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Structural Insight and *in vitro* Free Radical Scavenging Capacity of Arabinogalactan Polysaccharides from the Peels of *Punica granatum*

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The juice extracted from *Punica granatum* L. fruit has been used for ages as an important functional food that can endow with healthcare benefits besides fundamental nutritional food that we intake. Although demonstration of antioxidant activity has been shown here, the target molecule responsible for this phenomenon remains unidentified. In this present work, we report a structural insight and antioxidant activity of its polysaccharide (PF-1), purified from water extract (WE) by precipitation with ethanol, ethanol soluble fraction (PF-2) and acetone extracted fraction (PF-3). The purified polysaccharide (PF-1) contains mainly arabinose, galactose together with lesser amount of rhamnose and glucose residues, and the molecular mass was determined 180 kDa. This fraction consists of T-(1,5)- and (1,3,5)-linked Ara_f; T-(1,6)- and (1,3,6)-linked Gal_p alongside (1,2,4)-linked Rha_p residues. The glucan is found to be β-(1→3)-linked glucopyranosyl residues with a molecular mass of 9 kDa. The *in vitro* antioxidant activity of the active fractions was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), hydroxyl radical scavenging and total antioxidant activity (TAA) assays. The arabinogalactan showed the highest potential among the polysaccharides. This work involves an economically viable simple extraction method and reports promising antioxidant capacity. The studies suggest that this polysaccharide from *Punica granatum* L. fruit could be a natural antioxidant.

Keywords: *Punica granatum* peels, Arabinogalactan, Radical scavenging activity, Phenolic acid.

INTRODUCTION

Antioxidant activity is generally referred to as a measure of the reduction of the oxidation of proteins, deoxyribonucleic acid (DNA) or other molecules mainly by restricting the propagation step in the oxidative chain reactions. The primary antioxidants role is to remove the free radicals directly formed, while that of secondary antioxidants is to cease the free radical formation alternatively through Fenton's reaction [1]. The raising extent of reactive oxygen species (ROS) and free radicals causes damage to bio-macromolecules present, while the attacks by the free radicals would be terminated by the antioxidants consumed in diet and minimize the outcomes faced due to these diseases [2]. The ROS are instant reactive species which are continuously synthesized by cellular enzymatic reactions. They are also produced under usual physical conditions in minute level, which are essential for regulating the normal cellular functions, and the interior antioxidant resistance capacity of the body have the ability to prevent any harmful effects. In addition, the consequences of DNA, proteins, lipids, and small cellular molecules modification, mediated by these free radicals is reported to be cancer, atherosclerosis and rheumatoid arthritis [3].

Now, research reveals that the antioxidants are vital for the defense mechanism of the body to combat against the continuous oxidative stress. There have been many edible food sources including the spices and other ingredients, which have revealed the presence of antioxidants previously [4]. In some medicinal plants, the peels, leaf extracts or seeds are reported to possess a range of natural antioxidants, which also assist in protecting and preserving their physical integrity as well as their heredity. These extracts from plants are prospecting compounds, which are applied as candidates for limiting the early aging process on skin thereby inhibiting the oxidation process. The compounds

like vitamin C and E have become a part of our daily food diet as well as in cosmetic items owing to their effective antioxidant activity. The search for novel edible sources and plant is never ending, since the natural antioxidant compounds are being substituted by the synthetic antioxidants, mainly due to its side-effects, besides protecting the aging cells. The added advantage in finding out novel antioxidants from natural source is that relatively simple established bioassays are easily available for measurement of *in vitro* and *in vivo* antioxidant activity, which has the ability to reduce oxidative stress.

The edible fruit is generally sweet in taste and available throughout the year and the fruit juice is consumed considering it as an essential health benefit refreshing drink. Besides the edible fruit, the hard peels *Punica granatum*, generally the non-edible part which is obtained after the extraction of the juice, is being generally discarded as a waste material in huge amounts. The extracts from pomegranate fruit are reported to contribute in anticarcinogenic, antibacterial and antihypertensive potential amongst others [5]. The extract finds application as an ingredient in mouthwash solution used for oral hygiene [6]. Interestingly, scope for further research is possible since the phytophenolic composition was initially screened to vary in the edible and non-edible parts of the fruit and the analyses of these extracts from various parts of the fruit becomes more important for biological activity. However, no report on its macromolecules present in the peels has been traced. The juice from *Punica granatum* L. fruit is considered as a healthy drink and a rich source of vitamins, since ages. *Punica granatum* L. generally belongs to the Punicaceae family and is native to the Himalayas in northern India [5]. For the medical ailment, the edible part and in some cases the non-edible part finds use in folklore medicine for various pathological symptoms [7-9]. Therefore, with a target to highlight the chemical structure of the polysaccharides isolated and extracted from the peels of *Punica granatum*, the antioxidant capacity of different active fractions were analyzed.

EXPERIMENTAL

The total sugars (arabinose and galactose) and uronic acid (as galacturonic acid) contents were estimated by PhOH-H_2SO_4 [10] and 3-phenylphenol-sulfuric acid [11] assays, respectively. Neutral sugar compositions of the poly- and oligosaccharides were determined after hydrolysis with 2 M CF_3CO_2H at 100 ºC for 2 h. Protein contents were estimated according to Lowry and coworkers [12] using bovine serum albumin as standard. Reduction ($NaBH_4$) and acetylation (Ac_2O) were carried out according to Blakeney *et al*. [13] and the generated alditol acetates were analyzed by gas chromatography (GC) (Shimadzu GC-17A, Japan). Monosaccharide response factors relative to myo-inositol were determined using standard sugars. The monosaccharide composition of the polymer was also determined after methanolysis and the generated methyl glycosides were separated as their trimethyl silyl (TMS)-derivatives [14] by GC. UV-vis absorption spectra were measured on a Shimadzu UV-1800 UV-vis spectrophotometer. GC was performed with a Shimadzu GC-17A chromatograph fitted with a DB-225 column. The electron impact mass spectra were recorded with a QP 5050A instrument (Shimadzu) at 70 eV. The ^{1}H NMR spectrum was recorded on a Bruker DRX-400 NMR spectrometer at 30 ºC in D_2O solvent.

Fresh reddish brown colored fruits of *Punica granatum* were purchased from the local market of Durgapur, India in adequate amount. The peels were detached from the fruits and washed with tap water for repeated times to remove the dust attached to the surface and the foreign particles. The cleaned peels were subjected to drying under moderate conditions under sunlight for about 7-8 days until found dried completely. The peels were grinded to fine powder using laboratory grinder.

Extract preparation: The finely grinded powder (200 g) was dissolved in deionized water (500 mL) and left for 1 h in a boiling water-bath. The supernatant solutions were pooled, filtered through a sintered-glass filter (G2), dialyzed and then lyophilized to yield the water extract, PG-WE (27.4 g). Next, the extracted material (PG-WE) was dissolved in water (100 mL). The polysaccharide was precipitated with 95% EtOH (4 vol). The precipitate, recovered by centrifugation was washed thoroughly with 95% EtOH, and finally dissolved in water, dialyzed in deionized water and then lyophilized (PF-1; yield 20.7 mg). The soluble material obtained from PG-WE with EtOH precipitation was marked as PF-2, ethanol soluble fraction. The residue obtained after the water extract was further subjected to treatment with 100 mL acetone at room temperature for 4 h. The acetone extracted fraction, marked as PF-3 was lyophilized and preserved for further chemical and antioxidant assay.

Phenolics analysis: The total phenolic content was estimated according to the Folin-Ciocalteu method as described [15]. Briefly, the mixture of 300 µL of sample and 1.5 mL of Folin-Ciocalteu reagent diluted 10 times with distilled water were added to 1.2 mL of 7.5% (w/v) Na_2CO_3 in water between 1 and 8 min. The absorbance was recorded at 765 nm against blank (300 µL distilled water) after incubation for 1 h at 30 ºC. Quantification was obtained by reporting the absorbance in the calibration curve of gallic acid used as standard phenolics ($r > 0.99785$). All assays were carried out in triplicate. The result was shown as milligrams of gallic acid equivalent per gram of sample (mg GAE/g of sample). Ester-linked phenolic acids were saponified in 0.5 M NaOH under N_2 for 24 h in the dark at room temperature, and then acidified with 6 M HCl. The resultant solution was extracted with ether, and the Et_2O phase was washed with water and quantified by gas chromatography-mass spectrometry (GC-MS) (Shimadzu GC-17A & QP 5050A, Japan) [15].

Molecular weight determination: The water extracted fraction (PG-WE) was initially passed through a S-8 microporous resin and then chromatographed on a Sephacryl S-300 column (2.6 × 30 cm; Amersham Biosciences AB, Uppsala, Sweden) using 0.5 M CH_3COONa buffer (pH 5.6) as eluent. The flow rate of the column was 30 mL h^{-1} and fractions of 10 mL were collected. The amount of carbohydrate in the eluent was determined by PhOH-H_2SO_4 assay [10]. The column was calibrated with standard dextrans. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_0)/(V_t - V_0)$ with V_t and V_0 being the total and void

volume of the column determined as the elution volume of dextran (molecular weight 1000 kDa) and methyl-β-D-glucopyranoside, respectively and V_e is the elution volume of the sample].

Glycosidic linkage analysis: Permethylation of the polysaccharide was carried out according to Blakeney & Stone [16]. Ahead of permethylation, the carboxyl groups of uronic acid moieties of polysaccharide were reduced to primary alcohols on the polymer level (PF1-R) using the method of Kim & Carpita [17], apart from $NaBH_4$ was used in place of $NaBD_4$. The carboxyl reduction step was replicated once more. For permethylation, samples (3-5 mg) were dried overnight (P_2O_5 *in vacuo*) and then 0.7 mL of dry DMSO was added to each sample and the mixture ultrasonicated. Next, lithium dimethylsulfinyl carbanion in DMSO (0.8 mL) was added and the solutions were stirred for 1 h at 15-26 ºC. Ice-cooled CH_3I was then added with external cooling to the vessel and the resulting mixture was stirred for 1 h. The excess of CH_3I was removed under a stream of air and the methylated samples were purified by partitioning with H_2O:$CHCl_3$: 2:1 (v/v). This process of permethylation was repeated twice more. Subsequently, the permethylated samples were hydrolyzed (2 M CF_3CO_2H, 3 h, 100 ºC), reduced (1 M $NaBD_4$ in 2 M NH_4OH, 3 h, 15-26 ºC), acetylated (Ac_2O-$HClO_4$) and analyzed as their partially methylated alditol acetates (PMAAs) by GC and GC-MS. The PMAAs were identified by (i) measuring their retention times relative to myoinositol and (ii) comparing saccharide composition of the parental polymer [18].

NMR spectroscopy: The ^1H NMR spectrum was recorded on a Bruker 400 spectrometer (Bruker Biospin AG, Switzerland) operating at 400 MHz. The sample (~10 mg) was heated (at 80 ºC for 30 min) with water (1 mL), centrifuged and the resulting supernatant lyophilized. The freeze-dried sample was deuterium exchanged by lyophilization with D_2O (Sigma-Aldrich) and then examined in D_2O (99.96 atom % D) as described earlier [19].

Radical scavenging activity using DPPH• method: The method used for measurement of free radical scavenging capability was carried out as reported in Shimada *et al.* [20]. The free radical scavenging capacity of PF-1, PF-2 and PF-3 were measured in the concentration ranging from 20-100 µg/mL using a stable DPPH• and butylated hydroxyanisole (BHA) as standard. To equal volume of different concentrations of sample solution, 1 mL of freshly prepared methanolic solution of DPPH (0.5 mM) was added, mixed well, and then let stand for 1 h in a dark area at 30 ºC. The absorbance of the sample solution and DPPH• was recorded at 518 nm against blank. All assays were carried out in triplicate and the capability to scavenge the DPPH• was determined using the following equation:

$$\text{Inhibition (\%)} = \frac{A_1 - A_2}{A_1} \times 100$$

where A_1 is the absorbance of DPPH• solution and A_2 is the absorbance of DPPH solution in presence of the sample or standard after 1 h.

Ferric ion antioxidant power (FRAP) assay: The FRAP assay was done according to Benzie & Strain [21] as modified by Pulido *et al.* [22]. The concentrations of sample solution used for measurement were 20, 40, 60, 80 and 100 µg/mL. Initially, the oxidant was prepared by mixing 2.5 mL of a 10 mM TPTZ [2,4,6-tri(2-pyridyl-5-triazine)] solution in 40 mM HCl with 25 mL of 0.3 M acetate buffer (pH 3.6) and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$. The final solution has Fe(III) of 1.67 mM and TPTZ of 0.83 mM. To measure the FRAP value, 900 µL of freshly prepared FRAP reagent was warmed to 37 ºC and a reagent blank reading was taken at 593 nm; then 30 µL of test sample and 90 µL of distilled water were added. Absorbance readings were taken after 0.5 s and every 15 s until 30 min using a Shimadzu UV-1800 spectrophotometer. The change of absorbance ($\Delta A = A_{30\,min} - A_{4\,min}$) was calculated and related to ΔA of Fe(II) standard solution. Aqueous solutions of known Fe(II) concentrations (100-2000 µM/L $FeSO_4 \cdot 7H_2O$) were used for calibration. Butylated hydroxytoluene (BHT) was used as reference compound.

Hydroxyl radical scavenging activity: The hydroxyl radical scavenging capacity of sample extracts was measured according to the method of Klein *et al.* [23]. The concentration range (20-100 µg/mL) of extracts used for this measurements were added with 1.0 mL of iron-EDTA solution, 0.5 mL of EDTA solution (0.02%), and 1.0 mL of DMSO (0.8% DMSO (v/v) in 0.1 M phosphate buffer, pH 7.2), respectively. The mixture was further initiated by adding 0.5 mL of ascorbic acid (0.2%) and kept at 80-90 ºC for 15 min in a water bath. 1.0 mL of ice-cold TCA (17% w/v) was added to the reaction mixture followed by the addtion of 3 mL of Nash reagent and left at 27 ºC for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412 nm against reagent blank. In this assay, α-tocopherol was used as reference compound. The % hydroxyl radical scavenging activity (HRSA) was calculated using the following formula:

$$\text{HRSA (\%)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Total antioxidant activity assay by radical cation ($ABTS^{\bullet+}$) and chelating capacity: The stock solution of 10 mM ABTS concentration was prepared in water. Similarly, the $ABTS^{\bullet+}$ radical cation was generated by treating the ABTS stock solution with 2.5 mM $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at 27 ºC for 18 h prior to the use. In this assay, the solution was diluted in EtOH (~ 1:90 v/v) and equilibrated to 27 ºC to give an absorbance at 734 nm of 0.700 ± 0.02 in a 1 cm cuvette. The concentration of sample extract, which produced an inhibition between 20 and 100% of the blank absorbance was noted. Triplicate determinations were done at each dilution of the standard, and the % inhibition of the blank absorbance at 734 nm was plotted as a function of α-tocopherol concentration [24]. The unit of total antioxidant activity (TAA) was determined as the α-tocopherol concentration having comparable antioxidant activity expressed as µmol/g sample extracts.

The metal chelating property of the sample extract was performed according to the reported bipyridyl assay [25]. Initially, a reaction mixture was prepared containing 1 mg of

the sample extract, 0.25 mL of 1 mM $FeSO_4$ solution, 1 mL of 0.2 M Tris-HCl buffer (pH 7.4), 1 mL of 2,2′-bipyridyl solution, 0.4 mL of 10% NH_2OH-HCl and 2.5 mL of EtOH. Finally, the volume was made up to ~10 mL with deionised water and the absorbance was measured at 522 nm. The chelating activity of the samples was expressed as mg EDTA equivalent/g sample extracts and the experiment was performed using EDTA as standard.

Statistical analysis: For this purpose, all the experiments were repeated atleast three times and the data were presented as mean ± SD after replications in thrice per sample. The Origin 8.0 Pro software (Microcal Software Inc., USA) was used to analyze the data. The Fisher Least Significance Test was employed to test the equality of variances and oneway ANOVA was used to estimate the statistically significant difference ($p \leq 0.05$).

RESULTS AND DISCUSSION

Isolation, chemical analyses and molecular mass of arabinogalactan polysaccharide from *Punica granatum* peels: The main focus of this chemical profiling is to characterize the water, ethanol and acetone extracted fraction obtained from the powdered peels of *Punica granatum* and to analyze their radical scavenging capacity. The dry powdered peels upon extraction with water yielded a water soluble fraction (PG-WE). This fraction recovered an amount of 9.8% with respect to the starting materials weight and showed the presence of Gal, Ara, Rha, Glc, and GalA residues (Table-1). In this study, all the fractions were extracted using standard extraction protocols, as evident from Fig. 1. The water extracted fraction (PG-WE) showed mainly arabinosyl and galactosyl residues as the

TABLE-1
COMPOSITION OF THE NEUTRAL SUGAR, PHENOLIC ACID AND YIELD OF DRY PEELS OF *Punica granatum* POWDER WATER EXTRACT AND OF FRACTIONS[a] OBTAINED THERE FROM

Fractions	PG-WE	PF-1	PF-2	PF-3
Yield[b]	32	9.8	5.6	3.5
Total sugar[c]	84	90	71	59
Rhamnose[d]	1	2	2	1
Arabinose[d]	23	37	19	13
Xylose[d]	1	Trace	1	1
Mannose[d]	1	1	1	1
Galactose[d]	61	60	58	51
Glucose[d]	3	1	1	Trace
Ferulic acid[e]	Not determined	0.49 ± 0.01	Not determined	Not determined
Sinapic acid[e]	Not determined	0.08 ± 0.01	Not determined	Not determined
(*E*)-coumaric acid[e]	Not determined	0.12 ± 0.02	Not determined	Not determined

[a]Water extracted fraction (PG-WE), precipitated pure fraction (PF-1), ethanol soluble fraction (PF-2) and acetone soluble fraction (PF-3); [b]Weight % of dry weight; [c]% Weight of fraction dry weight; [d]Molar % of neutral sugars; [e]values are means of triplicate determinations (n = 3) ± SD and expressed in µg/mL.

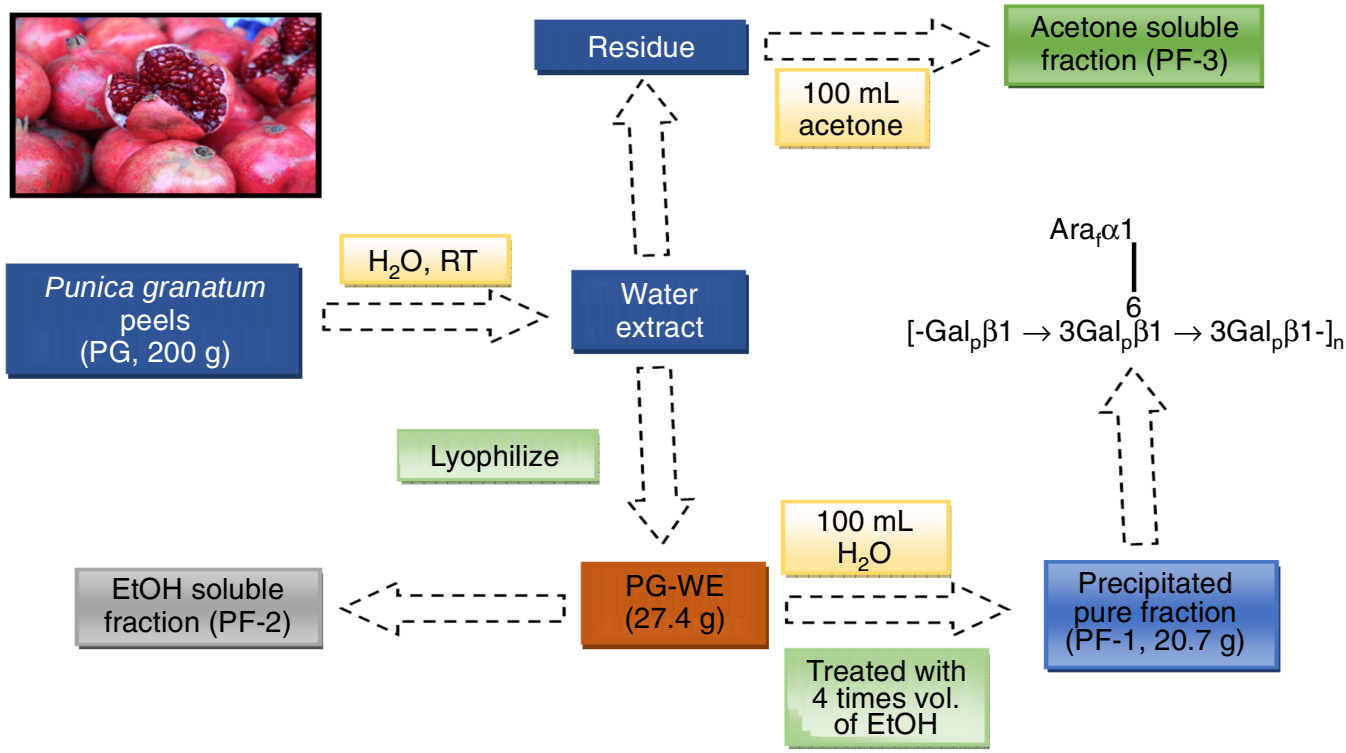

Fig. 1. Schematic representation of methods of extraction and purification of polysaccharides from the dry powdered peel of *Punica granatum*

major sugar constituents and its protein content was determined to be 16% (w/w). The amino acid composition in proteins in PG-WE fraction indicated the presence of alanine (20.4%), serine (10.5%) and glycine (6.7%). Thin layer chromatography (TLC) of the monosaccharides confirmed the presence of galactouronic acid.

Phenolic acid analysis: The presence of phenolic acids was detected in the polysaccharide (PF-1) under investigation. The total phenolic acid content of PF-1 was estimated to be 109 mg GAE/g of sample. The main component identified was ferulic acid (0.49 ± 0.01 µg/mL), sinapic acid (0.08 ± 0.01 µg/mL) (*E*)-coumaric acid (0.12 ± 0.02 µg/mL) as per the data of the liberated phenolic acids from the GC-MS analysis data (Table-1). The de-esterified derivative resulted from the polysaccharide upon treatment with an alkali solution.

Molecular weight determination: To identify the structure of the phytochemicals present in the peels of fruit, the water extracted fraction (PG-WE) was purified by size exclusion chromatography technique. The PG-WE solution was initially eluted through a S-8 macroporous resin and then passed through a Sephacryl S-300 packed column, yielded two fractions A (marked as PF-1) and B, which were eluted with NaOAc buffer (pH 5.5) of strength 0.2 and 0.5 M succes-sively (Fig. 2). These fractions accounted for 45% and 30% of the total sugars recovered from the column. The major fraction PF-1 contained rhamnose, arabinose, galactose, glucose and galacturonic acid in the molar ratio of 8:39:32:3:12. The remaining sub-fractions showed a similar trend of monosaccharide compositions (Table-1). The molecular weight of the major purified subfraction (PF-1) was determined as 180 KDa.

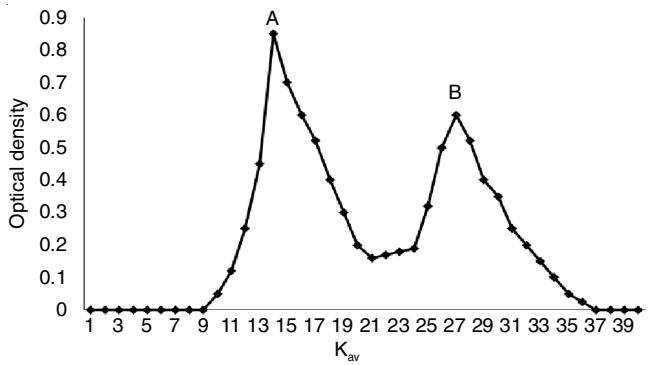

Fig. 2. Elution profile of the water extracted fraction (PG-WE) of the powdered peel of *Punica granatum* through the Sephacryl S- 300 column with 0.5 M sodium acetate buffer (pH 5.0) at 20 mL/h. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_0)/(V_t - V_0)$ with V_t and V_0 being the total and void volume of the column determined as the elution volume of glucose and standard dextran (500 kDa), respectively, and V_e is the elution volume of the sample]

Glycosidic linkage analysis: The data obtained from the methylation analysis of the purified fraction, PF-1 (Table-2), indicated the possibility of a highly branched polysaccharide and confirmed presence of Ara_f (2,4-Me_2-Ara) and Gal_p (2,3,4,6-Me_4-Gal) units as non-reducing end. The arabinofuranosyl residues were substituted at *O*-4,5 and *O*-3 in accordance with the results of 2,3-Me_2-Ara and 2-Me-Ara residues. The

TABLE-2
PARTIALLY METHYLATED ALDITOL ACETATES OBTAINED FROM THE PURE FRACTION (PF-1) ISOLATED FROM *Punica granatum* PEEL POWDER

Linkages[a]	*m/z*	Peak area[b]
T-Ara_f	43, 45, 102, 118, 129, 161	8
(1→5)-Ara_f	43, 102, 118, 129, 162, 189, 233	27
(1→3,5)-Ara_f	43, 118, 201, 261	13
T-Rha_p	43, 102, 118, 131, 162, 175	2
(1→4)-Glc_p	43, 45, 102, 113, 118, 130, 162, 233	1
T-Gal_p	43, 45, 101, 102, 118, 129, 145, 161, 162, 205	3
(1→3)-Gal_p	43, 45, 101, 118, 129, 161, 174, 234	13
(1→6)-Gal_p	43, 102, 118, 129, 162, 189, 233	1
(1→3,6)-Gal_p	43, 118, 174, 189, 234	22

[a]Linkage of monosaccharides. T-Ara_f denotes 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, *etc*.; [b]% of total area of the identified peaks.

linkage of galactopyranosyl units were found as T-(1,6)- and (1,3,6)-linked confirmed from the presence of 2,3,4-Me_3-Gal and 2,4-Me_2-Gal derivatives respectively. Additionally, the rhamnopyranosyl unit was determined as (1,2,4)-linked, due to the presence of 3-Me-Rha derivative. The glucopyranosyl residues were also detected in trace amount as 1,3-linked. Therefore, 3-*O*- and 4,5-*O*- substituted Ara_f and 6-*O*- and 3,6-*O*-substituted Gal_p are the constitutents present in this type of arabinogalactan polysaccharide. Overall, this arabinogalactan (AG) polysaccharide obtained from this fruit rind hold unique structural features compared to those extracted from other bioactive AG polysaccharides of different sources [19,26].

NMR analyses: From the NMR spectrum of the purified arabinogalactan polysaccharide fraction (PF-1) presented in Fig. 3, the signals around δ 5.35 was assigned to the anomeric H-atoms of T-α-Ara_f and that around δ 5.15 to the anomeric H-atoms of (1,3)- and (1,3,5)-linked Ara_f residues. This macromolecule depicted sharp signals from δ 4.4 to 4.49 region mainly due to the resonances of H-1 of the T-(1,6)- and (1,3,6)-

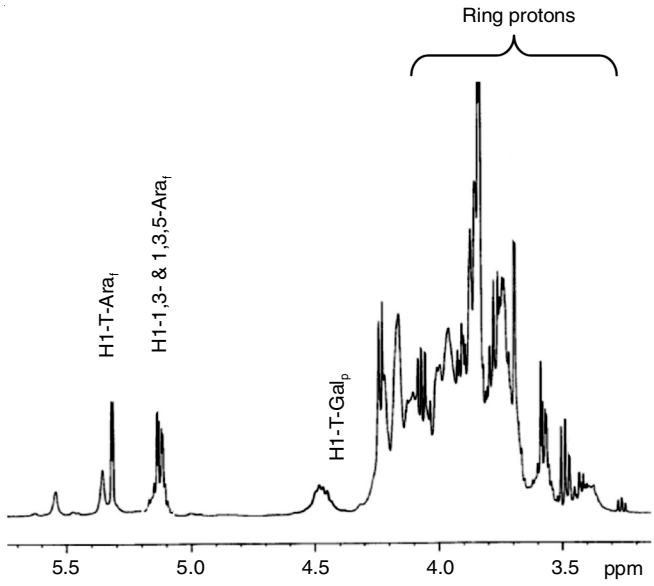

Fig. 3. ^{1}H NMR spectrum of the purified arabinogalactan polysaccharide (PF-1) from *Punica granatum* peels recorded in D_2O solution

linked Gal_p residues. The area with broad signals in the region δ 3.45-4.25 ppm may be attributed to the resonances due to the ring protons as compared to previously reported polysaccharides [19,20]. Usually, the ^1H NMR spectrums of polysaccharides are complex and overlapping, thus making it difficult to predict an accurate structure, but a temporary sketch of the polysaccharide present is always predicted.

Screening of antioxidant capacity

Scavenging effect on DPPH•: For measuring the antiradical scavenging capacity of the various extract from biological sources, the DPPH• scavenging assay is by far known to be a simple, accurate and effective method. The radical compound shows an absorption maximum peak at 518 nm (purple) [26]. The purple color was seen to lighten promptly as and when the H• was scavenged by the DPPH and finally turns to 1,1-diphenyl-2-picrylhydrazine [27]. The scavenging power of the antioxidant extract was determined from the extent of discoloration. A potent scavenging capability at a dosage of 100 µg/mL was detected with the arabinogalactan fractions PF-1 (84.2 ± 0.14 %) and PF-2 (68.33 ± 0.1 %) compared to the control BHA (96.5 ± 0.11 %) on DPPH• as evident from the plot presented in Fig. 4a. The overall order of scavenging capability of the sample extract and standard were found to be BHA > PF-1 > PF-2 > PF-3. It is the presence of the phenolic acid quantity that led to the DPPH• scavenging potential together with reducing potential activity. On the other hand, the other unidentified phytochemicals might be the cause for their potent antioxidant capacity, which triggers the possibility of future research. It is the due to the presence of electrons and their transfer ability along with the presence of phenolics, which is the outcome of the scavenging effect of sample extract on DPPH• radical [28].

FRAP assay: In FRAP assay, the reductant involved may be traced from the cleavage of a series of free radical by H-atom donation. A promising reducing ability of fraction PF-2 is marked over fraction PF-1 (Fig. 4b), owing to the polyphenolic content. The curve shows the reducing ability of the different fractions, the trend of which was found gradually increasing from low to high concentration. It is expected to quench the free radicals and get converted to more stable products, thereby acting as a reductant. Unexpectedly, the reduction potential of BHT, considered as a control showed the same value (92 ± 0.15%) as that of the ethanol soluble fraction (PF-2) at a dosage of 100 µg/mL. This was followed by the reduction potential was observed for the PF-1 (81.33 ± 0.12 %) and PF-3 (71.4 ± 0.11%) fractions sequentially at the same dosage. At other dosage, the reduction potential of BHT was followed the order PF-2 > PF-1 > PF-3. The reduction potential and the polyphenolics may be interrelated, which has been established in this work as was observed in many plant extracts previously [29]. The overall conclusion from this part that the polyphenolics in the sample extracts donates electron and can terminate radical chain reaction by converting the free radicals to a stable compound.

Hydroxyl radical scavenging activity: In the OH• scavenging assay, a combination of ascorbic acid-Fe(II) EDTA complex was used to generate the free radicals. To determine the OH•, the DMSO oxidation reaction followed by subsequent treatment with Nash reagent [30]. The OH• is one of the abundant oxygen centred species, and is responsible for severe damage owing to the free radical generated *in situ* within a biological system. The scavenging acitivity of the *Punica granatum* peel extracts with OH• concentration ranging from 20-100 µg/mL are plotted in Fig. 5a. It was observed that the PF-1 fraction depicted the most radical scavenging capacity at a dosage of 100 µg/mL. The ethanol (PF-2) and acetone (PF-3) extract showed comparable activity in between the concentrations of 40-100 µg/mL. The OH• scavenging power sequence of the extracts and that of the standard are as follows: α-Tocopherol > PF-1 > PF-2 > PF-3.

ABTS•+ cation radical scavenging activity and metal chelating activity (MCP): The potential of the extracts analyzed was expressed in terms of µM equivalent of α-tocopherol solution in this 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) ABTS•+ scavenging experiment, possessing antioxidant activity equivalent to per gram of the sample solution. This assay may be regarded as an important method to analyze the antioxidant capacity of H-donating moiety. The total antioxidant activities (TAA) values with varying dosage of the extract

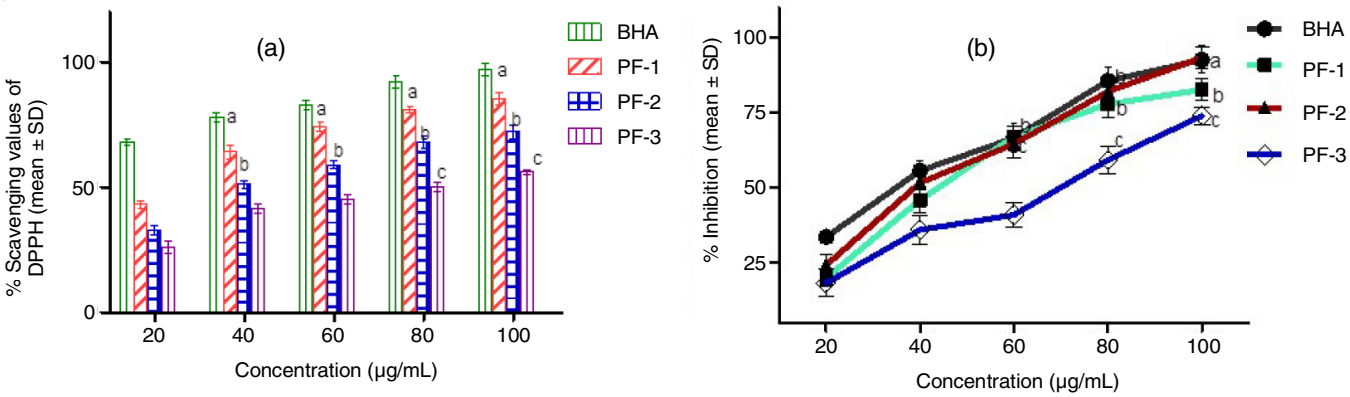

Fig. 4. Free radical scavenging activity of *Punica granatum* peel powder extracts analyzed by (a) DPPH method & (b) FRAP assay. Values are means of triplicate determinations (n = 3) ± SD. $p < 0.05$ was statistically significant. (a =< 0.001, b =< 0.01 c =< 0.05) Statistical analyses were performed with one way analysis of variance (ANOVA) followed by Posthoc Dunnet's test (Graph Pad Prizm software, version 5); BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene

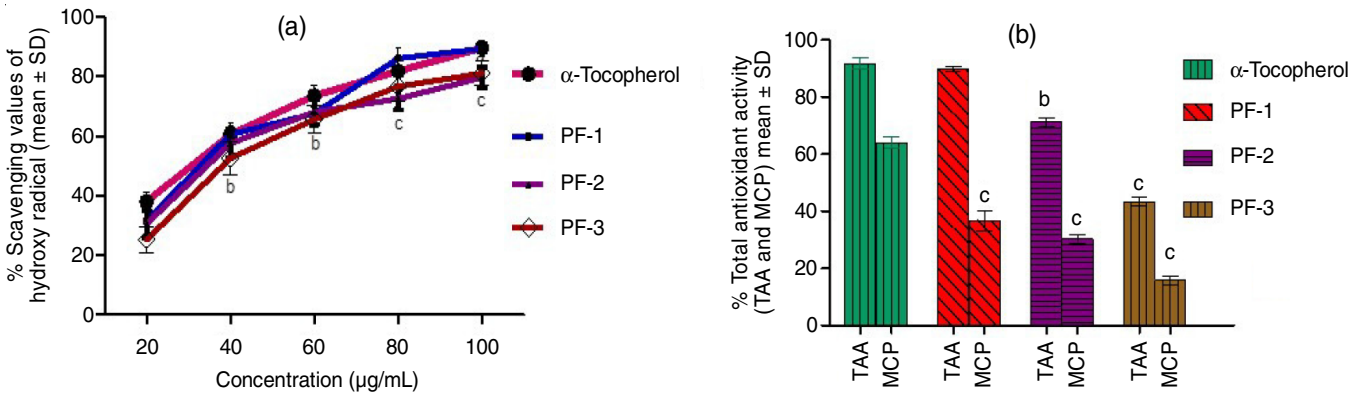

Fig. 5. Free radical scavenging activity of *Punica granatum* peel powder extracts analyzed by (a) hydroxyl radical method & (b) total antioxidant activity (TAA) using ABTS radical cation and metal chelating property (MCP) using Mg-EDTA complex. Values are means of triplicate determinations (n = 3) ± SD. $p < 0.05$ was statistically significant. (a =< 0.001, b =< 0.01 c =< 0.05)

ranging from 20-100 µg/mL (Fig. 5a). The TAA values of the extract was found in the range from 87.23 to 42.7 µmol α-tocopherol/g extract and the values are significantly ($p < 0.05$) different. It may be inferred that PF-1 exhibited the highest and comparable activity (> 50 µmol equivalent of α-tocopherol/g extract, respectively) followed by the PF-2 and PF-3 fraction on equivalence with α-tocopherol (90.8 ± 0.20%) as standard. The metal chelating agents, Mg-EDTA stabilizes the oxidized form of the metal ion and hence is considered as a secondary antioxidant due to the reduction in redox potential. Additionally, damage due to oxidation of the protein model system is initiated by Fenton's reagent, where a Fe^{2+} ion catalyzes the transformation of H_2O_2 to $OH^{\bullet}$ and ultimately gets converted to Fe^{3+} ion. From the metal chelating property, the extracts and α-tocopherol control, the value of the PF-1 fraction was the highest (35.43 ± 0.25%) and the PF-3 fraction to be the least (Fig. 5b). Overall, these data provides a solid basis towards the promotion of non-edible parts of pomegranate as dietary and pharmaceutical products. The available results suggest that these extracts might be a probable find for functional food health products and of various therapeutic benefit. Also, the pomegranate peel extracts enriched functional foods requires the developing technologies for better applications, thereby integrating the health promoting components into food without affecting their functionality.

Conclusion

The outcome of this work from powdered peels of *Punica granatum* fruit derived PF-1, PF-2 and PF-3 fraction with particular emphasis on its chemical structure and antioxidant activity may be summarized as: (i) an arabinogalactan polysaccharide of 180 kDa molecular mass with esterified phenolic acids was isolated employing simple, cheap extraction method, (ii) the phenolic acid constituents are considered to be the active sites for depicting antioxidant properties, (iii) the antioxidant activity of this macromolecule is equivalent to standard synthetic antioxidants, (iv) a promising antioxidant capacity of the extract was reported using $DPPH^{\bullet}$ radical assay, FRAP method, $OH^{\bullet}$ scavenging assay, $ABTS^{\bullet+}$ cation radical scavenging and metal chelating activity (MCP). Furthermore, the difference in the results of these scavenging assays may be attributed to the difference in functional group and structure in each fraction. This result establishes the fact that the investigated peels of pomegranate fruit cultivars depicted strong antioxidant activity. Also, instead of the peels being thrown away, which are an additional concern of environmental pollution, it may well be exploited as a scientific basis to promote added value for pharmaceutical and cosmetic purposes, owing to its potent natural antioxidant source. Although many beneficial properties of *Punica granatum* fruit were mentioned, yet there is a gap of research areas, which needs to be addressed and further explored for its pharmacological behaviour over a range of clinical applications and appropriate dosages in the therapy of human disorders with a special emphasis to maximize its benefits without any detrimental effects.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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