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Antibacterial Activity of Pomegranate (*Punica granatum*) Fruit Peel Extracts Against Antibiotic Resistant Gram- Negative Pathogenic Bacteria

Tarique Nazira Banu and Shyamapada Mandal*

Laboratory of Microbiology and Experimental Medicine, Department of Zoology, University of Gour Banga, Malda-732103, India

ABSTRACT

Fighting bacterial antibiotic resistance is a great challenge, and the researchers are in search of alternative therapies, the effective antibacterial biotherapeutics, in particular. This research aims to explore the antibacterial potentiality of pomegranate (*Punica granatum*) fruit peel extracts against gram-negative pathogenic bacteria having high MAR (multiple antibiotic resistance) indices. A total of 17 gram-negative pathogenic bacteria: *Escherichia coli* (n=5), *Proteus spp*. (n=4), *Klebsiella pneumoniae* (n=2), *Pseudomonas aeruginosa* (n=3), *Acinetobacter baumannii* (n=3), were subjected to susceptibility testing by disc diffusion method using 15 antibiotics, and the MAR indices were calculated. The antibacterial activities of APE (pomegranate fruit peel aqueous extract) and PEE (pomegranate fruit peel ethanolic extract), for the test bacteria, were determined by disc diffusion, while agar dilution technique was followed to determine the MIC (minimum inhibitory concentration) values of the extracts. The bacteria tested, displaying varied MAR resistance phenotypes, had resistance to 7-14 antibiotics, and the MAR indices for the bacterial isolates ranged 0.46–0.93. The PEE and APE both showed antibacterial activities, with respective ZDI (zone diameter of the inhibition) values of 14.7±5.32 mm and 17.53±5.72 mm (at 1 mg/disc), and 13.3±5.69 mm and 16.65±7.55 mm (at 2 mg/disc). The PEE and APE MICs ranged 2.5-3.3 mg/ml and 5–20 mg/ml, respectively, for the test bacteria. Thus, fruit peel of pomegranate might be useful in the preparation of antibacterial therapeutic agents, alternative to antibiotics, in order to combat the life-threatening infections of multiple antibiotic resistant gram-negative bacteria.

KEY WORDS: *POMEGRANATE FRUIT PEEL, ANTIBACTERIAL ACTIVITY, MINIMUM INHIBITORY CONCENTRATION, MAR INDICES, GRAM-NEGATIVE PATHOGENIC BACTERIA.*

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INTRODUCTION

The medicinal and food plants have been in use, for centuries, in treating infectious diseases, and have been considered as important source of antimicrobial agents, and for decades, their (plants) antimicrobial properties have been investigated in curing a variety of bacterial infections, and combating bacterial antibiotic resistances, as well (Alanis et al. 2005; Nozohour et al.2018, Matjuda and Aiyegoro (2019). The *Punica granatum* (pomegranate; family: Punicaceae; Bedana in Bengali) fruit peel is an important inedible part, possessing an enormous amount of flavonoids, tannins and other phenolic compounds (Khan et al. 2017; Janani et al. 2019) and thus displaying various kinds of bioactivities including antioxidative and antimicrobial properties.

(Devatkal et al. 2013; Voravuthikunchai et al. 2005; Reddy et al. 2007). Devatkal et al. (2013) reported the antibacterial activity of aqueous extract of pomegranate peel against poultry meat isolates of *Pseudomonas stutzeri*. Navidinia and Goudarzi (2017) demonstrated the MICs of aqueous and ethanolic extracts of *P. granatum* seeds that ranged 9.37- 150 mg/ml and 9.37- 75 mg/ml, respectively, for various gram-negative potential bacterial pathogens. The pomegranate edible and non-edible parts have been reported to be excellent antibacterial as well as antioxidative agents containing rich amount of polyphenolics (Rummun et al. 2013).The pomegranate fruit parts: peel, aril, seeds, and juice, have been reported to be rich in different bioactive components, as has been demonstrated by Jurenka et al. (2008).

The gram-negative bacteria, such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii*, are top listed WHO (World Health Organization) priority pathogens, and some of the members are included in the ESKAPE (*Enterococcus faecium,*

Table 1. Multiple antibiotic resistance (MAR) phenotypes and MAR indices for clinical bacterial isolates (n=17)

Bacteria	Resistance	MAR phenotypes	MAR index
A. baumannii AB1	7-drug	Vm-Am-Mc-Km-Tr-Cpd-Nx	0.46
E. coli EC3	8-drug	Cx-Vm-Am-Ip-Mc-Tr-Cpd-Nx	0.53
E. coli EC4	9-drug	Cx-Cf-Vm-Am-Ip-Mc-Tr-Cpd-Nx	0.6
P. vulgaris PV2	9-drug	Cf-Cp-Am-Ip-Mc-Km-Tr-Cpd-Nx	0.6
E. coli EC2	10-drug	Cx-Vm-Tc-Cp-Am-Ip-Mc-Tr-Cpd-Nx	0.66
A. baumannii AB2	11-drug	Cm-Cx-Cf-Vm-Am-Mc-Ak-Km-Tr-Cpd-Nx	0.73
K. pneumoniae KP2	11-drug	Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Ak-Km-Cpd	0.73
A. baumannii AB3	12-drug	Gm-Cm-Cx-Cf-Tc-Am-Ip-Mc-Ak-Km-Tr-Cpd	0.8
K. pneumoniae KP1	12-drug	Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Ak-Tr-Cpd-Nx	0.8
P. aeruginosa PA3	12-drug	Cm-Cx-Cf-Vm-Tc-Cp-Am-Mc-Km-Tr-Cpd-Nx	0.8
P. mirabilis PM1	12-drug	Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Km-Tr-Cpd-Nx	0.8
E. coli EC5	13-drug	Cm-Cx-Cf-Vm-Tc-Cp-Am-Ip-Mc-Km-Tr-Cpd-Nx	0.86
P. mirabilis PM2	13-drug	Gm-Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Km-Tr-Cpd-Nx	0.86
P. vulgaris PV1	13-drug	Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Ak-Km-Tr-Cpd-Nx	0.86
E. coli EC1	14-drug	Gm-Cx-Cf-Vm-Tc-Cp-Am-Ip-Mc-Ak-Km-Tr-Cpd-Nx	0.93
P. aeruginosa PA1	14-drug	Gm-Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Ak-Km-Tr-Cpd-Nx	0.93
P. aeruginosa PA2	14-drug	Gm-Cm-Cx-Cf-Vm-Tc-Cp-Am-Mc-Ak-Km-Tr-Cpd-Nx	0.93

Ak: amikacin; Am: ampicillin; Cf: cefotaxime; Cx: cefoxitin; Cpd: ceftazidime; Cp: ciprofloxacin; Cm: chloramphenicol; Gm: gentamycin; Ip: Imipenem; Km: kanamycin; Mc: methicillin; Nx: nalidixic acid; Tc: tetracycline; Tr: trimethoprim; Vm: vancomycin

Table 2. The ZDI (zone diameter of inhibition) values of pomegranate fruit peel extracts for clinical bacterial isolates (n=17)

Bacteria	ZDI (mm)			
	PEE (1 mg/disc)	APE (1 mg/disc)	PEE (2 mg/disc)	APE (2 mg/disc)
E. coli EC1	10	14	13	22
E. coli EC2	13	15	14	23
E. coli EC3	10	8	12	11
E. coli EC4	13	18	15	26
E. coli EC5	22	20	25	22
A. baumannii AB1	22	20	28	25
A. baumannii AB2	14	6	18	6
A. baumannii AB3	10	6	12	6
P. mirabilis PM1	19	16	22	20
P. mirabilis PM2	15	15	20	18
P. vulgaris PV1	22	24	26	27
P. vulgaris PV2	25	18	26	24
K. pneumoniae KP1	10	6	10	6
K. pneumoniae KP2	8	8	13	11
P. aeruginosa PA1	10	6	12	6
P. aeruginosa PA2	12	10	14	12
P. aeruginosa PA3	15	16	18	18
Mean	14.7	17.53	13.3	16.65
SD	5.32	5.72	5.69	7.55
p value	0.47		0.71	

The abbreviation of the plant extracts are mentioned in the text

Staphylococcus aureus, K. pneumoniae, A. baumannii, P. aeruginosa, and Enterobacter spp.) group (Rice, 2008; Smith et al. 2018; Perovic et al. 2018). For such bacterial pathogens, the gram-negative bacteria, in particular, having the capacity to cause severe nosocomial infections (and non-responsive to currently available antibiotics), newly developed effective therapies are required (Tacconelli et al. 2018). Both edible and non-edible parts of pomegranate plant have been reported to treat different pathological conditions in different traditional medicine (Derakhshan et al. 2018). Therefore, the current study was undertaken to authenticate the antibacterial capacity *P. granatum* fruit peel (available in the local niches: West Bengal, India) against *E. coli, A. baumannii, P. aeruginosa, K. pneumoniae*, and *Proteus spp.* (*P. mirabilis* and *P. vulgaris*) showing resistance to multiple antibiotics.

MATERIAL AND METHODS

Bacterial Strain and Media: A total of 17 clinical bacterial isolates: *Escherichia coli* (n=5), *Proteus spp.* (n=4), *K. pneumoniae* (n=2), *P. aeruginosa* (n=3), *A. baumannii* (n=3), which were maintained in the laboratory in cystine tryptone agar stabs, were utilized in the current study. The media (Hi-Media, India) used in the study were nutrient broth (for bacterial subculture and inoculums preparation) and nutrient agar (for antibiotic susceptibility and antibacterial activity testing).

Antibiotic Susceptibility: The antibiotic susceptibility testing, for the bacterial isolates, was done following disc diffusion (Bauer et al., 1966), using 15 antibiotics (Hi-Media, India): ampicillin (Am; 10-µg), amikacin (Ak; 30-µg), cefoxitin (Cx; 30-µg), cefotaxime (Cf; 30-µg), cefpodoxime (Cpd; 10-µg), chloramphenicol (Cm;

30-µg), ciprofloxacin (Cp; 10-µg), gentamycin (Gm; 30-µg), imipenem (Ip; 10-µg), kanamycin (Km; 30-µg), methicillin (Mc; 5-µg), nalidixic acid (Nx; 30-µg), tetracycline (Tc; 30-µg), trimethoprim (Tr; 5-µg), and vancomycin (Vm; 30-µg). The ZDI (zone diameter of inhibition) values from the antibiotic action against the test bacteria were recorded, and interpreted according to the CLSI protocol (CLSI, 2015). The MAR indices for the bacteria tested were calculated following the formula as stated by Nandi and Mandal (2016), and the results were interpreted according to the criteria published earlier (Krumperman, 1983). The MAR phenotypic profiles were determined for the bacterial isolates displaying resistance to three or more antibiotics (Adefisoye and Okoh, 2017).

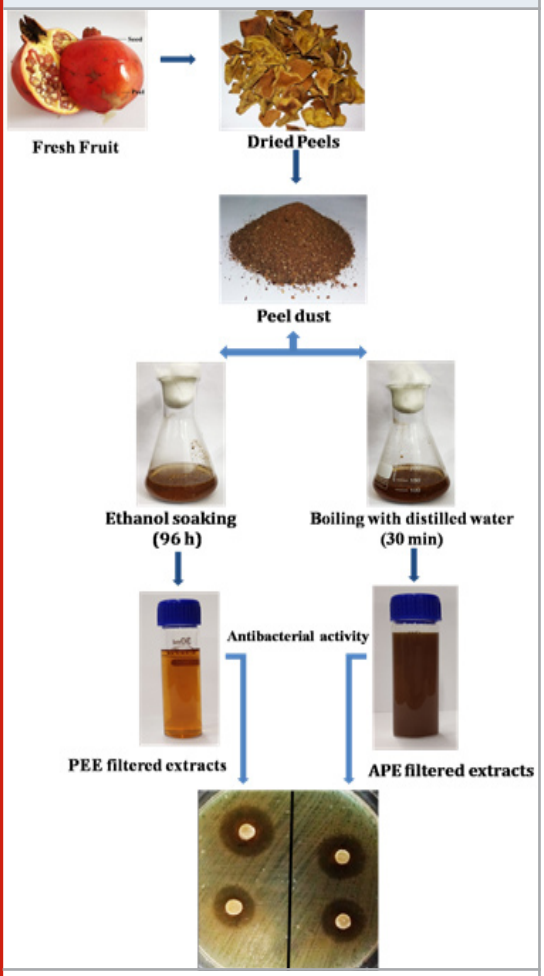
Plant Extract Preparation: The indigenous variety fruits of pomegranate, *Punica granatum* (family: Punicaceae) were collected from Rajapur village of Malda district (West Bengal, India), washed properly with distilled water, and the peels were separated and sliced for shade drying. The dried plant materials were granulated by electrical grinding machine and stored in airtight containers at room temperature for extract preparation. The pomegranate fruit peel ethanolic extract (PEE) and pomegranate fruit peel aqueous extract (APE), were prepared in line with a little modification of the protocol depicted by Sircar and Mandal (2016). Briefly, for PEE preparation, 5 g of dried pomegranate fruit peel granules was extracted

Table 3. The MIC (minimum inhibitory concentration) values of pomegranate fruit peel extracts for clinical bacterial isolates (n=17)

Bacteria	MIC (mg/ml)	
	PEE	APE
E. coli EC1	2.5	5
E. coli EC2	3.3	5
E. coli EC3	3.3	5
E. coli EC4	3.3	5
E. coli EC5	3.3	20
A. baumannii AB1	2.5	6.6
A. baumannii AB2	2.5	6.66
A. baumannii AB3	2.5	16.66
P. mirabilis PM1	2.5	6.6
P. mirabilis PM2	2.5	6.6
P. vulgaris PV1	2.5	6.6
P. vulgaris PV2	2.5	6.6
K. pneumoniae KP1	3.3	20
K. pneumoniae KP2	3.3	20
P. aeruginosa PA1	2.5	11.6
P. aeruginosa PA2	2.5	6.6
P. aeruginosa PA3	3.3	13.33
Mean	2.83	9.65
SD	0.41	±5.8
p value	0.00012	

The abbreviation of the plant extracts are mentioned in the text":

Figure 1. Flow diagram for antibacterial activity analysis of pomegranate fruit peel ethanolic and aqueous extracts



The abbreviation of the plant extracts are mentioned in the text":

by soaking with 100 ml of ethanol shaking at regular interval, for 96 h at room temperature, and sieved through cheese-cloth and Whatman No. 1 filter paper. For the preparation of APE, 5 gm granulated sample was dissolved in 100 ml of double distilled water, and boiled for 30 min in water bath, and filtered as mentioned above, after cooling. The concentration of each of the extracts (APE and PEE) in stock solution was 50-µg/µl. The extracts prepared were stored at 4°C until further used.

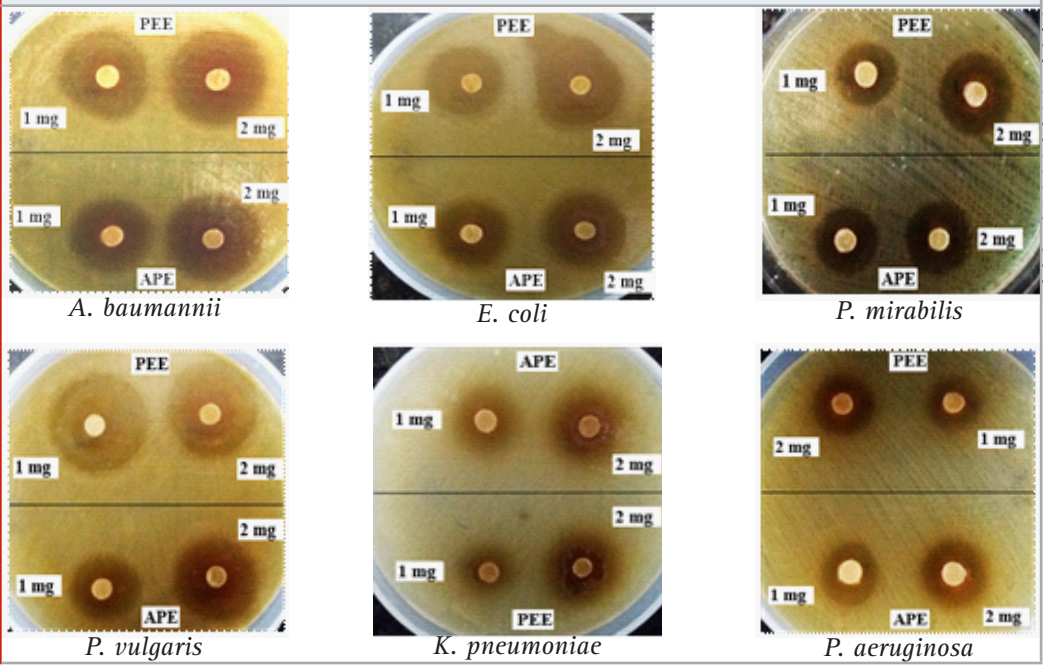
Antibacterial Property: The antibacterial activity of PEE and APE extracts were evaluated employing disc diffusion technique (in order to get the zone diameter of the inhibition; ZDI), as explained earlier by Das and Mandal (2016). Agar dilution method, the details of which was mentioned in previous publication (Mandal et al. 2007), was followed for the determination of MIC (minimum inhibitory concentration) values, using nutrient agar medium mixed with varied concentration of the extracts, ranging from 2.5 to 3.3 mg/ml and 5 to 20 mg/ml. The all incubations were done at 37°C for 24 h, and the testing was at once completed in triplicate. The antibacterial activity was recorded based on the ZDIs obtained around the plant extract impregnated discs on the agar plates inoculated with test bacteria, and the ZDI values ≥7 mm accounted sensitivity of the test extracts to the bacterial isolates (Nascimento et al. 2000). The lowest extract concentration that inhibited the visible growth of the test bacteria were defined as MICs (Mandal et al. 2007).

Statistical Analysis: To compare the antibacterial activity (in terms of ZDIs) by disc diffusion technique, and MICs of plant extracts: APE and PRE, against the gram-negative pathogenic bacteria tested, the data were expressed as the mean ± SD (standard deviation), and were evaluated by 't'-test , using MS Excel 2010 software; the statistical significance was projected by 'p' value of ≤ 0.05.

RESULTS AND DISCUSSION

The current research explores the antibacterial activity of pomegranate fruit peel ethanolic and aqueous extracts against antibiotic resistant

Figure 2. Disc diffusion technique representing the sensitivity pattern of pomegranate peel extracts. PEE: pomegranate fruit peel ethanolic extract; APE: pomegranate fruit peel aqueous extract.



gram-negative pathogenic bacteria (Figure 1). The multiple antibiotic resistance phenotypes for the test bacterial pathogens are represented in Table 1; the isolates showed 7-drug to 14-drug resistances, displaying the respective resistance patterns: 'Vm-Am-Mc-Km-Tr-Cz-Nx', for *A. baumannii* AB1 strain, and 'Gm-Cm-Cx-Cf-Vm-Tc-Cp-Am-Mc-Ak-Km-Tr-Cz-Nx' for *E. coli* EC1, *P. aeruginosa* PA1 and *P. aeruginosa* PA2 strains. As per the report of Matjuda and Aiyegoro (2019), among a total of 74 resultant MAR phenotypes (ranging from 3-drug to 12-drug resistances), the predominant patterns noted included "penicillin-sulphamethaxazole-Vm-Am-amoxicillin-apramycin-neomycin-tilmicosin-oxytetracycline-spectinomycin-linomycin-Tr" and "penicillin-sulphamethaxazole-Vm-amoxicillin-neomycin-tilmicosin-oxytetracyclin-spectinomycin-linomycin", for 15 and 6 test bacterial isolates, respectively. The MAR index for the test clinical bacteria ranged from 0.46 to 0.93 (Table 1). As has been reported earlier by Matjuda and Aiyegoro (2019), the MAR indices of pathogenic bacteria tested ranged from 0.2 to 1. Das et al., (2018).

Reported different MAR resistance phenotypes, among gram-negative clinical bacteria, which ranged up to 10-drug resistance, displaying the pattern: 'Am-Ce-Cp-Ct-Cx-Mp-Nx-Pc-PT-Tc' by E. coli CSD2 strain and in that study the MAR indices for the test bacteria ranged 0.15 – 0.77. The earlier authors (Tambekar et al. 2005; Kaneene et al. 2007) explained that the bacteria demonstrating MAR indices >0.4 might be originated from niches with human-faecal contamination, while the bacteria displaying MAR indices >0.2, have been regarded to be derived from niches with high antibiotic pollution (Krumperman, 1983; Matjuda and Aiyegoro, 2019). The high MAR indices (0.46 – 0.93) among the test gram-negative clinical bacteria demonstrated, in the current study, their origin from human-faecal contaminated niches with high antibiotic pollution.The antibacterial activity of pomegranate peel extracts against gram-negative pathogenic bacteria, following disc diffusion method, is shown in Figure 2. The ZDIs from the action of APE and PEE, against the test bacteria, are represented in Table 2. The PEE and APE had ZDIs 10–22 mm and 8–20 mm, respectively (at 1.0 mg/well), and 12–25 mm and 11–26 mm, respectively (at 2.0 mg/well), against *E. coli* isolates. The PEE was active against all the test *A. baumannii* isolates (ZDIs 10 – 28 mm), while the APE showed activity against *A. baumannii* AB1 isolate only. The pomegranate fruit peel showed anti-Proteus spp. activity with ZDIs 15 – 26 mm, for PEE, and 15 – 27 mm, for APE. The PEE had growth inhibitory activity against all the *K. pneumoniae* isolated tested (n=3; ZDIs: 8 – 14 mm). The pomegranate peel extract had ZDIs of 10 – 18 mm against *P. aeruginosa*; however, for *P. aeruginosa* PA1 the APE had no activity (ZDI: 6 mm). As per the report of Algurairy (2018) the pomegranate fruit peel ethanolic extract (10 – 100 %) had ZDIs of 22–36 mm, for Staphylococcus aureus clinical isolates. The respective ZDIs of pomegranate fruit peel methanolic and aqueous extracts (50 mg/ml) for *Enterobacter cloacae* were 14 mm and 10 mm, and for *Salmonella enterica* serovar Typhi, 20 mm and 10 5mm, while for the gram-positive (*S. aureus* and *Bacillus subtilis*) bacteria, the ZDIs ranged 22–26 mm and 24–28 mm, respectively (Kanoun et al. 2014).

The methanolic extract of pomegranate fruit peel showed antibacterial activity against food-borne bacteria, such as, *Listeria monocytogenes, S. aureus, E. coli* and *Yersinia enterocolitica* (Al-Zoreky, 2009). As has been reported by Kunte et al. (2018), the pomegranate fruit peel aqueous extract had antibacterial activity against potential cariogenic *Streptococcus mutans* isolates, displaying ZDIs of 15 – 17 mm. The pomegranate peel fresh aqueous extract showed growth inhibitory activity against *Pseudomonas stutzeri* isolates from poultry meat displaying ZDIs of 21 – 26 mm (Devatkal et al. 2013). The pomegranate peel extract showed antibacterial activity against S. mutans and *Streptococcus mitis* having ZDIs of 20 mm and 25 mm, respectively, while the leaf extract had ZDIs of 16 mm and 18 mm, respectively, for the bacterial isolates (Rummun et al. 2013). The *P. granatum* seed ethanolic extract showed antibacterial activity against gram-positive bacteria: *S. aureus*, with ZDIs 22–42 mm as well as gram-negative bacteria: *E. coli*, having ZDIs 27–42 mm, while the respective ZDIs of petroleum ether extract for the isolates ranged

16–34 mm and 15–27 mm (Bora et al. 2018). The two extracts, APE and PEE, by disc diffusion (Table 2), displayed growth inhibition activities against the test bacteria, wherein there was no significant difference between the antibacterial properties of APE and PEE (p values: 0.47 – 0.71). The MICs of PEE ranged 2.5-3.3 mg/ml for all the gram-negative pathogenic bacteria tested, while the APE MICs ranged from 5 mg/ml (for *E. coli* EC1, *E. coli* EC2, *E. coli* EC3 and *E. coli* EC4) to 20 mg/ml (for *E. coli* EC5), and the APE MICs for the remaining bacterial pathogens ranged 6.6 – 16.66 mg/ml (Table 3). The earlier authors also have demonstrated antibacterial activity of pomegranate extracts against clinically relevant bacteria, from different parts of the globe. The pomegranate peel methanolic and aqueous extract had MICs 0.39 and 0.195 for S. aureus, 1.56 and 0.78 mg/ml, for *B. subtilis*, 3.125 and 1.56 mg/ml, for *E. cloacae*, and 12.5 and 6.25 for S. enterica serovar Typhi (Kanoun et al. 2014). The respective MICs of aqueous and ethanolic pomegranate seed extracts for the bacteria tested were: *E. coli* (75 and 37.5 mg/ml), *Shigella sonnei* (37.5 and 18.75 mg/ml), *Shigella flexneri* (18.75 and 9.37 mg/ml), *Shigella dysentery* (18.75 and 9.37 mg/ml), *P. vulgaris* (18.75 and 9.37 mg/ml), *P. mirabilis* (9.37 and 9.37 mg/ml) and *Citrobacter fraundii* (150 and 75 mg/ml), as demonstrated by Navidinia and Goudarzi (2017).

Prashanth et al. (2001) reported antibacterial activity of pomegranate fruit extract against grem-negative bacteria: *E. coli*, *K. pneumoniae*, *P. vulgaris* and *S. enterica* serovar Typhi, in terms of MICs, which were 12 mg/ml, 12-25 mg/ml, 1.5-12 mg/ml, 12 – 50 mg/ml, for petroleum ether extract, chloroform extract, methanol extract and aqueous extract. The pomegranate fruit methanol and aqueous extracts had MICs, against gram-negative bacterial strains: *E. coli* ATCC 25922, *S. dysantriae* PTCC 1188 and *S. enterica* serovar Typhi ATCC 19430, of 6.25-12.5 and 3.12-12.5 mg/ml, respectively, as has been reported by Mahboubi et al. (2015). On the basis of the MIC determination (Table 3), the pomegranate fruit peel extracts, APE and PEE, were well active against the gram-negative multiple antibiotic resistant pathogenic bacteria; the APE, however, displayed greater activity compared to the PEE (p value: 0.00012).

CONCLUSION

The fruit peel ethanolic as well as aqueous extracts of pomegranate displayed antibacterial activity against gram-negative bacteria having high multiple antibiotic resistance indices, suggesting the usefulness of the of the plant parts in the preparation of antibacterial bio-therapeutics that might be utilized in the treatment of diseases caused due to the infection of multiple antibiotic resistant gram-negative bacteria. Further, phytochemical analysis and pharmacokinetic studies are required to explore the bioactive components responsible for antibacterial activity, and to determine the effective dosage

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