effective in treating hypertensive patients[3].

Infectious diseases pose a huge problem in recent era. These are mainly caused due to the entry of microbial pathogens into our body. Then their multiplication and growth in our body create hazardous health problems. Antibiotics are often preferred to treat these diseases, but nowadays the bacteria are becoming resistant to the conventional antibiotics. They acquire resistance due to their gene mutation capability or horizontal gene transfer ability. Hence, it is becoming difficult to treat these MDR strains with the available antibiotics.

The presence of different bioactive compounds like vitamin C, triterpenoid, steroids, flavonoids, tannins, carbohydrates, glycosides etc. in peel extract; triterpenoids, steroids, alkaloids, flavonoids, saponins, tannins, glycosides and vitamin C in whole fruit extract and triterpenoids, steroids, alkaloids, tannins, saponins, carbohydrate, glycosides along with vitamin C were reported to be present in seed extract[5]. The presence of alkaloids, terpenoids, steroids, tannins, coumarins, glycosides, flavonoids and proteins was confirmed in different extracts of *P. granatum* seed (table 1).

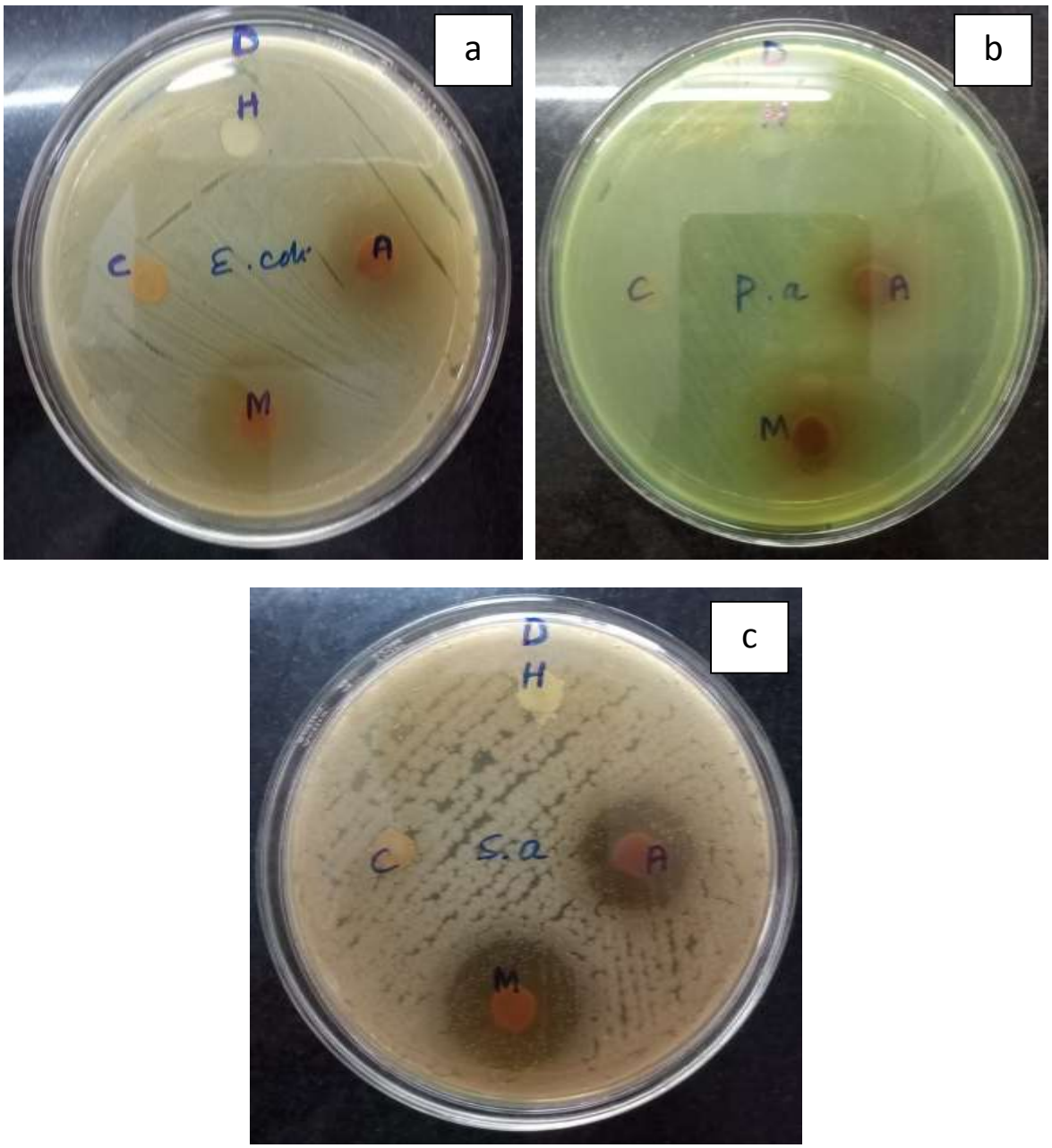
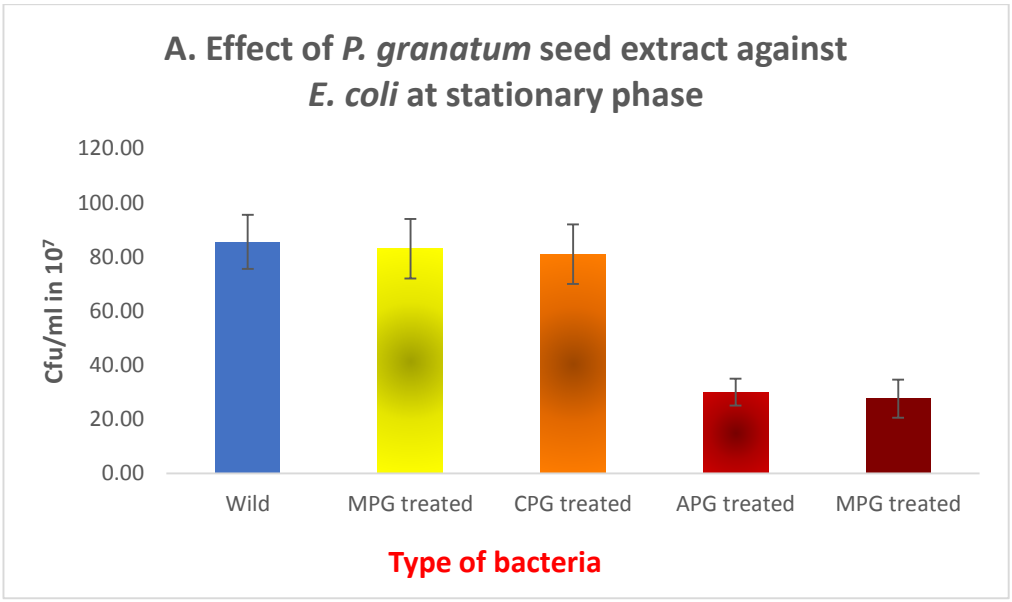
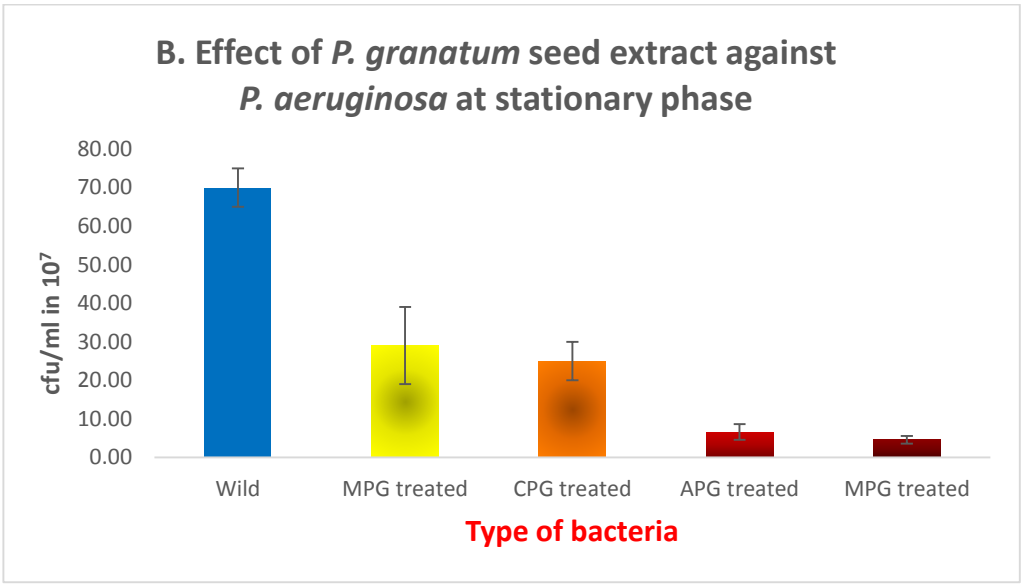


Plate 2: Growth inhibition study of HPG, CPG, APG and MPG by Disc diffusion method (a) *E. coli* (b) *P. aeruginosa* (c) *S. aureus*





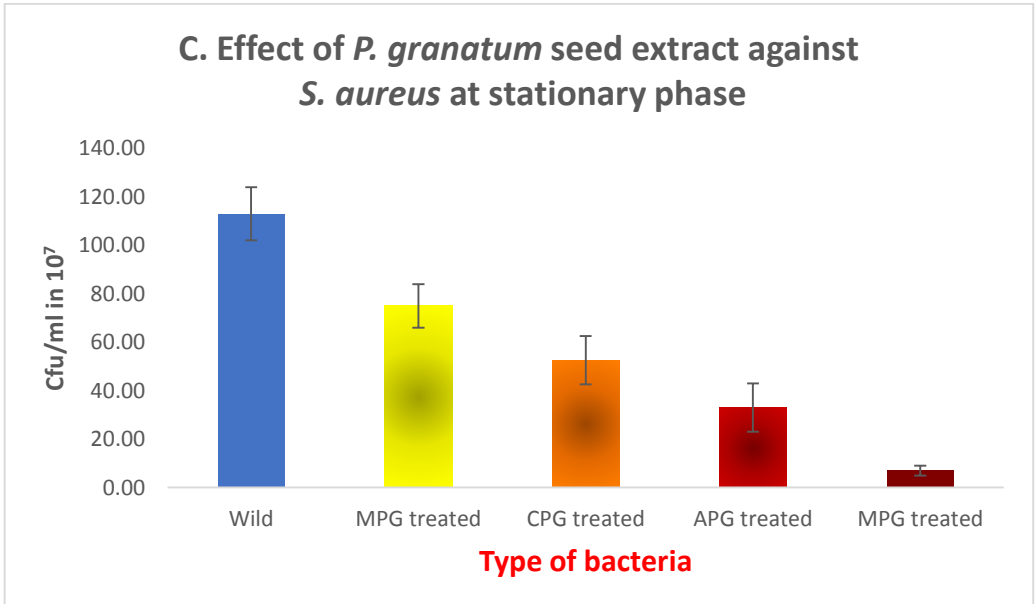


Figure 1: Cfu/ml determination at stationary phase (A) *E. coli* (B) *P. aeruginosa* (C) *S. aureus*

The antiuropathogenic effect of *P. granatum* L. seed extract was explored against UTI causing bacteria i.e. *E. coli, P aeruginosa* and *S. aureus* using different antibacterial techniques i.e. agar well diffusion, disc diffusion and modified agar well diffusion. The methanolic extract of *P. granatum* was reported to be most effective against different microorganisms in comparison to other extracts (petroleum ether, chloroform and water)[23]. Since different solvents vary in their polarity, solubility and accordingly dissolve different compounds, therefore we used four solvents for extraction and tried to compare their antibacterial potentiality. Many reports suggest that methanol has exceptional ability to dissolve phytochemicals which is also confirmed from this study.

Methanolic extract of pomegranate fruit had pelargonidine-3 galactose, cyanidine-3 glucose, gallic acid, quercetin and myricetin. All these compounds displayed significant bioactivity against spp. of *E. coli, Shigella, Salmonella, Vibrio cholerae, Streptococci, Staphylococci, Corynebacteria* and *Bacillus subtilis.* However, these compounds showed better activity against gram positive strains in comparison to gram negative strains[19].

APG and MPG had effective bacteriocidic/bacteriostatic effect against the MDR strains i.e. *E. coli* and *S. aureus* which could be observed from different antibacterial tests i.e. disc diffusion (plate 2), agar well diffusion (plate 3) and modified agar well diffusion results (plate 4). Plant extracts rich in different antimicrobial compounds can be used against MDR strains of pathogenic bacteria and may be beneficial in the treatment of dreaded infectious diseases caused by resilient microbes.

The present investigation showed potent urobactericidal activity of APG and MPG against all the test strains especially *E. coli* and *S.aureus* which were multidrug resistant strains. They were found to be more sensitive to APG and MPG in comparison to *P. aeruginosa* strain (figure 1) which was a comparatively sensitive strain (plate 1).

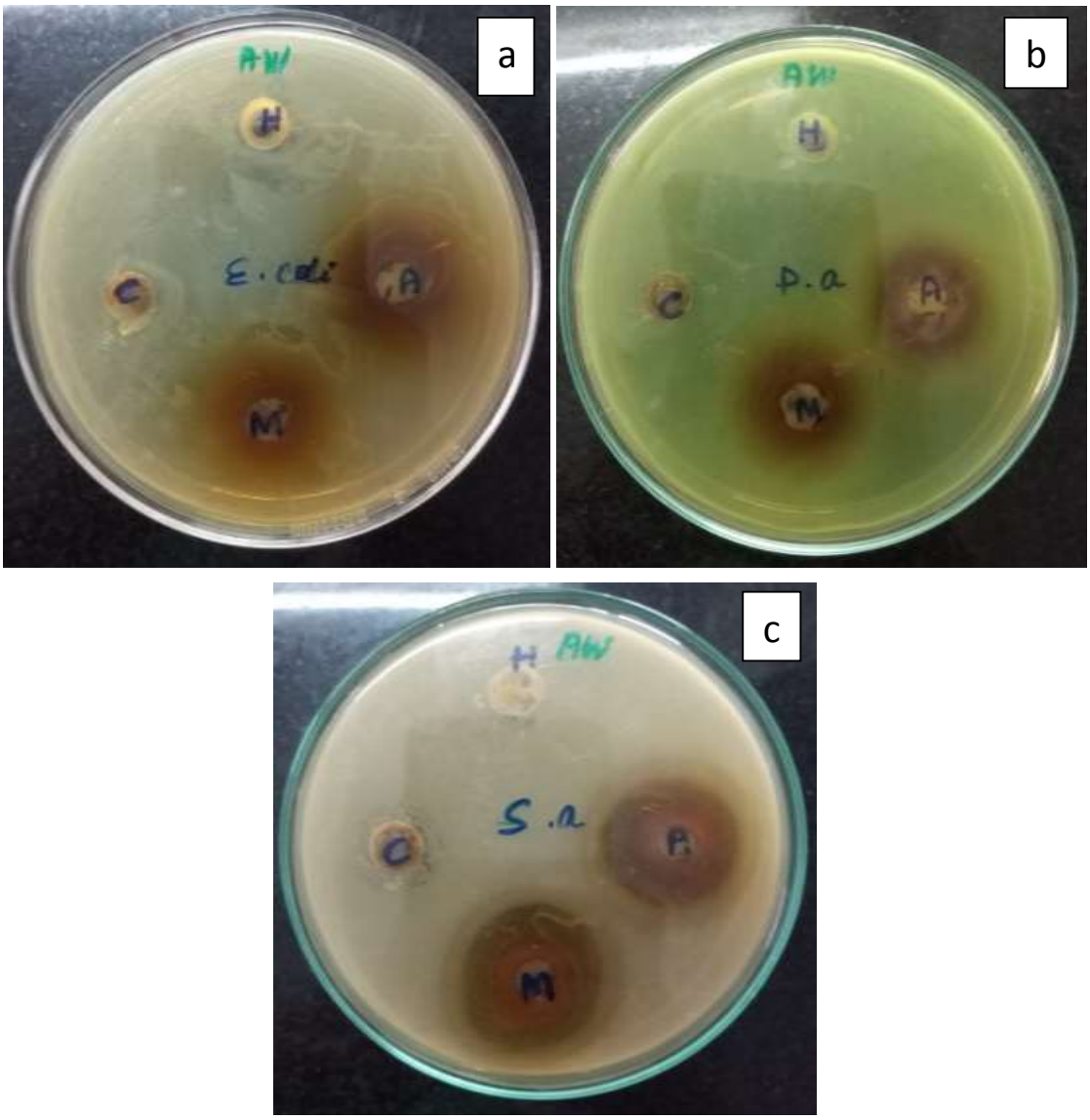


Plate 3: Growth inhibition study of HPG CPG APG and MPG by Agar well diffusion method (a) *E. coli* (b) *P. aeruginosa* (c) *S. aureus.*

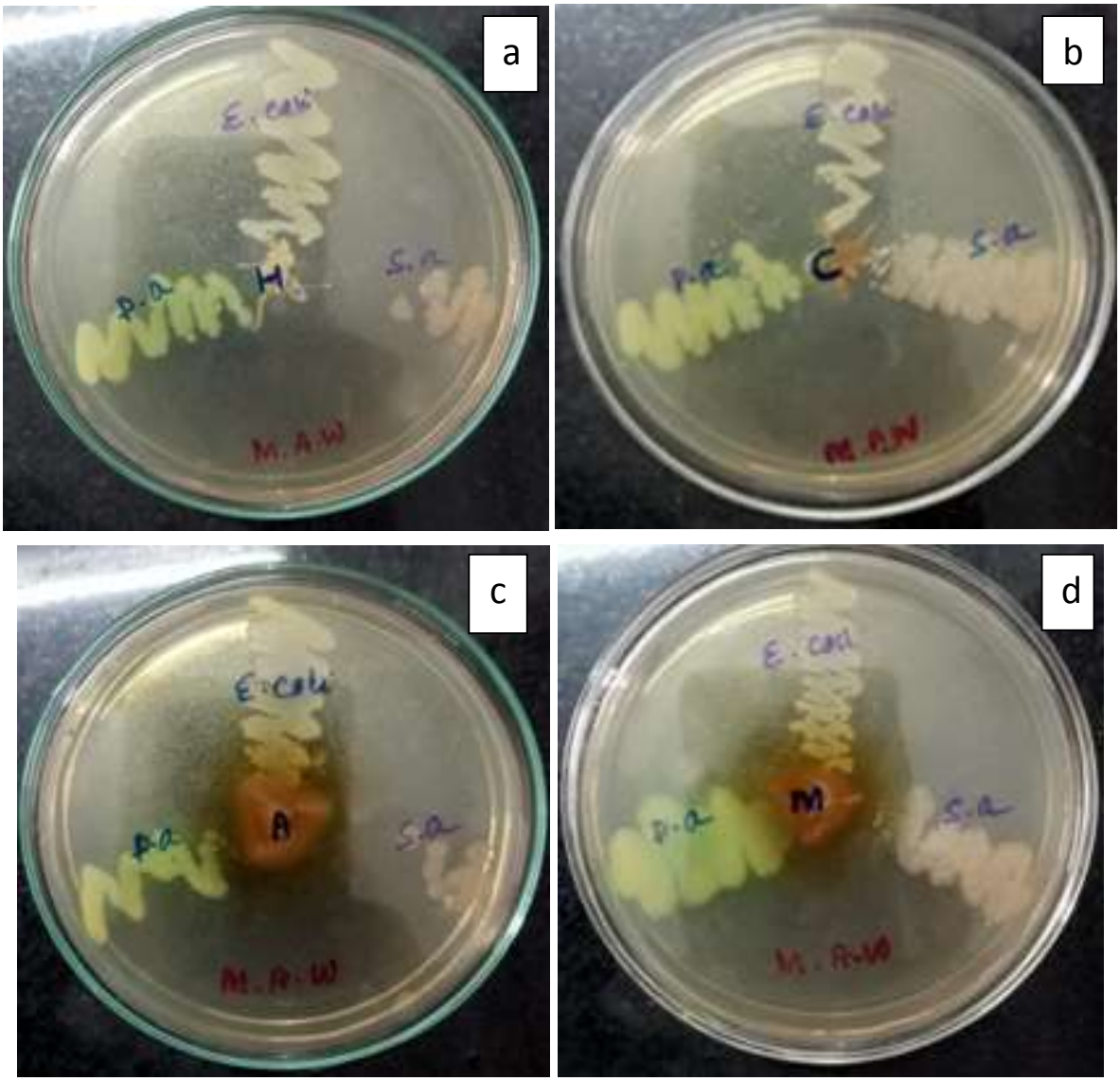


Plate 4: Bactericidal activity study by modified agar well diffusion method using different extracts (a) HPG (b) CPG (c) APG (d) MPG against *E. coli, P. aeruginosa* and *S. aureus*

The complementary and synergistic effect of antibiotic and plant extracts against resistant bacteria may initiate some novel treatment for infectious diseases. When antibiotics fail to work alone, it could be augmented with different antimicrobial plant extracts or their phytochemicals against resistant microorganisms[20].

The bacterial growth inhibitory studies can be conducted on both solid and liquid culture. In liquid culture, the bacteria are better exposed to the antibacterial agents and the sensitivity test is better implemented in broth culture than on agar plates. So, determination of cfu/ml in control and extract treated bacteria was carried out by serial dilution followed by spread plating.

Urobactericidal activity of methanolic extract of *P. granatum* seed was reported earlier against *E. coli* and *K. pneumoniae*[7]. In this present study it was observed that the TPC and TFC were found to be in the following descending order (MPG>APG>CPG>HPG) and ranged between 9.77±0.06 to 82.2±0.09mg/GA/g and 42.17±0.09 to 147.27±0.11mg/Rutin/g of the dry weight of extract. Both were significantly high in the hydrophilic extracts like APG and MPG and these fractions showed remarkable antiuropathogenic activity as evident from the different antibacterial tests conducted in this study.

Zulfiker et al[33] had reported the phenol content varying from 679.102 to 964.230mg/GA/g of the dry weight in the methanol extract of different plants including *H. indicus* root. Presence of different phytoconstituents such as steroid, tannin, alkaloid, coumarin, saponin, glycoside and terpenoid etc. was confirmed in different solvent fractions of *H. indicus* root, which might be acting synergistically to inhibit different urobacteria[9]. There were many reports that active phytochemicals were effective against MDR strains in comparison to sensitive strains[9]. In this present study, *S. aureus*, a MDR strain was found to be significantly inhibited by pomegranate seed extracts as marked from the results of different antibacterial studies.

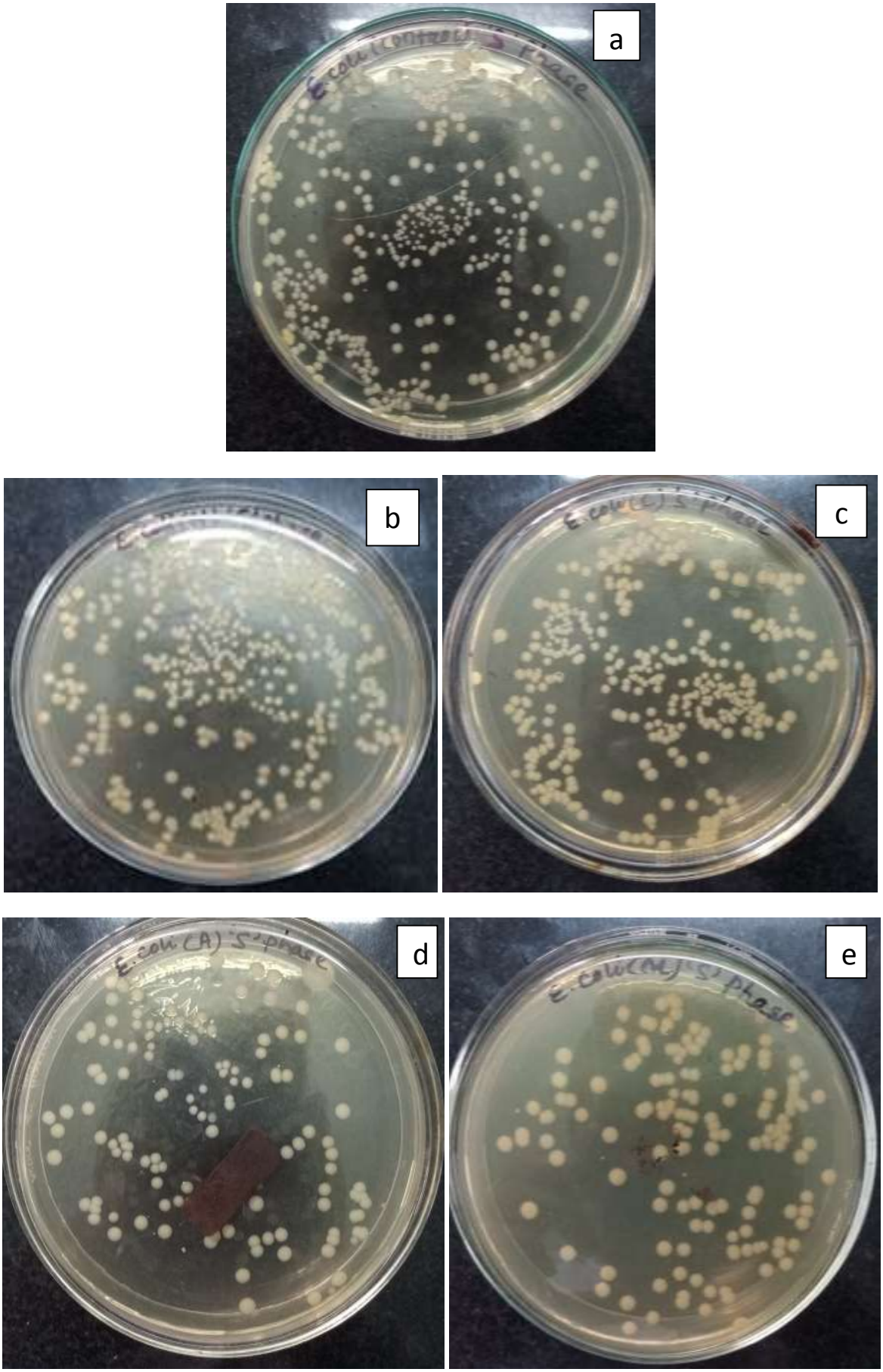


Plate 5: Cfu/ml determination at stationary phase
(a) Wild (b) HPG(c) CPG (d) APG (e) MPG treated *E. coli*

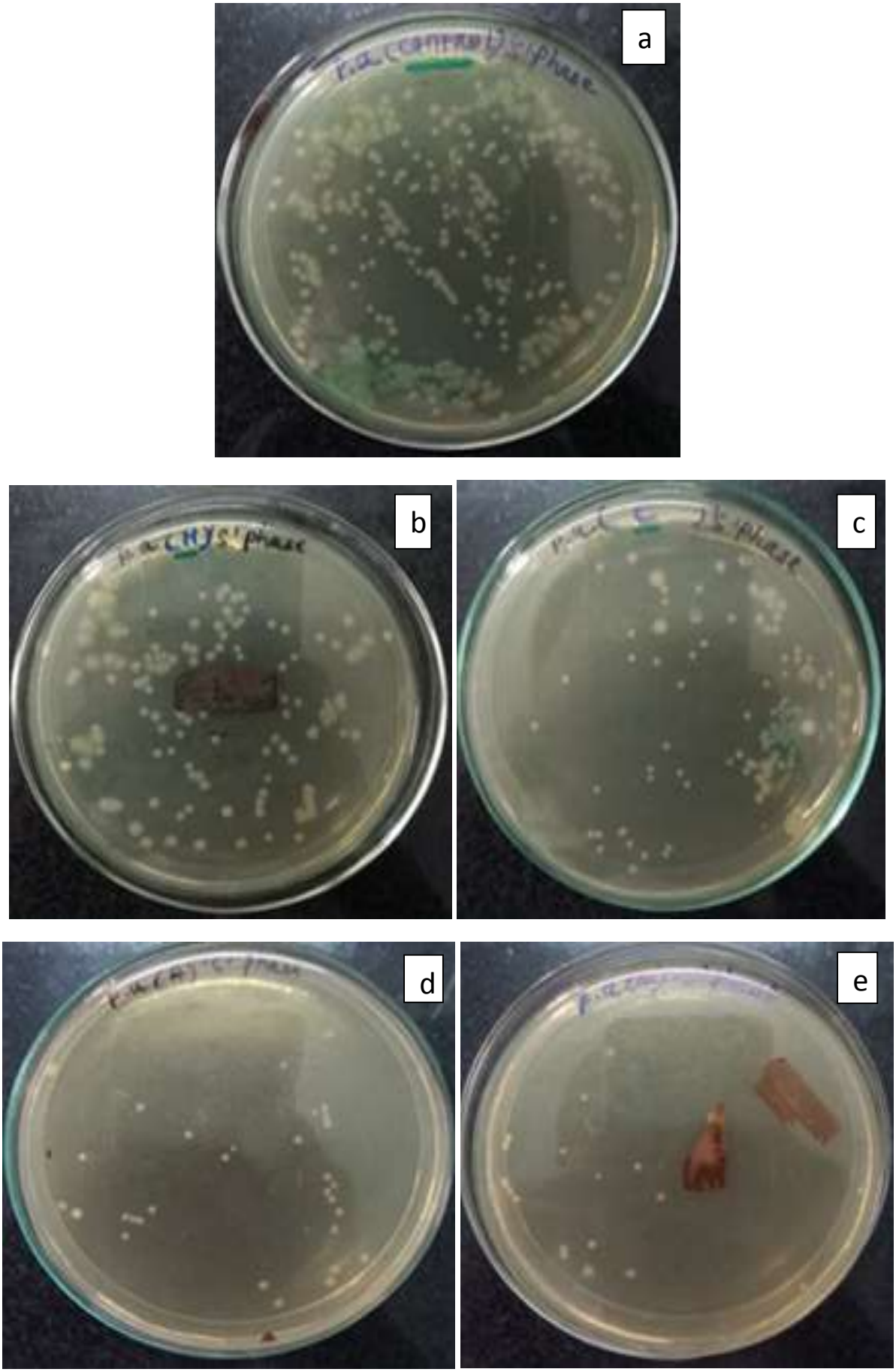


Plate 6: Cfu/ml determination at stationary phase
(a) Wild (b) HPG (c) CPG (d) APG (e) MPG treated P. *aeruginosa*

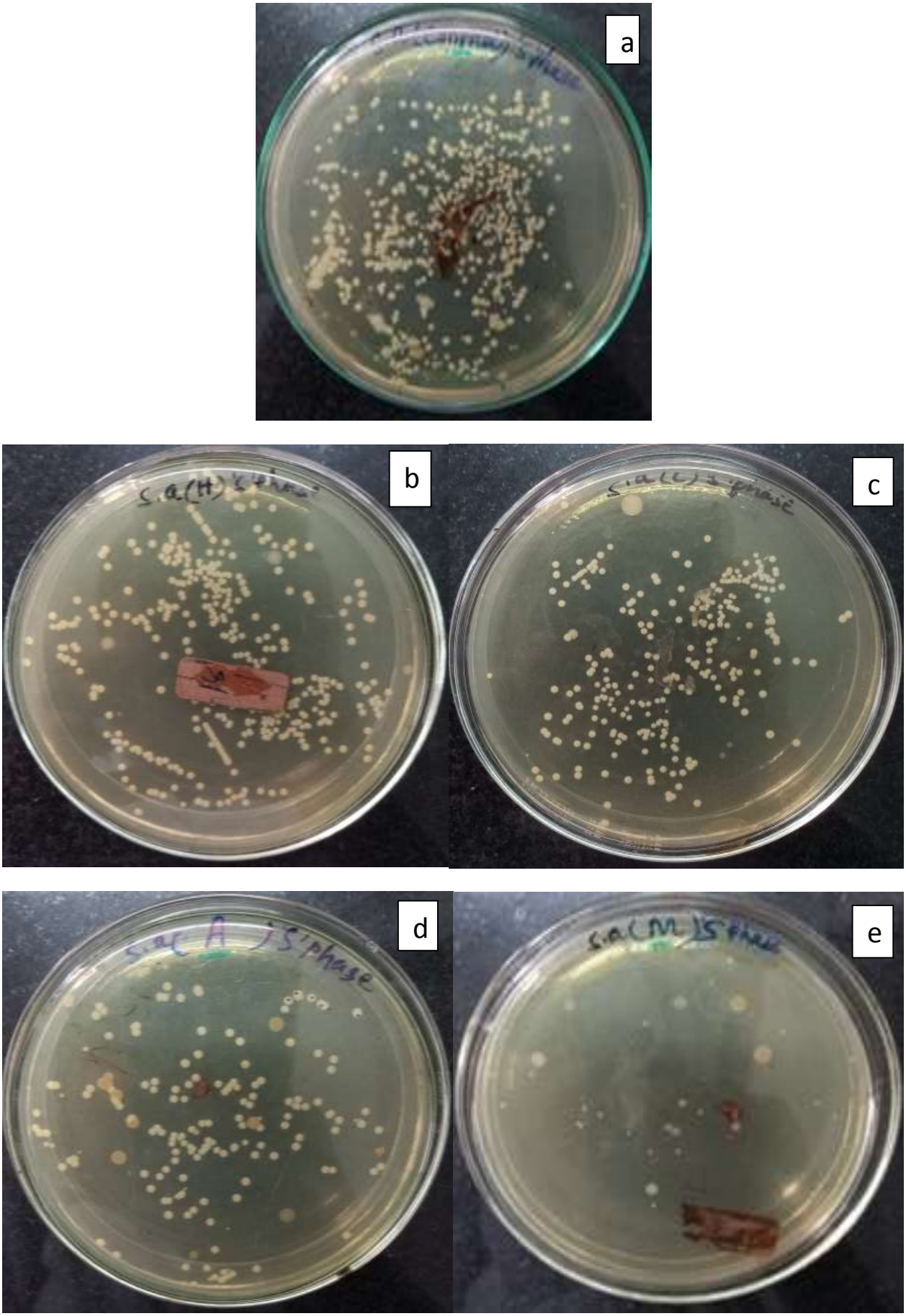


Plate 7: Cfu/ml determination at stationary phase
(a) Wild (b) HPG (c) CPG (d) APG (e) MPG treated *S. aureus*

Different bacteria were grown without and with the presence of different extracts for 18hr and their numbers were counted. This method is useful to calculate the number of live bacteria present in each culture. In consistent to our other antibacterial activity study results, the number of colonies were found to be less in all extract treated bacteria in comparison to wild/control bacteria and APG and MPG were found to have more effective urobactericidal activity against all the test strains in comparison to HPG and CPG which could be due to the presence of hydrophilic components in both APG and MPG.

HPG and CPG were rich in hydrophobic compounds, since they were dissolved in vegetable oil for their antibacterial study. In concordance to our previous report[9], in this present study we observed APG and MPG had better urobactericidal activity against the MDR strains possibly due to their higher TPC and TFC contents which are well known for their bactericidal activity.

Conclusion

P. granatum seed has exhibited potent urobactericidal activity. However, further study is needed to isolate and characterize the active constituents and explore their mode of action against various urotoxins and also their role in preventing or inhibiting uropathogenesis at a molecular level.

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