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Research Article

Antimicrobial and Anthelmentic Activity of *Punica granatum* Fruit Peel Extracts

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ABSTRACT

Punica granatum fruit is the widely consumed fruit in India. Peels of these fruit are used by traditional healers for treating stomach ailments and have many significant other medicinal effects. The present paper discusses antimicrobial and anthelminthic activity of *Punica granatum* fruit peels (PGFP) extracts prepared using different solvents (petroleum ether, ethylacetate, methanol, methanol:water and water) were evaluated. Antimicrobial activity was tested against gram positive bacteria (*Staphylococcus aureus, Salmonella typhi, Vibrio cholera, Klebsiella pneumonia, Shigella flexneri),* gram negative bacteria *(Pseudomonas aeruginos* and *Escherichia coli)* and a yeast (*Candida albicans*). Antimicrobial activity of these extracts was evaluated by using ditch plate method and agar well diffusion method. Ethylacetate, methanol, methanol:water and water extract inhibited bacterial and yeast growth at all concentrations. Anthelmentic activity was evaluated against *Pheritima posthuma* (Indian earthworm). Methanol extract of PGFP was found to possess maximum anthelmintic activity among all the extracts in dose dependent manner.

Key Words: Antimicrobial, Anthelmentic, *Punica granatum* fruit peel, cup plate.

INTRODUCTION

Worldwide, use of plants and its parts to cure specific aliments is expanding at an astonishing pace due to the fact that plants are known to be safe and contains many bioactive molecules.[1] Therapeutic effects of many plants in several disorders has been described by traditional medicine practitioners. Significant antimicrobial and anthelmentic properties of many plants have been increasingly reported from different parts of the world. There has been considerable interest in the use of plant extracts to control microbial and helminthic infection as major population is becoming aware about the side effects associated with the use of antimicrobial and anthelmentic drugs. Emergence of drug resistance and new pathogens has lead to systematic and intense research work so as to develop newer antimicrobial and anthelmentic drugs from plants.[2,3] High cost of antibiotics and anthelmentics drugs has also paved the way for plants as an alternative medicine.[4]

Punica granatum belonging to family Lythraceae, is a fruit bearing deciduous shrub. Fruits are consumed fresh or used for the preparation of fresh juice, jelly and jam, and beverage products.[5] In several systems of medicine *Punica granatum* fruit is used for variety of ailments. Its fruit juice have various phytoconstituents whose functional and medicinal effects such as hepatoprotective, antibacterial, antioxidant, anticancer, antidiabetic, anti-atherosclerotic effects, estrogen-like activity had been confirmed.[6,7,8] In Ayurveda, the peels of the fruit are used for stomach aliments including diarrhea and dysentery. The peels has wide range of therapeutic properties and can be used in treatment of diabetes, cancer, cardiovascular disease, dental conditions, erectile dysfunction and male infertility, infectious diseases, Alzheimer's disease and dermal wounds.[6] There are no reports on the antimicrobial and anthelmenthic activity of different extracts of PGFP. Thus the aim of the present study was to evaluate the antimicrobial and anthelmenthic activity of PGFP extracts.

MATERIALS AND METHODS

Collection and authetification of plant material: *Punica granatum* fruits were purchased from local market in Navi mumbai. The fruit peels were authenticated at Agharkar Research Institute, Pune, Maharashtra. The voucher specimen number is F-196.

Reagents and Chemicals*:* The chemicals and solvents used were of analytical grade. The solvents were purchased from Sigma Aldrich (USA). The nutrient agar and Sabouraud dextrose agar were purchased from Himedia (India).

Preparation of extracts: PGFP were separated from its seeds and dried at 40° C in hot air oven for 48 hours. Dried peels were grounded into powder using an electric blender (Remi). Petroleum ether, ethyl acetate and methanol extracts were prepared using Soxhlet extraction technique. Soxhlet extraction was carried for 18 hours at a temperature not exceeding the boiling point of the solvent. Aqueous (water) and methanol-water extracts were prepared by refluxing the dried peels powder with

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Table 1: Phytochemical analysis of PGFP extracts

Phytoconstituents	Aqueous	Methanol	Methanol-Water	Ethyl acetate	Petroleum ether
Carbohydrates	+	+	+	-	-
Proteins and amino acids	+	+	+	-	-
Glycosides	+	+	+	-	-
Saponins	-	-	-	-	-
Flavonoids	+	+	+	+	-
Alkaloids	-	+	+	-	-
Phenolic compounds and tannins	+	+	+	+	+
Fixed oils and fats	-	-	-	-	+
Sterols	-	-	-	+	+
Terpenoids	-	+	-	-	-

water and methanol:water (1:1) for 8 hours. The extracts were filtered using Whatman filter paper (No.1), concentrated in vacuum under reduced pressure using rotary flask evaporator and dried in a vacuum desiccator. The extracts were then kept in amber colored bottles at 2-4°C until further use.[13]

Phytochemical analysis: Phytochemical screening of the extracts was carried out for detection of different phytoconstituents including alkaloids, carbohydrates, proteins and amino acids, sterols and terpenoids, fixed oils and fats, saponins, phenolic compounds and tannins, flavonoids and glycosides.[9]

Antimicrobial activity-

Test Microorgainsms*:* The microorganisms selected for this study were, gram positive bacteria -*Staphylococcus aureus* (ATCC 29213), *Salmonella typhii* (MTCC 3214), *Vibrio Cholerae* (MTCC 3904), *Klebsiella pneumoniae* (NCIM 5082) and *Shigella flexneri* (MTCC 1457); gram negative bacteria- *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 9027), and yeast-*Candida albicans* (ATCC10231). The microbial cultures of these microorganisms were obtained from the Microbial Type Culture Collection and Gene Bank, Chandigarh and National Collection of Industrial Microorganisms, Pune.

Culture media and Preparation of Inoculum

The stock cultures were prepared by incubating pure cultures of bacteria on nutrient agar (Himedia, India) for 24 hours at 37±2°C and pure culture of yeast on Sabouraud dextrose agar (Himedia, India) for 72 hours at 25±2°C. The stock cultures were maintained at 4°C on agar slant. Culture inoculum was prepared by suspending the stock cultures in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 MacFarland standards (1X 10^6 CFU/ml).

Sample preparation*:* The methanol, methanol-water, aqueous and ethylacetate extracts were dissolved in sterile distilled water and petroleum ether extract was dissolved in Dimethyl Sulfoxide to facilitate the dissolution of the extracts in the agar.

Ditch Plate Technique*:* The susceptibility of microorganisms to various PGFP extracts was determined using Ditch Plate Technique. In this technique a ditch (1cm x3 cm) was aseptically made in the sterile agar plate using a sterile scalpal. Loopful of incoculum of each organism was streaked on the agar plate at right angle to ditch. 1ml of extract (50mg/ml) was added into the ditch. The plates were kept undisturbed at room temperature for diffusion period of 30 minutes. The nutrient agar plates containing bacterial cultures were incubated in incubator at 37±2ºC for 24 hours and the Sabouraud dextrose agar plates containing yeast at 25±2 º C for 72 hours. The susceptibility of microorganisms was determined by observing the zone of inhibition on the agar surface around the ditch.[10]

Agar well diffusion method

The susceptible microorganisms were tested further at various concentrations (20, 40 and 80 mg/well) by agar well diffusion method. In this method 1ml of culture suspension was added to 30ml of molten agar individually and cooled to 40°C. The molten agar was poured into a sterile petri plate and was allowed to set and harden. Wells of 11 mm diameter were cut aseptically using a sterile cork borer on the agar surface. Agar plates of each microorganisms were prepared. In wells of each plate, extract was loaded at different concentration and kept at room temperature for 30 minutes to allow diffusion of extract in the agar. The nutrient agar and Sabouraud dextrose agar plates were then incubated at 37ºC for 24 hours and at 25ºC for 72 hours respectively. Antimicrobial activity was evaluated by measuring the inhibition zone surrounding the well. Ciprofloxacin (2µg/ml) for bacterial strains and fluconazole (15ug/ml) for fungal strain were used as standards. Blank DMSO and distilled water served as control.[11]

Anthlementic activity: The anthelmintic activity was evaluated using Ghosh *et al method*. Indian adult earthworms *Pheritima posthuma* were used for the anthelmintic study. *Pheritima posthuma* were collected from moist soil and washed with normal saline to remove

Table 2: Antimicrobial activity of PGFP extracts by Ditch Plate Technique

PGFP extract (50mg/ml)	Aqueous	Methanol	Methanol:water	Ethyl Acetate	Petroleum ether
Microrganisms	Zone Of Inhibition (mm) Mean ± SEM				
S. aureus	1.83 ± 0.17	3.50 ± 0.29	2.50 ± 0.29	0.66 ± 0.17	-
E. coli	0.83 ± 0.17	5.83 ± 0.17	1.83 ± 0.17	0.83 ± 0.17	-
S. typhi	1.16 ± 0.17	3.66 ± 0.17	1.50 ± 0.29	1.00 ± 0.17	-
P. aeruginosa	1.00 ± 0.29	2.83 ± 0.17	1.33 ± 0.17	0.66 ± 0.17	-
V. cholera	0.50 ± 0.00	0.83 ± 0.17	0.83 ± 0.17	0.50 ± 0.00	-
K. pneumonia	1.83 ± 0.29	3.83 ± 0.17	2.33 ± 0.17	1.16 ± 0.17	-
S. flexneri	0.66 ± 0.29	1.83 ± 0.17	1.33 ± 0.33	0.66 ± 0.17	-
C. albicans	0.50 ± 0.00	2.83 ± 0.17	1.66 ± 0.17	1.00 ± 0.17	-

N=3, Values expressed as Mean±SEM

Table 3: Antimicrobial activity of PGFP Aqueous extract by Cup Plate Method

	Zone of Inhibition (mm) Mean±SEM		
Microorganisms	20mg/well	40mg/well	80mg/well
S. aureus	1.67±0.33	3.33±0.60	4.67±0.44
E. coli	1.67±0.18	4.33±0.33	13.00±0.5
S. typhii	2.00±0.58	3.00±0.00	8.67±0.441
P. aeruginosa	2.00±0.58	4.33±0.33	10.00±0.578
K. pneumonia	1.33±0.33	4.67±0.44	8.00±0.76
S. flexneri	2.00±0.00	2.67±0.33	7.00±0.57
V. cholera	1.33±0.18	3.00±0.58	4.67±0.33
C. albicans	1.67±0.33	2.33±0.67	5.67±1.20

Table 4: Antimicrobial activity of PGFP methanol extract by Cup Plate Method

	Zone of Inhibition (mm) Mean±SEM		
Microorganisms	20mg/well	40mg/well	80mg/well
S. aureus	4.66±0.33	5.66±1.20	9.33±1.45
E. coli	5.66±0.33	8.66±0.88	13.66±1.17
S. typhii	3.66±1.20	6.00±1.15	10.00±0.58
P. aeruginosa	4.33±0.33	7.00±0.58	11.00±1.15
K. pneumonia	4.33±0.33	7.00±0.00	10.33±1.01
S. flexneri	3.66±0.77	5.66±0.60	9.33±1.01
V. cholera	2.00±0.00	4.00±0.00	5.66±0.44
C. albicans	2.00±0.00	4.66±0.88	7.00±1.32

N=3, Values expressed as Mean±SEM

all faecal matter and soil. The earthworms of 3-5 cm in length and 0.1-0.2 cm in width were selected and used for the study. Earthworms were divided into different groups, each group containing six earthworms. Earthworms of test groups were released in extract solutions respectively

Table 5: Antimicrobial activity of PGFP Methanol:water extract by Cup Plate Method

	20mg/well	40mg/well	80mg/well
Microorganisms	Zone of Inhibition (mm) Mean±SEM		
S.aureus	3.33±0.33	3.33±0.17	6.33±0.67
E. coli	4.33±0.60	6.33±0.17	10.00±0.76
S. typhii	2.00±0.58	6.33±0.33	8.00±0.58
P. aeruginosa	3.00±0.58	5.00±0.58	9.00±0.76
K. pneumonia	3.33±0.44	5.67± 0.88	10.33±0.33
S. flexneri	2.00±0.29	3.00±0.58	7.33±0.67
V. Cholerae	1.33±0.33	2.67±0.88	3.33±0.17
C. albicans	1.67±0.17	2.67±0.33	5.33±0.44

N=3, Values expressed as Mean±SEM

Table 6: Antimicrobial activity of PGFP Ethyl acetate extract by Cup Plate Method

	Zone of Inhibition (mm) Mean±SEM			
Microorganisms	20mg/well	40mg/well	80mg/well	Ciprofloxacin (1ug/well)
	Zone of Inhibition (mm) Mean ± SEM			
S. aureus	0.83±0.17	4.83±0.44	7.17±0.44	12.00±1.04
E. coli	1.00±0.00	7.50±0.29	9.33±0.33	15.00±1.53
S. typhii	1.30±0.33	6.60±0.44	8.50±0.29	13.00±0.87
P. aeruginosa	0.67±0.17	6.00±0.29	7.00±0.29	13.33±1.20
K. pneumoniae	0.50±0.00	6.00±0.29	6.17±0.17	14.00±0.76
S. flexneri	1.67±0.33	6.50±0.29	6.00±0.57	13.00±0.87
V. Cholerae	0.67±0.17	4.50±0.50	6.17±0.17	11.00±0.76
				Fluconazole (15ug/well)
C. albicans	0.83 ± 0.167	5.33 ± 0.441	6.50 ± 0.287	12.00 ± 0.577

N=3, Values expressed as Mean±SEM

(10, 20, 40, 60, 80 and 100 mg/ml) and the volume was made upto 50ml with distilled water. Piperazine citrate (10mg/ml) was used as standard while water served as a normal control. Observations were made for the time taken for paralysis and death of the individual worms. *Time for the paralysis* (P) in minutes of each earthworm was noted when marked decrease in vigorous wriggling movement of the worm was observed. *Time of death* (D) in minutes was recorded when no movement of worm was seen after being pricked with pin. Slow movement of the worm after being pricked with pin indicates paralysis. Death was confirmed by dipping the worm in warm water at 50°C and shaken vigorously, no response and fading away of body color indicated earthworm death[12].

Statistical analysis: Antimicrobial activity was conducted in triplicate to confirm the reproducibility of the results. Values are expressed as Mean±SEM. One way ANNOVA was performed to determine significance level of anthelmentic activity and $p \leq 0.05$ was regarded as statistically significant.

RESULTS AND DISCUSSION

Phytochemical analysis of extracts: The phytochemical analysis of extracts revealed the presence of carbohydrates, proteins, glycosides, alkaloids, phenols and tannins, sterols and terpenoids, fixed oils and fats (Table 1).

Antimicrobial activity: Preliminary antimicrobial activity by ditch plate technique revealed that all the microorganisms were susceptible to ethyl acetate, methanol, methanol:water and aqueous extract of PGFP extracts . None of the microorganisms were susceptible to petroleum ether extract (Table 2). The detailed evaluation of antimicrobial activity of these extracts by agar well diffusion method showed dose dependent inhibitory action against bacteria and yeast. Aqueous extract was found to be most effective against *E.coli* and least

effective against *S. aureus* and *V. cholera* (Table 3). Methanol extract was found to be most effective against *E.coli* and least effective against *V. cholera* (Table 4). Methanol-water extract was found to be most effective against *K. pneumonia* and least effective against *V.cholera* (Table 5). Ethyl acetate extract was found to be most effective against *E.coli* and least effective against *S. Flexneri* (Table 6). The methanol extract of PGFP extract showed highest dose dependent antimicrobial activity by agar well diffusion method but was less as compared to that exhibited by standards.

S.flexneri and *V.cholera* are common pathogens causing GI infection. So PGFP methanol extract can be used to treat GI tract infection.[13]

Anthelmintic activity: *In vitro* anthelmintic activity was evaluated using adult Indian earthworm, *Pheretima posthuma* due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings and easy availability. The results revealed paralysis caused by all the extracts ranging from loss of motility to loss of response to external stimuli, which eventually progressed to death at all tested dose levels. Anthelmintic activity of the extracts was compared with reference standard piperazine citrate. The predominant effect of piperazine citrate on the worm is to cause a flaccid paralysis that result in expulsion of the worm by peristalsis. Piperazine citrate, by increasing chloride ion conductance of worm muscle membrane, produces hyper polarisation and reduces excitability that leads to muscle relaxation and flaccid paralysis. The worms were found to be most sensitive to the methanol extract as can be seen in Table 7. The aqueous at 100mg/ml, methanol at 20mg/ml, and methanol:water at 40mg/ml extracts of PGFP demonstrated paralysis compared to piperazine citrate (within 24.5 minutes) but the death occurred was after more time as compared to piperazine citrate (43.5 minutes). The ethyl acetate extract demonstrated paralysis or death time upto 100mg/ml less than piperazine citrate. The petroleum ether extract at 10, 20 40 and 60 mg/ml exhibited no paralysis and no death but at 80 and 100mg/ml exhibited only paralysis at 155.5 and 164 minutes.

Antimicrobial and anthelmenthic effects of PGFP are being reported from various parts of the world. In the present work, among all the extracts of PGFP prepared in different extraction solvents of variable polarity, the methanol extract showed prominent antimicrobial activity and anthelminthic activity in dose dependent manner. Phytoconstituents such as phenols, tannins, flavonoids and glycosides which are extracted in majorly in methanol (polar solvent) may be responsible for the antioxidant and anthelmintic activity.

Also the PGFP is the waste from agro-food industry, so the use of PGFP to treat microbial and helminths infection can be economical and helpful in waste management in better way.[14]

Table 7:

PGFP extract	Aqueous		Methanol		Methanol: Water		Ethylacetate		Petroleum ether	
Conc. (mg/ ml)	Para lysis time	Death time	Para lysis time	Death time	Para lysis time	Death time	Para lysis time	Death time	Para lysis time	Death time
	Mean±SEM									
10	40.60 ± 0.72**	125.5 ± 1.71**	27.16 ± 0.72**	85.33 ± 1.50*	36.5 ± 0.71**	109.66 ± 0.99**	67.66 ± 0.99**	163.16 ± 1.91**	No para Lysis	No Death upto 24 hours
20	36.66 ± 0.72**	117.83 ± 2.04**	24.5 ± 0.76ns	73.5 ± 1.38**	31.83 ± 0.60**	101.66 ± 1.23**	55.66 ± 1.05**	142.66 ± 0.95**		
40	31.66 ± 0.70**	104.26 ± 1.71**	18.16 ± 0.54**	62.00 ± 1.26**	27.08 ± 0.78**	87.33 ± 1.54**	46.5 ± 0.89**	132.5 ± 1.41**		
60	30.83 ± 0.86**	94.42 ± 1.54**	17.17 ± 0.40**	57.50 ± 1.57**	25.58 ± 1.05ns	85.17 ± 0.79**	36.00 ± 0.10**	125.42 ± 1.46**		
80	29.71 ± 0.86**	85.66 ± 1.12**	16.5 ± 0.77**	47.50 ± 1.34ns	19.16 ± 0.79ns	77.33 ± 1.94**	36.83 ± 0.83**	112.66 ± 1.82**	155.5 ± 0.85**	
100	26.33 ± 0.72**	81.33 ± 1.48ns	9.5 ± 0.43**	33.66 ± 1.26**	15.66 ± 0.67*	63.66 ± 1.28**	33.5 ± 0.77**	103.83 ± 1.25**	164 ± 1.03**	
Std (10)	24.5 ± 0.76	43.5 ± 1.31	-	-	-	-	-	-	-	

*N=6; ns=nonsignificant, *p≤ 0.5, **p≤0.01, One way ANNOVA followed by Dunnets test as compared to positive control group, Mean time for the paralysis (P) Time of death (D) in min*

In addition to this *S. aureus, S. typhi and P. aeruginosa* had been reported as the causal agents of food borne diseases. So the PGFP extracts can also be used as natural antimicrobials or preservatives in food products to control food borne pathogens.[15] The anthelmenthic activity of the extracts against worms suggests that it could be effective against parasitic infections of humans.[12] However studies using intestinal worms required to be done to confirm the activity.

CONCLUSION

Ethylacetate, methanol, methanol-water and aqueous of PGFP exhibited antimicrobial and anthelmintic activity**.** PGFP methanol extract showed most prominent antimicrobial activity and anthelmintic activity.

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