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Research Article

Antioxidant, antimicrobial and cytotoxic activities of rind of *Punica granatum*

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ABSTRACT

Punica granatum is traditionally used in diarrhea and dysentery. It is also used as refrigerant, stomachic and astringent. It is also reported to have anthelmintic, antioxidant and antiviral activities. Our present study investigated the antioxidant, antimicrobial and cytotoxic properties of rind of *P. granatum* using various *in vitro* methods. The mature fruit rind of *P. granatum* was extracted with methanol. The extract was then investigated for its possible antioxidant activities by DPPH radical scavenging activity assay, Cuprac reducing antioxidant activity assay and total antioxidant capacity assay. Antimicrobial activity and minimum inhibitory concentration (MIC) were performed by disc diffusion and broth dilution assay against 8 pathogenic bacteria. Brine shrimp lethality bioassay was performed to investigate the general cytotoxic potential of the extract. The plant extract showed strong DPPH radical scavenging capacity with IC_{50} 54.93 µg/ml compared to the IC_{50} of the standard Ascorbic acid 13.76 µg/ml. Total phenol, flavonoid, and antioxidant content of the extract were 20.1087 mg/g, 26.73469 mg/g and 210.6481 mg/g respectively. Cupric ion reducing capacity and total antioxidant capacity of the extract was also found very high compared to the standard. The antimicrobial activity exerted by the extract was also significant compared to the standard. The order of zone of inhibition was *Escherichia coli* (32.33 mm) > *Staphylococcus aureus* (25.33 mm) > *Salmonella typhimurium* (24.33 mm) > *Vibrio cholerae* (20.33 mm) > *Bacillus subtilis* (19.67) > *Klebsiella pneumoniae* (18.67 mm) > *Shigella boydii* (18.33 mm) > *Sarcina lutea* (17.33 mm).The MIC values was in the range 0.8 mg/ml to 3.2 mg/ml. The lowest MIC was found for *Salmonella typhimurium* and *Vibrio cholera* and the largest for *Bacillus subtilis, Shigella boydii* and *Staphylococcus aureus*. The plant extract also showed strong cytotoxic activity and the LC_{50} was found 3.99 µg/ml compared to the known anticancer drug Vincristine sulfate 0.66 µg/ml. The results in this work suggest that the rind of *Punica granatum* possesses significant antioxidant, cytotoxic and antimicrobial properties.

Keywords: *Punica granatum*, medicinal plant, antioxidant activity, cytotoxic activity, antimicrobial activity.

INTRODUCTION

Punica granatum Linn. (Pomegranate) locally known as 'Anar/Bedana' from the punicaceae family is a delicious fruit consumed widely among the Bangladeshi people. It has long been esteemed as food and medicine, originated from Iran and cultivated from ancient time throughout the Mediterranean region of Asia, Europe and Africa[1,2]. Various parts of this plant, like fruit, fruit juice, bark, leaves, peel, immature fruit have been reported to have various chemical constituents and various medical significance notably antioxidant activity, antibacterial activity, anticancer activity, anti-inflammatory activity, neuroprotective activity, antiatherogenic activity, antidiarrhoeal activity, antiglycemic and hypoglycemic activity and antispermatogenic activity[3-8]. Research on the chemical constituents of pomegranate have revealed the presence of ellagitannins (punicalagin), anthocyanin (delphinidin, cyanidin and pelargonidin 3- glucosides and 3,5-diglucosides), ellagic acid,

punicic acid, flavonoids, flavonols and many other constituents which are further correlated to the antioxidant potential of this plant[9]. Various *in vitro* studies of the various components of this plant have found significant antibacterial, antiviral and antiproliferative effects[10]. Ellagic acid, one of the constituents of *Punica granatum* juice and seed oils are reported to act in prostate, pancreas, intestine, skin, oesophagus, bladder, liver, breast, skin, colon, lung, neuroblastoma and leukaemia cancers through antioxidant, antiproliferation (growth inhibition, cell cycle disruption and apoptosis), antiangiogenisis and anti-inflammatory mechanisms of action[11-14].

Traditionally this plant has been used as astringent, refrigerant, blood purifier, tonic, cooling agent and also used against tapeworm, diarrhea, dysentery and hemorrhages[15]. To explore the therapeutic potential of *Punica granatum* in various disease conditions like male infertility, obesity, osteoarthritis and Alzheimer's disease numerous clinical trials are in progress[16]. Medicinal plants have secured a trustworthy position in the treatment of various diseases from time immemorial. Natural products derived from plant have also forms the basis of the treatment of human disease. The popularity of using plant derived medicines is increasing from the last century due to having lower side effects, low cost and availability of the product. Currently about 80% of world population relies on plant derived medication for the first line management of diseases[17]. Previous studies have suggested that the rind of *Punica granatum* has strong antidiarroheal activity[18], so our present study is designed to study the antioxidant, antimicrobial and cytotoxic activity of the rind of *Punica granatum* to authenticate the traditional uses and to enrich the information of the medicinal values this plant possess.

MATERIALS AND METHODS

Plant material and extract preparation

Mature fruit of *Punica granatum* was collected from Mohakhali Kacha Bazar, Dhaka, Bangladesh in the month of July, 2012. The sample was then identified by the taxonomist of the National Herbarium, Mirpur, Dhaka, Bangladesh (Accession Number 37848) where a Voucher specimen has been deposited for further reference. The rind part of the collected fruit was then dried and grinded. Then 250 gm of the sample was macerated with 500 ml methanol for seven days with occasional stirring. It was then filtered, dried and the yield was 14%[19].

Chemicals

Folin Ciocalteu reagent, gallic acid and methanol were purchased from Merck Co. (Germany). Griess reagent from Roch-light Ltd., Suffolk, England; Sodium Phosphate (Na3PO4) from Merck, Mumbai, India; Ammonium Molybdate from Merck DGaA, Germany and Quercetin from Sigma Chemicals, USA. Sodium nitroprusside was obtained from BDH Chemicals Ltd., Poole, England, Ascorbic acid from SD Fine chem. Ltd., Biosar, India and 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) and quercetin were purchased from Sigma Chemical Co. (St., Louis, USA).

Microorganisms

Pure culture of *Escherichia Coli*, *Bacillus subtilis* *Klebsiella pneumoniae*, *Vibrio cholerae*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Sarcina leutea*, and *Shigella boydii* were obtained from the Microbiology laboratory of Department of Pharmacy, Primeasia University, Dhaka, Bangladesh (106 CFU ml^{-1}).

Determination of Total Polyphenol contents

The total phenolic compounds content in the extract was determined by Folin-Ciocalteu reagent[20]. 500 µl of Folin- Ciocalteu reagent and 4 ml of 7.5% sodium carbonate solution were added in 1 ml of extract (200 µg/ml). The mixture was then incubated for 1 h at 20°C. The absorbance of the solution was measured at 765 nm using a UV spectrophotometer (Shimadzu, UV-1601PC) against blank. The total phenolic compounds content was calculated in gallic acid equivalents (GAE) using the following formula: $A = (C \times V) / m$; where A is the total content of phenolic compounds, mg/g plant extract in GAE; C is the concentration of gallic acid established from the calibration curve, mg/ml; V is the volume of extract in ml and m is the weight in gram of plant extract.

Determination of total flavonoid contents

The total content of flavonoid of the crude extract was determined by using standard procedure[21]. 3 ml methanol, 200 µl (10%) aluminium chloride solutions and 200 µl (1 M) potassium acetate solution were mixed with 1 ml of extract solution (100 µg/ml) or different concentrations of quercetin (standard). The mixture was then incubated for 30 min at room temperature after adding 5.6 ml of distilled water. The absorbance of the solution was measured at 415 nm using a UV spectrophotometer (Shimadzu, UV-1601PC) against blank. The total content of flavonoid compounds in plant extract was calculated and expressed as mg of quercetin equivalents (QE) / g of extract.

Determination of total antioxidant capacity

The phosphomolybdenum method was used to determine the total antioxidant capacity of the plant extract[22]. A mixture was made by adding 0.3 ml

extract with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The reduction of Mo (VI)-Mo (V) by the extract followed by the formation of a green phosphate/Mo (V) complex at acid pH forms the basis of this method. The absorbance was measured at 695 nm using a UV spectrophotometer (Shimadzu, UV-1601PC) against blank after cooling the reaction solution at room temperature. The antioxidant activity of the extract was expressed as mg of ascorbic acid equivalents / g of extract.

DPPH Assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging by the extract was determined by using standard procedure[23]. 2 ml of a 0.004% methanol solution of DPPH was mixed with 200 µl plant extract of different strength (5, 25, 50, 100, 500 µg/ml). After 30 min, the absorbance was determined at 517 nm using a UV spectrophotometer (Shimadzu, UV-1601PC) against a blank where absorbance of DPPH solution only without the extract or standard agent was used as control. The percentage scavenging activity of the extract was calculated using the formula: % scavenging activity = {(A0 – A1) / A0} × 100; where A0 is the absorbance of the control and A1 is the absorbance of the extract or standard.

Cuprac reducing antioxidant activity assay

The cupric ion reducing capacity of the plant extract was performed by using standard procedure[24]. 500 µl of plant extract or standard of different concentrations (5, 25, 50, 100, 200 µg/ml) was mixed with 1.0 ml of 0.01 M $CuCl_2.2H_2O$ solution. Then 1.0 ml of ammonium acetate buffer (pH 7.0) was mixed followed by the addition of 1.0 ml of 0.0075 M of neocaproine solution. Finally, 600 µl of distilled water was added and make the final volume of the mixture to 4.1 ml. After making final volume, the total mixture was incubated for 1 h at room temperature. Then the absorbance of the solution was measured at 450 nm using a UV spectrophotometer (Shimadzu, UV-1601PC) against blank.

Screening of antimicrobial activity

Antimicrobial activity of the plant extract was tested by disc diffusion method[25]. The extract solution was prepared at a concentration of 300 µg/ml. Then the paper discs were impregnated by using the extract and placed onto the nutrient agar media inoculated with the test bacteria and incubated at 37° C for 24 h. Amoxicillin (30 µg/disc) (oxoid, UK) were used as positive control and blank disc (impregnated with solvents followed by evaporation) were used as negative control. After incubation the culture plates were examined and the assessment of antimicrobial activity was based on the measurement of diameter of the inhibition zone formed around the disc. For each test the mean of triplicate results were taken.

Determination of the minimum inhibitory concentration (MIC)

Determination of the minimum inhibitory concentration (MIC) was carried using the broth dilution method[26]. Briefly, 1.0 ml of the reconstituted extract solution at a concentration of 200 mg/ml was added to another test tube containing 1 ml of sterile broth so as to obtain a concentration 100 mg/ml. 1 ml of this dilution was transferred to another test tube till the 10^{th} test tube was reached. The 11^{th} test tube did not contain any extract, but a solution of pure solvent served as negative control. Then 1 ml of an 18 h old culture of each of the organisms earlier adjusted at 10^6 CFU/ml was put into each tube and thoroughly vortexed. The tubes were incubated at 37° C for 24 h and observed for growth in form of turbidity. The test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC.

Cytotoxic activity

The cytotoxic activity of the crude extract was evaluated using Brine shrimp lethality bioassay according to standard procedure[27]. *Artemia salina* Leaches (brine shrimp eggs) was collected and taken in a small tank of water at 37°C with constant oxygen supply to allow hatching of the shrimps for two days and to mature as nauplii. 5 ml of seawater was taken in each vial. Specific volume of the test samples were taken and dissolved in specific volume of dimethyl sulfoxide (DMSO) to get stock solution. Serial dilution method was used to prepare a series of solutions of varying concentrations from the stock solution and was added to the vials. The concentrations of the obtained solutions in the vials were as 1 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 500 µg/ml. 10 nauplii were added by micropipette to each of the vials. After 24 h, a magnifying glass was used to inspect the vials and the number of survived nauplii in each vial was counted. The percentage of lethality of the brine shrimp nauplii was calculated for each concentration.

Statistical analysis

Analyses were performed in duplicates for antioxidant studies and triplicates for antimicrobial study. Data were presented as mean ± SD. SPSS 11.5 was used to calculate the LC_{50} in cytotoxicity study using probit chart.

RESULTS

Total phenol, flavonoid and antioxidant contents

Total phenol, flavonoid, and antioxidant contents of methanol extract of the rind of *P. granatum was* expressed as gallic acid equivalent (GAE) per gram of plant extract, quercetin equivalents(QE) per gram of plant extract and ascorbic acid equivalents(AAE) per gram of plant extract respectively. Table 1 shows the amount of total phenol, flavonoid, and antioxidant contents of the extract in mg/g of the respective standards. It is found from the table that the total antioxidant content is high in the plant extract.

DPPH radical scavenging activity

DPPH was used to evaluate the possible antioxidant potential present in the extract by its radical scavenging capacity measurement. The result of the present study shows that the plant extract possesses significant DPPH radical scavenging activity and the IC_{50} is 54.93 µg/ml compared to the IC_{50} of 13.76 µg/ml of the standard antioxidant ascorbic acid (Table 2).

Cuprac reducing antioxidant activity assay

Cupric ion reducing activity of the rind extract of *P.granatumm* was evaluated with the standard ascorbic acid. At the concentrations of 5, 25, 50, 100 and 200 µg/ml, the cupric ion reducing activity of the extract was significant compared to the standard and both the extract and standard showed a concentration dependent steady state increase in cupric ion reduction (Figure 1).

Antimicrobial Activity

The antimicrobial activity of methanol extract of the rind of *P. granatum* (PGR) was shown in Table 3. Amoxicillin (30 µg/disc) was used as standard and the zone of inhibition observed by the antibiotic against the tested organisms was also showed in the table. The result showed that the zone of inhibition observed by the extract against the tested bacteria was significant compared to the standard. The highest zone of inhibition observed by the extract was 32.33 mm for *Escherichia coli* and the lowest inhibition zone is 17.33 mm for *Sarcina lutea*. The order of zone of inhibition of PGR is: *Escherichia coli* (32.33 mm) > *Staphylococcus aureus* (25.33 mm) > *Salmonella typhimurium* (24.33 mm) > *Vibrio cholerae* (20.33 mm) > *Bacillus subtilis* (19.67) > *Klebsiella pneumoniae* (18.67 mm) > *Shigella boydii* (18.33 mm) > *Sarcina lutea* (17.33 mm).Standard antibiotic Amoxicillin produced significant zones of inhibition against all the tested bacterial while no zone of inhibition was observed for negative control which may be correlated with their no involvement in the antimicrobial activity of the extract.

Minimum inhibitory concentration (MIC)

The Minimum inhibitory concentration (MIC) of the methanol extract of rind of *Punica granatum* (PGR) against eight tested organisms was shown in Table 4. The inhibitory concentration was in the range 0.8 mg/ml to 3.2 mg/ml. The highest concentration required to inhibit the growth of the bacteria was 3.2 mg/ml for *Bacillus subtilis*, *Shigella boydii* and *Staphylococcus aureus.* The lowest concentration required to inhibit the growth of the bacteria was 0.8 mg/ml for *Salmonella typhimurium* and *Vibrio cholerae.*

Cytotoxic Activity

The result of cytotoxic activity of rind of *P. granatum* is shown in Table 5. The extract showed significant cytotoxic activity against brine shrimp nauplii with the LC_{50} value 3.99 µg/ml compared to the LC_{50} of standard anticancer drug Vincristine Sulfate 0.66 µg/ml.

DISCUSSION

Plant phenolic compounds like hydroxy-cinnamic acids (HCAs), flavonoids, anthocyanins and tannins are thought to exert different health benefits in coronary heart disease, cancer and diabetes. The possible mechanism behind these may be due to their role as antioxidants, anticarcinogens and cardioprotective agents[28]. The antioxidant activity of the polyphenolic compounds is believed to be mainly due to their redox properties[29]. Flavonoids are major plant secondary metabolites with good antioxidant properties. It is also reported to have antimutagenic and antimalignant activities[30]. Results obtained in the present study revealed that the level of these phenols and flavonoid compounds in the extract of rind of *P. granatum* were significant (Table 1). The total antioxidant potential of the extract was estimated from their capacity to reduce the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The total antioxidant activity of the extract was significant to give desired antioxidant activity. The scavenging of DPPH radical occurs either by a direct H-atom-abstraction process or a proton concerted electron-transfer process[31]. The free radical scavenging activity of flavonoids are well established and a major area of dietary antioxidant research. From our present study it is suggested that, *P. granatum* extract possess significant DPPH radical scavenging activity compared to the standard Ascorbic Acid. This may be due to the presence of flavonoids, as our previous work has revealed the presence of flavonoids in the rind extract of this plant[18]. Cuprac reducing capacity assay is a method for dietary polyphenols, vitamin C

and vitamin E. The Cuprac absorbance of the extracts could be due to the reduction of Cu (II)-neocuproine reagent which may be decreased in the presence of OH scavengers and this reduction may also supports the scavenging ability of the extract[32, 33]. The extract showed significant cuprac reducing capacity compared to the standard which can also support the antioxidant potential of this plant extract.

The presence of alkaloids in plant extract may contribute to the antibacterial activity[34] by intercalating the DNA and inhibiting DNA synthesis, cell lysis or by causing the morphological changes[34-36]. Antibacterial activity of plant extract may also be exerted due to the presence of plant secondary metabolites such as flavonoids[37]. Previous studies on this plant reported the presence of plant secondary metabolites alkaloids, tannins, flavonoids and glycosides in the rind extract of this plant[18]. The result of our present study suggests that, rind extract of *P. granatum* contains significant antimicrobial activity against a number of pathogenic bacterial strains (Table 3) which may be due to the presence of the phytochemical constituents present in the plant. MIC results also support the antimicrobial claim as very low concentration of the extract is effective against the tested bacterial strains to inhibit their growth (Table 4) and also suggests that this rind part of this plant possesses strong antimicrobial activity. The secondary metabolite present in the extract such as alkaloids may also be responsible for the cytotoxic potential of the extract[38]. From the results (Table 5) of the cytotoxic activity study, it can be said that the rind extract of *P. granatum* possesses significant cytotoxic potential compared to the standard anticancer drug Vincristine Sulfate. The phytoconstituents present in the plant extract may also be responsible for the given cytotoxic activity of this plant.

Table 1
Total phenol, flavonoid and antioxidant contents of the extract of *P. granatum*.

Extract	Total phenol (in mg/g , Gallic acid equivalents)	Total flavonoid (in mg/g, quercetin equivalents)	Total antioxidant (in mg/g, ascorbic acid equivalents)
PGR	20.11 ± 11.54	26.73 ± 5.89	210.65 ± 2.62

PGR refers to the rind extract of *Punica granatum.*
Results are expressed as mean ±SD (standard deviation).

Table 2
DPPH radical scavenging capacity of the extract of *P. granatum*.

Extract	IC_{50} values (µg/ml)
PGR	54.93
Ascorbic Acid	13.76

Table 3
Antimicrobial activity of the extract of *P. granatum* using disc diffusion method.

	Diameter of inhibition zone in mm Scale	
Test organisms	Amoxicillin (30 µg/disc) in mm	PGR (300 µg/disc) in mm
Escherichia coli	42	32.33 ± 2.05
Salmonella typhimurium	24	24.33 ± 0.94
Bacillus subtilis	28	19.67 ± 2.36
Shigella boydii	25	18.33 ± 2.62
Vibrio cholerae	22	20.33 ± 0.94
Klebsiella pneumoniae	26	18.67 ± 1.25
Staphylococcus aureus	40	25.33 ± 2.05
Sarcina lutea	38	17.33 ± 2.49

Data are expressed as mean ± SD of triplicate experiments.

Table 4
Minimum inhibitory concentration (MIC) of the extract of *P. granatum.*

Test organisms	MIC in mg/ml
Escherichia coli	1.6
Salmonella typhimurium	0.8
Bacillus subtilis	3.2
Shigella boydii	3.2
Vibrio cholerae	0.8
Klebsiella pneumoniae	1.6
Staphylococcus aureus	3.2
Sarcina lutea	1.6

Table 5
Cytotoxic activity of the extract of *P. Granatum.*

Extract	Conc. (µg/ml)	Log Conc.	% Mortality	LC_{50} (µg/ml)
PGR	1	0	0	3.99
	5	0.7	10	
	10	1	20	
	20	1.3	10	
	50	1.7	30	
	100	2	40	
	200	2.3	90	
	500	2.7	100	
Vincristine sulfate	0.078125	-1.10721	20	0.66
	0.15625	-0.80618	30	
	0.3125	-0.50515	40	
	0.625	-0.20412	50	
	1.25	0.09691	60	
	2.5	0.39794	80	
	5	0.69897	90	
	10	1	100	
	20	1.30103	100	

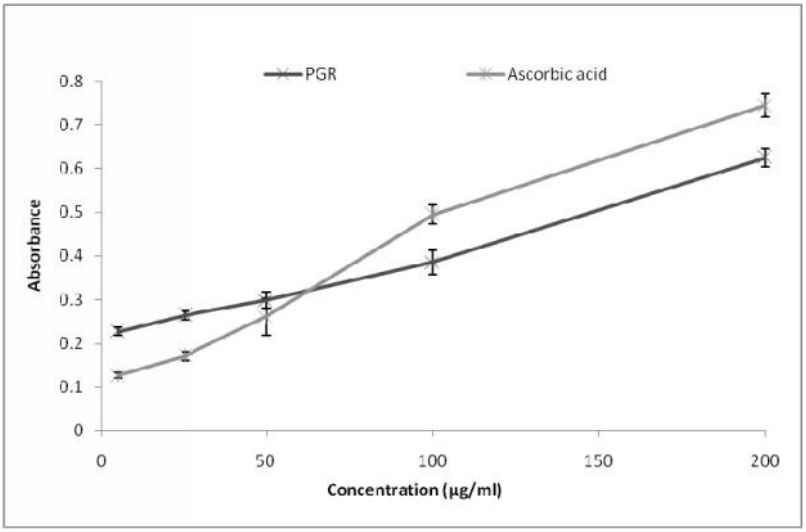

Figure 1
Cuprac reducing antioxidant activity of the extract of *P. granatum*.

CONCLUSION

The findings of our present work emphasizing on the antioxidant, antimicrobial and cytotoxic activity of the rind of *Punica granatum* have revealed that this plant have significant antioxidant, antimicrobial and cytotoxic activity which is further need to be authenticated by isolating and exploring the responsible chemical constituents for the given activities and this could lead to the discovery of some novel molecules in this particular field of interest.

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