

Antibacterial activity of *Punica granatum* peel extract

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Abstract

The current research aimed to explore the secondary metabolites and antibacterial activity of extracts prepared from peel part of *Punica granatum* L., family Punicaceae. The extract was prepared in different solvents including water, methanol, ethanol, petroleum ether and ethyl acetate. The antibacterial activity was examined against one gram positive (*Staphylococcus aureus*) and two gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) ATCC bacterial strains on the basis of zone of inhibition using agar well diffusion assay. Results revealed that maximum number of the phytochemical molecules were existing in methanolic, ethanolic and ethyl acetate, while minimum number of molecules were found in aqueous solvent. *P. granatum* exhibits significantly high activity against both gram negative and gram positive bacteria..

Keywords: Antibacterial activity, peel extract, phytochemistry, *Punica granatum*.

Introduction

To treat health diseases, plants have been used for thousands of years. In ancient time several herbs and spices were used in food, not only as a flavoring agent and food preservative but also as a folk medicine (Shan *et al.*, 2007). Recently, various natural substances have been determined as a source of effective antibacterial agents against a variety of microorganism. Plants are plentiful in a wide range of secondary metabolite like, alkaloids, flavonoids, tannins and terpenoids. These substances have been reported to have antimicrobials activities. Antibacterial activity of various plants had been received attention as one of the efficient means for controlling microorganism (Al-Saimary *et al.*, 2002). *Punica granatum* L. known as 'Annar' in Urdu and 'Pomegranate' in English is the famous edible fruit. In traditional medicine it has been used for the treatment of various diseases in America, Europe, Africa and Asia. In addition to past uses, *P. granatum* is used in several medicines for a variety of ailments (Olapour and Najafzadeh, 2010). Different parts of *P. granatum* contain a variety of chemicals. Tannin and alkaloid are present in both bark and roots. Antimicrobial activity of tannins, flavonoids and

polyphenols is well studied (Ahmad and Beg, 2001; Naz *et al.*, 2007; Shan *et al.*, 2007). Tannin-containing beverages consumption such as tea could be helpful in curing or prevention of several illnesses (Cowan, 1999). Different parts of *P. granatum* such as roots, peels and fruits have been used generally in herbal therapies by local therapists in many states. Peels of *P. granatum* have been used traditionally for treatment of dysentery and diarrhea (Ahmad and Beg, 2001; Braga *et al.*, 2005; Reddy *et al.*, 2007). The crude extracts of *P. granatum* peels were successfully used against *Agrobacterium tumefaciens*, causative agent of plant tumor (Sajjad *et al.*, 2015). One of the known pharmacological property of tannins is astringency (Cowan, 1999). The seed consist of steroids while, fruit pulp contains vitamins, minerals and macromolecules like fats, proteins and carbohydrates (Lama *et al.*, 2001).

The increase in antibiotic-resistant property of pathogens has led to the development of new tonic mediators that are active against these microbes. Now a days, there has been extensive attention in the practice of several plant ingredients as a substitute medicine to treat most of the enteric infections and various compounds

of plant products have been precisely used against resistant pathogens (Choi *et al.*, 2009). Therefore the use of indigenous medicinal plants as an alternative to antibiotics are being extensively evaluated these days and are considered to play a significant role (Ballal and Ramamurthy, 2005). Pomegranate is supposed “a pharmacy unto itself” in Ayurvedic medicine and is used as a blood tonic, antiparasitic mediator, and to heal aphthae and ulcers (Jurenka, 2008). However, to date, very few studies have been conducted on the antimicrobial activity of *P. granatum* peels. Since the prehistoric times, the *P. granatum* has been observed as medicinal food with several useful effects in numerous diseases (Vidal *et al.*, 2003). Therefore, the present study was aimed to assess the antibacterial activity of the ethanolic, methanolic, aqueous, ethyl acetate and petroleum ether extracts of *P. granatum* peel against different enteric pathogens *in vitro*.

Materials and Methods

The entire chemicals and reagents used in present work were of analytical grade and acquired from Sigma-Aldrich Chemical Co.

Sample collection and preparation

Peels of *P. granatum* were collected in fresh condition from different juice shops of Rawalpindi and Islamabad. Collected peels sample were processed in Microbiology Research Laboratory at Quaid-i-Azam University Islamabad. Collected peel samples were washed thoroughly with distilled water and white fruit sacs were cleaned off. The cleaned peels were air dried for two weeks with constant monitoring to avoid fungal contamination. Dried peels were ground to fine powder and stored in sterilized air tight container at room temperature for further analysis.

Preparation of crude extracts

Ethanol, methanol and deionized double distilled water were used as a solvent for the preparation of peels extract.

Ethanolic extract

Ethanol (99%) was used to prepare crude ethanol extract (CEE). Powdered sample material of 1.5 g was mixed in 50 mL of ethanol and incubated in shaking incubator at 25 °C with continuous shaking at 150 rpm for 7 days. By using Whatman filter paper, it was filtered and filtrate was kept at 40 °C to evaporate all the solvent so that we were left with CEE.

Methanolic extract

Powdered sample of 1.5 g were dissolved in 50 mL of methanol (80%) for crude methanolic extract (CME) preparation. The solution was incubated for a week under similar conditions as mentioned for CEE. Then the mixture was filtered and the filtrate was kept at 40 °C to evaporate all the solvent and leaving behind the CME.

Aqueous extract

For preparation of crude aqueous extract (CAE), simple double distilled water was used and same procedure was followed as for CME and CEE.

Preparation of soxhlet extract

Ethyl acetate and petroleum ether was used for the preparation of soxhlet extract.

Ethyl acetate extract

The sample was extracted with 200 mL of ethyl acetate using Soxhlet extractor for 5 hours at temperature not exceeding the boiling point of the solvent (77 °C). The ethyl acetate extract (EAE) collected in the collection flask was stored in sterilized tubes.

Petroleum ether extract

Petroleum ether of 200 mL amount was used to prepare petroleum ether extract (PEE) by following same procedure of EAE preparation.

Percentage yield

Percentage yield of all extracts was determined by following formula

$$\text{Yield (\%)} = \frac{\text{Extract weight}}{\text{Dried sample weight}} \times 100$$

Test microorganisms

The test bacterial strains used for screening were *E. coli* (ATCC 10536), *P. aeruginosa* (ATCC 15442) and *S. aureus* (ATCC 6538).

Antimicrobial assays

Extracts (CME, CEE, CAE, EEA and PEE) of 25 µg and 50 µg were dissolved in 1 mL of dimethyl sulfoxide (DMSO). 1 mg mL⁻¹ rifampicin solution in DMSO was used as a positive control and pure DMSO as a negative control. Agar well diffusion method was used to evaluate the antibacterial activity of peel extracts against test microorganism (NCCLS, 1999). Nutrient agar medium (pH 7.0) was prepared and autoclaved. It was allowed to cool up to 45 °C. Then it was seeded aseptically with 500 µL of freshly prepared inoculums (10⁶ colony forming

unit, cfu) and immediately mixed. For inocula preparation, the colonies of bacteria were suspended in nutrient broth and turbidimetrically adjusted to a McFarland 1. Thirty five milliliters of seeded nutrient agar media was transferred into each Petri plate and solidify. Four wells were made with sterile cork borer (8 mm) per plate. Test solution of 50 μL was poured into each respective well. Two concentrations of extract (25 and 50 $\mu\text{g mL}^{-1}$), one positive control (rifampicin) and one negative control (DMSO) were poured into each Petri plate. These plates were incubated at 37 °C. After 24 h of incubation, the diameter of the clear zones that showed inhibition of bacterial growth was measured in millimeter (mm). Experiment was done in triplicate and mean value of zone inhibition was calculated with standard error.

Phytochemical assessment of extract

All the extracts were qualitatively analyzed for various secondary metabolites. Carbohydrate presence was confirmed by Molish's test, glycoside was confirmed by Borntrager's test (Evan, 1997), alkaloids was evaluated by Mayer's test, saponins were analyzed by Froth's Test (Kokate, 1999), tannins were studied by gelatin test (Mace, 1963), flavonoid was investigated by lead acetate test, amino acid was assessed by Ninhydrin's test (Yasuma and Ichikawa, 1953), Anthraquinones was confirmed by Borntrager's test and steroids by Salkowski's test.

Statistical analysis

Zone of inhibition measuring experiment was done in triplicate sets and results were reported in mean value \pm SD (Steel *et al.*, 1997).

Results and Discussion

Medicinal plants are in use to treat different diseases since ancient time. Increasing resistant pattern and associated side effects of antibiotics have evolved the importance of medicinal plants to be used as an antibacterial agent. This property is because of the production of several secondary metabolites by different parts of plants. Therefore, for all the present day medical complications these metabolites can act as an alternative source. Among these, *Punica granatum* is best known for its pharmacological properties. In present study we described the qualitative study of different metabolites and antibacterial activity of *P. granatum* peel extract against ATCC strains of some common pathogens including *E. coli*, *S. aureus* and *P. aeruginosa*. Peel of *P. granatum*

was extracted with five different solvents i.e. ethanol, methanol, water, ethyl and petroleum ether. The obtained yields were based on the dried weight of sample raw materials. Among these extracts, the highest (6%) and the lowest (3.3%) yields of extraction were observed for Ethanolic and Ethyl acetate respectively. Previously aqueous methanolic (Braga *et al.*, 2005; Dell'Agli *et al.*, 2009) and polyphenolic (Haidari *et al.*, 2009) extract have been used for studying antibacterial and anti-inflammatory activity of *P. granatum* extract. Percent yield of the five solvents extracts was studied in comparison and the highest (6%) and the lowest (3.3%) yields of extraction were observed for Ethanolic and Ethyl acetate respectively. Sultana *et al.* (2008) reported the percent yield of dry pomegranate peels after extracting with 80% methanol was 16.4%.

Antibacterial activity of crude methanolic, ethanolic and aqueous extract of *P. granatum* was tested against one gram positive (*S. aureus*) and two gram negative (*P. aeruginosa* and *E. coli*). Two concentrations i.e. 50 μg and 25 μg of all extracts were used against test organisms that showed different level of antibacterial activity based on zone of inhibition size. All these extracts exhibited a significant antibacterial activity against all the test organisms. At 50 μg concentration PEE showed highest activity (17 ± 0.82 mm) against *E. coli* followed by CME (16 ± 1.63 mm) whereas CEE has lowest antibacterial activity (7 ± 1.63 mm). At 25 μg CME showed highest zone of inhibition (15 ± 0.82 mm) followed by EAE (14 ± 1.63 mm) and least was CEE (4 ± 0.41 mm) (Fig. 1). *P. aeruginosa* extracts showed a very potent antibacterial activity. Among all extracts at 50 μg concentration, CME has maximum zone of inhibition (14 ± 1.63 mm) followed by EAE and PEE (11 ± 0.81 and 10 ± 0.82 mm respectively) each, whereas minimum zone was shown by CEE (5 ± 0.83 mm). At 25 μg concentration CME showed maximum zone size (11 ± 0.82 mm) followed by EAE (10 ± 1.63 mm) and CEE has minimum zone size (3 ± 0.41 mm) (Fig. 2). Potent antibacterial activity of *Punica granatum* peel extract against *S. aureus* was reported. At concentration 50 μg , CME clear zone was maximum (15 ± 0.82 mm) followed by EAE (14 ± 1.63 mm) and least was shown by CEE (7 ± 0.82 mm). At concentration 25 μg , maximum zone of inhibition (13 ± 0.81 mm) was shown by PEE followed by CME (11 ± 1.63 mm) and least by CEE (4 ± 0.41 mm) Fig. 3. Similar results was reported previously by Prashanth *et al.* (2001) that PEE showing good MIC among all extracts. However, at 25 μg maximum activity was

observed with CME (15 ± 0.82 mm) which can depict the picture that CME can be a better choice at low concentration as compared to PEE which is better for higher concentration according to present study. Mathabe *et al.* (2005) described that ethanol, methanol and aqueous extracts from pomegranate were significantly active against *E.coli*. CME showed potent activity against *P. aeruginosa* at both 50 and 25 μg concentrations. Our results coincide with Al-Zoreky (2009) that reported highest zone of inhibition (18 mm) among all *P. granatum* extracts. In the case of *S. aureus* again CME showed maximum zone of inhibition (15 ± 0.82 mm) that is similar to 20.4 mm zone of inhibition reported by Naz *et al.* (2007) with methanol extract of *P. granatum*. The study conducted by Braga *et al.* (2005), found that pomegranate extract at a higher concentration completely inhibited the *S. aureus* FRI 722 and subsequent enterotoxin production. Five different solvent extracts were studied of which CME showed more promising results even at low concentration of extract. Generally medicinal plants always produce bioactive molecules used for curing of several diseases. Consequently, comprehensive study is necessary on both phytochemistry and pharmacology of plant harvests because this may lead to the discovery of new medicines of high therapeutic importance and additional studies may help their quantitative assessment and qualitative separation of pharmaceutically active bio compounds.

Different phytochemical tests were conducted for metabolites confirmation in methanolic, ethanolic, aqueous, ethyl acetate and petroleum ether peel extracts. This study discovered the presence of certain important secondary metabolites in extracts. All the five extracts were studied for these metabolites and some of the metabolites were present in one extract but absent in the other Table 1.

Phytochemical analysis in this study shown the existence of glycosides, tannins, steroids, flavonoids and carbohydrate in the *P. granatum* peel extract. Similar results were also reported by Satheesh (2012); Uma *et al.* (2012); Hegde *et al.* (2012); Chebaibi *et al.* (2013); Kannaiyan *et al.* (2013). The presence of these metabolites in plants extracts are responsible for therapeutic properties. Tannins and flavonoids are primary antioxidant found in various plants. Flavonoid might be responsible as anti-inflammatory (Jasim *et al.*, 2010). In short, these bioactive molecules in plants has high pharmaceutical worth. The solvent has great effect on the composition of extract and different phytochemical were found in different solvents. Most of the molecules were present in ethanolic, methanolic and ethyl acetate extracts. According to El-Falleh *et al.* (2012), there were variations in phytochemistry of different extracts of *P. granatum*. Also the composition of peel depends on various factors like environmental factors, processing, cultivar and post harvesting (Houston, 2005).

It was concluded from the present study that the crude extracts of *P. granatum* has significant inhibitory effect against both gram positive and gram negative ATCC bacterial culture. Among different extracts, methanolic extract was very effective. The phytochemistry of different extracts were not similar. Increasing challenges of microbial resistance is complicating the selection for drug of choice day by day. Such studies of medicinal plants can lead to open new windows for novel antibacterial agents and can be quite helpful in developing new antibacterial regime. However, additional comprehensive phytochemical studies are compulsory to detect the active principles and exact mechanisms of action.

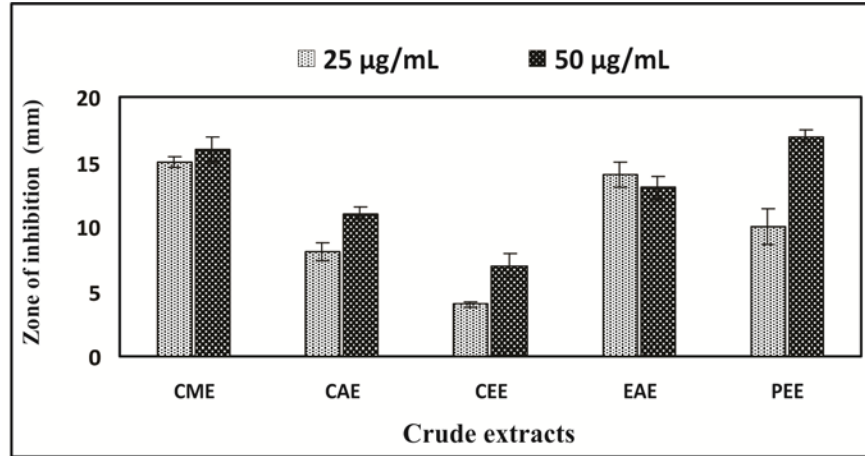


Fig. 1: Activity of various *P. granatum* extracts against *E. coli*. **CME:** Crude Methanolic extract; **CAE:** Crude aqueous extract; **CEE:** Crude ethanolic extract; **EAE:** Ethyl acetate extract; **PEE:** Petroleum ether extract.

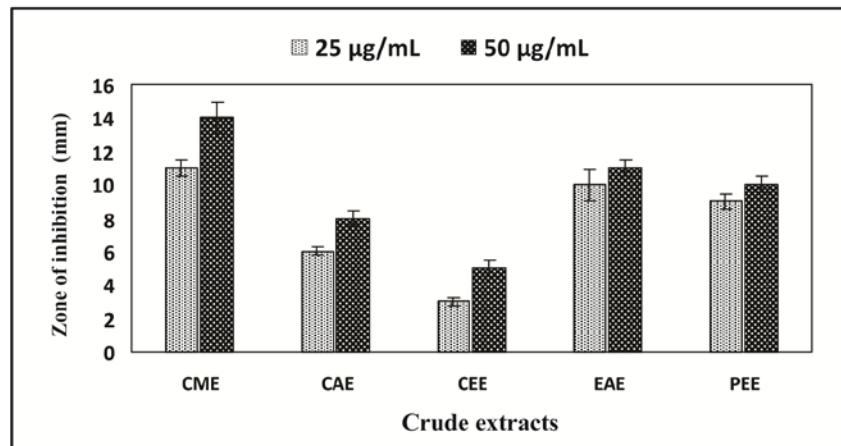


Fig. 2: Activity of various *P. granatum* extracts against *P. aeruginosa*. **CME:** Crude Methanolic extract; **CAE:** Crude aqueous extract; **CEE:** Crude ethanolic extract; **EAE:** Ethyl acetate extract; **PEE:** Petroleum ether extract.

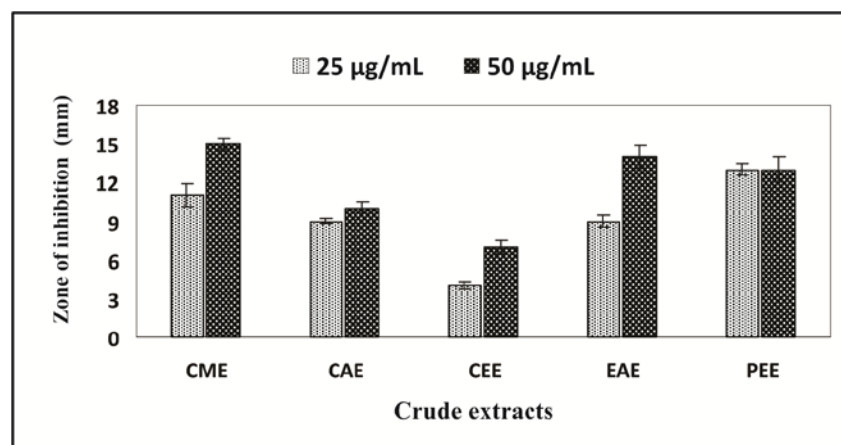


Fig. 3: Activity of various *P. granatum* extracts against *S. aureus*. **CME:** Crude Methanolic extract; **CAE:** Crude aqueous extract; **CEE:** Crude ethanolic extract; **EAE:** Ethyl acetate extract; **PEE:** Petroleum ether extract.

Table 1: Qualitative phytochemical screenings of pomegranate peel extracts.

S.No	Test for	Phytochemical Test	Crude Aqueous Extract	Crude Methanolic Extract	Crude Ethanolic Extract	Petroleum Ether Extract	Ethyl Acetate Extract
1	Saponins	Froth's test	-	-	-	-	-
2	Glycosides	Borntrager's test	-	+	+	+	+
3	Tannins	Gelatin test	+	+	+	-	+
4	Anthraquinones	Borntrager's test	-	+	+	+	-
5	Carbohydrates	Molish's test	+	+	+	+	+
6	Amino acids	Ninhydrin's test	-	+	+	-	-
7	Alkaloids	Mayer's test	-	+	+	+	+
8	Steroids	Salkowski's test	-	+	+	-	+
9	Flavonoids	Lead acetate test	-	+	+	-	+

+ = Presence of metabolites, - = Absence of metabolites

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