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Research Article

ANTIOXIDANT POTENTIALS OF *PUNICA GRANATUM* FRUIT RIND EXTRACTS

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ABSTRACT

Most of the degenerative diseases are caused by free radicals. Antioxidants are the agents responsible for scavenging free radicals. The aim of present study was to evaluate the phytochemical and *in vitro* antioxidant properties of *Punica granatum* fruit rind extract. DPPH assay, reducing power assay, nitric oxide radicals scavenging, superoxide radical scavenging, ABTS scavenging, hydrogen peroxide scavenging assay were the method adopted to study antioxidant potentials of extracts. Standard methods were used to screen preliminary phytochemistry and quantitative analysis of tannin, phenolics & flavanoids. Aqueous and alcoholic extracts were showed good antioxidant effect with IC_{50} ranges from 34.78±14.04 to 135.27±35.5µg/ml for aqueous and 40.03±14.72 to 105.93±17.19µg/ml for alcoholic extracts. Phenolic compounds, tannins and flavonoids were the major phytochemicals present in both the extracts. Percentage of inhibition increased with the increased concentration of extracts. The aqueous and alcoholic extract yielded 122.33±6.42 & 176±5.29mg/g gallic acid equivalent phenolic content 135.33±8.08 & 81.33±6.1mg/g quercetin equivalent flavonoid and 81.66±3.51 & 114.23±12.16mg/g tannic acid equivalent tannins respectively. The present study provides evidence that both extracts of *Punica granatum* fruit rind is a potential source of natural antioxidant.

Keywords: *Punica granatum*, Fruit rind, Antioxidants, Phytochemicals

INTRODUCTION

Oxidative stress is responsible for many of today's diseases that result from an imbalance between formation and neutralization of pro oxidants. Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid per oxidation. These changes contribute to cancer, atherosclerosis, cardiovascular diseases ageing and anti- inflammatory diseases[1, 2]. All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase [SOD] and catalase or compounds such as ascorbic acid, tocopherol and glutathione[3]. Sometimes these protein mechanisms are disrupted by various pathological processes and antioxidant supplements are vital to combat oxidative damage. Recently, much attention has been directed towards the development of ethno medicines with strong antioxidant properties but low cytotoxicities.

Punica granatum (Punicaceae) fruit rind [commonly called pomegranate] is rich in antioxidant of polyphenolic class which includes tannins[4] & flavonoids[5]. Antioxidant activity has been proposed to play vital role in various pharmacological activities such as anti-aging, anti-inflammatory, anti atherosclerosis and anti-activities[6,7]. Inhibition of free radical induced damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of diseases[8]. Several synthetic antioxidants are available, but are quite unsafe and their toxicity is of concern[9]. Natural products with antioxidant activity may be used for human consumption because of their safety. The present study evaluated antioxidant activity of pomegranate fruit rind extracts.

MATERIALS & METHODS

Plant material

The Fruit rind of Pomegranate was collected from Tiruchirappalli, Tamilnadu, India during the month of June 2010. The plant material was identified by Dr. John Britto, Professor, Department of Botany, St. Joseph's College, Tiruchirappalli, Tamilnadu, India and specimen was deposited in department of Microbiology, Srimad Andavan Arts and Science College, Tiruchirappalli, Tamilnadu, India.

Preparation of extracts

The powdered plant material (150gm) was extracted with water and alcohol using cold maceration method. Both the extracts were filtered with a muselin cloth and the filterate was concentrated in vacuum evaporator. Dried extracts were used for further studies[10].

Phytochemical analysis

The aqueous and alcoholic extracts of *Punica granatum* fruit rind were studied for their phytoconstituents using different phytochemical tests[11].

Quantitative phytochemical analysis

Determination of tannins

The total tannin content in the lyophilized plant extract was determined by modification of a previous method[12]**.** The water and ethanolic extract (0.1mL) was mixed with 0.5mL of Folin- Denis reagent followed by 1mL of Na_2CO_3 (0.5% w/v) solution and made up to 10mL with distilled water. The absorbance was measured at 755nm within 30 minutes of the reaction against the reagent blank. Standard curve was prepared using 20, 40, 60, 80 and 100µL. Total tannins in extracts were expressed as equivalent to tannic acid (g TE/g extract).

Total flavanoids determination

Aluminum chloride colorimetric method was used for flavonoids determination[13]. Each plant extracts (0.5mL of 1:10g/mL-1) in methanol were separately mixed with 1.5mL of methanol, 0.1mL of 10% aluminum chloride, 0.1mL of 1M potassium acetate and 2.8mL of distilled water. It remained at room temperature for 30 minutes; the absorbance of the reaction mixture was measured at 415nm with a single beam Systronics UV/Visible spectrophotometer (India). The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100g/mL^{-1} in methanol.

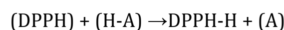
Total phenols determination

Total phenols were determined by Folin Ciocalteu reagent[14]. A diluted plant extract (0.5mL of 1:10g/mL-1) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5mL, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4mL, 1M). The mixtures were allowed to stand for 15 minutes and the total phenols were determined by colorimetry at 765nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250mg L^{-1} solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), which is a common reference compound.

In-vitro antioxidant assay

DPPH assay: (2, 2-diphenyl-1-picrylhydrazyl)

The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as



(DPPH) + (H-A) →DPPH-H + (A)

(Purple) (Yellow)

Antioxidants react with DPPH, a stable free radical which was reduced to DPPH-H and as consequence the absorbance were decreased from the DPPH radical to the DPPH-H form. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Antioxidant activity by DPPH staining

An aliquot (3μL) of each sample and standard (Quercetin and Ascorbic acid) were carefully loaded onto a 10cm X 10cm Silica gel plate (silica gel 60 F254; Merck) and allowed to dry for 3 minutes. Drops of each sample were loaded in an order of decreasing concentration along the row. After 5 minutes, the TLC plate was sprayed with 0.2% DPPH in methanol. Discolouration of DPPH indicates scavenging potential of the compound tested[15].

DPPH assay by TLC

This preliminary test was performed with a rapid TLC screening method using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) as a spray reagent[16, 17]. Analytical TLC silica gel plate (10cm X10cm) was developed using chloroform : methanol : water (61:32:7) after application of 5μL of each test compound solution (1mg/mL), dried and sprayed with DPPH solution (0.2%, MeOH). After 5 minutes, the active compounds appeared as yellow spots against a purple background. The purple stable free radical 2, 2-diphenyl-1-picrylhydrazyl was reduced to yellow diphenylpicryl hydrazine. Quercetin was used as a positive control.

DPPH radical scavenging activity (Spectrophotometer)

The free radical scavenging capacity of the extracts of *Punica granatum* aqueous and alcoholic extract was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. Extracts of *Punica granatum* was mixed with 95% methanol to prepare the stock solution (10mg/100mL). The concentration of extract solution was 10mg/100mL or 100μg/mL. From stock solution 2mL, 4mL, 6mL, 8mL and 10mL of the solution were taken in five test tubes and serially diluted, final volume of each test tube was made up to 10mL whose concentration was then 20μg/mL, 40μg/mL, 60μg/mL, 80μg/mL and 100μg/mL respectively. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing extracts (20μg/mL, 40μg/mL, 60μg/mL, 80μg/mL, 100μg/mL) and after 10 minutes, the absorbance was taken at 517nm using a spectrophotometer (Systronics UV-Visible Spectrophotometer 119, INDIA). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10mg/100mL or 100μg/mL) of extracts. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank. % scavenging of the DPPH free radical was measured using the following equation $(A_{control}-A_{Test})/A_{control} \times 100$. Where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts. The IC_{50} value was defined as the concentration (in μg/mL) of extracts that produced 50% antioxidant effect[18].

Reducing power assay

Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form Potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700nm. This experiment was carried out as described previously[19]. 1mL of plant extract solution (final concentration 100-500mg/L) was mixed with 2.5mL phosphate buffer (0.2M, pH 6.6) and 2.5mL potassium ferricyanide [$K_3Fe(CN_6)$] (10g/L), then the mixture was incubated at 50^{0}C for 20 minutes. To this 2.5mL of trichloroacetic acid (100g/L) was added, and centrifuged at 3000rpm for 10 minutes. Finally, 2.5mL of the supernatant solution was mixed with 2.5mL of distilled water and 0.5mL $Fecl_3$ (1g/L) and the absorbance was measured at 700nm in UV-Visible Spectrophotometer (Systronics UV-Visible Spectrophotometer 119, India). Ascorbic acid was used as standard and phosphate buffer as blank solution. The absorbance of the final reaction mixture of two parallel experiments was expressed as mean ± standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

$$\% \text{ increase in Reducing Power} = \frac{A \text{ test}}{A \text{ Blank}} - 1 \times 100$$

A_{test} is the absorbance of test solution; A_{blank} is absorbance of blank. The antioxidant activity of the rind extract was expressed as IC_{50} and compared with standard.

Nitric oxide scavenging activity[15]

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of NO compete with oxygen leading to reduce production of NO.

Nitric oxide was generated from sodium nitroprusside, which at physiological pH liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions (NO_2-) which diazotize with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink color, which can be measured at 546nm. Sodium nitroprusside (10mM, 2mL) in phosphate buffer saline was incubated with the test compounds in different concentrations at room temperature for 30 minutes. After 30 minutes, 0.5mL of the incubated solution was added with 1mL of Griess reagent and the absorbance was measured at 546nm. The nitric oxide radicals scavenging activity was calculated according to the following equation (Acontrol-ATest)/Acontrol X 100.

Superoxide radical scavenging activity (PMS-NADH System)

Superoxide anions were generated using PMS / NADH system. The superoxide anions are subsequently made to reduce nitroblue tetrazolium, which yields a chromogenic product, which is measured at 560nm. Phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system was used for the generation of superoxide anion. It was assayed by the reduction of nitroblue tetrazolium (NBT). About 1mL of nitro blue tetrazolium (156μM), 1mL NADH (468μM) in 100mM phosphate buffer of pH 7.8 and 0.1mL of sample solution of different concentrations were mixed. The reaction started by adding 100μl PMS (60μM). The reaction mixture was incubated at 25°C for 5 minutes and absorbance of the mixture was measured at 560nm against blank samples. The percentage inhibition was determined by comparing the results of control and test samples. (Acontrol-ATest)/Acontrol X 100 is a formula adopted to measure % of free radical scavenging activity[20].

ABTS radical scavenging assay

ABTS (2, 2'-azinobis-3-ethylbenzothiozoline- 6-sulphonic acid) assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process that is associated with a change in absorption. The relatively stable ABTS radical was green and it was quantified spectrophotometrically at 734nm.

ABTS radical cations were produced by reacting ABTS and APS. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and percentage inhibition was calculated. The stock solutions included 7mM ABTS solution and 2.4mM potassium per sulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowed them to react for 14 hrs at room temperature in the dark. The solution was then diluted by mixing 1mL ABTS solution with 60mL methanol

to obtain an absorbance of 0.706 ± 0.01 units at 734nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1mL) were allowed to react with 1mL of the ABTS solution and the absorbance was taken at 734nm after 7 minutes using a spectrophotometer. $(A_{control}-A_{Test})/A_{control} \times 100$ formula is used to assess ABTS radical scavenging activity where $A_{control}$ is the absorbance of ABTS radical in methanol. All determinations were performed in triplicate (n = 3)[21].

H_2O_2 scavenging activity

H_2O_2 scavenging ability of aqueous and alcoholic extracts of *Punica granatum* fruit rind was determined according to the method of Ali *et al*.,[22]. A solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH 7.4). The aqueous and alcoholic extracts at the 30µg/mL concentration in 3.4mL phosphate buffer were added to a H_2O_2 solution (0.6mL, 40mM). The absorbance value of the reaction mixture was recorded at 230nm. Blank solution was containing the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of aqueous and alcoholic extracts and standard compounds were calculated using the formula:

$$[(A_{Control}-A_{Sample}) / A_{Control}] \times 100$$

Where $A_{Control}$ is the absorbance of the control, and A_{Sample} is the absorbance in the presence of the sample of aqueous and alcoholic extracts.

RESULTS

Phytochemical analysis

Phytochemical evaluation was performed for qualitative detection of various chemical constituents which aid in tracing the presence of active entity that elicit a major pharmacological response. The results proved the presence of alkaloids, flavonoids, phenolic compounds, tannins, lignins, fats & oil, inulin, cardiac glycosides and carbohydrates, which were tabulated in Table-1.

Table 1: Results of preliminary phytochemical analysis of *Punica granatum* fruit rind

S. No	Test	Aqueous extract	Alcoholic extract
1	Alkaloids	Negative	Positive
2	Steroids	Negative	Negative
3	Terpenoids	Positive	Negative
4	Flavonoids	Positive	Positive
5	Saponins	Positive	Negative
6	Phenolic compounds	Positive	Positive
7	Tannins	Positive	Positive
8	Lignin	Positive	Positive
9	Phlobatannins	Negative	Negative
10	Fat and Oil	Positive	Positive
11	Saponins	Negative	Negative
12	Inulin	Negative	Positive
13	Anthroquinones	Negative	Negative
14	Cardiac glycosides	Negative	Positive
15	Proteins	Positive	Positive
16	Carbohydrates	Positive	Positive
17	Aminoacids	Negative	Negative

Quantitative phytochemical analysis

Table 2 showed the availability of quantifiable tannins, phenolicacids and flavonoids. The aqueous and alcoholic extract yielded 122.33±6.42 & 176±5.29mg/g gallic acid equivalent phenolic content, 135.33±8.08 & 81.33±6.1 mg/g quercetin equivalent flavonoid and 81.66±3.51 & 114.23±12.16mg/g tannic acid equivalent tannins respectively.

DPPH scavenging

DPPH test provides simplified version to detect the antioxidant properties of various molecules present in the extracts. A DPPH solution is decolourized when the odd electron becomes paired off in the presence of a free radical scavenger. The colour becomes light yellow from deep violet. Dose dependent dot assay and chromatographical assay clearly indicated the DPPH scavenging nature of the extracts (Figure 1 and Figure 2).

Table 2: Results of quantitative phytochemical analysis of *Punica granatum* fruit rind

S. No	Phyto constituents	Concentration in mg/g	
		Ethanol extract	Aqueous extract
1	Flavonoids	135.33±8.08	81.33±6.1
2	Tannins	81.66±3.51	114.23±12.16
3	Phenols	122.33±6.42	176.00±5.29

Corresponding increase in absorbance is noted in extracts as well as standard when the concentrations of extracts and standard were increased. The percentage of DPPH radical scavenging activity of aqueous and alcoholic extracts (at 100µg/ml) and standard (at 50µg/ml) were 50.59±2.85, 60.10±1.82 and 52.67±0.55 respectively (Table 3).

Table 3: *In vitro* free radical scavenging effect of *Punica granatum* fruit rind by DPPH method

Percentage scavenging (mean±SD) of Triplicates						
Concentration	20µg/ml	40µg/ml	60µg/ml	80µg/ml	100µg/ml	IC_{50} µg/ml
Aqueous extract	4.97±2.65***	16.08±1.07**	22.23±0.46**	34.17±1.87 *	50.59±2.85**	135.27±39.30
Alcoholic extract	8.71±0.73**	15.64±1.47**	27.79±1.61**	41.73±1.37 *	60.10±1.82*	105.93±17.19
Concentration	10µg/ml	20µg/ml	30µg/ml	40µg/ml	50µg/ml	
Ascorbic acid	5.34±0.64**	14.39±0.98*	24.97±0.61*	37.95±1.68*	52.67±0.55*	64.67±18.18

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

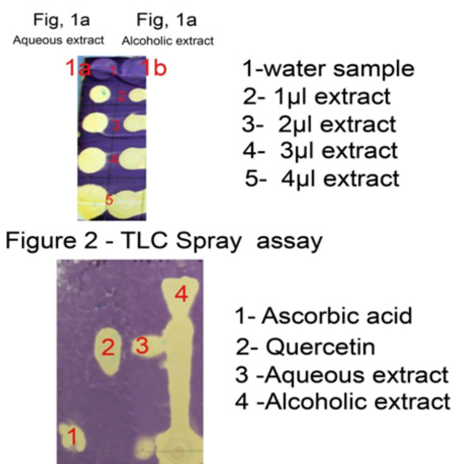


Fig. 1 & 2: DPPH scavenging activity of extracts and standard

Table 4: *In vitro* free radical scavenging effect of *Punica granatum* fruit rind by reducing power assay

Percentage scavenging (mean±SD) of Triplicates						
Concentration	20μg/ml	40μg/ml	60μg/ml	80μg/ml	100μg/ml	IC_{50} μg/ml
Aqueous extract	30.17±3.28**	42.36±2.07*	62.79±2.76*	87.66±4.78*	93.17±3.88*	45.48±7.54
Alcoholic extract	50.96±2.76*	61.63±1.09*	74.93±4.28*	74.80±9.10*	91.54±2.78*	40.03±14.72
Concentration	10μg/ml	20μg/ml	30μg/ml	40μg/ml	50μg/ml	
Ascorbic acid	28.15±3.6**	76.8±1.94*	83.85±2.70*	86.63±4.80**	96.26±1.1*	19.54±5.05

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

Table 5: *In vitro* free radical scavenging effect of *Punica granatum* fruit rind by nitric oxide scavenging assay

Percentage scavenging (mean±SD) of Triplicates						
Concentration	20μg/ml	40μg/ml	60μg/ml	80μg/ml	100μg/ml	IC_{50} μg/ml
Aqueous extract	45.23±3.98**	50.63± 0.94*	53.38±0.84 *	57.94±0.63*	65.44±4.2**	52.64±22.08
Alcoholic extract	56.31±1.55*	60.63±1.33*	71.06±2.29 *	73.73±3.59*	73.02±0.87*	43.12±19.47
Concentration	10μg/ml	20μg/ml	30μg/ml	40μg/ml	50μg/ml	
Ascorbic acid	13.43±0.77**	21.56±1.43**	33.2±2.24**	31.86±0.02*	36.42±4.6**	52.04±13.12

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

Reducing power assay

Aqueous and alcoholic extracts of *Punica granatum* fruit rind showed potent antioxidant power by reducing power ability. Aqueous extract yielded better antioxidant power (93.17±3.8%) than alcoholic extract (91.54±2.78%) at 100μg/ml concentrations. Ascorbic acid produced 96.26±1.1% reducing power at 50μg/ml concentrations. Results of reducing power assay significantly different (p<0.001) among various concentrations tested (Table 4).

Nitric oxide scavenging assay

Significant Nitric oxide scavenging activity was exhibited by aqueous and alcoholic extracts of *Punica granatum* fruit rind (Table 5). Alcoholic extract showed 73.03±0.87% Nitric oxide scavenging activity at 100μg/mL concentration. Similarly aqueous extract exhibited 65.44±4.2% inhibition and only 36.42±4.6% inhibition was noted for standard at 50μg/mL concentration.

Superoxide radical scavenging assay

Aqueous extract of *Punica granatum* fruit rind showed significant free radical scavenging activity against superoxide ions. The percentage of scavenging was found to be 59.90±2.37 which is slightly higher than alcoholic extract (46.88±1.61). Ascorbic acid was used as a reference standard which exhibited 50.70±1.24% supeoxide radical scavenging power at 50μg/mL concentration (Table 6).

Table 6: *In vitro* Free Radical scavenging effect of *Punica granatum* fruit rind by superoxide radical scavenging assay method

Percentage scavenging (mean±SD) of Triplicates						
Concentration	20μg/ml	40μg/ml	60μg/ml	80μg/ml	100μg/ml	IC_{50} μg/ml
Aqueous extract	11.33±0.79**	15.89±3.14***	31.05±3.50**	44.7±1.59 *	59.90±2.37*	97.11±16.97
Alcoholic extract	11.64±1.96**	19.05±1.42**	37.22±0.82*	41.76±2.1*	46.88±1.61*	94.78±11.46
Concentration	10μg/ml	20μg/ml	30μg/ml	40μg/ml	50μg/ml	
Ascorbic acid	4.57±1.55***	11.03±1.08**	35.58±4.93**	41.85±1.08*	50.70±1.24*	67.86±30.22

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

Table 7: ***In vitro*** **Free Radical scavenging effect of** ***Punica granatum*** **fruit rind by ABTS radical scavenging assay method**

Percentage scavenging (mean±SD) of Triplicates						
Concentration	20µg/ml	40µg/ml	60µg/ml	80µg/ml	100µg/ml	IC_{50} µg/ml
Aqueous extract	62.89±2.47*	72.1±0.72*	87.63±2.00*	90.88±0.62*	96.09±0.41*	34.78±14.04
Alcoholic extract	51.74±1.52*	59.06±0.28*	66.43±0.90*	77.01±0.70*	89.48±0.83*	41.16±14.85
Concentration	10µg/ml	20µg/ml	30µg/ml	40µg/ml	50µg/ml	
Ascorbic acid	55.57±7.64*	71.09±7.51*	81.69±3.03*	86.70±5.36*	92.79±2.16*	18.25±7.10

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

Table 8: ***In vitro*** **Free Radical scavenging effect of** ***Punica granatum*** **fruit rind by H2O2 method**

Percentage scavenging (mean±SD) of Triplicates						
Concentration	20µg/ml	40µg/ml	60µg/ml	80µg/ml	100µg/ml	IC_{50} µg/ml
Aqueous extract	34.24±9.61***	60.29±3.51**	68.39±1.8*	73.46±2.3*	79.76±2.59*	44.63±14.20
Alcoholic extract	7.52±3.03***	23.52±2.45**	47.33±4.06**	54.06±2.53*	59.39±0.71*	87.91±26.68
Concentration	10µg/ml	20µg/ml	30µg/ml	40µg/ml	50µg/ml	
Ascorbic acid	28.65±5.06**	41.53±2.29*	50.38±2.54*	58.21±8.38*	74.67±0.59*	27.82±7.07

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

Extracts of *Punica granatum* showed better ABTS scavenging activity, which was evident in table 7. Aqueous extract produced 96.09±0.41% ABTS scavenging power with 34.78±14.04µg/ml IC_{50} value followed by alcoholic extract 89.48±0.83% with 41.16±14.85 IC_{50} value. IC50 value for ascorbic acid was found to be 18.28± 7.10µg/ml

ABTS radical scavenging assay

H_2O_2 scavenging assay

Antioxidant compounds present in the extracts/standard can donate electrons to H_2O_2 and converted to H_2O. Both extracts of *Punica granatum* fruit rind showed promising antioxidant activity (79.76±2.59%for aqueous extract, 59.39±0.71% for alcoholic extract). Reference standard yielded 74.67±0.59% H_2O_2 scavenging activity (Table 8).

DISCUSSIONS

Biological and chemical research in Life Science evidenced that free radical and reactive oxygen species can be involved in a high number of diseases[23]. Numerous physiological and biochemical processes in the human body may produce oxygen centered free radical and other reactive oxygen species and byproducts. Over production of such free radical cause oxidative damage to biomolecules leading to may chronic diseases[24]. Plants are the important source for free radical scavenging molecules. Intake of natural antioxidant has been associated with reduced risk of cancer; cardiovascular diseases, diabetes and other diseases associated ageing.

Antioxidant is one of the most essential ingredient of today's menu/therapy because the antioxidative system protects the animal against reactive oxygen species (H_2O_2, superoxide, OH, singlet oxygen & nitrogen species) induced oxidative damage.Various synthetic antioxidants (BHT) are on the use, but they are suspected to be carcinogenic[25]. Natural antioxidants, therefore, have gained importance. Aqueous & alcoholic extracts of *Punica granatum* fruit rind has been studied for its antioxidant properties using different in vitro antioxidant methods. Flavonoids, phenolic acids, tannins, steroids are found in the extracts of *Punica granatum* fruit rind. *Punica granatum* fruit rind extracts showed good antioxidant effect, which could be due to the available phytoconstituents. In this respect, poly phenolic compounds commonly found in plants have been reported to have multiple biological effects like Anticancer[26], Antiproliferative[27], Antimicrobial[28], wound healing[29] and Antibacterial[30] activities including Antioxidant activity [26,31,32].

Potent antioxidant activity of *Punica granatum* fruit rind extracts was analysed by making use of 6 different methods. However, the efficiency of each extract differed against various free radicals depending on the specific assay methodology, which reflects the complexity of the mechanisms and diversity of the chemical nature of the plant material. Numerous scientists have reported the potent antioxidant capacity of pomegranate fruit juice and its components using multiple assay systems[31, 33, and 34]. However this is the first of this kind of work on fruit rind antioxidant assay. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating activity[35]. The aqueous & alcoholic extracts had significant radical scavenging effect on the DPPH radical. As DPPH is considered as the lipophilic radical, it readily accept electron from the antioxidant compound and converts its colour from violet to yellow which is detected at 517nm. The IC_{50} value of aqueous extract of *Punica granatum* was found to be 135.27±39.30µg/ml and for alcoholic extract 105.93±17.19µg/ml. Although the IC_{50} values of *Punica granatum* fruit rind extracts were higher than standard (32.33±9.08µg/ml), it was comparable to other tropical fruits which are considered to have a good antioxidant powers (Guava IC_{50} 2.1±0.63mg/ml; papaya IC_{50} 3.5±0.9mg/ml)[36]. Hydrogen donor in the extract may responsible for DPPH radical scavenging power of the extracts increased with the increased concentration of the extract.

Antioxidants present in the sample reduce $Fe^{3+}$to$Fe^{2+}$by donating electrons. Amount of Fe^{2+} can be assessed by measuring OD at 700nm. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging[37]. Table 4 indicated superior antioxidant power of extracts i.e., more than 90% at 100µg/ml concentrations. Nitrite was detected in nitric oxide scavenging assay method. Antioxidants compete with oxygen and reduce nitric oxide production which intern reduces the burden of nitric oxide. *P. granatum* extracts revealed significant antioxidant activity with increase concentration of extracts. The % of inhibition of Nitric oxide being 73.03±0.87% and 65.44±4.2% for alcoholic and aqueous extracts respectively at 100µg/ ml. Nitric oxide play an vital role in various inflammatory process. Higher levels of these radical are toxic to tissue and contribute to the vascular collapse. Hyper level expression of nitric oxide radical is associated with various carcinoma and ulcerative colitis. The toxicity of nitric oxide increases when it reacts with superoxide radical forming highly reactive peroxy nitrate anion (ONOO-). Reactive oxygen species, nitric oxide is implicated in inflammation, cancer and other pathological conditions[38].

The results of the present study suggested that the extracts of *Punica granatum* rind is a more potent scavenger of superoxide radical with IC_{50} value <100µg/ml (Table 6). Superoxide anions are highly toxic to cellular components. Hazra *et al.,* reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions. Chromatophore $ABTS^{+}$ was formed by the reaction between ABTS and potassium persulphate and reduced to ABTS by the action

of antioxidants available in the extracts. IC_{50} value for the extracts on ABTS scavenging process were 34.78±14.04μg 41.16±14.85μg[37]. Hydrogen peroxide inactivates a few enzymes directly, usually by oxidation of essential thiol group (-SH). It can cross membranes and reacts with Fe^{2+} and Cu^{2+} ions to form hydroxy radical and are responsible for various toxic effects. Antioxidant compounds react with H_2O_2 and converted into H_2O, which involved in normal metabolic activities of the cell[37]. Quantitative phytochemical analysis indicated that the plant contains significant amounts of phenolic compounds such as Total phenolic acids, tannin and flavonoids. These classes of compounds were responsible for antioxidant and free radical scavenging effect of plant material[27,32,37,38]. Furthermore all of our results were significantly different among various concentrations as well as different extracts and standard ($p<0.05$).

CONCLUSION

On the basis of the results it is concluded that the extracts contain higher quantities of phenolic compounds, which exhibit antioxidant and free radical scavenging activity. It also chelates iron and possesses reducing power. *In vitro* assay systems confirm *Punica granatum* fruit rind as natural antioxidants but it is doubtful that specific components responsible for antioxidant activity. Further *In vivo* assessment also needed to confirm antioxidant nature of *Punica granatum* fruit rind.

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