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International Journal of Pharmaceutical and Phytopharmacological Research

(ICV-5.09)

ISSN (Online) 2249 – 6084

ISSN (Print) 2250 – 1029

Int.J.Pharm.Phytopharmacol.Res. 2012, 2(3): 160-166

(Research Article)

In-vitro Antioxidant Potential from the Leaves of *Punica granatum* Linn. Grown in Bangladesh

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Received on: 09/11/2012 ***Accepted on:*** 30/12/2012

ABSTRACT

The crude ethanolic extract of the leaves of Punica granatum Linn. (Family: Punicaceae) was evaluated for its possible antioxidant activities growing in northeast part of Bangladesh. Five complementary test systems, namely DPPH free radical scavenging, nitric oxide scavenging, reducing power, Fe^{++} ion chelating ability and total phenolic content were used for determining antioxidant activities of the leaf extract. In DPPH free radical scavenging test, IC_{50} value for ethanolic crude extract was found fairly significant 23.45±0.062 µg/ml while compared to the IC_{50} value of the reference standards ascorbic acid and Butylated Hydroxy Anisole (BHA) (5.23±0.034 and 6.15±0.027 µg/ml) respectively. The ethanol extract showed maximum nitric oxide (NO) scavenging activity of 76.55±0.89% at 100 µg/ml, where as ascorbic acid exhibited 88.43 ± 0.81% inhibition at the same concentration. The IC_{50} value for nitric oxide scavenging test was also found significant (55.65±0.072 µg/ml) while compared to the IC_{50} value of the reference standard ascorbic acid (31.38±0.047 µg/ml). The maximum absorbance for reducing power assay was found to 2.015 at 100 µg/ml while compared to 2.817 and 2.031 for standard ascorbic acid and BHA respectively. The IC_{50} value of the extract as percentage of Fe^{++} ion chelating ability was determined as 65.109 µg/ml where that of EDTA showed 8.75µg/ml. The total phenolic amount was also calculated as quite high in ethanolic crude extract 378.37 mg/g of gallic acid equivalent. Therefore, the obtained results tend to suggest the antioxidant activities of the crude ethanolic extract of the leaves of P. granatum and justify its use in folkloric remedies.

Key Words: *Punica granatum,* DPPH free-radical scavenging, Nitric oxide scavenging, Reducing power, Total phenolic.

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[1]. The leaves are shiny and about 7.6 cm long. Different parts of the plant such as bark, leaves, immature fruits and fruit rind have medicinal significance[2]. Flavone glycosides, apigenin and luteolin[3], tannin, punicafolin, granatins A and B, corilagin, strictinin[4] ellagic acid, brevifolin, gallic acid[5] have also been isolated from the leaves extract of *P. granatum*. . *P. granatum* has been extensively used as a traditional medicine in many countries for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory pathologies[6]. It has also been used in anti viral, anti bacterial, and anti diabetic agents[7-10].

Cancer and atherosclerosis, two major causes of death, are salient "free radical" diseases in human. Reactive oxygen species (ROS) have a tendency to donate oxygen to other substances. Many such reactive species are free radicals and have a surplus of one or more free-floating electrons rather than having matched pairs and are, therefore, unstable and highly reactive includes the hydroxyl radical (OH.), the superoxide radical ($O._2$), the nitric oxide radical (NO.) and the lipid peroxyl radical (LOO.) cause severely deleterious effects on the human body [11]. Enzymatic and non-enzymatic reactions like respiratory chain reaction, the phagocytosis, prostaglandin synthesis, cytochrome P450 system and oxidative phosphorylation (i.e. aerobic respiration) in the mitochondria[12]. ROS are the products of normal cellular metabolism, having both deleterious and beneficial effect in the body[13]. The balance between the production of free radicals and the antioxidant defenses in the body has important health implications. If there are too many free radicals produced and too few antioxidants, a condition of "oxidative stress" develops which may cause chronic damage body[13]. Antioxidants play an excellent role in preventing cell damage. They donate their own electrons to

free radicals. Free radical accepts the electron from antioxidant and they do not attack the cell and the chain reaction of oxidation is inhibited [14]. Phenolic compounds, flavonoid and triterpenoids containing foods and beverages with antioxidant activity have been reported[15]. Very recent, health risks and toxicity have been reported using synthetic antioxidants restricted[16]. Some well known natural antioxidants like rosemary and sage are already exploited commercially either as antioxidant additives or as nutritional supplements stipulating the antioxidant potential of plant species[17]. In recent years, the interest in natural antioxidant, especially of plant origin, has greatly increased[18].

Since there is no sufficient data currently available to substantiate antioxidant activities from leaf extract of *P. granatum* that abundantly grown in Bangladesh, therefore the present study was designed to provide scientific evidence for its use as a traditional folk remedy by investigating the antioxidant activities that also confirm its use in pathological conditions where free radicals are implicated.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

The plant (leaves) *Punica granatum* Linn. was collected at November, 2010 from Barisal, southeast distrct of Bangladesh and was identified by Bangladesh National Herbarium, Mirpur, Dhaka. (Accession number-DACB-34562).

Preparation of Ethanolic Extract

The leaves of *P. granatum* were freed from any of the foreign materials. Then the plant materials were chopped and air-dried under shed temperature followed by drying in an electric oven at 40º C. The dried plant materials were then grounded into powder. About 500g of powdered material was taken in a clean, flat-bottomed glass container and soaked in 1.2 liters of 80% ethanol. The container with its contents was sealed and kept for a period of 4 days accompanying occasional shaking and stirring. The ethanolic extract was filtered by Buchner funnel and the filtrate was concentrated with rotary evaporator at bath temperature not exceeding 40˚ to have gummy concentrate of greenish black extract (Yield approx. 13.86%).

Test Animals

For the screening of acute toxicity activity male rats of Wister strain weighing 175-202 g were used. The animals were housed under standard Laboratory (at Pharmacology Laboratory of BCSIR, Chittagong) conditions maintained at 25±1°C and under 12/12 h light/dark cycle and feed with Balanced Trusty Chunts and water *ad libitum.* All experimental protocols were in compliance with BCSIR Ethics Committee on Research in Animals as well as internationally accepted principles for laboratory animal use and care.

Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH), L-Ascorbic acid, BHA (Butylated Hydroxy Anisole), Gallic acid, Folin-ciocalteu phenol reagent, Ferrozine and Griess reagent were obtained from Sigma Chemical Co.[(St. Louis, MO, USA)]. Trichloroacetic acid (TCA), Phosphate buffer (pH 6.6), Potassium ferricyanide [$K_3Fe(CN)_6$], $FeCl_2$, $FeCl_3$, Sodium nitroprusside, Ethanol, Sodium phosphate, EDTA, Tween 80, Ammonium molybdate and Sodium carbonate were of analytical grade and purchased from Merck (Darmstat, Germany).

Phytochemical Screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents, by using the following reagents and chemicals, for example, alkaloids were identified by the Dragendorff's reagent; flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions; steroids with Libermann-Burchard reagent and reducing sugars with Benedict's reagent[19-21].

Total Phenolic Content Determination

The modified Folin-Ciocaltu method[22] followed to determine the total phenolic content of the extract of leaves of *P. granatum*. A 0.5 ml of each extract (1 mg/ml) was mixed with 5 ml Folin-Ciocaltu reagent (1:10 v/v distilled water) and 4 ml (75g/l) of Sodium carbonate and the mixture was then vortexed for 15 second for the development of color the mixture was allowed to stand for 30 min at 40°C.Then the absorbance was read at 765 nm with the same spectrophotometer. Total phenolic content was calculated as mg of Gallic acid equivalent per gram using the equation obtained from a standard Gallic acid calibration curve y=6.2548x -0.0925, R^2=0.9962.

Acute Toxicity Test

The acute toxicity of *P. granatum* ethanolic extract was determined in male rats of Wister strain according to the method of Hilaly et al[23] with slight modifications. Rats fasted for 16 h were randomly divided into groups of five rats per group. Graded doses of the extract (200, 400, 800, 1600 and 3200 mg/kg p.o.) were separately administered to the rats in each of the groups by means of bulbed steel needle. All the animals were then allowed free access to food and water and observed over a period of 48 h for signs of acute toxicity. The number of deaths within this period was recorded.

Antioxidant Activities

DPPH free radical scavenging activity

The method of Chang et al [24] was used for performing the DPPH radical scavenging activity. A stock solution (5mg/ml) of ethanolic extract of leaves of *P. granatum* (5 mg/ml) was prepared in respective solvent systems. A serial dilutions were the carried out to obtain concentrations of 5, 10, 20, 40, 60, 80, 100 µg/ml. An equal amount of sample solution was mixed with an equal amount of 0.1 mM methanolic solution of DPPH, The mixture was vortex and allowed to stand at the dark at 25 °C for 30 min. After 30 min incubation, the absorbance of the mixture was read against a blank at 517 nm using a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). The radical scavenging activity was expressed as the inhibition percentage (I%) and calculated as per the equation: $I\ (\%) = (A_{blank} - A_{sample} / A_{blank}) \times 100$

Where A_{blank} is the absorbance of the control (containing all reagents except the test compound), and A_{sample} is the absorbance of the experimental sample with all reagents. IC_{50} value (the concentration of sample required to scavenge 50% DPPH free radical) was calculated from the plot of inhibition (%) against the concentration of the extract. All

determination was carried out in triplicate and average of the results was noted. Ascorbic acid and BHA was used as standard for this study.

Nitric Oxide (NO) Scavenging Activity

Nitric oxide scavenging activity was measured spectrophotometrically25. Sodium nitroprusside (5 mmol) in phosphate buffered saline was mixed with different concentrations of the extract of leaves of P. granatum (5–100 µg/ml) dissolved in methanol and incubated at 25 °C for 30 min. In control there was not test sample but an equivalent amount of methanol was used. After 30 min, 1.5 ml of incubation the solution was taken and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). The nitric oxide (NO) radical scavenging activity was expressed as the inhibition percentage (I%) and calculated as per the equation: I (%) = $(A_{blank} - A_{sample} / A_{blank}) \times 100$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the experimental sample with all reagents. IC_{50} value is the concentration of sample required to scavenge 50% nitric oxide free radical and was calculated from the plot of inhibition (%) against the extract concentration. All the determinations were carried out in triplicate and average of the absorptions was noted. Ascorbic acid was used as positive control standard for this study.

Reducing Power Assay

The method of Dehpour et al[26] was followed to determine the reducing power of *P. granatum* leaves of ethanolic extract. Different concentrations of the extract (5-100 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was then incubated at 50°C for 20 min and a 10% solution of trichloroacetic acid (2.5 ml) was added to it. It was then centrifuged at 3000 rpm for 10 min. The upper layer of the mixture (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml, 0.1% $FeCl_3$ and the absorbance of the mixture was measured at 700 nm with the same spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. All the determinations were carried out thrice and average of the results was taken. Ascorbic acid and BHA were used as the standard reference compounds in this study.

Ferrous Ion Chelating Ability

The ferrous ions chelating activity of ethanol extract of leaves of *P. granatum* and standards were investigated according to the method of Dinis et al[27]. Briefly, different concentrations of the extract (5-100 µg/ml) were added to 0.1ml solution of 2 mM ferrous chloride ($FeCl_2$). Then, the reaction was initiated by the addition of 0.2ml of 5 mM Ferrozine and mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured at 562 nm in spectrophotometer, wherein the Fe^{+2} chelating ability of extracts was monitored by measuring the ferrous ion-Ferrozine complex. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was given in the below formula:

Ferrous ions chelating ability (%) = $[(A_0 - A) / A_0] \times 100$

Where A_0 is the absorbance of the control solution (containing all reagents except extract); A is the absorbance in the presence of the sample of plant extracts. All the tests were carried out in triplicate and EDTA was used as standard.

Statistical Analysis

For antioxidant determination, data were presented as mean ± Standard deviation (S.D). 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 < 0.05 were considered to be statistically significant (*p* indicates probability).

RESULTS

Chemical Group Test

Results of different chemical tests on the ethanolic extract of *P. granatum* leaves showed the presence of alkaloid, reducing sugars, steroid, tannins and significantly presence of flavonoid (Table-1).

Table-1: Results of different group tests of ethanolic extract of *P. granatum* leaves.

Phytoconstituents	Ethanol extract of *P. granatum*
Alkaloid	+
Reducing sugars	+
Tannins	+
Gums	-
Flavonoids	++
Saponin	-
Steroid	+

+: Positive result; - : Negative result; ++: significantly positive

Total Phenolic Content

The amount of total phenolic content was calculated as quite high in the ethanolic crude extract of *P. granatum* (378.37±0.92 mg/g of gallic acid equivalent) (Table-2).

Table-2: Total phenolic content of the ethanol extract of *P. granatum* leaves.

Extract	Avg. absorbance at 765 nm	Total phenolic content
		mg of gallic acid equivalent (GAE) per g of dry extract
Ethanol extract of *P. granatum* leaves	2.5210±0.02	378.37±0.92

The values are expressed as mean ± standard deviation (n=3).

Acute Toxicity Test

In acute toxicity study, oral administration of graded doses (200, 400, 800, and 1600 mg/kg p.o.) of the ethanol extract of *P. granatum* to rats did not produce any significant changes in behaviour, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects during the observation period. No mortality or any toxic reaction was recorded in any group after 48h of administering the extract to the animals. *P. granatum* was safe upto a dose level of 1600 mg/kg of body weight.

Antioxidant Activities

Ethanolic extract of *P. granatum* was screened for evaluation of its possible antioxidant activities. Five complementary test systems, namely DPPH free radical scavenging, nitric oxide scavenging activity, reducing power, ferrous ion chelating ability and total phenolic contents determination were followed for this analysis.

DPPH Free Radical Scavenging Activity

DPPH free radical scavenging activity of the *P. granatum* was found to be increased with the increase of concentration of the extract (Table-3). The extract exhibited 93.72±0.072% radical inhibitions at 100 µg/ml whereas at the same concentration the standards ascorbic acid and BHA exhibited 95.96 ±0.031% and 93.09 ±0.019% inhibitions respectively. IC_{50} value of the extract was found to be very fairly significant (23.45±0.062µg/ml). When compared to the IC_{50} value of the reference compounds ascorbic acid and BHA (5.23±0.034 and 6.15±0.027 µg/ml) respectively.

Table-3: DPPH radical scavenging activity of the ethanolic extract of *P. granatum* leaves and standards.

Concentration (µg/ml)	% Inhibition of ethanol extract and Standards at different concentration		
	Ethanol Extract of *P. granatum* leaves	Ascorbic acid (standard)	BHA (standard)
5	25.46±0.069	48.44 ±0.027	41.76 ±0.038
10	38.24±0.032	71.39 ±0.011	65.23 ±0.016
20	47.67±0.058	82.76 ±0.019	82.18 ±0.018
40	61.67±0.075	89.48 ±0.014	87.11 ±0.039
60	74.48±0.085	94.38 ±0.017	92.33 ±0.013
80	82.45±0.053	95.68 ±0.043	92.61 ±0.051
100	93.72±0.072	95.96 ±0.031	93.09 ±0.019
IC_{50} (µg/ml)	23.45±0.062	5.23±0.034	6.15±0.027

The values are expressed as mean ± standard deviation (n=3).

Nitric Oxide (NO) Scavenging Assay

The scavenging of NO by the ethanol extract of *P. granatum* was increased in dose dependent manner. Table-4 illustrates a significant decrease in the NO radical due to the scavenging ability of the extract and ascorbic acid. The ethanol extract showed maximum scavenging activity of 76.55 ± 0.89% at 100 µg/ml, where as ascorbic acid at the same concentration exhibited 88.43 ± 0.81% inhibition. The IC_{50} value for ethanolic extract was found fairly significant (55.65±0.072 µg/ml) while compared to the IC_{50} value of the reference standard ascorbic acid (31.38±0.047 µg/ml).

Table-4: Nitric oxide radical scavenging activity of the ethanolic extract of *P. granatum* leaves and standard.

Concentration (µg/ml)	% NO inhibition of the extract and standard at different concentration	
	Ethanol Extract of *P. granatum*	Ascorbic acid (standard)
5	17.85 ± 0.56	23.28 ± 0.43
10	28.78 ± 0.38	34.55 ± 0.75
20	34.59 ± 0.88	42.78 ± 0.66
40	46.38 ± 0.67	56.49 ± 0.78
60	51.63 ± 0.91	63.47 ± 0.81
80	63.23 ± 0.92	70.28 ± 0.95
100	76.55 ± 0.89	88.43 ± 0.81
IC_{50} (µg/ml)	55.65±0.72	31.38±0.47

The values are expressed as mean ± standard deviation (n=3).

Reducing Power Assay

In determination of reducing power of ethanolic crude extract of *P. granatum* ascorbic acid and BHA was used as positive control (Table-5). The maximum absorbance for ethanolic extract was found to be (2.015 ±0.009) at 100 µg/ml concentration while compared to standard ascorbic acid (2.817 ±0.013) and BHA (2.031 ±0.019) respectively, at the same concentration. With the increase of concentration, the absorbance of the extract was found to be increased and those for the standards were also increased with increasing concentration.

Table-5: Reducing power assay of the ethanolic extract of *P. granatum* leaves and standards.

Concentration (µg/ml)	Average absorbance at 700nm of extract and Standards at different concentration		
	Ethanol Extract of *P. granatum* leaves	Ascorbic acid (standard)	BHA (standard)
5	0.285 ±0.007	0.380 ±0.016	0.430 ±0.011
10	0.724 ±0.004	0.830 ±0.013	0.776 ±0.013
20	1.233 ±0.009	1.483 ±0.017	1.452 ±0.012
40	1.405 ±0.003	1.935 ±0.012	1.749 ±0.017
60	1.592 ±0.008	2.645 ±0.015	1.842 ±0.013
80	1.932 ±0.005	2.778 ±0.014	1.976 ±0.015
100	2.015 ±0.009	2.817 ±0.013	2.031 ±0.019

The values are expressed as mean ± standard deviation (n=3).

Fe^{++} ion Chelating Ability

Fe^{++} ion chelating ability of ethanol extract of leaves of *P. granatum* is shown in Table-6. The extract showed 65.109 ±0.054% Fe^{++} ion chelating ability at 100 µg/ml where as the standard EDTA showed 99.87±0.015% at the same concentration. The IC_{50} value of the extract was also found significant (73.80±0.063 µg/ml) while compared to the IC_{50} value of the reference standard EDTA (8.75±0.027 µg/ml) (Table-6).

Table-6: Fe^{2+} ion chelating ability of ethanol extract of *P. granatum* leaves and EDTA (Standard).

Concentration (µg/ml)	% Chelating Ability of different solvent extract and Standard	
	Ethanol extract of *P. granatum* leaves	Na_2EDTA (Standard)
5	6.108 ±0.045	34.67±0.043
10	9.334 ±0.056	55.81±0.023
20	12.996 ±0.023	82.79±0.062
40	21.258 ±0.034	92.47±0.028
60	33.866 ±0.086	98.19±0.020
80	59.341 ±0.043	99.20±0.031
100	65.109 ±0.054	99.87±0.015
IC_{50} (µg/ml)	73.80±0.063	8.75±0.027

The values are expressed as mean ± standard deviation (n=3).

DISCUSSION

A method based on the scavenging of the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) has been used extensively to predict the antioxidant activities of extracts of plants[28- 29]. The high inhibition value of *P. granatum* ethanol extract may due to the presence of tannins and significant amount of flavonoids in the extract as phytochemicals. Tannins and Flavonoids, commonly found in plants have been reported to have significant antioxidant activity[30].

NO scavenging capacity of the extract may help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to the human health. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions[31]. NO works as a atypical neural modulator that is involved in neurotransmitter release, neuronal excitability and learning and memory. Besides its role in physiologic processes, it also participates in pathogenic pathways underlying a large group of disorders including muscle diseases, inflammatory bowel disease, sepsis and septic shock, primary headaches and stroke. Additionally, increasing evidence shows that NO modulates neurotoxin induced cell damage and is involved in neuronal cell death in Parkinson's disease (PD) and other neurodegenerative disorders such as Alzheimer disease[32]. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and treatment of diseases associated with oxidants or free radicals[33]. Preliminary phytochemical studies of the ethanol extract of *P. granatum* showed the presence of tannins and significant amount of flavonoids therefore suppression of released NO may be attributed to direct NO scavenging. Furthermore it has also been reported that the ethanolic extract of *P. granatum* leaves possess apigenin and luteolin in where luteolin suppress nitric oxide production possibly through reduction of inducible nitric oxide synthase (iNOS) enzyme expression[34]and apigenin suppressed COX-2 or inducible nitric oxide synthase (iNOS) expression as well as NO and PGE_2 levels in lipopolyssacharide-stimulated macrophages35 [2]. Furthermore, the antioxidant potential of luteolin is twice stronger than that of vitamin E[36-37] and more potent antioxidant than the synthetic antioxidant butylated hydroxytoluene (BHT)[38]. The presence of ellagic acid in extract also exerts its antioxidant effects by inhibiting NADPH oxidase-induced overproduction of superoxide, suppressing the release of NO by down-regulating iNOS[39].

A direct correlation between antioxidant capacity and reducing power of certain plant extracts has been reported[40]. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[41]. The maximum absorbance for the ethanolic extract of *P. granatum* was found to be 1.531 at 100 µg/ml while that of standard ascorbic acid and BHA was found as 2.8111 and 2.031, respectively (Table 5).

Bivalent transition metal ions (e.g. Fe^{++}) play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry[42]. These processes can be delayed by iron chelation. Iron can generate free radicals from peroxides and may be implicated in human cardiovascular disease[43]. Therefore, minimizing its concentration affords protection against oxidative damage. Ferrozine can quantitatively form complexes with Fe^{2+}. The absorbance of Fe^{2+}-ferrozine complex was decreased dose-dependently, that is, the activity was increased on increasing concentration from 5 to 100 µg/ml. Table 6 is exhibiting the comparative percentage Fe^{++} ion chelating ability of ethanol extract and standard compound (Na_2EDTA). Therefore, the presence of apigenin and luteolin[3] in the extracts also have scavenging activity of free radicals generated by H_20_2 or or chelating activity with metal ions (e.g. Fe^{++})[44].

Phytochemical components, especially phenolic compounds (such as flavonoids, phyenyl propanoids, phenolic acids, tannins etc.) are very important components for the free radical scavenging and antioxidant activities of plants. Polyphenols are generally of the chemical patterns; phenolic groups react as hydrogen donors and neutralize the free radicals[29, 40]. In the present study the total amount of phenolic compounds was calculated as quite high in the ethanol extract of *P. granatum* leaves. The result of present study revealed that the presence of high concentration of phenolic components in the extract might cause the high inhibition value of the extract. Phenols are important components of plants. It is reported that the hydroxyl group of the phenolic compounds to eliminate radicals and they contribute directly to antioxidant effect of the system[41].

CONCLUSION

In conclusion it can be revealed that the crude ethanolic extract of *P. granatum* leaves possess significant antioxidant activity. The potential of the extract of *P. granatum* as antioxidant agents may be due to the presence of phytoconstituents like apigenin, luteolin[3], tannin[4], ellagic acid, brevifolin, gallic acid[5] might be responsible for its activity and justify its use as a traditional folk remedy.

However, extensive researches are necessary to search for active principles responsible for these activities.

REFERENCES

1) Ghani A. Medicinal Plants of Bangladesh Chemical constituents and uses, 2nd edition, The Asiatic Society of Bangladesh, Dhaka, 2003, pp. 362-3, 502-4.
2) Arun N and Singh DP. "*Punica granatum*: a review on pharmacological and therapeutic properties" *Int J Pharm Sci Res*, 2012, 3(5): 1240-1245.
3) Mahmoud Nawwa AM, Sahar Hussein AM and Merforta I. "Leaf phenolics of *Punica granatum*" *Phytochem*, 1994, 37(4): 1175–1177.
4) Tanaka T, Nonaka GI and Nishioka I. "Punicafolin, an ellagitannin from the leaves of *Punica granatum*" *Phytochem*, 1985, 24(9): 2075–2078.
5) Jia L, Yan L and Li-zhen X. "Chemical constituents from leaves of *Punica granatum* L" *Cen S Phar*, 2005, 02.
6) Choi JG, Kang OH, Lee YS, Chae HS, Oh YC, Brice OO, Kim MS, Sohn DH, Kim HS, Park H, Shin DW, Rho JR and Kwon DY. "In vitro and in vivo antibacterial activity of *Punica granatum* peel ethanol extract against Salmonella" *Evid Based Complement Alternat Med*, 2011, 1-8.
7) Zofar R and Singh J. "Anti-diabetic activity of *Punica granatum* Linn." *Science and Culture*, 1990, 56: 303-304.
8) Bala S and Grover IS. "Anti-mutagenic effect of Pomegranate (*Punica granatum* variety Anardana) fruit extract on direct acting and 59- dependent mutagens in *Salmonella typhimurium*" *J Plant Sci Res*, 1992, 8: 14-16.
9) Naqvi SAH, Siddigi T, Hamdard ME and Hameed A. "Anti-amoebic activity of rind and flowers of *Punica granatum* Linn." *J Sci Islam Repub Iran*, 1992, 4: 1-13.
10) Pradhan KD, Thakur DK and Sudhan NA. "Therapeutic efficacy of *Punica granatum* and *Cucurbita maxima* against clinical cases of neatodiasis in cloves" *Indian J Indig Med*, 1992, 9: 53-55.
11) Bagchi K and Puri S. "Free radicals and antioxidants in health and disease" *Eastern Med Health J*, 1998, 4(2): 350-360.
12) Tiwari AK. "Antioxidants: new-generation therapeutic base for treatment of polygenic disorders" *Current Sci*, 2004, 86: 1092-1102.
13) Valko M, Izakovic M, Mazur M, Rhodes CJ and Telser J. "Role of oxygen radicals in DNA damage and cancer incidence" *Mol Cell Biochem*, 2004, 266: 37–56.
14) Dekkers JC, Van Doornen LJP and Kemper HCG. "The Role of Antioxidant Vitamins and Enzymes in the Prevention of Exercise-Induced Muscle Damage" *Sports Med*, 1996, 21: 213-238.
15) Brown JE and Rice-Evans CA. "Luteolin rich artichoke extract protects low-density lipoprotein from oxidation in vitro" *Free Radical Res*, 1998, 29: 247-255.
16) Buxiang S and Fukuhara M. "Effects of co-administration of butylated hydroxytoluene, butylated hydroxyanisole and flavonoide on the activation of mutagens and drug-metabolizing enzymes in mice" *Toxicol*, 1997, 122: 61-72.
17) Rahman MH, Alam MB, Chowdhury NS, Jha MK, Hasan M, Khan MM, Rahman MS and Haque ME. "Antioxidant, Analgesic and Toxic Potentiality of *Stephania japonica* (Thunb.) Miers. Leaf" *Int J Pharmacol*, 2011, 7: 257-262.
18) Jayaprakasha GK and Jaganmohan R. "Phenolic constituents from lichen *Parmontrema stuppeum*. Hale and antioxidant activity" *Zeitschrift Für Naturforscung*, 2002, 55: 1018-1022.
19) Ghani A. Medicinal Plants of Bangladesh, 1st edition, Asiatic Society, Dhaka, 1998, 13.
20) Evans WC. Trease and Evan's Textbook of Pharmacognosy, 13th ed., Cambidge University Press, London, 1989, 546.
21) Harborne JB. Phytochemal methods (A guide to modern techniques to plantanalysis), 3rd ed., Chapman and Hall, London, 1984.
22) Wolfe K, Wu X and Liu RH. "Antioxidant activity of apple peels" *J Agric Food Chem*, 2003, 51: 609-614.
23) Hilaly JE, Israili ZH and Lyoussi B. "Acute and chronic toxicological studies of *Ajuga iva* in experimental animals" *J Ethnopharmacol*, 2004, 91: 43-30.
24) Chang ST, Wu JH, Wang SY, Kang PL, Yang NS and Shyur LF. "Antioxidant activity of extracts from *Acacia confusa* bark and heartwood" *J Agric Food Chem*, 2001, 49: 3420–3424.
25) Govindarajan R, Rastogi S, Vijayakumar M, Shirwaikar A, Rawat AKS, Mehrotra S and Palpu P. "Studies on the Antioxidant Activities of *Desmodium gangeticum*" *Biol Pharm Bull*, 2003, 26: 1424-1427.
26) Dehpour AA, Ebrahimzadeh MA, Nabavi SF and Nabavi SM. "Antioxidant activity of methanol extract of *Ferula assafoetida* and its essential oil composition" *Grasasy Aceites*, 2009, 60(4): 405-412.
27) Dinis TC, Madeira VM and Almeida LM. "Action of phenolic derivatives (acetaminophen, salicylate, and 5-amino salicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers" *Arch Biochem Biophys*, 1994, 315(1): 161-169.
28) Yen GC and Duh PD. "Scavenging effect of methanolic extracts of peanut hulls on free-radical and active oxygen species" *J Agric Food Chem*, 1994, 42: 629–632.
29) Kulisic T, Radonic A, Katalinic V and Milos M. "Use of different methods for testing antioxidative activity of oregano essential oil" *Food Chem*, 2004, 85: 633–640.
30) Vinson JA, Dabbagh YA, Serry MM and Jang J. "Plant flavonoids, especially tea flavonoid, are powerful antioxidants using an in vitro oxidation model for heart diseases" *J Agric and Food Chem*, 1995, 43: 2800-2802.
31) Moncada A, Palmer RMJ and Higgs EA. "Nitric oxide: Physiology, pathophysiology and pharmacology" *Pharmacol Rev*, 1991, 43: 109-142.
32) Aliev G, Palacios HH, Lipsitt AE, Fischbach K, Lamb BT, Obrenovich ME, Morales L, Gasimov E and Bragin V. "Nitric Oxide as an initiator of brain lesions during the development of Alzheimer disease" *Neurotox Res*, 2009, 16: 293-305.

33) Soares JR, Dinis TCP, Cunha AP and Almeida LM. "Antioxidant activities of some extracts of *Thymus zygis*" *Free Rad Res*, 1997, 26: 469-478.
34) Kim HK, Cheon BS, Kim SY and Kim HP. "Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships" *Biochem Pharmacol*, 1999, 58: 759-765.
35) Raso GM, Meli R, Di Carlo G, Pacilio M and Di Carlo R. "Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1" *Life Sci*, 2001, 68: 921–31.
36) Rice-Evans CA, Miller NJ and Paganga G. "Antioxidant properties of phenolic compounds" *Trends Plant Sci*, 1997, 2: 152-159.
37) Miller NJ. The relative antioxidant activities of plant-derived polyphenolic flavonoids. In: J.T. Kumpulainen, and J.T. Salonen (eds.), Natural Antioxidants and Food Quality in Atherosclerosis and Cancer Prevention. The Royal Society of Chemistry, Cambridge, 1996, 256-259.
38) Igile GO, Oleszek W, Jurzysta M, Burda S, Fafunso M and Fasanmade AA. "Flavonoids from *Vernonia amygdalina* and their antioxidant activities" *J Agric Food Chem*, 1994, 42(11): 2445-2448.
39) Lee WJ, Ou HC, Hsu WC, Chou MM, Tseng JJ, Hsu SL, Tsai KL and Sheu WH. "Ellagic acid inhibits oxidized LDL-mediated LOX-1 expression, ROS generation, and inflammation in human endothelial cells" *J Vasc Surg*, 2010, 52(5): 1290-300.
40) Tanaka M, Kuie CW, Nagashima Y and Taguchi T. "Applications of antioxidative Maillard reaction products from histidine and glucose to sardine products" *Nippon. Suisan Gakkaishi*, 1988, 54: 1409-1414.
41) Duh PD. "Scavenging effect of methanolic extracts of peanut hulls on free-radical and active oxygen species" *J Agric Food Chem*, 1994, 42: 629–632.
42) Halliwell B. "Antioxidants and human diseases: a general introduction" *Nutr Rev*, 1997, 55: S44-S52.
43) Halliwell B and Gutteridge JMC. "Role of free radicals and catalytic metal ions in human disease: an overview" *Meth. Enzymol*, 1990, 186: 1-85.
44) Romanová D, Vachálková A, Cipák L, Ovesná Z and Rauko P. "Study of antioxidant effect of apigenin, luteolin and quercetin by DNA protective method" *Neoplasma*, 2001, 48(2): 104-7.

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