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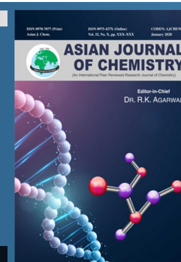

Chemical Profiling of Polysaccharides Present in Peels of *Citrus limetta* and Bioassay based Screening of *in vitro* Antioxidant Activities

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In this analyses, the chemical compositions of polysaccharides isolated from the peels of *Citrus limetta* had been studied and discussed its antioxidant activity of different active fractions. To emphasize the chemical structure of polysaccharides, a rhamnoglucan polysaccharide was identified with probable ester linked phenolic acid. The sugar composition and purification by size exclusion chromatography (SEC) has been presented. The antioxidant capacities of the extracts prepared from *Citrus limetta* peel powder were determined using well known *in vitro* systems and standard procedure for ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl ($DPPH^•$), 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonicacid) ($ABTS^{•+}$), hydroxyl radical ($OH^•$), nitric oxide (NO) assay, total antioxidant activity (TAA) and metal chelation property. The rhamnoglucan (A) present exhibited the highest bioactivity potentiality succeeded by traces of uronic acid and galactan. From the investigation, it could be emphasized that water extracted polysaccharide, which brings forth potent pharmacological activities figures out the importance as alternative natural compounds as to-be-exploited leads for low-cost sources of efficient bioactive molecules with strong antioxidant activities in different pharmaceutical and cosmoceutical formulations.

Keywords: ***Citrus limetta* peels, Chelating capacity, FRAP assay, Polysaccharides, Antioxidant activity, Rhamnoglucan.**

INTRODUCTION

The search for new antioxidants exclusively from natural sources including plants, edible food sources or discarded waste materials with radical scavenging properties has led to a number of experiments and wide range of findings therefrom in the last decade [1]. The role of antioxidants is to resist the process of oxidation by free radicals scavenging, inhibiting the lipid per oxidation and thereby preventing the disease progression. The prolonged use of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butyl hydroquinone and propyl gallate has been found to be an old practice and their safe use is questionable to the consumers [2]. Hence, the need of hour is to bring forth new effective formulations with antioxidative activities preferably from natural origin, cheaper in price with hardly any side effects happens to be the need of the hour and very active domain of research to reckon with. There have been several reports of official medicinal herbs from natural product sources in the last couple of decades that have ignited the area of natural products research with added structural elucidation of the active component and its applications. In many cases, it has been found that they have been established as a cure medicine for the diagnosis of several infections and as food preservatives from the adverse effect of the oxidants present [3]. The free radicals are the basic components to any biochemical process and highlight an important aspect of aerobic life and metabolism [4]. The most commonly available reactive oxygen species (ROS) includes superoxide anion, peroxyl (ROO^-), hydrogen peroxide (H_2O_2) and reactive hydroxyl (OH^-) radicals. Similarly, nitric oxide and per oxynitrite anion ($ONOO^-$) are the most commonly available reactive nitrogen species (RNS), which are the outcome of normal cellular metabolism. These ROS and RNS are the main contributors in the detection of a number of oxidative stressed related human diseases like carcinogenesis, neurological degenerative diseases, ulcerative colitis, rheumatoid arthritis and cardiovascular diseases [5].

Considering the side effects and limitations of the synthetic antioxidants, there has been a trend to locate novel alternative

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natural compounds with efficient antioxidant activities. Several studies have depicted that natural phenolic phytochemicals possesses, anticarcinogenic or antimutagenic activities. In addition, there have been quite a number of reports about the edible and non-edible plants, where the phenolic compounds was found responsible for its antioxidant capacity [6]. Among them, not only plants but a large number and types of plant material, *viz*., seeds, hulls, barks, leaves, fruits, flowers and roots were examined in these years and found excellent potential source for new antioxidant compound with profound activity [7]. Musambi, *Citrus limetta* (also known as sweet lime locally) is most commonly used as a very popular fruit in different parts of the world owing to its unique colour, flavor and taste. For centuries, this fruit is traditionally used as a health drink rich in vitamin sources [8]. The fruit juice is reported to be rich in vitamin A and C along with a considerable amount of dietary minerals. Vitamin C present has been regarded as a vigorous antioxidant that protects our cells from the harmful free radicals, which is the case in most of the long-standing disease and age related issues. Studies and reports are aplenty to advocate this fruit as a reservoir of high levels of biologically active components. No previous report neither on its structural features of the bioactive compounds present in it nor antioxidant activities of the same hereof, although some group have reported on the larvicidal efficacy of its extracts and pectinase production ability [9]. Accordingly, the target of the present research was to characterize and estimate their sugar content and measure their antioxidant activities employing the use of several bioactivity guided purification, isolation and *in vitro* assay systems for characterization of the active components present herein. This work will provide a deep insight regarding the chemical information of bioactives present within, which could be exploited judiciously reference to their medicinal value as future drugs.

EXPERIMENTAL

2,2′-Azino*bis*(3-ethylbenzothiozoline-6-sulfonic acid) disodium salt (ABTS), 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{•+}$), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^•$), ethylenediamine tetracetic acid (EDTA) disodium salt, 2,4,6-tri(2-pyridyl-5-triazine) (TPTZ), 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox), 2,2′-bipyridyl, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), rutin, α-tocopherol, hydroxylamine hydrochloride ($NH_2OH·HCl$), trichloroacetic acid (TCA) and other chemicals were obtained from either of the reputed well known brands *viz*. Himedia, Fluka, Merck or Sigma.

Plant materials: Fresh yellowish green colored fruits of *Citrus limetta* were purchased from Chinsurah market, Chinsurah, Hooghly, India in adequate amount. The peels were detached from the fruits and washed with tap water for repeated times to clean the attached foreign particles and dust attached. It is needless to mention that nicely examined and good quality peels were used to carry out the desired work and any infected material was discarded. The cleaned peels were subjected to drying under moderate conditions under sunlight for about 7-8 days until found dried completely.

Preparation of peel extract: The dried peels were mechanically grinded and sieved to get fine powder (CP). Then 15 g of powdered CP were extracted by stirring with water, followed by addition of 50 mL of 50% methanol and 50% ethanol each (1:5 w/v) in separate fractions at 27 ºC for 24 h and centrifuged (using REMI 24 centrifuge) at 9,000 rpm for 15 min to obtain fraction A,B and C, respectively. The air-dried residue, was re-extracted by occasional shaking with 50 mL of 50% acetone for fraction D. The solvents of the respective extracts from each fractions were evaporated under reduced pressure, using a rotary vacuum evaporator in reduced pressure at 45 ºC (Eyela Rotary evaporator, Model-VN-1100) and the residual moisture was removed by lyophilization (VirTis BenchTop Pro Freeze Dryer, Model-XTXL-55). The dried extracts were used directly for assessment of antioxidant capacity using standard protocols.

Size exclusion chromatography (SEC): The water extracted fraction (A) 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 sodium acetate buffer (pH 5.6) as eluent. The flow rate of the column was 0.5 mL/min, and fractions of 10 mL were collected and checked by phenol-sulfuric acid reaction [10]. The column was calibrated with standard dextrans. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_o)/(V_t - V_o)$ with V_t and V_o being the total and void volume of the column determined as the elution volume of potassium hydrogen phthalate and dextran, respectively and V_e is the elution volume of sample].

Chemical analysis: All experimental determinations were repeated and readings were used at least in duplicate. Large volumes of aqueous solution were concentrated under diminished pressure at ~45 ºC (bath) and the reduced volume was lyophilized. Total sugars and uronic acids were determined by the phenol-sulfuric acid [10] and *m*-hydroxydiphenyl [11] assay, respectively. For the determination of sugar composition, the monosaccharide residues released by acid hydrolysis were converted into their alditol acetate [12] and analyzed by gas-liquid chromatography (GLC; Shimadzu GC-17A, Shimadzu, Kyoto, Japan). Monosaccharides were identified by thin-layer chromatography and gas-liquid chromatography-mass spectrometry (GLC-MS; Shimadzu QP 5050 A, Shimadzu, Kyoto, Japan) as described elsewhere [13]. Alternatively, TMS-derivatives of methyl glycosides were analyzed by GLC [14].

Linkage analysis: Triethylamine form of purified fraction rhamnoglucan (AP1) amounting 5 mg was exposed for three rounds of methylation [15]. Permethylated samples were hydrolyzed, converted into their partially methylated alditol acetates and analyzed by GLC and GLC-MS as described earlier [16].

NMR analysis: The ^{1}H NMR spectrum was recorded on a Bruker 400 spectrometer (Bruker Biospin AG, Switzerland) operating at 400 MHz, respectively. 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 elsewhere [16].

Radical scavenging activity using DPPH method: The method used for measurement of free radical scavenging capability was carried out as reported by Shimada *et al*. [17]. The concentrations of sample solution used for measurement were 20, 40, 60, 80 and 100 µg/mL. To each 4 mL of sample solution, 1 mL of freshly prepared methanolic DMSO solution of DPPH (0.5 mM) was added, mixed well, and then let stand for 30 min at room temperature in dark. The absorbance of the result was recorded at 517 nm. Butylated hydroxyanisole (BHA) was used as reference compound. The capability to scavenge the DPPH$^{\bullet}$ was calculated using the following equation:

$$\text{Scavenging effect } (\%) = \left(1 - \frac{\text{Absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}}\right) \times 100$$

Ferric ion reducing/antioxidant power (FRAP) assay: The FRAP assay was performed according to Benzie & Strain [18] as modified by Pulido *et al*. [19]. The concentrations of sample solution used for measurement were 20, 40, 60, 80 and 100 µg/mL. Initially, an oxidant was prepared by mixing 2.5 mL of 10 mM TPTZ 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-1601 (PC) spectrophotometer. The change of absorbance ($\Delta A = A_{30\,min} - A_{4\,min}$) was calculated and related to ΔA of an Fe(II) standard solution. Aqueous solutions of known Fe(II) concen-trations (100-2000 µM/L $FeSO_4 \cdot 7H_2O$) were used for calibration. Butylated hydroxytoluene (BHT) was used as reference.

Hydroxyl radical scavenging activity (HRSA): The hydroxyl radical scavenging capacity of sample extracts was measured according to the method of Klein *et al*. [20]. 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. Ice-cold TCA (1.0 mL, 17% w/v) was added to the reaction mixture. A 3 mL of Nash reagent was added and left at 27 ºC for 15 min. The reaction mixture without sample was used as control. The intensity of 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 was calculated using the following formula:

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

Nitric oxide radical scavenging (NOS) assay: The varying concentrations (20-100 µg/mL) of sample extracts were prepared along with the standard rutin. For this purpose, Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations (20-100 µg/mL) and incubated at 27 ºC for 180 min. The sample extracts were mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. A volume of 150 µL of the reaction mixture was transferred to a quartz cell cuvette. The absorbance was measured at 546 nm. The % inhibition of the extract and standard was calculated. The percentage nitrite radical scavenging activity of sample extracts and rutin were calculated using the following formula:

$$\text{NOS } (\%) = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

where, $A_{control}$ = absorbance of control sample; A_{test} = absorbance in the presence of samples of extract or standard.

Total antioxidant activity assay by radical cation ($ABTS^{\bullet +}$): 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, 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 [21]. The unit of total antioxidant activity (TAA) was determined as α-tocopherol concentration having comparable antioxidant activity expressed as µmol/g sample extracts.

Chelating capacity: Metal chelating property of sample extract was done as per the bipyridyl assay [22]. 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 deionized water and the absorbance was measured at 522 nm. The chelating activity of the samples was expressed as mg EDTA equiv./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 ± S.D. after replications in thrice per sample. The Origin 8.0 Pro software (Microcal Software Inc., USA) was used by the authors 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 and chemical composition: The target of this work mainly focused on the chemical profiling of water soluble

polysaccharides extracted from the peels of *Citrus limetta* and to analyze its radical scavenging capacity. With an aim to chemically characterize the polysaccharides present in the peels, standard extraction procedures were adopted as depicted in Fig. 1. The water extracted polysaccharide (A), yielded 50 mg/g of *Citrus limetta* powder (CP) after it was fractionally precipitated with EtOH. The presence of rhamnose and glucose as major sugars were confiimed from the sugar composition analysis of fraction A (Table-1) and some trace amount of galactose, mannose, arabinose and xylose units. Very small amount of uronide was also detected in fraction AP2 which amounted to 6% (w/w). Thin layer chromatography (TLC) of the monosaccharides were done to confirm the presence of glucuronic acid with appropriate R_f values. The above result was confirmed from the GLC analysis of the TMS derivatives of derived methyl glycosides, along with the traces of galacturonic acid.

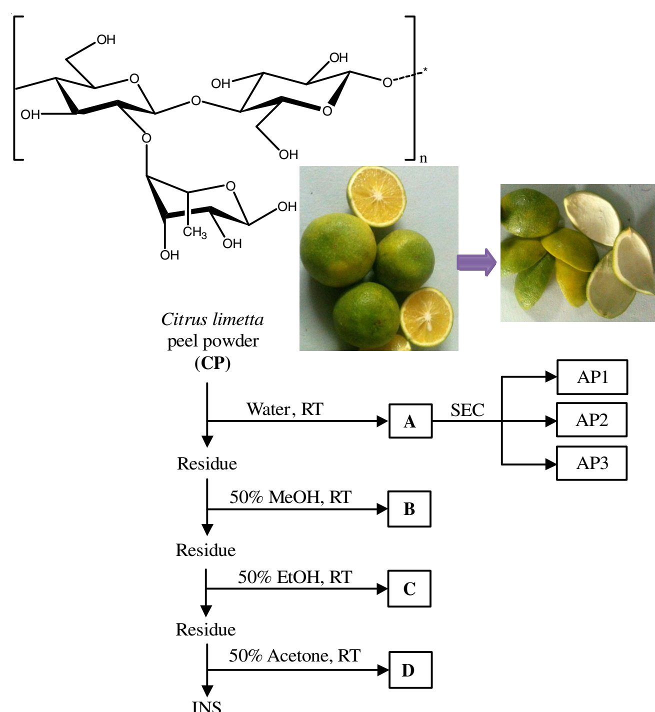

Fig. 1. Scheme for the extraction and purification of polysaccharides from the dry powdered peel of *Citrus limetta*

The glucosyl and rhamnosyl residues were found to be the main sugar constituents of the water extracted polysaccharidic fraction A, whereas its protein content was found to be 13% (w/w). The compositional analyses of sugar showed the presence of glucose residues as major units in addition to rhamnose residues as minor units, thereby confirming rhamnoglucan proteins (Table-1). The composition of amino acids in proteins present in fraction A showed the presence of serine (29.7%), alanine (10.8%), glutamine (8.5%) and glycine (4.4%).

Purification by size exclusion chromatography (SEC): In this part, the water extracted fraction A was further subjected to purification. Initially, this fraction was passed through a S-8 macroporous resin and then on to a Sephacryl S-300 packed column for SEC (Fig. 2), which yielded three overlapping sub-fractions (AP1, AP2 and AP3). All the sub-fractions had similar monosaccharide compositions (Table-1). The three purified

TABLE-1
SUGAR COMPOSITION AND YIELD OF *Citrus limetta* PEEL POWDER WATER EXTRACT AND OF FRACTIONS[a] OBTAINED THERE FROM

	A	AP1	AP2	AP3
Yield[b]	25	2.5	5	2
Total sugar[c]	88	85	70	65
Uronic acid[c]	7	5	3	2
Rhamnose[d]	29	26	25	28
Arabinose[d]	2	2	3	3
Xylose[d]	1	1	1	Trace
Mannose[d]	1	1	Trace	Trace
Glucose[d]	67	62	65	60
Galactose[d]	2	1	2	1
GlcA[d]	Trace	Trace	1	Trace
GalA[d]	3	Trace	5	Trace

[a]Water extracted polysaccharides containing fraction (A) isolated from *Citrus limetta* peels, and of fractions (AP1-AP3) derived there from by SEC; [b]Weight % of dry weight; [c]% Weight of fraction dry weight; [d]Molar % of neutral sugars.

fractions AP1, AP2 and AP3 showed similar sugar compositions and represented 74, 16 and 10%, respectively of the total sugar recovered. The molecular weight of three purified sub-fractions varied from 100-150 KDa. The range of fractionation for Sephacryl S-300 column was 1,000-3,00,000 Da for globular protein and dextrans.

Methylation analysis: From the results of glycosidic linkage analysis of purified fraction (AP1), it may be predicted that 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol residue is present, *i.e.* glucan has a (1→4)-linked backbone with terminal rhamnose residues (Table-2). It was also found that large proportion of terminal rhamnose residues was present, suggesting the polymer to be branched at position 6 (Table-2). Trace amounts of other glucopyranosyl residues and rhamnose residues were also detected.

^{1}H NMR analysis: From the ^{1}H NMR spectrum of purified rhamnoglucan polysaccharidic fraction (AP1) from *Citrus limetta* peel powder as shown in Fig. 3, two broad anomeric resonances, one at 5.08 ppm and the other at 4.50 ppm were observed. The signals appearing at 5.08 ppm and 4.50 ppm (Fig. 3) were tentatively assigned to α-(1→4) linked glucopyranosyl and

TABLE-2
PARTIALLY METHYLATED ALDITOL ACETATES DERIVED FROM THE WATER EXTRACTED POLYSACCHARIDE CONTAINING FRACTION (AP1) ISOLATED FROM *Citrus limetta* PEELS

Linkages[a]	*m/z*	Peak area[b]
T-Rha*p*	43, 102, 118, 131, 162, 175	22
(1→2)-Rha*p*	43, 130, 131, 174, 175, 190, 234	2
(1→3)-Rha*p*	43, 118, 131, 174, 234	2
(1→2,4)-Rha*p*	43, 130, 143, 190, 203	2
(1→4)-Glc*p*	43, 45, 102, 113, 118, 130, 162, 233	30
T-Glc*p*	43, 45, 101, 102, 118, 129, 145, 161, 162, 205	Trace
(1→3)-Glc*p*	43, 45, 101, 118, 129, 161, 174, 234	1
(1→6)-Gal*p*	43, 102, 118, 129, 162, 189, 233	3
(1→3,6)-Gal*p*	43, 118, 174, 189, 234	2

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

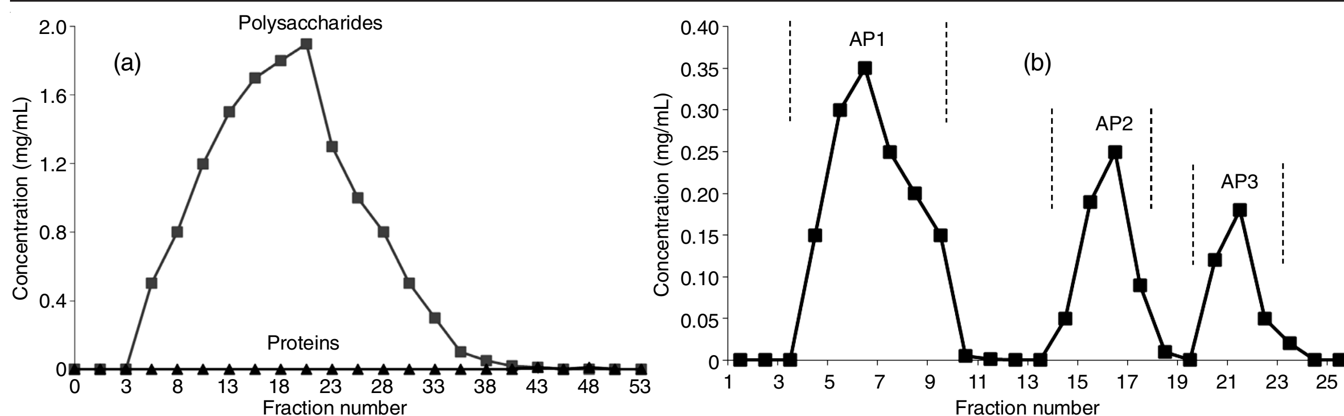

Fig. 2. (a) Dynamic elution curve of the water extracted polysaccharide obtained from peels of *Citrus limetta* on S-8 macroporous resin. (b) Size exclusion chromatogram of the crude polysaccharides on a Sephacryl S-300 column. Collected fractions were analyzed for total sugar content by phenol-sulfuric acid

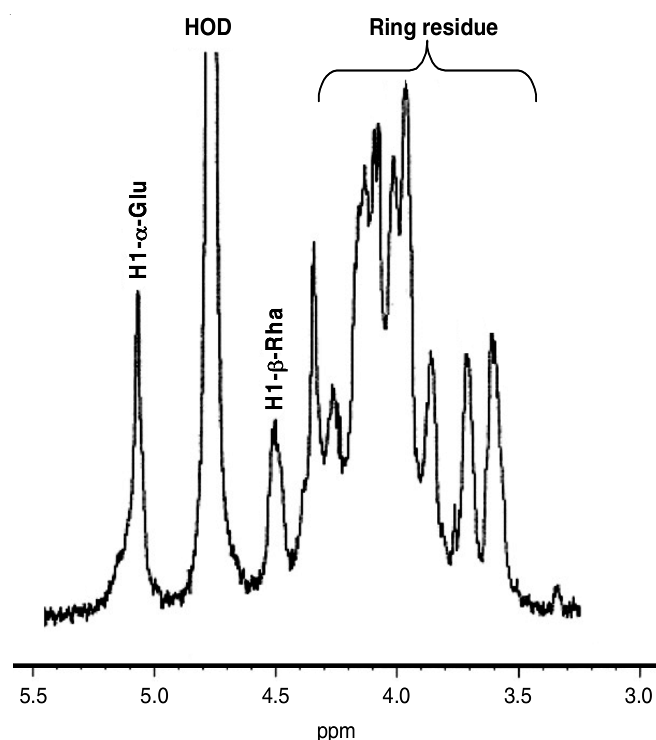

Fig. 3. ^{1}H NMR spectrum of the purified rhamnoglucan fraction (AP1) from *Citrus limetta* peels. The spectrum for the sample was recorded in D_2O solution. The signal for the residual water was designated as HOD

β-linked terminal rhamnose residues, respectively. The anomeric proton of β-galactopyranosyl residues was assigned for the signals which appeared in the region δ 4.35 ppm. A number of signals that appeared in the region δ 3.55-4.20 ppm may be due to the resonances of the ring protons. Since, ^{1}H NMR spectra of polysaccharides are complexed and overlapping for precise structural information, hence a tentative structure of polysaccharide skeleton may be predicted, but precise linkage may not be assigned always.

Antioxidant activity

Scavenging effect on DPPH$^{•}$: The radical compound shows an absorption peak at 517 nm (purple) [23]. The purple color was seen to fade quickly as soon as the proton radical was scavenged by the DPPH and finally turns to 1,1-diphenyl-2-picrylhydrazine [24]. The scavenging capacity of antioxidant extract was marked from the magnitude of discoloration. From the plot (Fig. 4a), good scavenging capability at a dosage of 100 µg/mL (84.25 ± 2.14 %) was detected with the rhamnoglucan fraction A compared to the control BHA (100.08 ± 2.11 %) on DPPH radicals. In current findings, the scavenging capacity order of the sample extract and standard are as follows: BHA > A > B > C > D. The DPPH$^{•}$ scavenging potential was predicted to depend on the phenolic quantity just as reducing power activity. In addition, other phytochemicals present that could not be identified might be responsible for their powerful antioxidant activity, which might be an interesting area of further research. The scavenging effect of sample extract on DPPH$^{•}$ radical is mainly due to their electron transfer ability and due to the presence of phenolics [25].

FRAP assay: Data shown in Fig. 4b highlights the marked reducing activity of these fractions, possibly due to the presence of polyphenols. It reacts with free radicals to convert to more stable products thereby functioning as reductants. The graphical representation depicts the reducing power of extracts, which was found increasing gradually on moving from low to high concentration as indicated by their absorbance values. As expected, the reduction potential of BHT, considered as a control was observed as the highest. It is also visible that a strong reduction potential for the methanolic extract (B) of *Citrus limetta* peel powder (CP) in variable concentration with reference to standard BHT. Comparatively, a lesser reduction potential was observed for the remaining ethanolic (C) and acetonic (D) extracts of peel powder. For many plant extracts similar to this, a relationship between the reduction potential and the polyphenolics have been established previously [26]. Overall, it may be inferred that the data conformed to the fact that the polyphenolics present in the sample extracts acts as electron donor and has the capacity to terminate radical chain reaction by transforming the free radicals to a stable compound, although the structure of the particular polyphenolics present in this case is not elucidated .

Hydroxyl radical scavenging activity: In this OH$^{•}$ scavenging capacity assay, the free radical were synthesized by

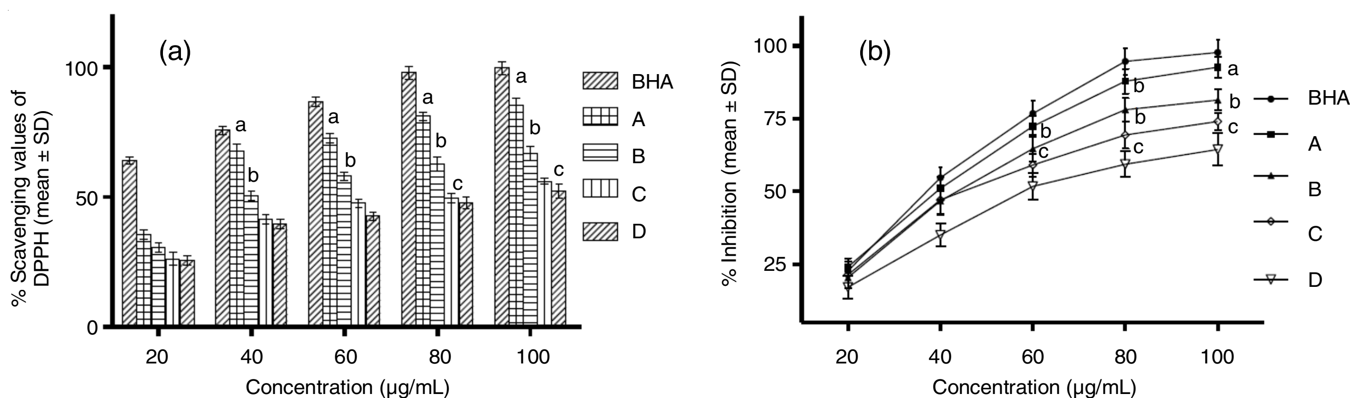

Fig. 4. Free radical scavenging activity of *Citrus limetta* powder extracts analyzed by (a) DPPH method & (b) FRAP assay. Values are means of triplicate determinations (n = 3) ± SD. P < 0.05 was statistically significant (a = < 0.001, b = < 0.01 c = < 0.05); BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene

employing ascorbic acid-Fe(II) EDTA complex. The oxidation reaction with DMSO followed by reaction with Nash reagent is a very handy method to determine $OH^{\bullet}$ as described earlier [27]. The scavenging potential of the extracts with varying concentration ranging from 20-100 µg/mL on $OH^{\bullet}$ were plotted graphically in Fig. 5a. From the results, it was seen that water extract of *C. limetta* depicted the most radical scavenging capacity at the concentration of 100 µg/mL. The ethanol, methanol and acetone extract showed comparable activity at the concentrations of 40-100 µg/mL. Overall from this analysis, it may be inferred that the effective scavenging capacities of these sample extracts may be assigned mainly because of the active H-donor power of $OH^{\bullet}$ moiety. The order of hydroxyl radical scavenging capacity of the sample extracts and standards are as follows: α-tocopherol > A > B > C > D.

Nitric oxide radical ($NO^{\bullet}$) scavenging activity: The polysaccharide containing sample extracts (A-D) from *Citrus limetta* powder along with a standard, rutin was used for $NO^{\bullet}$ scavenging assay. The sample extracts inhibits the process by scavenging peroxynitrite. From Fig. 5b, it was seen that scavenging potential on $NO^{\bullet}$ at a concentration of 100 µg/mL was observed for the water extracted fraction (A) with reference to rutin. From the comparison exhibited in Fig. 5b, it may be inferred that the scavenging potential order of the extracts and control used are as follows: Rutin > A > B > C > D.

$ABTS^{\bullet+}$ cation radical scavenging activity: The total antioxidant activities (TAA) of the sample extract with varying concentration of the samples ranging from 20-100 µg/mL are shown in Fig. 6. The total antioxidant activities (TAA) of sample extract ranges from 87.9 to 45.1 µmol α-tocopherol/g extract and the values were significantly ($p < 0.05$) different. From the experiment, it was found that H_2O, MeOH and EtOH extracts of *Citrus limetta* powder exhibited higher and comparable activity (> 50 µmol equivalent of α-tocopherol/g extract, respectively) on an equivalence with α-tocopherol as standard. The values obtained from the total antioxidant activities using different extracts of *Citrus limetta* peel powder suggest it to be an effective natural oxidant in food industries [28].

Metal chelating activity: The data of metal chelating activity for the sample extracts and the standard, α-tocopherol were plotted (Fig. 6), which was found to vary significantly ($p < 0.05$). The metal chelating capability of methanolic extract (B) was found to be the highest, whereas the acetone extracted fraction D to be the least. Overall, the metal chelating ability order of the different extracts and the control used are as follows: α-tocopherol > B > A > C > D.

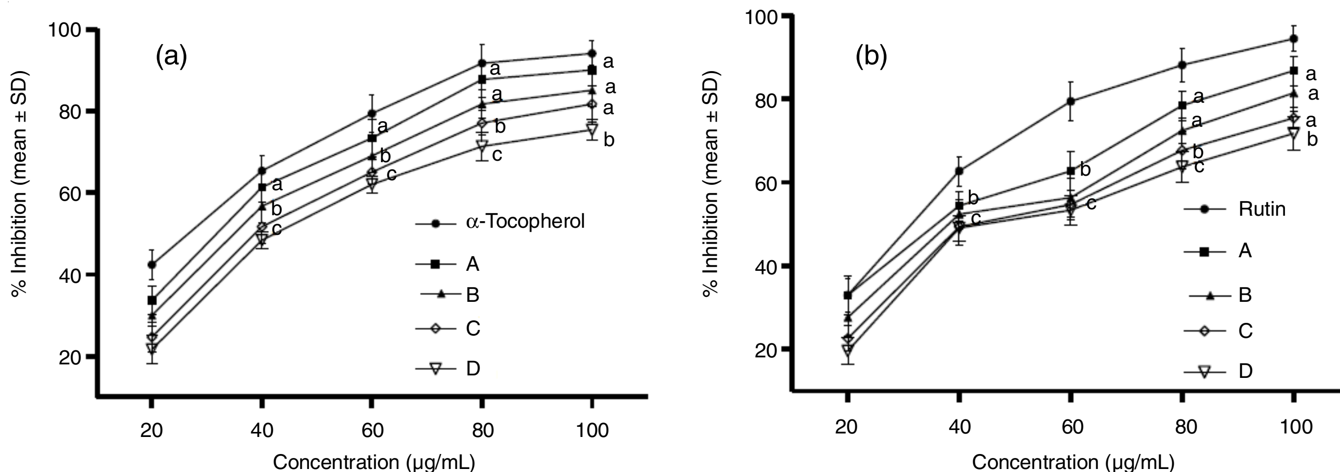

Fig. 5. Free radical scavenging activity of *Citrus limetta* peel powder extracts analyzed by (a) hydroxyl radical method & (b) nitric oxide assay. Values are means of triplicate determinations (n = 3) ± SD. $p < 0.05$ was statistically significant (a = < 0.001, b = < 0.01 c = < 0.05)

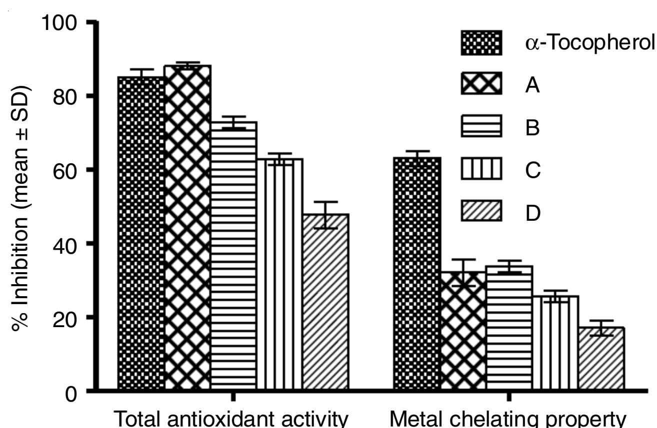

Fig. 6. Total antioxidant activity (TAA) using ABTS radical cation and metal chelating property (MCP) using Mg-EDTA complex of *Citrus limetta* peel powder extracts. Values are means of triplicate determinations (n = 3) ± SD. $p < 0.05$ was considered to be statistically significant

Conclusion

In this work, the chemical structure elucidation of polysaccharides present in the aqueous and other solvent extract of *Citrus limetta* peels powder was emphasized. The water extracted fraction (A) was then subjected to purification *via* size exclusion chromatography (SEC) technique into three sub-fractions (AP1-AP3), where the sugar composition of the major fraction AP1 was rhamnoglucan. From the antioxidant assay, water soluble polysaccharide and methanolic fraction of *Citrus limetta* highlited profound free radical scavenging capacities using various methods in comparison to the other fractions. The difference in these scavenging activities may be assigned to the structural differences in each fraction and the functional group available, which are actually held responsible in all the structures. This findings where the commonly discarded peels of *Citrus limetta* fruit were found to posses substantial quantities of polysaccharides with excellent antioxidant activities which can be extracted easily without any use of toxic chemical reagents during extraction process.

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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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