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Antioxidant, Antidiarrhoeal and Cytotoxic Properties of *Punica granatum* Linn.

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SUMMARY. The present study was designed to investigate antioxidant, antidiarrhoeal and cytotoxic potential of hydromethanolic extract of the fruit rind of *Punica granatum* Linn. A dose dependent scavenging of DPPH radical and NO was observed with significant total antioxidant capacity with the plant extract in 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, total antioxidant capacity and nitric oxide (NO) scavenging assays. The extract was also studied for antidiarrhoeal property using castor oil and $MgSO_4$-induced diarrhoeal model, and charcoal induced gastrointestinal motility test in mice. At the doses of 200 and 400 mg/kg body weight, the extract reduced the frequency and severity of diarrhoea in test animals throughout the study period. At the same doses, the extracts significantly ($p < 0.001$) delayed the intestinal transit of charcoal meal in test animals as compared to the control. The extract also displayed strong cytotoxic potential with LC_{50} value of 10 μg/ml in brine shrimp lethality bioassay.

INTRODUCTION

Punica granatum (Family-Punicaceae), locally known in Bangladesh as 'Dalim' or 'Bedana', is a highly ornamental large deciduous shrub or small tree. It is planted all over Bangladesh as a fruit plant. Different chemical substances, particularly from the bark, fruit rind and fruit juice, have been reported in the plant [1]. Phytochemical analysis of the seeds have revealed the presence of ursolic acid, β-sitosterol, estrogens and phenolic glycosides [2] while the rind has been reported to contain tannins (ellagitannins, punicalagin), flavonoids and colouring matters [3]. Root bark, stem bark and rind are astringent and anthelmintic; fruit rind is particularly useful in the treatment of diarrhoea [4] and dysentery. Pulp of fruit is also astringent and stomachic. Methanolic extract of fruit rind exhibits significant antibacterial and antioxidant activity [1,5].

Literature reviews indicated that no studies combining the antioxidant, antidiarrhoeal and cytotoxic activities of the fruit rind have so far been undertaken. Taking this in view and as part of our ongoing search [6-10] on Bangladeshi medicinal plants the present study aimed at evaluating the antioxidant, antidiarrhoeal and cytotoxic properties of the fruit rind extract of *Punica granatum*.

MATERIALS AND METHODS

Chemicals and drugs

DPPH (1, 1-diphenyl, 2-picryl hydrazyl) was obtained from Sigma chemical co. USA, Ascorbic acid from SD Fine chem. Ltd., Biosar, India, Naphthyl ethylene diamine dihydrochloride from Roch-light Ltd., Suffolk, England and Sodium nitro prusside was obtained from Ranbaxy Lab., Mohali, India. Loperamide and Atropine were purchased from local market.

KEY WORDS: Antioxidant, antidiarrhoeal, cytotoxicity, DPPH, *Punica granatum*.

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Plant material

The fruits of *Punica granatum* were collected from the local market in Mohammadpur, Dhaka, Bangladesh in April, 2008 and identified by experts in Bangladesh National Herbarium, Mirpur, Dhaka where the Voucher specimen no: 32895 has been retained for future reference.

Extraction

The rind of *Punica granatum* was manually separated from the whole fruits, dried in hot-air woven, powdered and extracted with a mixture of methanol: water (7:3, v/v) by a Soxhlet apparatus at 65 ºC. The solvent was completely removed and the dried crude extract thus obtained was used for investigation

Animals

Swiss albino mice of either sex, 3-4 weeks of age, weighing between 20-25 gm, were collected from the animal research branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDRB). Animals were maintained under standard environmental conditions (temperature: (24.0 ± 1.0 °C), relative humidity: 55-65% and 12 hlight/12 h dark cycle) and had free access to feed and water . The animals were acclimatized to laboratory condition for one week prior to experiments.

Phytochemical Screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. These were identified by characteristic color changes using standard procedures [1].

Tests For Antioxidant Activity

DPPH radical scavenging activity

The free radical scavenging activity of the extract, 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.* [11]. Plant extract (0.1 ml) was added to 3 ml of a 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage inhibition activity was calculated from $[(A_0–A_1)/A_0]$ x100, where A0 is the absorbance of the control, and A_1 is the absorbance of the extract/ standard. The inhibition curves were prepared and IC_{50} values were calculated.

Determination of total antioxidant capacity

The total antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al.* 1999 [12]. 0.3 ml of extract solution in various concentrations was combined with 3 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Nitric oxide scavenging assay

Nitric oxide radical scavenging was estimated on the basis of Griess Illosvoy reaction using method followed by Govindarajan *et al.* [13]. In this investigation, Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5 %). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and *P. granatum* extract (5 to 250 µg/ml) or standard solution (ascorbic acid, 0.5 ml) was incubated at 25 °C for 150 min. After incubation, 0.5 ml of the reaction mixture mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25 °C. A pink coloured chromophore formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

Tests For Antidiarrhoeal Activity

Castor oil-induced diarrhoea

The experiment was performed according to the method described by Shoba & Thomas [14]. Briefly, mice fasted for 24 h were randomly allocated to four groups of five animals each. The animals were all screened initially by giving 0.5 ml of castor oil. Only those showing diarrhoea were selected for the final experiment. Group I received 1% CMC (10 ml/kg, *p.o.*), groups III and IV received orally the drug extract (200 and 400 mg/kg), respectively. Group II was given Loperamide (3 mg/ kg, *p.o.*) in suspension. After 60 min, each animal was given 0.5 ml of castor oil, each animal was placed in an individual cage, the floor of which was lined with blotting paper which was changed every hour, observed for 4 h and the characteristic diarrhoeal droppings were recorded.

Magnesium sulphate-induced diarrhoea

Diarrhoea was induced by oral administration of magnesium sulphate at the dose of 2 g/kg to the animals 30 min after pre-treatment with vehicle (1% Tween 80 in water, 10 ml/kg, *p.o.*) to the control group, loperamide (3 mg/kg) to the positive control group, and the methanol extract at the doses of 200 and 400 mg/kg to the test groups [15].

Effect on gastrointestinal motility

Animals were divided into four groups of five mice each and each animal was given orally 1 ml of charcoal meal (5% activated charcoal suspended in 1% CMC) 60 min after an oral dose of drugs or vehicle. Group I was administered 1% CMC (10 ml/kg) and animals in groups III and IV received extract at the dose of 200 mg/kg and 400 mg/kg body weight respectively. Group II received atropine sulfate (0.1 mg/kg,) as the standard drug. After 30 min, animals were killed by light ether anaesthesia and the intestine was removed without stretching and placed lengthwise on moist filter paper. The intestinal transit was calculated as a percentage of the distance travelled by the charcoal meal compared to the length of the small intestine [16].

Cytotoxic Activity Test

Brine shrimp lethality bioassay was used for probable cytotoxic action [17,18]. The eggs of Brine shrimp (*Artemia salina* Leach) was collected and hatched in a tank at a temperature around 37 °C with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solution the sample was prepared by dissolving required amount of extract in specific volume of pure dimethyl sulfoxide (DMSO). Four ml of seawater was given to each of the vials. Then specific volumes of sample were transferred from the stock solution to the vials to get final sample concentrations of 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320 µg/ml. In the control vials same volumes of DMSO (as in the sample vials) were taken. With the help of a Pasteur pipette 10 living nauplii were put to each of the vials. After 24 h the vials were observed and the number of nauplii survived in each vial was counted. From this, the percentage of lethality of Brine Shrimp nauplii was calculated for each concentration of the extract.

Statistical Analysis

Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnet's multiple comparisons. The results obtained were compared with the control group. p values < 0.001 were considered to be statistically significant.

RESULTS AND DISCUSSION

Phytochemical analyses of the crude extract revealed the presence of alkaloid, tannin and flavonoid (Table 1).

In DPPH radical scavenging assay, the extract showed dose dependent scavenging of DPPH radical as was with the reference ascorbic acid (Fig. 1); the IC_{50} value of the extract was 11.7 µg/ml while the IC_{50} value for the reference ascorbic acid was 5.1 µg/ml. Figure 2 shows the total antioxidant capacity of the *P. granatum* extract. The result is expressed as the number of equivalents to ascorbic acid. Scavenging of nitric oxide was also found to increase with increasing concentration of the extract and the result was comparable to ascorbic acid which was used as the reference (Fig. 3). The IC_{50} values of

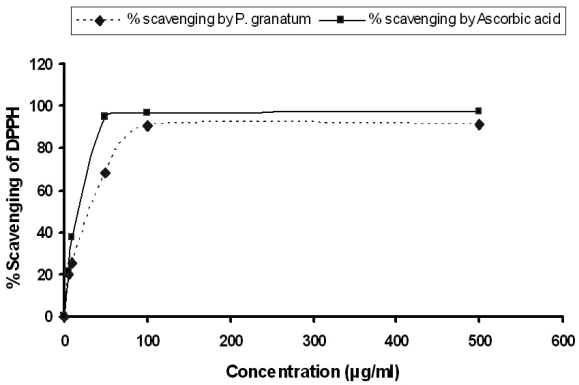

Figure 1. DPPH radical scavenging activity of the hydromethanol extract of *P. granatum*. Values are the average of duplicate experiments and represented as mean ? SD.

Extract	Steroid	Alkaloid	Reducing sugar	Tannin	Gum	Flavonoid	Saponin
HME of *P. granatum*	-	++	-	+++	-	++	-

Table 1. Result of phytochemical group test of the crude extract of Punica granatum. HME: Hydromethanolic extract; (+): Present; (-): Absent.

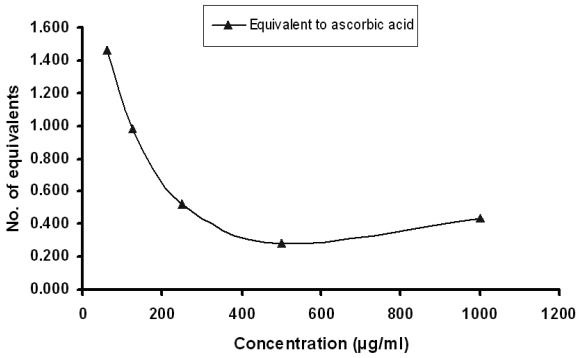

Figure 2. Total antioxidant capacity of the hydromethanol extract of *P. granatum*. Values are the average of duplicate experiments and represented as mean ± SD.

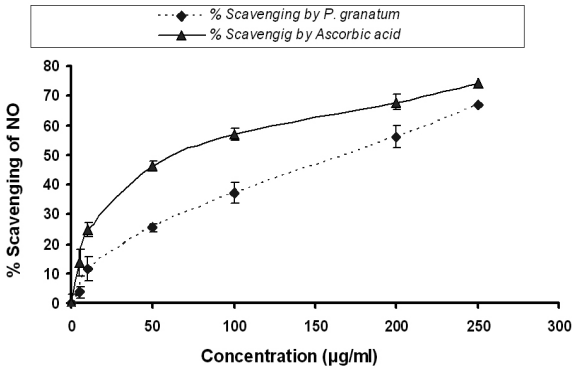

Figure 3. Nitric oxide scavenging activity of the hydromethanol extract of *P. granatum*. Values are the average of duplicate experiments and represented as mean ± SD.

the extract and ascorbic acid were12.5 µg/ml and 17.2 µg/ml respectively. In the past few years, there has been growing interest in the involvement of reactive oxygen species (ROS) in several pathological situations. The oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular disease [19]. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and treatment of diseases associated with oxidants or free radicals.

Groups	Treatment	Dose *(p.o.)*	No. of faeces in 4 h	% Inhibition of defaecation
Group-I	1% Tween 80 in water	0.4 ml/mouse	22.2 ± 1.366	-
Group-II	Loperamide	10 mg/kg	7.2 ± 0.532**	67.57**
Group-III	HME of *P. granatum*	200 mg/kg	13.6 ± 1.176**	38.74**
Group-IV		400 mg/kg	9.8 ± 1.396**	55.86**

Table 2. Effect of *P. granatum* extract on castor oil-induced diarrhoea in mice. Values are presented as mean ± SEM, (n = 5); ** $p < 0.001$, Dunnet test as compared to control. HME: Hydromethanolic extract.

Groups	Treatment	Dose *(p.o.)*	No. of faeces in 4 h	% Inhibition of defaecation
Group-I	1% Tween 80 in water	0.4 ml/mouse	19.4±1.245	-
Group-II	Loperamide	3 mg/kg	5.4±0.847**	72.16**
Group-III	HME of *P. granatum*	200 mg/kg	11.4±0.983**	41.24**
Group-IV		400 mg/kg.	7±1.041**	63.92**

Table 3. Effect of *P. granatum* extract on MgSO4-induced diarrhoea in mice.Values are presented as mean ± SEM, (n = 5); ** p < 0.001, Dunnet test as compared to control. HME: Hydromethanolic extract.

Treatment	Dose *(p.o.)*	Mean Intestinal length (cm)	Mean distance traveled by charcoal (cm)	% GI transit
1% Tween 80 in water	0.4 ml/mouse	68.2±0.931	51±2.00	74.95±3.644
Atropine	0.1 mg/kg	62.6±1.794	20.6±1.176**	32.84±1.345**
HME of *P. granatum*	200 mg/kg	64.6±2.924	35.6±1.372**	55.28±1.381**
	400 mg/kg.	66.2±2.604	25.6±2.210**	39.05±3.849**

Table 4. Effect of *P. granatum* extract on charcoal meal-stimulated gastrointestinal transit. Values are presented as mean ± SEM, (n = 5); ** p < 0.001, Dunnet test as compared to control. HME: Hydromethanolic extract.

Test solution	Conc. (µg/ml)	Log Conc.	% Mortality	LC_{50} (g/ml)	LC_{90} (µg/ml)
	1.25	0.097	25		
	2.5	0.398	30		
	5	0.699	40		
	10	1	45		
P. granatum	20	1.301	50	10.00	125.9
	40	1.602	70		
	80	1.903	85		
	160	2.204	100		
	320	2.505	100		

Table 5. Cytotoxic potential of crude hydromethanolic extract of fruit rind of *P. granatum*.

Preliminary phytochemical screening of the extract revealed the presence of flavonoid, alkaloid and tannin. Polyphenolic compounds, like flavonoids, tannins and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity. Flavonoids and tannins present in the plant extract, as evident from phytochemical screening, may be responsible for the antioxidant action in the tested models. Moreover, nitric oxide is implicated for inflammation, cancer and other pathological conditions. Hence, nitric oxide scavenging capacity of the extract may help to arrest the chain of reactions initiated by excess generation of nitric oxide that are detrimental to the human health [20].

In castor oil-induced diarrhoeal model, the methanol extract of *P. granatum,* at the doses of 200 and 400 mg/kg, reduced the total number of faeces in a dose dependent manner (Table 2). The results were found to be statistically significant ($p < 0.001$). The extract at both dose levels significantly ($p < 0.001$) reduced the extent of diarrhoea in test animals in magnesium sulphate-induced diarrhoeal experiment (Table 3).

Both the doses were shown to reduce the total number of faeces when compared to control. In the gastrointestinal motility test, the methanol extract, at the doses of 200 and 400 mg/kg, retarded ($p < 0.001$) the intestinal transit of charcoal meal in mice when compared to the control (Table 4).

Several mechanisms have been previously proposed to explain the diarrhoeal effect of castor oil including inhibition of intestinal Na^+, K^+-ATPase activity to reduce normal fluid absorption [21], activation of adenylate cyclase or mucosal cAMP mediated active secretion [22], stimulation of prostaglandin formation [23], platelet activating factor and recently nitric oxide has been claimed to contribute to the diarrhoeal effect of castor oil [24]. However, it is well evident that castor oil produces diarrhoea due to its most active component recinoleic acid which causes irritation and inflammation of the intestinal mucosa, leading to release of prostaglandins, which results in stimulation of secretion [25]. Since the methanol extract of *Punica granatum* successfully inhibited the castor oil-induced diarrhoea, the extract might have exerted its antidiarrhoeal action via antisecretory mechanism which was also evident from the reduction of total number of wet faeces (not shown separately) in the test groups in the experiment. Again, flavonoids present in the plant extract are reported to inhibit release of autacoids and prostaglandins, thereby inhibit motility and secretion induced by castor oil [26]. The antidiarrhoeal activity of the extract may also be due to denature proteins forming protein tannates which make intestinal mucosa more resistant and reduce secretion. On the other hand, magnesium sulphate has been reported to induce diarrhoea by increasing the volume of intestinal content through prevention of reabsorption of water. It has also been reported that it promotes the liberation of cholecystokinin from the duodenal mucosa, which increases the secretion and motility of small intestine and thereby prevents the reabsorption of sodium chloride and water [23,27]. The methanol extract was found to improve the diarrheal condition in this model. The extract may have increased the absorption of water and electrolyte from the gastrointestinal tract, since it delayed the gastrointestinal transit in mice as compared to the control. The delay in the gastrointestinal transit prompted by the extract might have contributed, at least to some extent, to their antidiarrhoeal activity by allowing a greater time for absorption.

Moreover, the extract produced concentra-

tion dependent increment in percent mortality of Brine Shrimp nauplii (Table 5). LC_{50} and LC_{90} values of the extract solution were 10 µg/ml and 125.9 µg/ml respectively. Nevertheless, the plant is reported to contain triterpinoids [1]. There is growing interest in natural triterpinoids caused as much by the scientific aspects extraction and structural analysis of these compounds, as by the fact of their wide spectrum of biological activities; they are bactericidal, fungicidal, antiviral, cytotoxic, analgesic, antiinflammatory, anticancer and antiallergic [28]. So, the observed cytotoxic activity may be attributed to these triterpinoids.

CONCLUSION

The results of the present study provides further support to and reinforces the traditional use of the plant in diarrhoea, dysentery and other allied disorders in which free radicals are implicated. In addition, positive result in cytotoxic activity test led us to the inference that the plant extract may contain bioactive compounds which may aid ongoing anticancer drug discovery from floristic resources. Since the extract is reported to contain a myriad array of compounds, it is difficult to ascribe these observed activities to any specific group of compounds. Hence, further studies are suggested to be undertaken to pin point the exact compound(s) and to better understand the mechanism of such actions scientifically.

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