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Pectic Polysaccharide from Seeds of *Lagenaria Siceraria* (Lau): Structural Characterization and Study of Immunoenhancing and Antioxidant Properties

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ABSTRACT

A water-soluble pectic polysaccharide was isolated from the aqueous extract of the seeds of *Lagenaria siceraria.* is composed of methyl-α-D-methyl galacturonate, α-D-galactose and β-D-galactose in a ratio of nearly 1:1:1.Compositional analysis, methylation analysis, periodate oxidation study and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC) revealed the presence of the following repeating unit in the polysaccharide:

→4)-β-D-Gal*p*-(1→4)-α-D-Gal*p*A6Me-(1→2)-α-D-Gal*p*-(1→

This polysaccharide showed splenocyte, thymocyte as well as macrophage activations. Moreover, it exhibited antioxidant activities.

Keywords: Lagenaria siceraria; Pectic polysaccharide; structure; NMR spectroscopy; Antioxidant activities.

1. Introduction

The plant *Lagenaria siceraria* (Lau) is a herb, which grows throughout the year. Its leaves, the stem, mesocarp and endocarp of young fruit (pepo) are edible. *L. siceraria* seeds contain saponins, essential oils, vitamins and used in migraine type headache and pain [1-2]. A novel ribosome inactivating protein, lagenin isolated from water extract of seeds reported to show antitumor, antiviral and anti-HIV activities[3].From the point of pharmacological function the seeds are advised for consumption, as they have more nutrition and omega fatty acids [2].Plant polysaccharide from *Moringa oleifera* (sajina) showed anticancer activity against human epidermoid, carcinoma of nasopharynx in tissue culture and P_{388} lymphocytic leukemia in mice [4].Several plant polysaccharides isolated from fruit juice of *Morinda citrifolia* (noni) [5], *Morus alba, Chlamydomonas mexicana* and *Poria cocos* [6]show immunomodulatory and antitumor activities.

Two homogeneous polysaccharides were obtained from hot water extract of stems and fruits of ***L. siceraria*** respectively. The structures of these polysaccharides have been published Ghosh. K. et al. in journal of *Carbohydrate Research*, 2008 [7] and 2009[8].The polysaccharide isolated from fruits of *L. siceraria* showed cytotoxic activity

in vitro against human breast adenocarcinoma cell line MCF-7. In the present investigation a pectic polysaccharide composed of methyal galacturonate and galactose was isolated from hot water extract of seeds of *L. siceraria* and therefore, a detailed structural characterization and study of its immunoenhancing properties and antioxidant activity were carried out and reported herein.

2. Result and discussion

2.1. Chemical analysis of the polysaccharide

The pure polysaccharide showed specific rotation $[\alpha]_D^{25}$ +10.4 (c 0.68,water). The molecular weight was determined using calibration curve of standard dextrans [9] (T-100, T-70 and T-40) and found to be ~70,000 Da. Total neutral sugars were estimated as 68.5 % by phenol-sulfuric acid method[10].

The paper chromatographic analysis [11] of the hydrolyzed product of this polysaccharide showed the presence of galacturonic acid and galactose only. The GLC analysis of the alditol acetates of the sugars showed the presence of galactose and carboxyl reduced polysaccharide [12] on hydrolysis followed by GLC examination of the corresponding alditol acetates also showed the presence of galactose. The absolute configuration [13] of the sugar residues was determined as D by the GLC analysis of acetylated glycosides with (+) 2-octanol according to a modified method.

To know the mode of linkages present in the polysaccharide methylation analysis was carried out. In this connection, the polysaccharide was methylated by Ciucanu and Kerek method [14] and then hydrolyzed. The alditol acetates of the methylated products were identified by GLC using column A (3% ECNSS-M) and column B (1% OV-225) and GLC-MS analysis using HP-5 capillary column using a temperature program from 150 °C (2min) to 200 °C (5min) at 2 °C min^{-1}. and the polysaccharide showed the presence of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl galactitol (m/z: 43, 45, 71, 87, 99, 101, 113, **129**,145,161,189) and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol (m/z: 28,43,45,71,75,85,87,99,101,113,**117**,129,131,143,161,173,233) in a molar ratio of nearly 1:1. This result indicates that (1→2)-linked galactopyranosyl and (1→4)-galactopyranosyl moiety may be present in the polysaccharide. The carboxyl reduced polysaccharide [12] was methylated and alditol acetates of methylated sugars were identified by GLC-MS analysis, which showed the presence of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl galactitol (m/z: 43,45,71,87,99,101,113,**129**,145,161,189) and 1,4,5-tri-*O*-acetyl-2,3,6-tri-methylgalactitol (m/z:45,58,71,87,99,101,113,**117**,129,161,173,233) in a molar ratio of nearly 1:2. This result indicates (1→2)-linked galactopyranosyl, (1→4)-linked galactopyranose and (1→4)-linked galacturonic acid may be present in the polysaccharide. Periodate-oxidation experiment was carried out with this polysaccharide for the confirmation of the mode of linkage obtained from methylation study. Periodate-oxidised-reduced material of the polysaccharide, upon hydrolysis showed the absence of D-galactose. It indicates that the (1→2)-linked and (1→4)-linked D-galactose moieties are consumed during oxidation. A part of periodate-oxidised polysaccharide on hydrolysis showed the absence of D-galactouronic acid in paper chromatographic examination indicating that it has been destroyed during oxidation since D-galactouronic acid is present as (1→4)-linked residue in the repeating unit of polysaccharide.

2.2. NMR and structural analysis of polysaccharide

The ^{1}H NMR (500 Hz) spectrum of the polysaccharide (Fig. 1) showed three peaks in the anomeric region at δ 4.61, δ 4.96, and δ 5.07 ppm in a ratio of nearly 1:1:1. The sugar residues were assigned as **A, B** and **C** according to their increasing anomeric chemical shifts (Table 1). In the ^{13}C NMR (125 MHz) spectrum (Fig. 2, Table 1) three anomeric carbon signals appeared at δ 104.8, δ 100.8 and δ 99.9 ppm in a ratio of nearly 1:1:1.Further δ 53.3 ppm was assigned for carbomethoxy carbon. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HMQC and HMBC (Fig. 3) NMR experiments.

Sugar residue	H-1/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5/ C-5	H-6a/6b C-6	COOMe
→4)- β-D-Gal*p*-(1→ **A**	3.61	3.66	3.68	4.15	3.78	3.75,3.64	
	104.8	72.1	68.8	78.8	70.7	61.2	
→2)-α-D-Gal*p*-(1→ **B**	4.96	3.71	3.82	3.98	4.10	3.69,3.69	
	100.8	74.9	72.1	68.8	73.7	61.2	
→4)-α-D-Gal*p*A6Me-(1→ **C**	5.07	3.83	3.98	4.43	5.13		3.78
	99.9	68.8	68.1	79.3	72.1	171.1	53.3

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.74 ppm.
[b] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm. at 30 ºC.

Table 1: ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of the polysaccharide isolated from seeds of *Lagenaria siceraria* recorded in D_2O at 30 ºC.

Residue **A** has anomeric proton chemical shift at 4.61 ppm. A large coupling constant $J_{H-1, H-2}$ value (~ 7.9 Hz) and $J_{H-1, C-1}$ value (~ 161 Hz) indicate that it is a β-linked residue. The small $J_{H-2, H-3}$ value (~ 3.5 Hz) and the large $J_{H-3, H-4}$ value (~ 9.0 Hz) for residue **A** indicated that it is a β -D-galactosyl residue. The C-1 signal of residue C at 104.8 ppm was confirmed by the presence of cross peak **A**C-1, **C**H-4 in HMBC experiment (Fig. 4, Table 3). The carbon signals of residue **A** were observed at δ 72.1, 68.8, 70.7 and 61.2 for C-2, C-3, C-5 and C-6 respectively. The downfield shift of C-4 (δ 78.8) with respect to standard methyl glycosides [15] indicates that residue **A** is (1→4)-β-D-Gal*p*

Residue **B** has an anomeric proton chemical shift at 4.96 (unresolved) and $J_{H-1, C-1}$ ~ 171 Hz indicating that it is an α-linked residue. The anomeric carbon chemical shift of moiety **B** at δ 100.8 was confirmed by the presence of cross peak **B**C-1, **A**H-4 in HMBC experiment (Fig. 4, Table 3). The down field shift of C-2 (δ 74.9), compared to that of standard methyl glycosides[15]was due to the effect of glycosylation. The carbon signals of residue **B** were observed at δ 72.1, 68.8, 73.7 and 61.2 corresponding to C-3, C-4, C-5 and C-6 respectively. These indicate that residue **B** is (1→2)-α-D-Gal*p*.

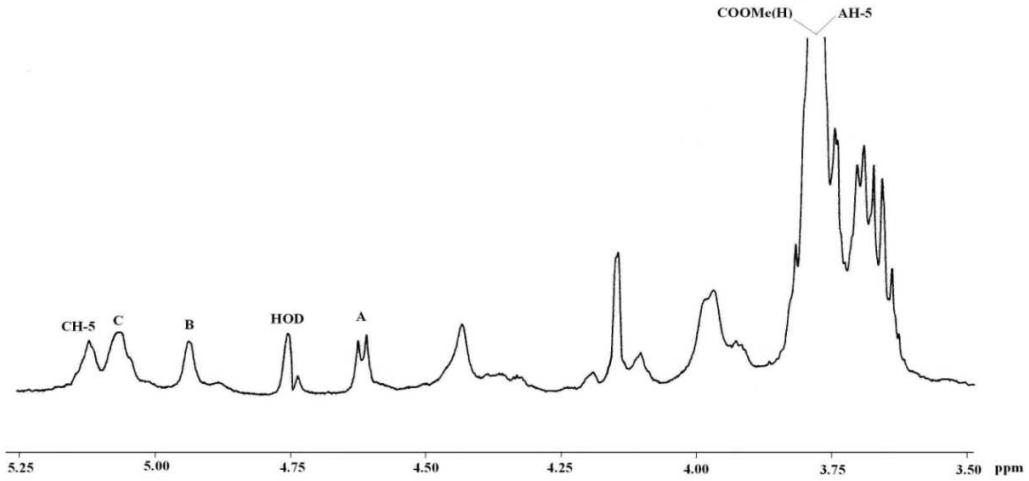

Figure 1: ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of polysaccharide, isolated from seeds of *Lagenaria siceraria.*

Residue **C** has an anomeric chemical shift at δ 5.07and $J_{H\text{-}1,\ H\text{-}2}$ ~ 2.8 Hz and $J_{H\text{-}1,\ C\text{-}1}$ ~ 170 Hz, indicating an α-linked residue. The spin system of this residue, which consisted of only five protons with a relatively high chemical shift of the H-5 signal (δ 5.13) [16] and weak coupling between H-3, H-4 and H-5 indicated that residue **C** is D-galacturanosyl moiety. The C-4 peak of residue **C** at δ 79.3 showed a down field shift compared to that of standard methyl glycosides[15] Indicating that the C is (1→4)-linked residue.The C-1 signal of residue **C** at δ 99.9 was confirmed by the appearance of cross peak **C**C-1, **B**H-2 in HMBC experiment (Fig. 4, Table 3). The carbon signals of residue **C** were observed at δ 68.8, 68.1, 72.1 and 171.1 for C-2, C-3, C-5 and C-6 (carbonyl carbon) respectively.

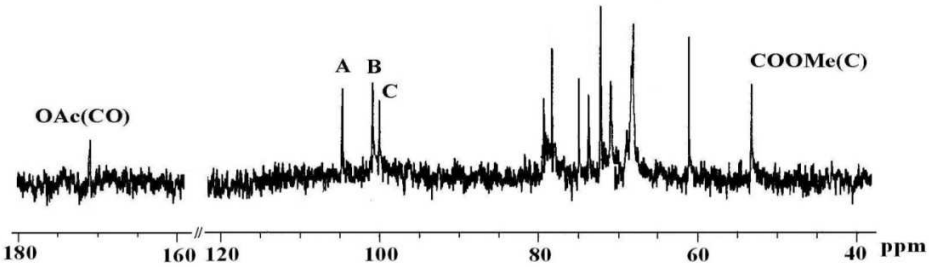

Figure 2: ^{13}C NMR (125 MHz, D_2O, 30°C) spectrum of polysaccharide, isolated from seeds of *Lagenaria siceraria.*

In HMBC experiment (Fig. 4, Table 3) the C-6 carboxyl carbon signal (δ 171.1) couple with carboxy methyl proton (δ 3.78) and also **C**H-5(δ 5.13) indicating that the residue **C** is methyl ester of galacturonic acid. These results indicate that residue **C** is

(1→4)-α-D-Gal*p*A6Me.

The sequence of glycosyl residues of the polysaccharide was determined from NOESY (Fig.3, Table 2) as well as ROESY experiments followed by confirmation with an HMBC experiment

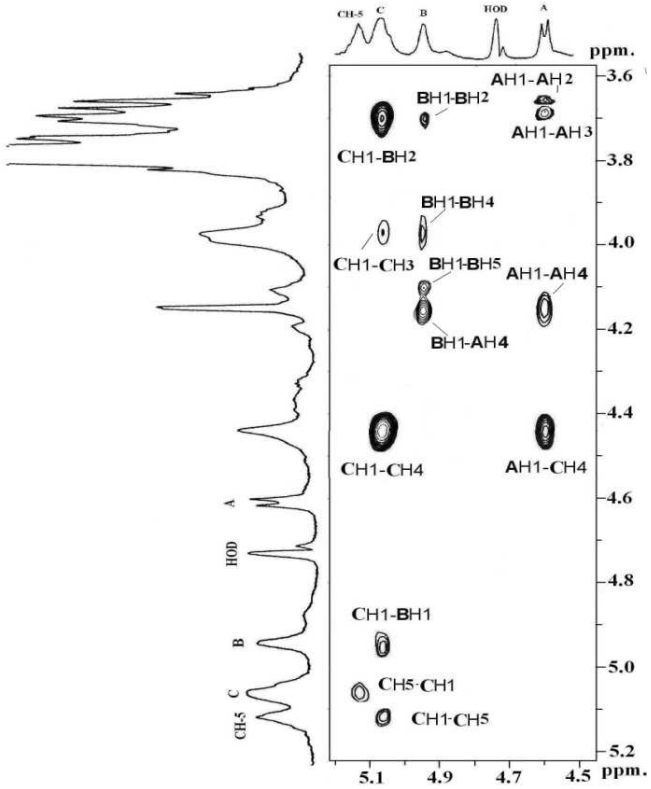

Figure 3: NOESY spectrum of polysaccharide, isolated from seeds of *Lagenaria siceraria.* The NOESY mixing time was 300 ms.

In NOESY experiment, the inter-residual contacts AH-1/ CH-4;BH-1/AH-4;CH-1/BH-2 along with other intra-residual contacts were also observed.thus from NOESY experiment the following sequences are established:

A(1→4)**C**; **B**(1→4)**A**;**C**(1→2)**B**

The sequence was further confirmed by ^{13}C-^{1}H correlation in HMBC spectrum (Fig. 4, Table 3).Inter residual cross peaks AH-1/CC-4,AC-1/CH-4; BH-1/CC-4,BC-1/CH-4;CH-1/BC-2and CC-1/BH-2 along with other intra residual peaks were also observed.Therefore, based on the results obtained from monosaccharide composition, methylation studies and NMR experiments the following repeating unit of the pectic polysaccharide of seeds of *Lagenaria siceraria* was assigned as:

→4)-β-D-Gal*p*-(1→4)-α-D-Gal*p*A6Me-(1→2)-α-D-Gal*p*-(1→

A **C** **B**

	Anomeric proton	NOE contact to proton	
Glycosyl residue	δ_H	δ_H	Residue, atom
→4)- β-D-Gal*p*-(1→ **A**	4.61	3.66 3.68 4.15 4.43	**A**H-2 **A**H-3 **A**H-4 **C**H-4
→2)-α-D-Gal*p*-(1→ **B**	4.96	3.71 3.98 4.15 4.10	**B**H-2 **B**H-4 **A**H-4 **B**H-5
→4)-α-D-Gal*p*A6Me-(1→ **C**	5.07 5.13(**C**H-5)	3.71 4.96 4.43 3.98 5.13 5.07	**B**H-2 **B**H-1 **C**H-4 **C**H-3 **C**H-5 **C**H-1

Table 2: NOE data for the polysaccharide isolated from seeds of *Lagenaria siceraria*

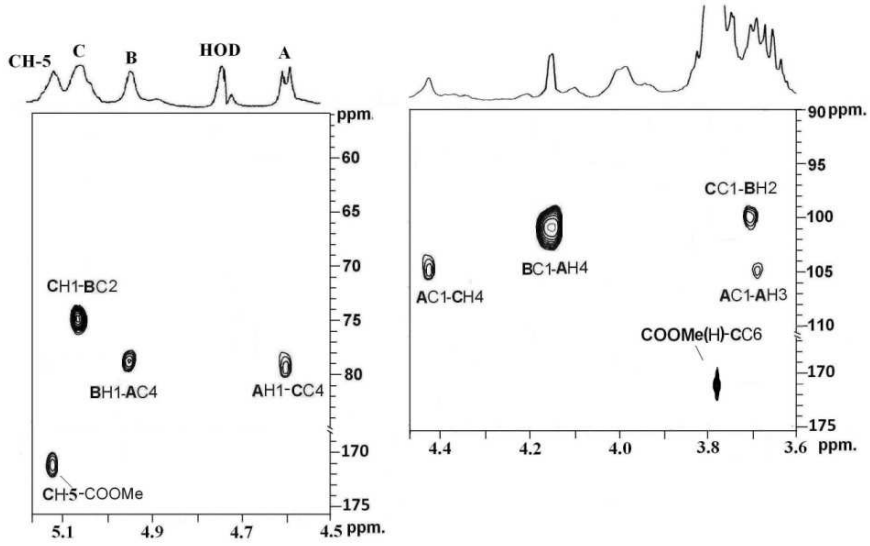

Figure 4: HMBC spectrum of polysaccharide, isolated from seeds of *Lagenaria siceraria.* The delay time in the HMBC experiment was 80 ms.

2.3. Immunostimulating properties of the polysaccharide

Some biological studies were carried out with polysaccharide. Macrophage activation by polysaccharide was observed in vitro. On treating different concentrations of polysaccharide, an enhanced production of NO was observed in a dose dependent manner

Residue	Glycosyl linkage	H-1/C-1 δ_H /δ_C	Observed connectivities δ_H /δ_C	Residue,	atom
A	→4)- β-D-Gal*p*-(1→	4.61 104.8	79.3 4.43 3.68	**C** **C** **A**	C-4 H-4 H-3
B	→2)-α-D-Gal*p*-(1→	4.96 100.8	78.8 4.15	**A** **A**	C-4 H-4
C	→4)-α-D-Gal*p*A6Me-(1→	5.07 99.9	74.9 3.71	**B** **B**	C-2 H-2
Residue	Glycosyl linkage	COOMe (δ_H)	Observed connectivities δ_C	Residue	atom
C	→4)-α-D-Gal*p*A6Me-(1→	3.78	171.1	**C**	C-6
		COOMe (δ_C)	Observed connectivities δ_H	Residue	atom
C	→4)-α-D-Gal*p*A6Me-(1→	171.1	5.13	**C**	H-5

Table 3: The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from seeds of *Lagenaria siceraria*

with optimum production of 8.2µM NO per 5x 10^5 macrophages at 25µg/mL(Fig.5).Hence, the effective dose of polysaccharide was observed at 25µg/mL.

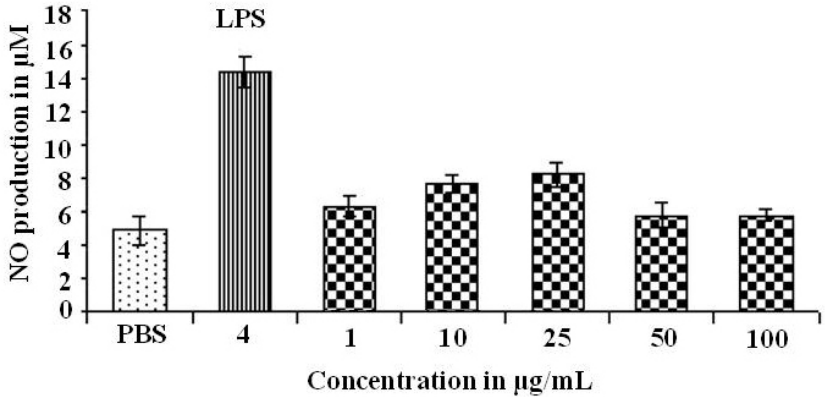

Figure 5: In vitro activation of peritoneal macrophage stimulated with different concentrations of the polysaccharide in term of NO production.

Splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with polysaccharide by the MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [17]Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. Polysaccharide was found to stimulate splenocytes and thymocytes as shown in Figure 6A and 6B, respectively. The splenocytes proliferation index (SPI) and thymocytes proliferation index (TPI) as compared to phosphate stimulatory effect on the immune system. At 10µg/mL of polysaccharide, splenocytes and thymocytes proliferation index were found maximum as compared to other concentrations. Hence, 10µg/mL of polysaccharide can be considered as efficient splenocyte and thymocyte stimulator.

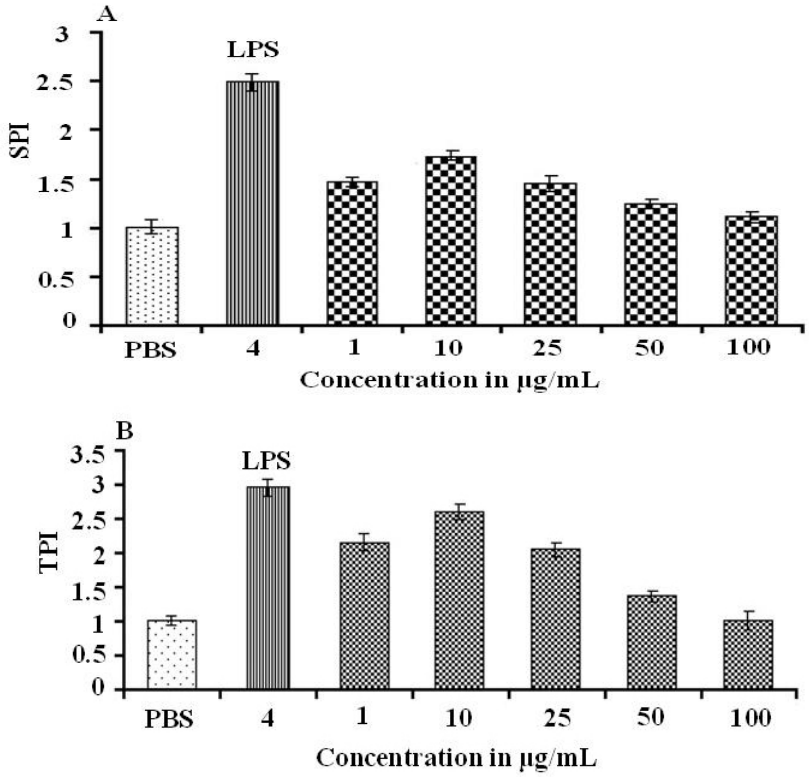

Figure 6: Effect of different concentrations of the polysaccharide on splenocyte **A** and thymocyte **B** proliferation.

2.4. Antioxidant properties

The antioxidant activity of the polysaccharide was measured on the basis of the scavenging activity of DPPH free radical test [18].A maximum of 65% DPPH radical scavenging activity (Fig.7).was observed at 8mg/mL of polysaccharide. The EC_{50} value of polysaccharide for DPPH radicals was 3mg/mL.the scavenging activity of the polysaccharide increased from 0.5 to 6mg/Ml, while it reached a maximum plateau from 0.5 to 2mg/mL for ascorbic acid, which indicates the scavenging activity of polysaccharide against DPPH radical was less than that of ascorbic acid.

3. Conclusion

A water soluble pectic polysaccharide was isolated from the hot water extract of the seeds of *Lagenaria siceraria* (Lau).The structure of this polysaccharide was elucidated on the basis of total hydrolysis, methylation analysis and 1D/2D NMR studies. These result indicated that the repeating unit of the polysaccharide contained a backbone of one (1→4)-linked β-D-galactopyranosyl residue, one (1→4)-linked α-D-methyl galacturonate residue and one (1→2)-linked α-D-galactopyranosyl residue. The polysaccharide activated the macrophages, splenocytes and thymocytes and also showed antioxidant activities. Hence, on the basis of these activities it could be used as a natural immunostimulant and antioxidant material.

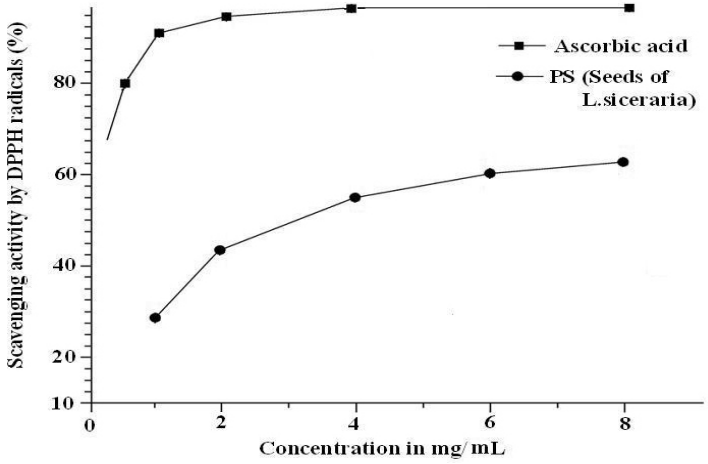

Figure 7: Ability of polysaccharide against DPPH radical. Result were presented as Mean±SD (n=3).

4. Materials and methods

4.1. Isolation and purification of the polysaccharide

Seeds of fresh pepos of *Lagenaria siceraria* (Lau) were extracted with hot water and collected. The supernatant was obtained through centrifuge and freeze-dried. The freeze-dried material was then dissolved in distilled water and precipitated in ethanol (1:5, v/v). The precipitated material (Polysaccharide) was lyophilized, dissolved in 1% acetic acid solution and boiled for 30 min. to remove lipid part. The solution was centrifuged at 8000 rpm. The filtrate was re precipitated in EtOH and collected through centrifugation. Now, the crude polysaccharide was dissolved in water and dialyzed through dialysis tubing cellulose membrane (Sigma-Aldrich, retaining M.W.> 12,400) for 12h to remove acid and small carbohydrate molecules; freeze dried, yield 3.2g.

The crude polysaccharide (35 mg) was purified by gel permeation chromatography on column (90 × 2.1 cm) of Sepharose 6B in water as eluant (0.4 mL min^{-1}) using Redifrac fraction collector. 95 test tubes (2 mL each) were collected and monitored spectrophotometrically at 490 nm with phenol-sulfuric acid reagent [10] using Shimadzu UV-VIS spectrophotometer, model-1601. One homogeneous fraction (test tubes 34-60)

was collected and freeze-dried, yield-30 mg. The purification process was carried out in four lots and polysaccharide fraction was again purified and collected, yield-110 mg.

4.2. Monosaccharide analysis

The polysaccharide sample (3.0 mg) was hydrolyzed with 2M CF_3COOH (2 mL) in a round-bottom flask at100°C for 18 h in a boiling water bath. The excess acid was completely removed by co-distillation with water. Then the hydrolyzed product was divided into two parts. One part was examined by paper chromatography in solvent systems X and Y. Another part was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure MeOH to remove excess boric acid. The reduced sugars (alditol) were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GLC using column (A) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) and column (B) 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170°C.Gas-liquid chromatography-mass spectrometric (GLC-MS) analysis was also performed on Hewlett-Packard 5970A automatic GLC-MS system, using an HP-5 capillary column (25 m × 25 mm). The program was isothermal at 150°C; hold time 2 min, with a temperature gradient of 4 °C min^{-1} up to a final temperature of 200°C. Quantitation was carried out from the peak area, using response factors from standard monosaccharides.

4.3. Preparation of Carboxy methyl reduced polysaccharide [12]

The Polysaccharide (1.0 mg) was dissolved in 1M imidazole- hydrochloric acid buffer, *p*H 7.0 (200µL / mg) and cooled on ice. Sodium borohydride (40 mg) was then added and reacted on ice for at least 1 h. The excess borohydride was destroyed by adding glacial acetic acid (100 µL / 40 mg borohydride) slowly to the cooled sample. An equal volume of redistilled water was then added, and the reduced polysaccharide was precipitated by adding 3 to 4 volume of 95 % (v/v) ethanol (2 mL). The sample was reprecipitated two more times with 95 % ethanol and freeze-dried. The carboxy-reduced polysaccharide was hydrolysed with 2M CF_3COOH for 18 h at 100 °C and after usual treatment, the sugars were estimated by GLC.

4.4. Methylation analysis

The Polysaccharide (4.0 mg) was methylated using Ciucanu and Kerek method[14].The methylated products were isolated by partition between $CHCl_3$ and H_2O (5:2, v/v). The organic layer containing products was washed with 3 mL water for three times and dried. The methylated products were then hydrolyzed with 90 % formic acid (1mL) at 100 °C for 1 h, reduced with sodium borohydride, acetylated with (1:1) acetic anhydride-pyridine and analyzed by GLC (using columns A and B) and GLC-MS (using HP-5 fused silica capillary column) and the same temperature program indicated above. The carboxy-reduced polysaccharide[12] was also methylated according to the Ciucanu and Kerek method[14].

4.5. Periodate oxidation study

The Polysaccharide (5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark during 48 h. The excess periodate was destroyed by adding 1,2-ethanediol and the solution was dialyzed against distilled water. The dialyzed material was reduced

with $NaBH_4$ for 15 h and neutralized with acetic acid. The resulting material was obtained by co-distillation with methanol. The periodate reduced material was divided into two portions. One portion was hydrolyzed with 2M CF_3COOH for 18 h and alditol acetate was prepared as usual. Another portion was methylated by Ciucanu and Kerek[14] method and alditol acetate of this methylated product was prepared. Alditol acetates were analyzed by GLC using column A and B.

4.6. Optical rotation

Optical rotation was measured on a Perkin-Elmer model 241 MC spectropolarimeter at 25 °C.

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4.7. Absolute configuration of monosaccharides

The method used was based on Gerwig et.al[13]. The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A solution of 250µL of 0.625 (M) HCl in R- (+)-2-butanol was added and heated at 80°C for 16 h.Then the reactants were evaporated and TMSi-derivatives were prepared with N,O-bis (trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30m × 0.26mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMSi- (+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides. The same procedure was applied with carboxy-reduced polysaccharide [12]where the methyl ester of galacturonic acid was converted to galactose and the determination of configuration of galactose indicated that galacturonic acid was present as D-configuration.

4.8. Paper chromatographic studies

Paper partition chromatographic studies were performed on whatmann nos.1 and 3 mm sheets. Solvent systems used were: (X) BuOH-HOAc-H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc-pyridine-H_2O (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution[11].

4.9. Determination of molecular weight

The molecular weight of polysaccharide was determined by a gel– chromatographic technique. Standard dextrans[9]T-200, T-70, and T-40 were passed through a sepharose 6B column and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of polysaccharide was then plotted in the same graph and molecular weight of polysaccharide was determined.

5. NMR studies

The polysaccharide was kept over P_2O_5 in vacuum for several days and then exchanged with deuterium [19] by lyophilizing with D_2O (99.96 % atom ^2H, Aldrich) for four times. With a Bruker Avance DPX-500 spectrometer,^1H, TOCSY, DQF-COSY, NOESY and HMBC NMR spectra were recorded in D_2O at 30ºC. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.74) using the WEFT pulse sequence[20].The 2D-DQF-COSY experiment was carried out using standard Bruker software at 30 ºC. The TOCSY experiment was recorded at mixing time of 150 ms, and

complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY mixing delay was 200 ms. The ^{13}C NMR spectrum of polysaccharide, solution in D_2O was recorded at 27 ºC using acetone as internal standard, fixing the methyl carbon signal at δ 31.05 ppm. The delay time in the HMBC experiment was 80 ms.

6. Biological studies

6.1. Test for macrophage activity by nitric oxide assay

Peritoneal macrophages (5 x 10^5 cell mL^{-1}) after harvesting were cultured in complete RPMI (Rose well park Memorial Institute) media in 96 -well plates[21-22].The purity of macrophages was tested by adherence to tissue culture plates. The polysaccharide was added to the well in different concentrations (1-100µg/mL).The cells were cultured for 24 h at 37 ºC in a humidified 5% CO_2Incubator. Production of nitric oxide was estimated by measuring nitrite levels in cell supernatant with Greiss reaction[23].Equal volumes of Greiss reagent (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample cell supernatant were incubated together at room temperature for 10 min. Absorbance was observed at 550nm. Lipopolysaccharide(LPS), L6511 of *Salmonella enteric* serotype typhimurium (sigma,St. Louis,USA) was used as positive control.

6.2. Splenocyte and thymocyte proliferation assay [22-24]

A single cell suspension of spleen and thymus was prepared from the normal mice under aseptic conditions by frosted slides in PBS (Phosphate Buffered Saline). The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells were removed by hemolytic Gey's solution. After washing two times in PBS the cells were resuspended in complete RPMI medium. Cell concentration was adjusted to 1 x 10^5 cells/mLand viabilityof the suspended cells (as tested by trypan blue dye exclusion) was always over 90%. The cells (180µL)were plated in 96-well flat-bottomed plates and incubated with 20 µLof various concentrations (1-100µg/mL) of the polysaccharide. The same lipopolysaccharide as used in macrophage activation was also used here as positive control. The cultures were set-up for 72 h at 37 ºC in a humidified atmosphere of 5% CO_2. Proliferation was checked by MTT assay method[17]. Data were reported as the mean ±standard deviation of five different observations and compared against PBS control.

6.3. DPPH radical scavenging activity of Polysaccharide

The antioxidant activity of the polysaccharide was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical test according to the method described by Yen et al.[19]with some modification. Samples were dissolved in distilled water at 0.2,0.5,1,2,4 and 8mg/mL. One milliliter test sample was mixed with two milliliter of freshly prepared DPPH (0.1mM) in 50% ethanol. After shaking vigorously, the mixture was incubated at 25 ºC for 30 min in the dark and then the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Ascorbic acid was used as a standard antioxidant material. The scavenging activity of the DPPH radical was calculated as:

$Q = (A_o - A_c) / A_o \times 100\%$

Q = percentage reduction of the DPPH. Where A_o= initial absorbance, A_c= absorbance after added sample concentration c.

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