ANTIMICROBIAL ACTIVITY OF *PUNICA GRANATUM*

Kalpita Mulye* and Jayashree Pawar

*In-Charge, Department of Biotechnology and Microbiology, VPM's B. N. Bandodkar College of Science, Thane, Maharashtra India,
Coordinator, Biotechnology, Department of Biotechnology and Microbiology, VPM's B. N. Bandodkar College of Science, Thane, Maharashtra India, 400601
Email: kbmulye@vpmthane.org; Contact Number: 91 9819597054

ABSTRACT

Pomegranate (*Punica granatum* L.) is used as herbal medicine in India and other countries. Pomegranate is an important source of bioactive properties. In Ayurveda, it is been used to stimulate the appetite and for treating digestive problems.

The present study is focused on assessing the antimicrobial activity of pomegranate leaves and peel extracts using two representative human pathogens and to check the synergistic activity of pomegranate peel extract and pomegranate leaf extract using 'newly' designed 'Grid method'. The Grid method has been used to determine the synergistic action of these extracts in multiple combinations on a single plate using autoclaved Whatman paper strips having dimensions 3mm width by 8cm length that were impregnated with extracts.

The study revealed antioxidant properties associated with leaves extract. The peel and leaves extracts displayed antimicrobial activity when tested for inhibition of *S. aureus* and *E. coli*. When checked using newly designed Grid method these two extracts in combination did not have synergistic action and showed better antimicrobial activity when used individually.

Key words: Pomegranate, antimicrobial activity, synergistic action, Grid method

INTRODUCTION

The present scenario of emergence of multiple drug resistance to human pathogenic organism has initiated a search for new antimicrobial substances from plant sources. The use of higher plants and preparations made from them to treat infections is an age-old practice in large part of world population. The antibiotics available have various side effects on human body which has revived an interest in search of higher plants of natural origin having antimicrobial activity. A medicinal plant has similar properties as pharmaceutical drugs (Matjuda *et al.*, 2019).

Pomegranate (*Punica granatum* L.) is used as herbal medicine in India and other countries. Pomegranate is an important source of bioactive properties. Pomegranate fruit is about 5 inches wide with deep red, leathery skin, grenade shape with a pointed calyx (Khan *et al.*, 2017). The pomegranate belongs to punicaceae family and is distributed all over the world; it also has high nutritional value. It has many therapeutic uses. They are used in making

pomegranate leaf tea, healthy juices and salads and medicinal supplements. A healthy leaf is flat and a glossy light green.

Pomegranate has potent antioxidant, antimicrobial and antifungal activity. A range of phytochemicals compounds in pomegranate such as phenols, carotenoids, tannins, flavonoids, tocopherols and other secondary metabolites which show biological activity (Janani, *et al.*, 2019). In Ayurveda, it is been used to stimulate the appetite and for treating digestive problems.

Pomegranate Leaves aids in weight loss, treating a number of disorders and ailments such as insomnia, abdominal pain, dysentery, cough, jaundice, mouth ulcers, skin ageing and inflammation of the skin like eczema. Pomegranate leaves are used in supplementary and alternative therapy. Supplementary therapy is the use of vitamin and minerals. Human studies have shown that daily consumption of pomegranate juice lowers blood pressure in hypertensive subjects, delays the atherosclerotic process and increases the total antioxidant status of the blood.Many studies indicate that pomegranate extracts may be employed as natural alternative for the treatment of a wide range of bacterial and viral infections due to their antimicrobial activity (Jurenka, 2008).

The present study is focused on assessing the antimicrobial activity of pomegranate leaves and peel extracts using two representative human pathogens and to check the synergistic activity of pomegranate peel extract and pomegranate leaf extract using newly designed Grid method. The Grid method has been used to determine the synergistic action of these extracts in multiple combinations on a single plate using autoclaved Whatman paper strips having dimensions 3mm width by 8cm length that were impregnated with extracts.

METHODS AND MATERIALS

1**. Collection of sample:** Pomegranate leaves were collected for study of antimicrobial activity. The leaves were collected from Railadevi Lake, Thane (West). Pomegranate peels were collected from Gaondevi Market, Thane (West).

2. Processing of sample: The leaves were cleaned, rinsed with water and allowed to shade dry for 5 days, grinded to fine powder and stored in air tight container in cool place. The peels were cleaned, washed with water and shade dried for 5 days before grinding to form fine powder.

3. Preparation of extract: The powder (5 grams) of leaves was dissolved in 50 ml of distilled water. It was boiled for 5 – 10 minutes and was filtered using muslin cloth. Different concentrations of stock were prepared using St. distilled water as diluent *viz*. 2%, 4%, 6%, 8%, 10%. The pomegranate 'peel' extract was prepared in similar manner.

4. Microorganisms and Culture: Two bacterial strains *viz Staphylococcus aureus* and *Escherichia coli*were procured from laboratory.The cultures were maintained on Nutrient agar medium.

5. Analysis of antibacterial activity: Agar cup diffusion method was used to evaluate the antibacterial activity of leaf extracts against test organisms. Mueller-Hinton agar medium (pH 7.3) was prepared and autoclaved. A saline suspension of *Escherichia coli* and *Staphylococcus aureus* was prepared (density was adjusted as 0.2 at 530 nm) that was seeded aseptically in st. molten MH agar butt (18 ml). The seeded Mueller-Hinton agar media was transferred to sterile petriplate and was allowed to solidify. Four wells were made in each plate. Test solution of 50µl was poured into each respective well. These plates were incubated at 37◦C. After 24 hours of incubation, the diameter of zone of inhibition was measured in millimeter (mm).The experiment was performed in duplicate.

6. Analysis of synergistic action: The grid method was designed to determine the synergistic activity of drugs in multiple combinations on a single petriplate. To check the activity, Whatman filter paper strips were used. The strips were cut having dimensions 3mm (width) by 8cm (length) and were autoclaved. For antibacterial analysis, Mueller - Hinton agar butts were prepared and autoclaved. The media was transferred to petriplate and allowed to solidify. A saline suspension of *Escherichia coli* and *Staphylococcus aureus* was prepared and density was adjusted as 0.2 at 530 nm. The culture was swabbed evenly on the St. MH agarusing Sterile cotton swabs.The experiment was performed by dipping autoclaved strips in aqueous extracts of leaf and peel of concentration 8% and 10% and placing immediately on pre-swabbed Mueller-Hinton agar plateone after the other perpendicular to each other. These plates were incubated at 37◦C for 24 hours.

7. Phytochemicals analysis: The following tests were carried out for phytochemical analysis of pomegranate peels extract and leaves extract.

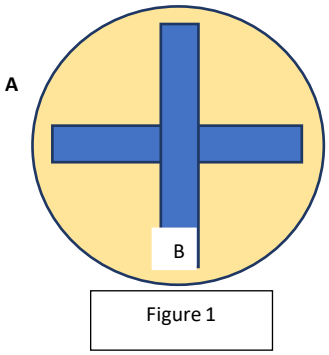

Figure 1

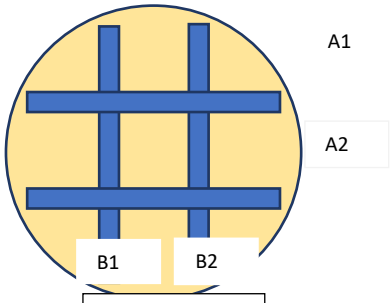

Figure 2

Figure 1: Conventional placing of Whatman filter paper strips impregnated with different drug preparations allowing analysis of single combination

Figure 2: Grid pattern placing of Whatman filter paper strips impregnated with different drug preparations allowing analysis of four combination

Test for Amino acids:

- Amino acid: 3 drops of nitric acid was added to from the corner of the test tube to 2ml of extract. Absence of yellow color indicates absence of proteins and free amino acids.

Test for Ninhydrin:

- Ninhydrin test: To 1 ml of extract,3 drop of Ninhydrin solution is added in attest tube. A characteristic blue color indicated the presence of amino acids.

Test for Tannins:

- Lead Acetate test: To a few ml of extract, few drops of 1% Lead acetate is added. The mixture is shaken well. A yellowish precipitate indicated the presence of tannins.

Test for Carbohydrates:

- Benedict's test: to 0.5 ml of the filtrate, 0.5 ML of Benedict's reagent was added. Mixture was heated on BWB for 2minutes.

Test for Proteins:

- Biuret test: Test solution was treated with 10% sodium hydroxide solution and two drops of 0.1% copper solution and treated and observed for the formation of violet or pink color.

Test for Total Phenols:

- Total Phenols: To 2ML of extract, 3% of FeCl2 is added. Formation of deep blue color indicates the presence of total phenol.

Test for Quinone:

- Quinone test: Few drops of extract added 5ML of HCL. Formation of yellow precipitate indicates the presence of quinone.

8. Preparation of standard Ampicillin stock

Ampicillin belongs to antibiotic class of penicillin. It exerts bactericidal cell wall synthesis by binding to one or more penicillin binding proteins (PBPs). It is a broad-spectrum antibiotic which acts against wide range of gram positive bacteria and gram negative bacteria. Ampicillin is used to treat or prevent different types of infections such as gastrointestinal infections, Genito-urinary tract infection, Otitis media, Respiratory infections, Bacterial meningitis etc.

The standard ampicillin stock was prepared using 500 mg ampicillin tablet. The 500 mg ampicillin tablet was dissolved in 10 ml of sterile distilled water and was heated for few minutes to prepare 50 mg / ml concentration of stock.

From 50 mg / ml stock, 10 mg / ml concentration of stock was made. Further, different dilutions (1:500, 1:1000, 1:1500 and 1:2000) were prepared using 10 mg / ml concentration of stock.

Agar well diffusion method was used to determine the antimicrobial activity of ampicillin. The agar cultures of Staphylococcus aureus and Escherichia coliwere prepared to assess the standard ampicillin inhibitory effects. The Mueller Hinton Agar was used to check the activity. The wells were cut (8.0 mm in diameter) from the agar at sterile condition. Different dilutions (1:500, 1:1000, 1:1500 and 1:2000) including 10 mg / ml concentrations were added to the wells. The zone of inhibition was measured in mm after 24 hours incubation at 37°C.

9. Antioxidant Activity

Free radicals are inevitably produced in biological systems and also encountered exogenously, and are known to cause various degenerative disorders, like mutagenesis, carcinogenesis, cardiovascular disturbances and ageing. Antioxidants are the compounds, which combat the free radicals by intervening at any one of the three major steps of the free radical mediated oxidative process, viz., initiation, propagation and termination. These antioxidants are also produced by biological system and occur naturally in many microorganism, algae, plants etc.

DPPH assay

To determine the antioxidant activity of extracts a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M=394.33) is used. The assay is based on the measurements of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine.

DPPH is characterized as a stable free radical by the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerize, like most other free radicals. The delocalization also gives rise to the deep violet color, with an absorption in methanol solution at around 520 nm. On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet color. Representing the DPPH radical by Z* and the donor molecule by AH, the primary reaction is Where ZH is the reduced form and A* is free radical produced in the first step.

Table 1: Addition table for DPPH assay

Reagents	**Blank**	**Reference**	**Test**
Methanol	4 ml	1 ml	-
Sample	-	-	1 ml
DPPH	-	3 ml	3 ml
Incubate at room temperature (in dark) for 30 minutes			
Absorbance at 520 nm			

RESULT AND DISCUSSION

1. Antimicrobial activity

A study has reported Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels (Al-Zoreky, 2009). Antibacterial activity of aqueous extract of pomegranate peel against *Pseudomonas stutzeri i*solate has been analyzed (Devatkal, 2013). Prashanth, *et al.,* (2001) has also studied Antibacterial activity of *Punica granatum*.

The leaf extracts of *Punica granatum* had been tested for their antibacterial activities and an antibacterial profile has been observed against Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) (Algurairy, 2018). The activities of extracts are mentioned in terms of zone of inhibitions (mm).

Table 2: Antimicrobial activity of aqueous extract of pomegranate leaves

Concentration of pomegranate leaf extract (%)	Diameter of zone of inhibition(mm)	
	Escherichia coli	*Staphylococcus aureus*
2	19	12
4	20	13
6	21	14
8	21	15
10	21	16

The diameter of zone of inhibition against *Escherichia coli* was 19mm, 20mm, 21mm, 21mm and 21mm for concentration of pomegranate leaf extract stock of 2%, 4%, 6%, 8% and 10% respectively. The diameter of zone of inhibition against *Staphylococcus aureus* was 12mm, 13mm, 14mm, 15mm and 16mm for concentration of pomegranate leaf extract stock of 2% , 4%, 6%, 8% and 10% respectively.

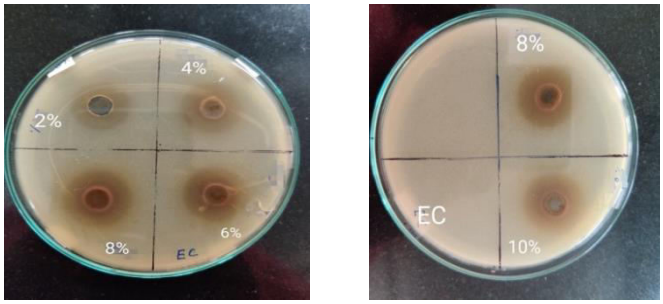

Plate 1: Results showing antimicrobial activity of different concentrations of pomegranate leaf extracts against *Escherichia coli*.

The standard antibiotic ampicillin showed good activity at 1:500 concentration of antibiotic. The diameter of zone of inhibition of *Escherichia coli* and *Staphylococcus aureus* for ampicillin was 20mm and 21mm respectively. The standard ampicillin results were compared with the test result which indicates that pomegranate leaf extract has comparative good antimicrobial activity.

From the result, it was observed that zone of inhibition of *Escherichia coli* is higher (21mm) at high concentration (10%) whereas zone of inhibition of *Staphylococcus aureus* is 16mm at high concentration (10%). Pomegranate leaf extracts of different concentrations were active and effective against the growth of tested organisms.

2. Phytochemical Screening

Plants are recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities. The biologically active compounds present in plants are called photochemical. These phytochemicals are derived from various parts of plants such as leaves, flowers, seeds, barks, root and pulps. Phytochemicals are chemicals that are present naturally in plants.

The phytochemical screening of various extracts shows the presence of certain important components such as amino acids, tannin, carbohydrates, proteins, phenol and quinone. Phytochemical constituents afford imperative pharmaceuticals properties for human health. These compounds can be used as drugs or as dietary supplements to heal or to prevent various diseases.

The results of phytochemical screening were obtained as follows

Table 3: Phytochemical analysis of pomegranate peels extract and leaves extract

Phytochemicals	**Peels**	**Leaves**
Amino acid	+	+
Tannin	+	-
Benedicts (Carbohydrate)	+	-
Biuret (Protein)	-	-
Ninhydrin	-	-
Phenol	-	-
Quinone	+	-

3. Antioxidant Activity

Antioxidant activity and total phenolic content of ethanolic extract of pomegranatepeels, juice and seeds has been analyzed earlier by Derakhshan, (2018) The antioxidants of Pomegranate leaf extract was analyzed using DPPH reagent.

Table 4: Observation table for DPPH assay

Tube	**Absorbance at 520nm**
Blank	0.0002
Control	1.5339
Test	0.33

Radical Scavenging Activity was determined using following formula:

$$\text{Antioxidant Activity (\%)} = \frac{100 - [(\text{Abs (Sample)} - \text{Abs (Blank)}]\times 100}{\text{Abs(Control)}}$$

The DPPH (%) of pomegranate leaf extract was 78.49%. It shows maximum DPPH percent indicating it has good antioxidant activity.

4. Analysis of Synergistic action

Combination drug therapy is recommended to have better disease management. Times when the infectious agent is drug resistant strain, when single drug concentration cannot be increased considering its toxicity, when multiple target sites are to be hit for efficient bacterial control in host body multiple drugs are recommended. However, the interaction between two drugs needs to beanalyzed before their actual usage to ensure that the combination of two is more efficient than individual drugs.

Combinations of two antimicrobial agents are considered to be synergistic if the effect of the combination is greater than the effect of either agent alone or greater than the sum of the effects of the individual agents. Antagonism results if the combination provides an effect worse than the effect of either agent alone or worse than the sum of the effects of the individual agents (Cappelletty and Rybak, 1996).

The synergistic action of different concentrations of pomegranate leaf extract and peel extract were checked on single petri plate using Grid method.

Table 5: Zone of inhibition in mm for synergistic action

Organisms	Combinations	Zone size for Peel extract	Zone size for Peel extract	Zone size at intersection in mm
Escherichia coli	P10, L10	4mm	4mm	3mm
	P8, L8	4mm	4mm	4mm
	P10, L8	4mm	4mm	3mm
	P8, L100	4mm	4mm	3mm
Staphylococcus aureus	P10, L10	5mm	5mm	3mm
	P8, L8	5mm	5mm	3mm
	P10, L8	5mm	5mm	3mm
	P8, L100	5mm	5mm	3mm

Plate 2: Results showing antagonist effect of pomegranate leaf and peel extract of different concentration using grid Whatman filter paper method against *Escherichia coli* and *Staphylococcus aureus* respectively

The leaves and peel extract in combination were observed to display antagonistic effect. The leaves and peels have better antimicrobial activity individually, rather than the combination.

CONCLUSION

Pomegranate is a medicinal plant and has many pharmaceutical and therapeutic uses. Antibacterial activity, Antioxidant activity, Phytochemicals screening and Synergistic activity were performed.

From this study, it can be concluded that pomegranate leaves have good antimicrobial activity and they are effective against growth of Gram Positive (*Staphylococcus aureus*) and Gram Negative bacteria (*Escherichia coli*) having zone of inhibition as 16mm and 21mm respectively. The aqueous extract of Pomegranate leaves exhibited high antimicrobial activity against Gram negative organism.

The Antioxidant activity of leaf extract was determined by DPPH method and was estimated to be 78.49% .The high percent of antioxidant activity indicates that it has principles similar to an antioxidant drug.

The Standard phytochemical tests were performed. Presence of phytochemicals indicates that different constituents (amino acids, carbohydrate, tannins, quinones) present enhances the biological activity of Pomegranate leaves and thus it can be used in medicines to treat disorders.

The synergistic activity was checked using Grid method.The Grid method used is introduced for the first time to check the synergistic activity of different extracts in multiple

combinations on a single plate on a suitable medium using Whatman paper strips. The pomegranate leaf and peel extract showed antagonist activity; although pomegranate leaf and pomegranate peels have good antimicrobial activity when used separately.

World has been following traditional medicines since long and hence it is important to investigate such plants having antimicrobial activity and generate clinically important data. Promising and can be investigated further to have better

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REFERENCES

1. Algurairy, A. T. M. (2018). Assessing the antibacterial activity of pomegranate against *Staphylococcus aureus* obtained from wound infections. *Research Journal of Pharmaceutical, Biological and ChemicalSciences*, 9(4): 1602-1606.
2. Al-Zoreky, N. S. (2009). Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. *International Journal of Food Microbiology*, 134(3): 244-248.
3. Cappelletty D. M. and Rybak M J. (1996), Comparison of Methodologies for Synergism Testing of Drug Combinations against Resistant Strains of *Pseudomonas aeruginosa*. *Antimicrobial Agents And Chemotherapy*, 40(3): 677–683.
4. Derakhshan, Z., Ferrante, M., Tadi, M., Ansari, F., Heydari, A., Hosseinei, M. S., Conti, G. O., and Sadrabad, E. K. (2018). Antioxidant activity and total phenolic content of ethanolic extract of pomegranatepeels, juice and seeds. *Food and Chemical Toxicology*, 114: 108-111
5. Devatkal, S. K., Jaiswal, P., Jha, S.N., Bharadwaj. R., and Viswas, K. N. (2013). Antibacterial activity of aqueous extract of pomegranate peelagainst *Pseudomonas stutzeri* isolated from poultry meat. *Journal of Food science and Technology*, 50(3): 555–560.
6. Janani, J., Rajiv, P., Gopalan, R., and Lakshmanapermalsamy, P. (2019). An overview of phytochemicaland pharmacological potentials of *Punica granatum* L. *Pharmacognosy Journal*, 11(5): 1167-1171.18
7. Jurenka, J. (2008). Therapeutic applications of pomegranate (*Punica granatum* L.): A review. *Alternative Medicine Review*, 13: 128–144.
8. Khan I *et al.* (2017). Punica granatum peel extracts: HPLC fractionation and LC MS analysis to questcompounds having activity against multidrug resistant bacteria. *BMC Complementary and AlternativeMedicine*, 17: 247.

9. Matjuda, D.S. and Aiyegoro, O.A. (2019). Analysis of bacteriological pollution and the detection of antibiotic resistance genes of prevailing bacteria emanating from pig farm see page. *Microbiology Open*, 8:737.

10. Prashanth, D., Asha, M.K. and Amit, A. (2001). Antibacterial activity of *Punica granatum*. *Fitoterapia*, 72(2): 171 – 173.