

In-vitro antimicrobial and antioxidant activities of aqueous pericarp extract of *Punica granatum*

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ABSTRACT

The study aimed to evaluate the anti microbial against human pathogenic organisms and antioxidant activities of aqueous pericarp extract of *Punica granatum*. The antimicrobial activity was tested against human pathogenic bacteria and fungi. The aqueous pericarp extract of *Punica granatum* was showed significant inhibitory activity. 11 mm was the highest inhibitory zone against *Staphylococcus aureus* but not active against *C. albicans*. The MIC of pericarp extract was found to be the range between 10- 100 µg/ml. 10 µg/ml was the lowest MIC to most of the bacteria. Radical scavenging activity of pericarp extract of *P. granatum* was carried out to assess antioxidant activity with employing ascorbic acid as standard drug. 6.8, 36.3, 4, 65.2, 10.0, 185 µg/ml were the IC₅₀ values of seed coat extract to Superoxide, Hydroxyl, Hydrogen peroxide, DPPH, Nitric oxide, Lipid peroxide radical scavenging activities respectively. The antimicrobial as well as antioxidant activities were dose dependently increased with pericarp extract concentration. The present study revealed that pericarp of *Punica granatum* has shown significant antimicrobial and antioxidant activities.

INTRODUCTION

Nowadays herbal drugs are prescribed widely even when their biologically active compounds are unknown because of their effectiveness, minimum side effects and relatively low cost (Valiathan, 1998). Medicinal plants are the important source of life saving drugs for the majority of the world's population. The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial bio-molecules. If they are not effectively scavenged by cellular constituents, they lead to disease conditions (Halliwell & Gutteridge, 1990). The harmful action of the free radicals can, however, be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism. Recently, the most important reported biological property of flavonoids is due to their antioxidant activity (AA) by scavenging oxygen radicals and inhibiting peroxidation (Hanasaki *et al.*, 1994). The potential value of such antioxidants prompted investigators to study

new flavonoids to improve the treatment of various diseases. Due to side effects and emerging antibiotic resistance, the need for developing new anti microbial compounds. Plants are the best source for the identification of new drug compounds.

Punica granatum L. (Punicaceae), commonly known as pomegranate, is a shrub or a small tree native to the Mediterranean region is widely used for therapeutic formulae, cosmetics, and food seasoning. Pomegranate, also easily acquired from traditional medicine markets. Pomegranate peel is a rich source of tannins, flavonoids, polyphenols and some anthocyanins as Delphinidins, Cyanidins, etc. The pharmacological functions of pomegranate include antioxidation (Rajan. *et al.*, 2011), antitumour (Khan N *et al.*, 2007), antihepatotoxicity (Kaur.G., 2006), antidiarrhoeal (Das *et al.*, 1999) and antiliperoxidation (Reddy, *et al.*, 2007).

MATERIALS AND METHODS

Chemicals

FeCl₃, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals were purchased from Sigma-Aldrich. Methanol, chloroform, acetic acid and ethyl acetate were of analytical grade.

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Plant Materials

Pomegranate fruits was collected from local market. Peels were then cut into smaller pieces and then first washed with tap water followed by washing with distilled water. It was then dried under shade. Dried pericarp was then taken for grinding by the help of mixer grinder. Powdered form of plant sample was used throughout the study.

Test microorganisms

Salmonella typhi, *Vibrio cholerae*, *Shigella dysenteriae* and *Enterococcus faecalis*, *Escherichia coli* are gastrointestinal pathogens. *Klebsiella pneumoniae* is causative agent of pulmonary infections. *Staphylococcus aureus*, *Candida albicans* are causative agents of skin infections. *Salmonella typhi*, *Vibrio cholerae*, *Shigella dysenteriae* and *Enterococcus faecalis* are clinical isolates and remaining cultures are purchased from IMTEC, Chandigarh, India and NCL, Pune, India.

Antioxidant activity

Determination of superoxide scavenging Activity

Riboflavin photoreduction method: Superoxide scavenging activity of the extract was determined by McCord and Fridovich method, which depends on light induced superoxide generation by riboflavin and the corresponding reduction of NBT. The assay mixture contained 0.3 ml of different concentrations of the extract and 0.2 ml ethylene diamine tetra acetic acid (6 μ M containing 3 μ g NaCN), 0.1ml NBT (50 μ M), 0.05 ml riboflavin (2 μ M) and 2.35 ml phosphate buffer (58 mM, pH 7.8) added to give total volume of 3 ml. The tubes were uniformly illuminated with an incandescent light for 15 minutes and the optical density was measured at 560 nm. The percentage inhibition by the extract of superoxide production was evaluated by comparing the absorbance values of control and experimental tubes. (McCord and Fridovich, 1969).

Determination of hydroxyl radical scavenging activity

Deoxyribose method: Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radical generated from the Fe³⁺/ascorbate/EDTA/H₂O₂ system. The hydroxyl radical attacks deoxyribose and eventually results in formation of thiobarbituric acid reacting substances (TBARS). (Elizabeth and Rao, 1990). The reaction mixture containing deoxyribose (2.8 mM), ferric chloride (0.1 mM), EDTA (0.1 mM), H₂O₂ (1mM), ascorbate (0.1 mM), phosphate buffer (20 mM, PH 7.4) and 0.2 ml different concentrations of the extract in a final volume of 1 ml was incubated for 1 hour at 37°C. Deoxyribose degradation was measured as TBARS by the method of Ohkawa (Ohkawa *et al.* 1974) and percentage of inhibition was calculated from the control where no test compound was added (Elizabeth and Rao, 1990).

DPPH radical scavenging activity

The antioxidant activity of the extracts, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl

(DPPH) free radical, was determined by the method described by Braca *et al.* (2001). Plant extract (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Water (0.1 ml) in place of the plant extract was used as control. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated as $[(A_0 - A_1)/A_0] \cdot 100$, where A₀ was the absorbance of the control, and A₁ was the absorbance of the extract/standard.

Hydrogen peroxide radical scavenging assay

The ability of extracts to scavenge hydrogen peroxide was determined by little modification here the solution of hydrogen peroxide (100mM) was prepared instead of 40mM in phosphate buffer saline of (PH 7.4), at various concentration of aqueous pericarp extract (10 -50 μ g/ml) were added to hydrogen peroxide solution (2 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction. In case of control takes absorbance of hydrogen peroxide at 230 nm without sample extracts. Results are provided in the percentage inhibition activity was calculated from $[(A_0 - A_1)/A_0] \times 100$, where A₀ is the absorbance of the control and A₁ is the absorbance of extract/standard taken as Ascorbic acid (10 - 50 μ g/ml) (Gülçin, 2005).

Determination lipid Peroxidation scavenging activity

Lipid peroxidation induced by Fe²⁺-ascorbate system in rat liver homogenate by the method of Bishayee and Balasubramaniyam was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al.* The reaction mixture contained rat liver homogenate 0.1 ml (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); FeSO₄ (NH₄)₂SO₄·7H₂O (0.06 mM); and various concentrations of *Punica granatum* extract in a final volume of 0.5ml. The reaction mixture was incubated at 37°C for 1 h.

After the incubation period, 0.4ml was removed and treated with 0.2ml sodium dodecyl sulphate (SDS) (8.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed and its absorbance at 532 nm was measured to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of treatments with that of the control. Quercetin and L-ascorbic acid were used as standard.

Assay of nitric oxide-scavenging activity

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent.

Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of CAE dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the CAE but with an equivalent amount of water, served as control.

After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H₃PO₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Curcumin was used as positive control. (Sreejayan & Rao, 1997).

Antimicrobial studies

Anti microbial assay by Agar Well Diffusion Method

The bacteria were grown in Muller-Hinton media (HiMedia Pvt. Ltd., Mumbai, India) at 37^o C while fungi were grown in Saboured Dextrose Agar media at 28^o C and maintained on nutrient agar slants at 4^o C and stored at -20^o C. Inoculum of bacteria was prepared by growing pure isolate in nutrient broth at 37^o C for overnight.

The overnight broth bacterial cultures was sub-cultured in fresh nutrient broth and grown for 3hrs to obtain log phase culture. 21 days old grown fungi culture was scraped with sterile scalpel and dissolved in sterile saline solution to make different dilutions. The diluted suspension which has the absorbance of 0.600 at 450nm determined spectroscopically (Electronics India) then it was used as inoculums for fungi. The agar plates were prepared by pour plate method using 20ml of agar medium. The sterile agar medium is cooled to 45^o C and mixed thoroughly with 1ml of growth culture of concerned test organism (1 x 10⁸ cells) and then poured into the sterile petri dishes and allowed to solidify. Wells of 6 mm size were made with sterile cork borer and test extracts were added. The agar plates were incubated at for 4days at 28^o C for fungi wile 24hours at 37^o for bacteria.

The diameter of inhibition zones was measured in mm using HiMedia zone reader. Ciprofloxacin (Antibiotic) used as Standard while Solvent (DMSO) used for control (Govinda *et al.*, 2011).

Determination the Minimum Inhibitory Concentration by Broth Dilution Assay

The minimum inhibitory concentration (MIC) of the plant extract was determined using broth dilution assay. The medium containing different concentrations of plant extracts viz., 1mg -1µg per ml prepared by serial dilution (10⁻¹ dilution). After inoculation of culture, the tubes were incubated for 72 hours at 28^o C for fungi while 24hours at 37^o for bacteria. The MIC of each sample was determined by measuring the optical density in the spectrophotometer (Electronics India) at 520nm and compared the result with those of the non-inoculated broth used as blank. Control was prepared using media and inoculum without plant extract (Andrews JM., 2001; M07-A9, 2012).

RESULTS & DISCUSSION

Antioxidant activity

Superoxide radical scavenging activity

Table 1 shows the superoxide radical (O₂⁻) scavenging activity of the extract, as measured by the riboflavin- NBT-light system in vitro. Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species. Photochemical reduction of flavins generates O₂⁻, which reduces NBT, resulting in the formation of blue formazan (Beauchamp & Fridovich, 1971). The extract was found to be a moderate scavenger of superoxide radical generated in riboflavin-NBT-light system in vitro. The extract inhibited the formation of the blue formazan and the % inhibition was proportional to the concentration with an IC₅₀ value of 6.8±0.02 (Table 1). These results indicated that the tested extract had a notable effect on scavenging of superoxide when compared with ascorbic acid, which was used as positive control.

Table. 1: Antioxidant activity of APPG extract :

Radical	IC ₅₀ (µg/ml)			R ²	
	APPG	Ascorbic Acid	APPG	Ascorbic Acid	
Superoxide	6.8±0.02	4.6±0.11	0.988	0.970	
Hydroxyl	36.3±0.24	28.2±0.11	0.979	0.956	
Hydrogen peroxide	4±0.24	3.8±0.21	0.930	0.890	
DPPH	65.2±0.11	65.6±0.99	0.941	0.980	
Nitric oxide	10.0±0.16	11.3±0.27	0.849	0.922	
Lipid peroxidation	185±0.24	48.0±0.26	0.940	0.980	

Table. 2: Anti bacterial activity of APPG extract on human pathogens.

s. no.	Human pathogens	Causing Disease	Zone of inhibition at various doses (mm)					MIC (µg/ml)	
			25µg	50µg	75µg	100µg	Antibiotic* (30µg)	APPG extract	Antibiotic*
1	<i>Salmonella typhi</i>	Typhoid fever	4	5	6	9	10	10	10
2	<i>Vibrio cholerae</i>	Cholera	4	5	6	9	10	10	10
3	<i>Shigella dysenteriae</i>	Dysentery	4	5	5	7	10	10	10
4	<i>Enterococcus faecalis</i>	Gastro intestinal infections	3	4	6	8	12	10	10
5	<i>Escherichia coli</i>	Gastro intestinal infections	6	7	8	9	13	10	1
6	<i>Klebsiella pneumoniae</i>	Pulmonary infections	7	8	9	10	12	10	10
7	<i>Staphylococcus aureus</i>	Wound infections	6	8	9	11	11	10	10
8	<i>Candida albicans</i> (MTCC 180)	Dermatophycosis	3	3	4	4	12	100	10

*Ciprofloxacin for bacteria, Griseofulvin for fungi.

Hydroxyl radical scavenging activity

The ability of APPG extract to scavenge hydroxyl radicals was measured by studying competition between deoxyribose and test compounds for hydroxyl radical generated from ferric-ascorbate-EDTA-H₂O₂ system. Hydroxyl radicals attack deoxyribose starting a set of reactions, which eventually results in TBARS formation. When a molecule scavenges hydroxyl radicals the TBARS formation is decreased. The IC₅₀ (36.3±0.24) of APPG extract was found to be a lower scavenging activity than ascorbic acid (28.2±0.11)(Table 1).

DPPH radical scavenging activity

The CAE showed a concentration-dependent antiradical activity by inhibiting DPPH radical with an IC₅₀ value of 65.21±0.11 (Table 1). DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants (Oyaizu, 1986). The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine.

It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g., p-phenylene diamine, p-aminophenol), reduce and decolorise 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability (Blois, 1958). It appears that the APPG possesses hydrogen donating capabilities and acts as an antioxidant. The scavenging effect increased with increasing concentration of the extract. However, scavenging activity of Ascorbic Acid, a known antioxidant, used as positive control, was relatively pronounced than that of APPG.

Hydrogen peroxide scavenging activity

Biological systems can produce hydrogen peroxide. Hydrogen peroxide can attack many cellular energy-producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Hyslop *et al.*, 1988). With regard to IC₅₀ values of hydrogen peroxide scavenging ability, APPG (IC₅₀ = 4±0.24) had the highest radical scavenging ability, (Table 1). However, it had the lowest hydrogen peroxide-scavenging effect than ascorbic acid.

Nitric oxide scavenging activity

The APPG extract also showed a moderate nitric oxide-scavenging activity between 10 to 50 µg/ml in a dose dependent manner (IC₅₀-10.0±0.16g/ml) (Table 1). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada, Palmer, & Higgs, 1991).

The plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to

arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. The % inhibition was increased with increasing concentration of the extract. Ascorbic Acid, a natural antioxidant was used as a positive control for comparison (Sreejayan & Rao, 1997).

Lipid peroxidation scavenging activity

The effect of APPG and commercially available antioxidants namely quercetin and L-ascorbic acid on the *in vitro* inhibition of lipid peroxidation is shown in Fig 1. The generation of lipid peroxidase by Fe²⁺-ascorbate in rat liver homogenate seems to be inhibited by APPG with IC₅₀ value of 185±0.24 µg/ml. A similar effect was produced by L-ascorbic acid (IC₅₀=48.0±0.26 µg/ml).

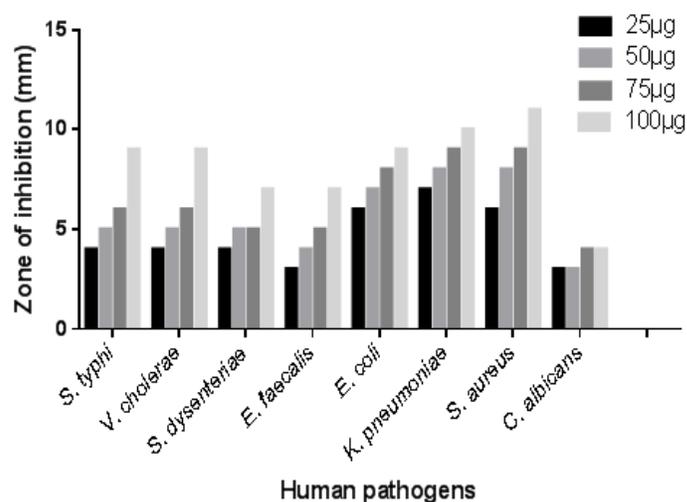


Fig. 1: Dose dependent inhibitory activity of APPG extract on human pathogens:

Antimicrobial activity on human pathogens

Antimicrobial studies were carried out on human pathogenic bacteria and fungi. *Salmonella typhi*, *Vibrio cholerae*, *Shigella dysenteriae* and *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae* *Staphylococcus aureus* are bacterial sps. *Candida albicans* is a dermatophytic fungus. APPG extract showed significant antibacterial activity and moderate antifungal activity. As shown in table, 11mm was the highest zone of inhibition against *S. aureus* at 100 µg concentration of APPG extract. MIC of APPG extract found to be between 10 - 100 µg/ml. 10µg/ml was the lowest MIC value to bacteria except *Shigella dysenteriae*. APPG extract showed comparable antibacterial activity with Ciprofloxacin (antibiotic) (Fig. 2).

S. aureus, *E. faecalis* are Gram positive bacteria which were showed sensitive to extract compared to gram negative bacteria. APPG extract was not much effective against clinical isolates compared to non-clinical isolates. APPG extract was not active against *C. albicans*. 3mm was the zone of inhibition against dermatophytic *C. albicans* but 100 µg/ml was MIC, this indicates that PG showed microbial growth static activity rather than microbicidal activity.



A. *Salmonella typhi*



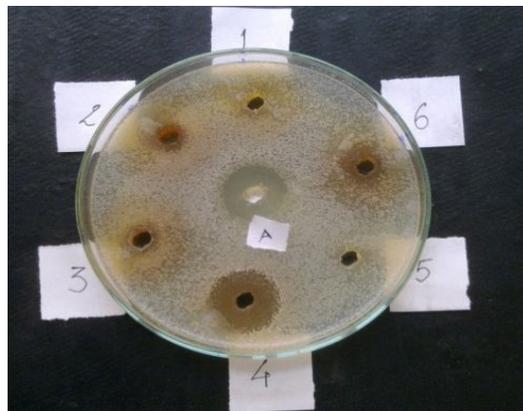
B. *Vibrio cholerae*



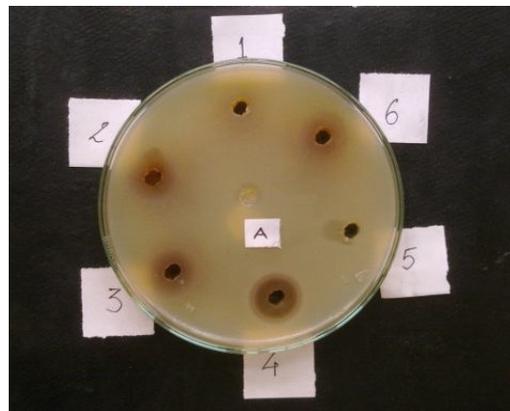
C. *Shigella dysenteriae*



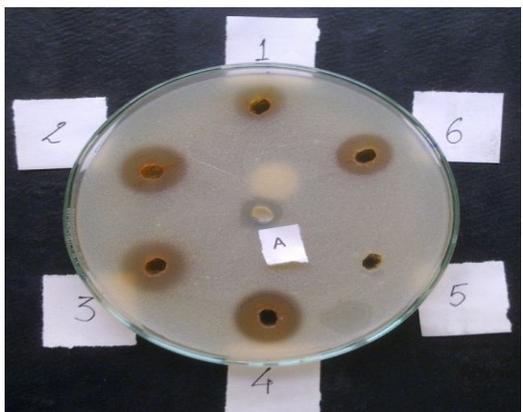
D. *Enterococcus faecalis*



E. *Escherichia coli*



F. *Klebsiella pneumonia*



G. *Staphylococcus aureus*



H. *Candida albicans*

Fig. 2: Dose dependent antimicrobial activity of APGG extract on human pathogens:

*In E, F, G images 1(10µg), 2 (25 µg), 3 (50 µg), 4 (100 µg), 5 (DMSO), 6 (75 µg), A (antibiotic).

CONCLUSION

Aqueous pericarp extract of *Punica granatum* showed significant antimicrobial and antioxidant activities further investigation under progress to isolate and identify bioactive compounds.

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