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Structural studies of immunoenhancing heteropolysaccharide from *Azadirachta indica* leaves

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Abstract: An immunoenhancing water-soluble heteropolysaccharide (PS) with molecular weight ~1.96 × 10^5 Da was isolated from the hot water extract of the leaves of *Azadirachta indica*. Sugar analysis revealed that PS was made up of L-arabinose, L-rhamnose, D-galactose, and D-galacturonic acid. This polysaccharide exhibited splenocyte, thymocyte as well as macrophage activations**.** The structure of the molecule was investigated using acid hydrolysis, methylation analysis, periodate oxidation, Smith degradation, and 1D/2D NMR studies and the repeating unit of the polysaccharide was established as:

A
→4)-α-D-Gal*p*A-(1→
2
↑
1
α-L-Ara*f*-(1→6)-β-D-Gal*p*-(1→2)-β-L-Rha*p*
B **C** **D**

Keywords: *Azadirachta indica*; Heteropolysaccharide; NMR Spectroscopy; Immunological study

1. Introduction

Polysaccharides possess immense biological and medicinal values and exhibit immunomodulating (Brochers, Stern, Hackaman, Keen, & Gershwin, 1999; Wasser & Weis, 1999), antitumor (Maeda & Chihara, 1999; Schepetkin & Quinn, 2006), and anti-oxidant properties (Liu, Fang, Li, & Xiao, 2002). Presently, extracts of various parts of the plant have drawn the attention of chemists and immunobiologists due to their various uses in medicinal purposes (Stavric, 1994). Also, plant extracts consisting of polysaccharides are used in industry for wide applications (Gross, 1986). Several polysaccharides from fruit juice of *Morinda citrifolia* (Hirazumi & Furusawa, 1999), *Morus alba*, *Chlamydomonas mexicana*, and *Poria cocos* (Shuxiu, Yuanying, & Changxu, 1995) show immunomodulatory and antitumor activities. Structural characterizations of some plant (Patra et al., 2010; Mandal et al., 2011) and mushroom (Maity et al., 2011; Mandal et al., 2010; Patra et al., 2011; Dey et al., 2013) polysaccharides have been carried out by our group and reported. Pectic polysaccharides (pectins), a structural component of fruits and vegetables (Ridley, O'Neil, & Mohnen, 2001; Voragen & Pilnik, 1989), are complex heterogeneous polysaccharides containing galacturonic acid or its ester in the main chain having different pharmaceutical activities (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). These also play an important role in the regulation of some physiological process to prevent hyperlipidemia and bowel cancer (Lim, Yamada, & Nonaka, 1998; Willats, McCartney, Mackie, & Knox, 2001). An effective immunostimulatory pectic polysaccharide active on T and B lymphocytes was isolated from the stems of *Dendrobium nobile* and reported (Wang, Luo, & Zha, 2010). Two different immunoenhancing pectic polysaccharides were isolated and reported by our group (Patra et al. 2013, Patra, Das, Behera, Maiti, & Islam, 2012).

Azadirachta indica (locally known as neem), a fast-growing evergreen tree ranging in height from 12 - 24 m belongs to the family Meliaceae. It grows in the tropical countries of the world. The different parts of the neem tree are used in traditional medicine for the treatment of a variety of human ailments, particularly against the disease infected by bacteria and fungus. Neem bark extract can control gastric hyperacidity and ulcer (Maity, Biswas, Chattopadhyay, Banerjee, & Bandyopadhyay, 2009). Leaf extract has anti-malarial and anti-cancer activities (Udeinya, 1993; Subapriya & Nagini, 2005). The aqueous extract of neem leaves is very effective for curing the viruses of small pox, polio, and HSV (Rao et al. 1969). Here, we report the detailed structural

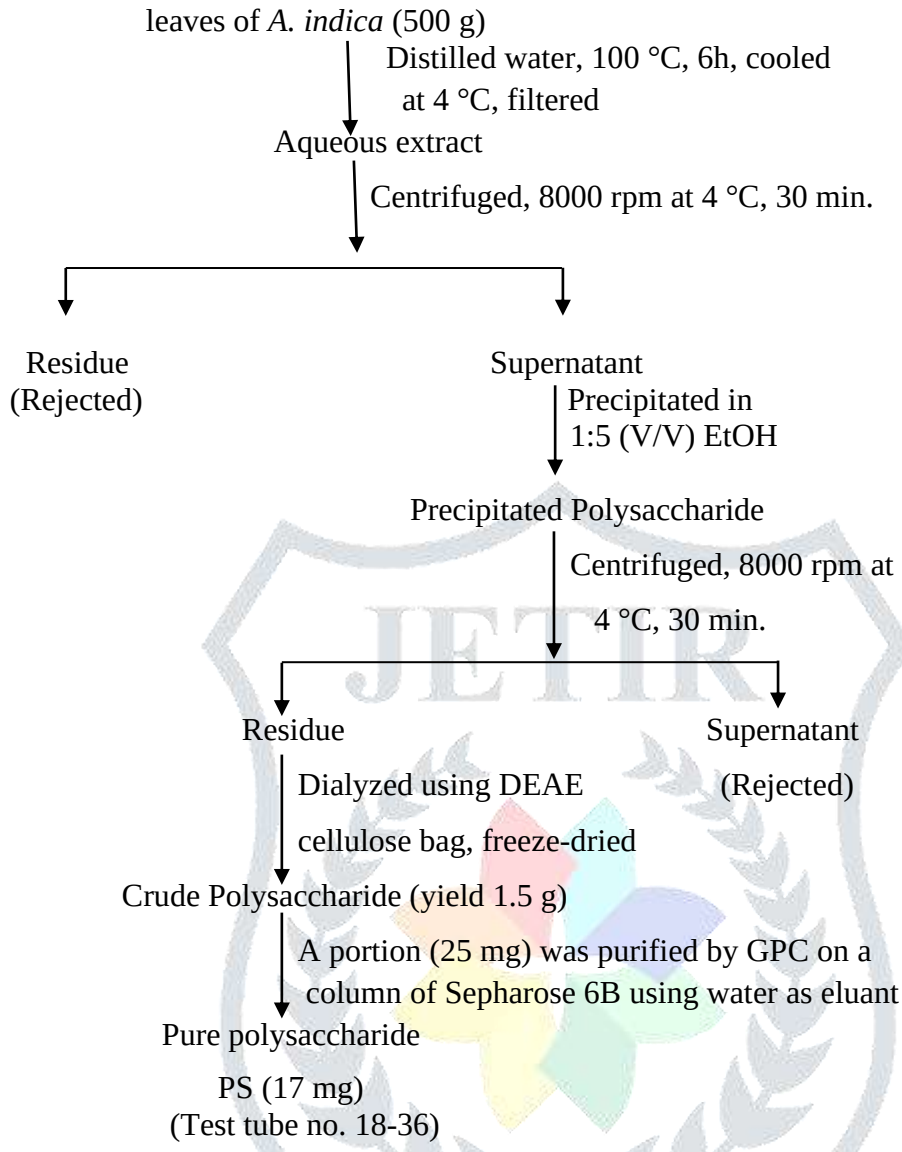
characterization and also some immunoenhancing properties of the polysaccharide isolated from the leaves of *A. indica* and reported herein.

2. Materials and methods

2.1. Isolation, fractionation, and purification of the crude polysaccharide

The fresh leaves (500 g) of *A. indica* were washed properly with water. Its leaves were cut into fine pieces and washed with distilled water, then boiled with distilled water at 100 ^{o}C for 6 h. The whole mixture was kept overnight at 4 °C and filtered through a linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) for 30 min at 4 ^{o}C. The supernatant was collected and precipitated in ethanol (1:5, v/v). It was kept overnight at 4 °C and again centrifuged as above. The precipitated material (polysaccharide) was washed with ethanol for five times and then freeze-dried. The freeze-dried material was dissolved in 40 mL of distilled water and dialyzed through cellulose membrane (Sigma-Aldrich, retaining > M.W. 12 kDa) against distilled water for 10 h to remove low molecular weight materials. The aqueous solution was then collected from the dialysis bag and freeze-dried. The crude polysaccharide (25 mg) was purified by gel permeation chromatography on column (90 × 2.1 cm) of Sepharose-6B using water as eluent (0.4 mL min^{-1}) by Redifrac fraction collector. 95 test tubes (2 mL each) were collected and assayed aliquots of the fractions using the phenol-sulphuric colorimetric assay method (York, Darvill, McNeil, Stevenson, & Albersheim, 1985). The absorbance was recorded at 490 nm by UV-vis spectrophotometer. And one homogeneous peak for the polysaccharide (PS) was obtained. The fraction containing polysaccharide was collected and freeze-dried. The same procedure was repeated for several times to get more pure PS. The fractionation and purification steps are shown below.

Flow diagram of isolation and purification of the polysaccharide



2.2. Monosaccharide analysis

2.2.1. Alditol acetate analysis

The PS (3 mg) was hydrolyzed with 2.0 M CF_3COOH (2 mL) at 100 °C for 16 h in a boiling water bath and excess acid was completely removed by repeated co-distillation with water. Then, the hydrolyzed product was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars (alditol) were acetylated with 1:1 pyridine–Ac_2O in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GLC. All gas liquid chromatography experiments were performed on a Hewlett-Packard Model 5730 A gas chromatograph, having a flame ionization detector and glass columns (1.8m x 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q

(100-120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100-120 mesh). All GLC analyses were performed at 170 °C.

2.2.2. Preparation of carboxyl reduced polysaccharide

The Polysaccharide (3.5 mg) was dissolved in water and then 1-cyclohexyl-3-(2-morpholino-ethyl)-carbo-di-imide-*p*-toluene sulfonate (CMC, 94 mg) was added to it with stirring and the pH was maintained at ~ 4.75 by the addition of 0.01 M hydrochloric acid. After 2h, 2 M aqueous sodium borohydride (2 mL) was added drop wise during 45 min, and the pH maintained at ~ 7.0 by simultaneous addition of 4 M hydrochloric acid. After 1h, the solution was dialyzed against distilled water and freeze dried. The procedure was repeated once again for complete reduction. The carboxyl-reduced polysaccharide was hydrolyzed with 2.0 M CF_3COOH for 16 h at 100 °C and after usual treatment the sugars were analyzed by GLC.

2.3. Methylation analysis

PS (4.0 mg) was methylated using the procedure described by Ciucanu and Kerek (1984) where distilled DMSO and finely grounded NaOH were used. The methylated product was isolated by making partition between $CHCl_3$ and water (5:2, v/v). The organic layer-containing product was washed with water for several times; divided into two parts and dried. One portion of the methylated polysaccharide was hydrolyzed with 90% HCOOH (1 mL) at 100 °C for 1 h and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with $NaBH_4$ and acetylated with pyridine and Ac_2O (1:1). The alditol acetates of the methylated sugars were analyzed by GLC (using columns A and B) and GLC-MS (using ZB-5MS capillary column). Another portion of the methylated PS was dissolved in dry THF (2 mL), refluxed with $LiAlH_4$ (Abdel-Akher & Smith, 1950) (40 mg) for 5 h and kept overnight at room temperature. The excess of the reductant was decomposed by drop wise addition of EtOAc and aqueous THF. The inorganic materials were filtered off. The carboxyl-reduced methylated material was hydrolyzed with formic acid as before. The alditol acetate of the carboxyl-reduced, methylated sugar was prepared in usual way and analyzed by GLC-MS. Gas-liquid chromatography-mass spectrometric (GLC–MS) analysis was also performed on Shimadzu GLC-MS Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m × 0.25 mm). The program was

isothermal at 150 °C; hold time 5 min, with a temperature gradient of 2 °C min^{-1} up to a final temperature of 200 °C.

2.4. Periodate oxidation

PS (5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark for 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-oxidized-reduced (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965) material was hydrolyzed with 2 M CF_3COOH at 100 °C for 18 h, and this hydrolyzed material was used for paper chromatographic examination as well as alditol acetate preparation as usual for the GLC analysis.

2.5. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatman 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 (Hoffman, Lindberg, & Svensson, 1972).

2.6. Absolute configuration of monosaccharides

The method used was based on Gerwig et al. (Gerwig, Kamarling, & Vliegenthart, 1978). PS (1.0 mg) was hydrolyzed with CF_3COOH and then the excess acid was removed by co-distillation with water. A solution of 250 μl 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 TMS-derivatives were prepared with BSTFA. The products were analyzed by GC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.7. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 25.6 °C.

2.8. Determination of molecular weight

The molecular weight of the PS was determined by gel-chromatographic technique. Standard dextrans (Hara, Kiho, Tanaka, & Ukai, 1982) 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 PS was then plotted in the same graph, and molecular weight of polysaccharide was determined.

2.9. Smith degradation

The polysaccharide (25 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 25 °C in the dark during 48 h. The oxidation process was stopped by addition of 1,2- ethanediol, and the solution was dialyzed through cellulose bag against distilled water. The dialyzed material was freeze dried. Then the freeze-dried material was dissolved in minimum volume of water and then it was reduced with $NaBH_4$ for 15 h. at 25°C, neutralized with 50% acetic acid, and again dialyzed against distilled water and freeze dried. The product was subjected to mild hydrolysis with 0.05 M CF_3COOH for 15 h. at 25 °C to eliminate residues of oxidized sugars attached to the polysaccharide chain (Smith degradation). Acid was removed after repeated addition and lyophilized with water. A portion of the material was collected and kept with P_2O_5 in vacuum for several days and then exchange with deuterium followed by lyophilization with D_2O. Then ^{1}H and ^{13}C NMR spectra of Smith degraded material were examined. Another portion of the material was collected and performed acid hydrolysis and methylation analysis as discussed above.

2.10. NMR studies

The ^{1}H and ^{13}C NMR experiments were carried out at 500 MHz and 125 MHz Bruker Avance DPX-500 spectrometer respectively using a 5 mm broad-band probe. For NMR studies, the PS was dried in vacuum over P_2O_5 for several days, and then exchanged with deuterium (Dueñas Chasco et al., 1997) by lyophilizing with D_2O for three times. The deuterium-exchanged polysaccharide (4 mg) was dissolved in 0.7 mL D_2O (99.96% atom ^{2}H, Aldrich). The ^{1}H and ^{13}C (both ^{1}H coupled and decoupled) NMR spectra were recorded at 27 °C. Acetone was used as an internal standard (δ 31.05) for ^{13}C spectrum. The ^{1}H NMR spectrum was recorded fixing HOD

signal at δ 4.73 at 27 °C using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992). 2D (DQF-COSY) NMR experiment was performed using standard Bruker software. 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 and ROESY mixing delay were 300 ms.

2.11. Test for macrophage activity by Nitric oxide assay

RAW 264.7 growing in Dulbecco's modified Eagle's medium (DMEM) was seeded in 96 well flat bottom tissue culture plates (Ohno, Hasimato, Adachi, & Yadomae, 1996; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006) at 5 x 10^5 cells/mL concentrations (180 µL). Cells were left overnight for attachment and different concentrations (12.5, 25, 50, 100 or 200 µg/mL) of PS were treated. After 48 hrs of treatment culture supernatant of each well were collected and NO content was estimated using Griess Reagent (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) (Green et al. 1982).

2.12. Splenocyte and Thymocyte Proliferation Assay

A single cell suspension of spleen and thymus were prepared from the normal mice by sacrificing under aseptic conditions and suspended in 10mM phosphate buffer saline (pH 7.4), The contaminating RBC obtained in the cell suspension was treated by hypotonic lysis buffer (Maiti et al., 2008). After two washes the cells were resuspended in complete medium (RPMI) and adjusted to a concentration of 1×10^5 cells/mL and viability of the suspended cells (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 µL) were plated in 96-well flat-bottom plates and incubated with 20 µL of various concentrations (12.5 µg/mL to 200 µg/mL) of the PS. PBS (10 mM, pH-7.4) was taken as negative control whereas lipopolysaccharide (LPS, L6511 of Salmonella enterica serotype typhimurium, Sigma, 4 µg/mL) and Concavalin A (Con A, 10 µg/mL) served as positive controls. Cultures were set-up for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation was checked by MTT assay method (Ohno et al. 1993). Data are reported as the mean ± standard deviation of six different observations and compared against PBS control (Maiti et al. 2008; Sarangi et al. 2006).

3. Result and discussion

3.1. Isolation and purification of the polysaccharide

The hot aqueous extract of fresh leaves of *A. indica* (500 g) was cooled, filtered, and precipitated in alcohol and then centrifuged. The residue was dissolved in a minimum volume of distilled water, dialyzed, centrifuged, and then freeze dried to yield 1.5 g of material. 25 mg crude polysaccharide on fractionation through sepharose-6B using water as eluant yielded 16 mg pure PS (Fig. 1a). For more purification, PS was again fractionized through sepharose-6B in aqueous medium.

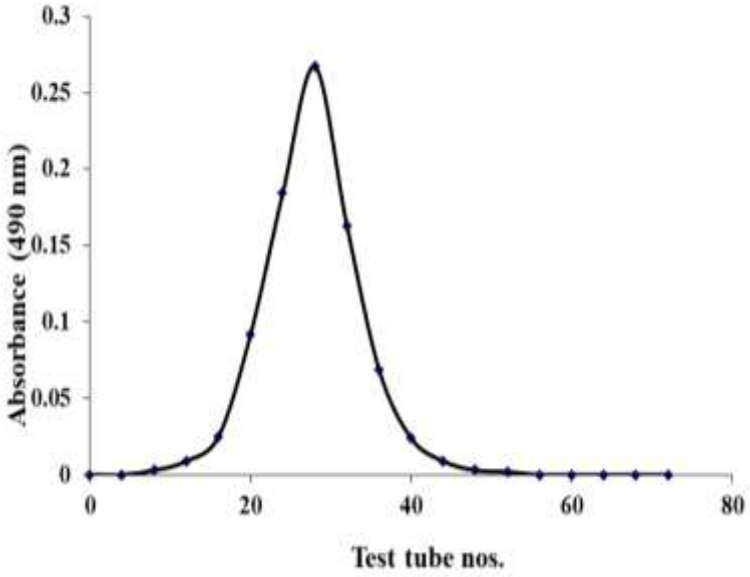


Fig. 1a. Gel permeation chromatogram of crude polysaccharide isolated from *Azadirachta indica* using Sepharose 6B column.

3.2. Chemical analysis of the polysaccharide

The molecular weight (Hara et al., 1982) of the PS was found to be ~1.96 × 10^5 Da (Fig. 1b). It showed a specific rotation of $[\alpha]_D^{32.5}$ +8.96 (*c* 0.05, water). Paper chromatographic analysis (Hoffman, Lindberg, & Svensson, 1972) of the hydrolyzed PS showed the presence of galactose, and galactouronic acid and two slow moving spots nearer to arabinose and rhamnose. The polysaccharide was hydrolyzed with 2 M CF_3COOH for 18 h at 100 °C. The GLC analysis of the alditol acetates of the hydrolyzed PS showed the presence of arabinose, rhamnose, and galactose in the molar ratio of nearly 1:1:1, but the carboxyl-reduced polysaccharide (Maness, Ryan, & Mort, 1990) on hydrolysis followed by GLC analysis showed the presence of the above mentioned

sugars in a molar ratio of nearly 1:1:2, which confirmed the presence of 1 mol of galacturonic acid in the PS. The absolute configuration of the sugar units present in the PS were determined by the method of Gerwig et al, (Gerwig, Kamarling, & Vliegenthart, 1978) taking intact and carboxyl-reduced polysaccharide, and it was found that arabinose and rhamnose had L-configuration and galactose and galacturonic acid had D-configuration. The modes of linkages of PS were determined by methylation analysis using Ciucanu and Kerek method (Ciucanu & Kerek, 1984) followed by hydrolysis and alditol acetates preparation. The GLC and GLC-MS analysis of alditol acetates of methylated product revealed the presence of 2,3,5-Me_3-Ara, 3,4-Me_2-Rha, and 2,3,4-Me_3-Gal in a molar ratio of nearly 1:1:1. These results indicated the presence of terminal Ara*f*, (1→2)-Rha*p*, and (1→6)-Gal*p* moieties in the PS. The alditol acetates of methylated carboxyl-reduced polysaccharide (Abdel-Akher & Smith, 1950) were identified by GLC-MS analysis, which showed the presence of the above peaks along with a new peak of 3-Me-Gal in a molar ratio of nearly 1:1:1:1. This result indicated that 1 mol of (1→2,4)-linked galacturonic acid was also present in the PS. The methylated sugars were identified with respect to their retention times and comparing with standard derivatives. Thereafter, these linkages were further confirmed by periodate-oxidation experiment (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965). The periodate-oxidized reduced material upon hydrolysis with trifluoro acetic acid followed by GLC analysis showed no peaks corresponding to neutral sugars but paper chromatographic studies showed the presence of galacturonic acid only. Thus, the periodate oxidation experiment confirmed that galacturonic acid was present as (1→2,4)-linked moiety.

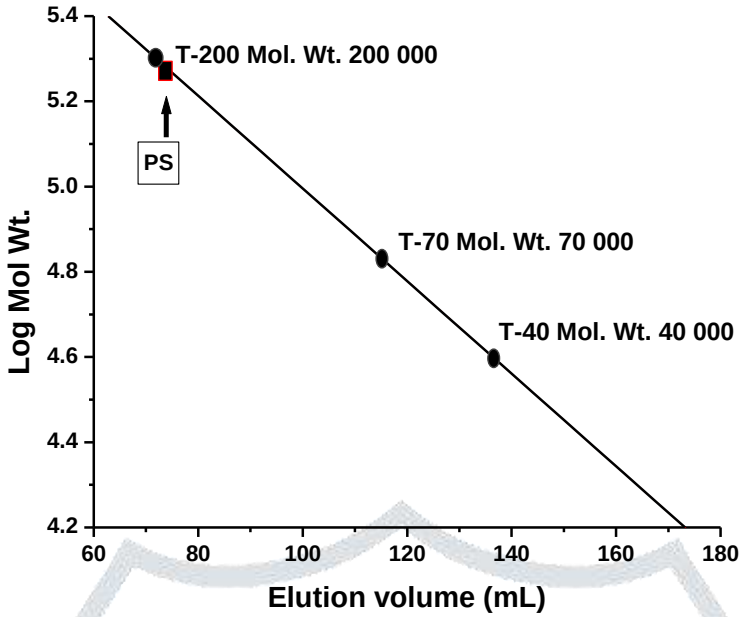


Fig. 1b. Determination of molecular weight of PS by gel permeation chromatography in Sepharose 6B column.

3.3. NMR and structural analysis of the PS

The 500 MHz ^{1}H NMR (Fig. 2a, Table 1a) spectrum of PS recorded at 27 ^{o}C, gave four anomeric signals at δ 5.23, 5.22, 4.50, and 4.44 in a ratio of nearly 1:1:1:1. The sugar residues were designated as **A-D** according to their decreasing anomeric proton chemical shifts (Table 1). The ^{13}C NMR spectrum (125 MHz, Fig. 2b, Table 1a) of PS at 27 ^{o}C also revealed four signals at δ 98.8, 103.5, 103.7, and 107.9 which corresponded to the anomeric carbons of **A**, **C**, **D**, and **B**, respectively, from HSQC spectrum (Fig. 2c). All the ^{1}H and ^{13}C signals (Table 1a) were assigned using DQF-COSY, TOCSY, and HSQC NMR experiments.

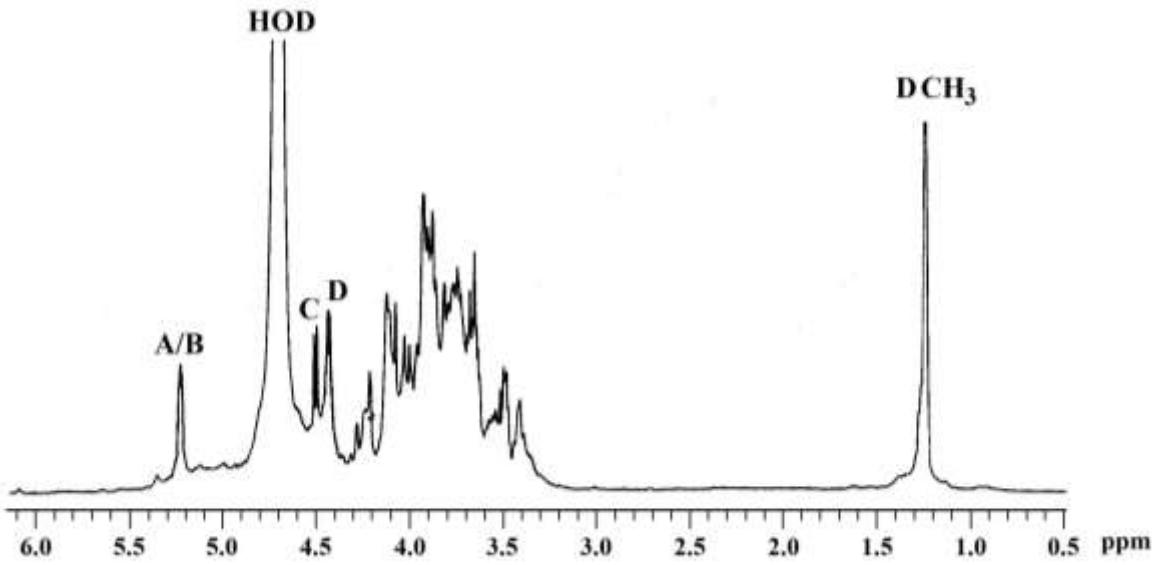


Fig. 2a. ^{1}H NMR spectrum (500 MHz, D_2O, 27 ºC) of PS, isolated from *Azadirachta indica*.

Table 1a

^{1}H NMR and ^{13}C NMR chemical shifts (ppm) of PS, isolated from *Azadirachta indica* [a,b] recorded in D_2O at 27 °C

Glycosyl residue	H-1/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5a, H-5b/ C-5	H-6a, H-6b/ C-6
→2,4)-α-D-Gal*p*A-(1→	5.23	3.71	4.21	4.12	4.44	
A	98.8	75.2	70.1	76.0	71.0	171.2[c]
α-L-Ara*f*-(1→	5.22	4.13	4.44	4.10	3.75, 3.86	
B	107.9	81.3	77.5	83.9	61.1	
→6)-β-D-Gal*p*-(1→	4.50	3.50	3.66	3.75	3.71	3.68, 3.91
C	103.5	71.9	72.5	69.9	75.2	66.4

→2)-β-L-Rha*p* (1→	4.44	4.03	3.93	3.75	3.41	1.24
D	103.7	79.2	73.5	69.9	71.9	16.6

[a]Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal set at δ 4.73 at 27 °C.

[b]Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and set at δ 31.05 at 27 °C.

[c]Value of the carbon of acid group.

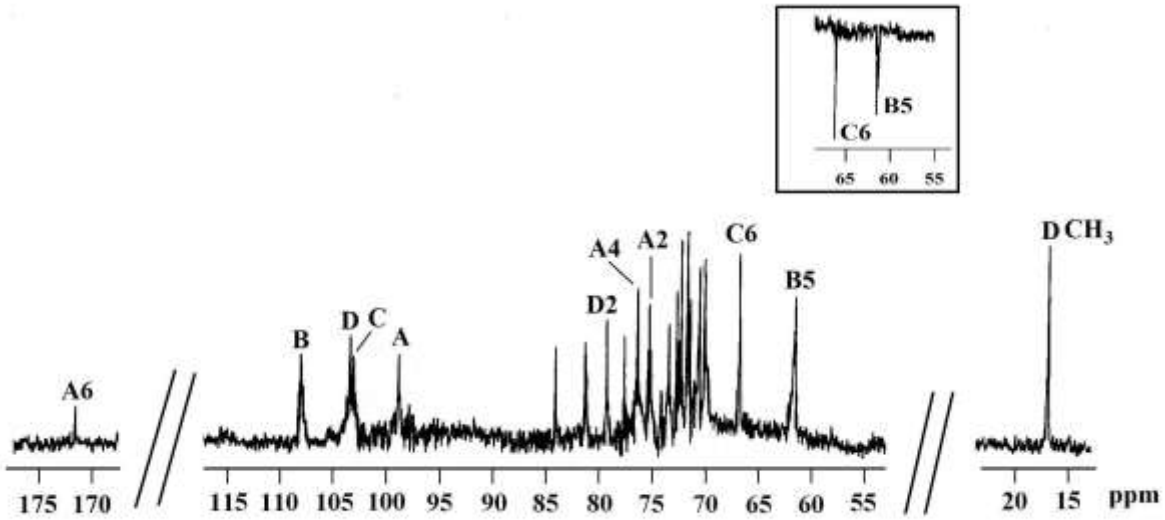


Fig. 2b. Combination of ^{13}C NMR and DEPT-135 spectrum (125 MHz, D_2O, 27 ºC) of PS isolated from *Azadirachta indica*.

The anomeric proton (δ 5.23) and carbon (δ 98.8) chemical shift values indicated that residue **A** was α-linked sugar residue. The C-2 (δ 75.2) and C-4 (δ 76.0) signals of residue **A** showed downfield shifts compared to the standard methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982). The appearance of a carbon signal at δ 171.2 due to the carboxylic acid (-COOH) group clearly indicated that the residue was galacturonic acid. These data confirmed that residue **A** was (1→2,4)-α-D-Gal*p*A.

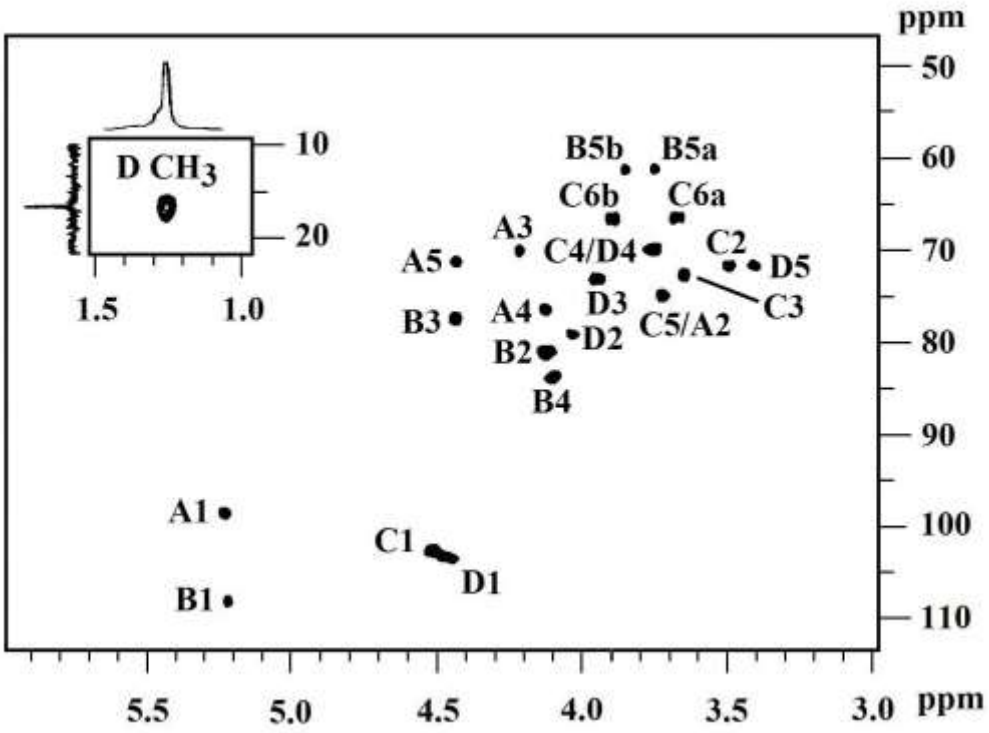


Fig. 2c. HSQC spectrum of PS, isolated from *Azadirachta indica*.

Residue **B** with anomeric proton signal at δ 5.22 and very high anomeric carbon chemical shift at δ 107.9 indicated that it was α-linked sugar residue existing as a furanose structure. The signals at δ 81.3, 77.5, 83.9, and 61.1 corresponded to C-2, C-3, C-4, and C-5, respectively. All the carbon values with respect to the standard methyl arabinofuranosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that the residue **B** was terminal-α-L-arabinofuranosyl moiety.

In the case of residue **C**, the anomeric proton chemical shift at δ 4.50 and anomeric carbon chemical shift at δ 103.5 indicated that it was β-linked sugar residue. The downfield shift of C-6 (δ 66.4) with respect to the standard methyl glycosides indicated that it was present as (1→6)-β-D-galactopyranosyl moiety. The C-6 linkage was further confirmed from DEPT-135 spectrum (Fig. 2b).

The anomeric proton chemical shift at δ 4.44 and anomeric carbon signal appeared at δ 103.7 confirmed that the residue **D** was present in β-configuration. The. A very upfield carbon signal (δ 16.6) and proton signal (δ

1.24) of the exocyclic CH_3 group present in residue **D**. Downfield chemical shift of C-2 (δ 79.2) with respect to the standard value of methyl glycosides indicated that it was (1→2)-β-L-rhamnopyranosyl moiety.

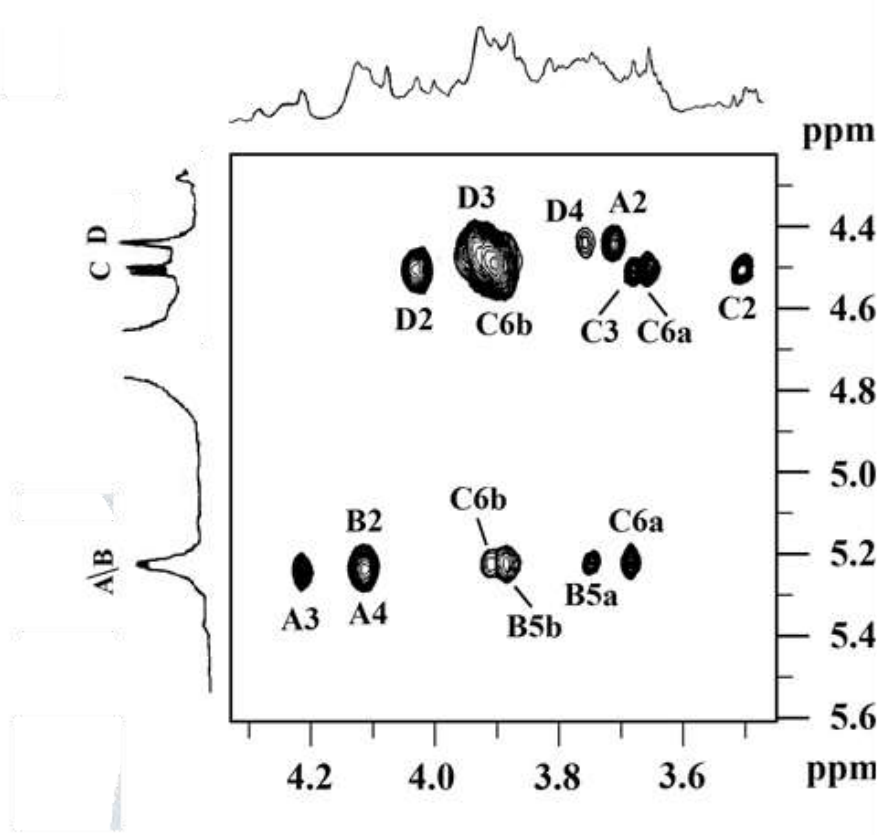


Fig. 3. Part of NOESY spectrum of the polysaccharide. The NOESY mixing time was 300 ms.

The sequence of glycosyl residues (**A** to **D**) of the polysaccharide was determined from NOESY (Fig. 3a, Table 2b) as well as ROESY (Figure not shown) experiments. The inter-residual NOESY contacts between **A** H-1 to **A** H-4; **B** H-1 to both **C** H-6a and **C** H-6b; **C** H-1 to **D** H-2; and **D** H-1 to **A** H-2 were observed along with other intra-residual contacts. The NOESY connectivities established the following sequences:

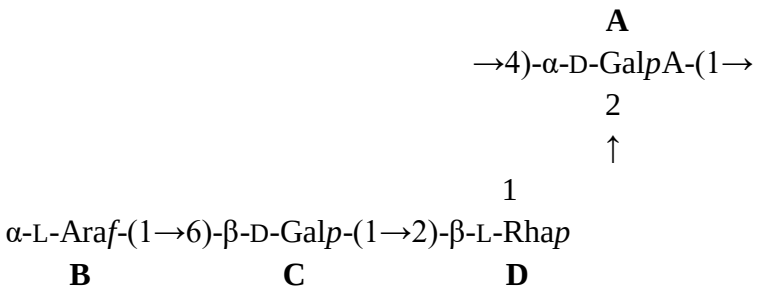
A-(1→4)-**A; B**-(1→6)-**C; C**-(1→2)-**D; D**-(1→2)-**A**

Table 2b

NOESY data for the polysaccharide, isolated from *A. indica*

Anomeric proton		NOE contact protons		
Glycosyl residue	δ_H	δ_H	Residue	Atom
→2,4)-α-D-Gal*p*A-(1→	5.23	4.12	**A**	H-4
A		4.21	**A**	H-3
α-L-Ara*f*-(1→	5.22	3.68	**C**	H-6a
B		3.91	**C**	H-6b
		4.13	**B**	H-2
		3.75	**B**	H-5a
		3.88	**B**	H-5b
→6)-β-D-Gal*p*-(1→	4.50	4.03	**D**	H-2
C		3.50	**C**	H-2
		3.66	**C**	H-3
		3.68	**C**	H-6a
		3.91	**C**	H-6b
→2)-β-L-Rha*p* (1→	4.44	3.71	**A**	H-2
D		3.75	**D**	H-4
		3.93	**D**	H-3

Based on all these evidences the structure of repeating unit of the pectic PS was proposed as:



In order to obtain information on the sequence of the sugar residues in the repeating unit, the polysaccharide was subjected to Smith degradation (Abdel-Akher, Hamilton, Montgomery, & Smith, 1952) studies, and the products were separated on a Sephadex G-25 column using water as the eluant, resulting in one fraction (SDPS). GLC analysis of the alditol acetates of the acid-hydrolyzed product from SDPS showed no peaks corresponding to neutral sugars, but the alditol acetates of the acid-hydrolyzed carboxyl-reduced product from SDPS showed the presence of only galactose indicating that only the galacturonic acid is survived during oxidation. The alditol acetates of the methylated product from carboxyl-reduced SDPS were analyzed by GLC–MS analysis using ZB-5MS capillary column which showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol indicating the presence of (1,4)- linked galacturonic acid in SDPS. The ^{13}C NMR (125 MHz) experiment (Table 2c, Fig. 3b) of SDPS showed only one anomeric carbon signal at δ 99.5 corresponding to →4)-α-D-Gal*p*A-(1→ (**E**), indicating all the other residues were consumed during oxidation. The signals at δ 68.8, 70.9, 77.0, 71.6, and 171.1 corresponded to C-2, C-3, C-4, C-5, and C-6 respectively. Hence, from all these data the structure of the Smith-degraded homopolysaccharide is established as →4)-α-D-Gal*p*A-(1→ which further proves that the backbone of the polysaccharide is composed of galacturonic acid with rhamnogalactoarabinan side chain.

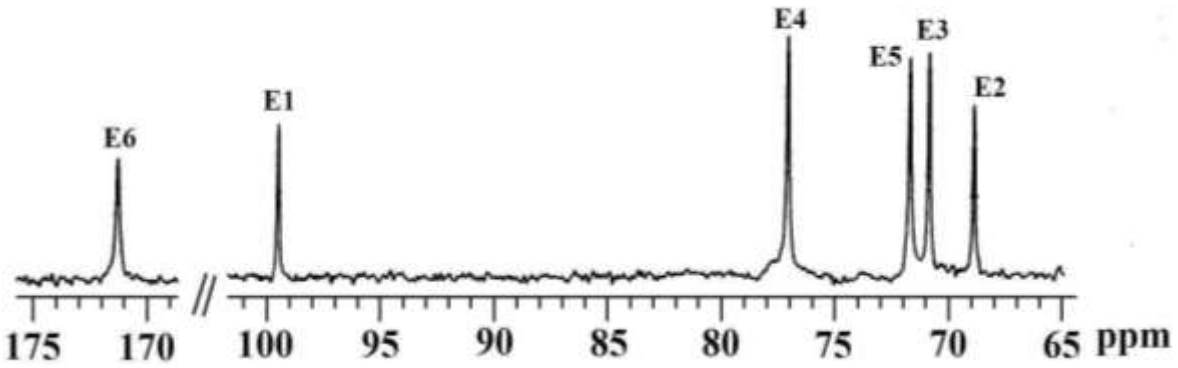


Fig. 3b. ^{13}C NMR spectrum (125 MHz, D_2O, 27 ºC) of the Smith-degraded polysaccharide.

Table 2c

^{13}C NMR chemical shifts (ppm) of Smith degraded polysaccharide, isolated from *Azadirachta indica* recorded in D_2O at 27 °C

Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
→4)-α-D-Gal*p*A-(1→ **E**	99.0	68.8	70.9	77.0	71.6	171.1[a]

Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and set at δ 31.05 at 27 °C.

[a]Value of the carbon of acid group.

3.4. Immunostimulating properties

3.4.1. Test for macrophage activity by Nitric oxide assay

Macrophage activation by this polysaccharide has been studied by nitric oxide (NO) production in culture supernatant in vitro. Upon treatment with different concentrations of PS, an enhanced production of NO was observed with optimum production of 20.7 µM NO with initial seeded cell number 5×10^5 macrophages at 50 µg/mL (Fig. 4a) and then decreases. Hence 50 µg/mL was the effective dose of PS for NO production. The

various types of polysaccharides like lentinan inhibits tumor growth by stimulating the immune system (Chihara, 1978) through activation of macrophages, T-helper, NK, and other cells.

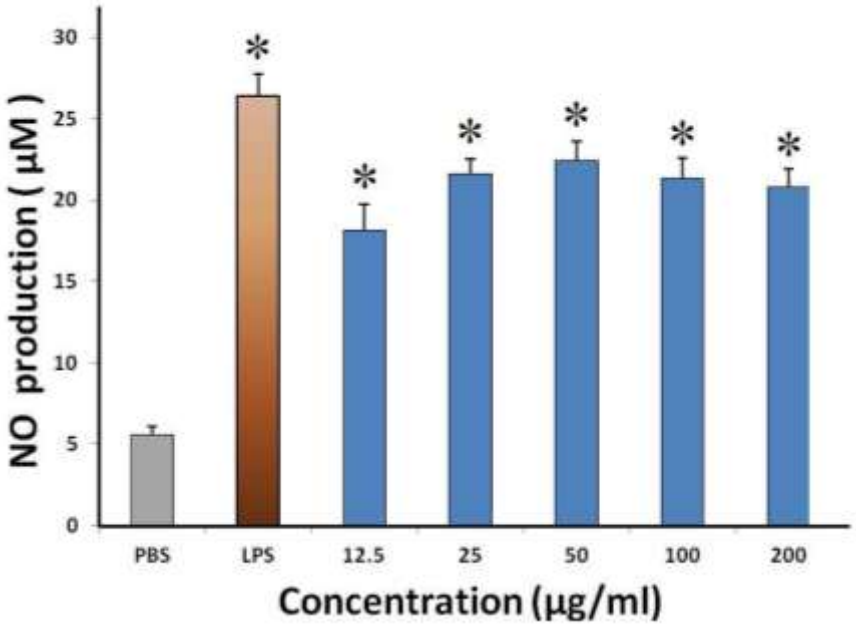


Fig. 4a. In vitro activation of peritoneal macrophage stimulated with different concentrations of the polysaccharide in terms on NO production.

3.4.2. Splenocyte and Thymocyte Proliferation Assay

Splenocytes are the cells present in the spleen that include T cells, B cells, dendritic cells, etc. that stimulate the immune response in living organisms where as thymocytes are hematopoietic cells present in thymus and the primary function of which is the generation of T cells. Splenocyte and thymocyte proliferations are the measure of immunoactivation. The activation of splenocyte and thymocyte tests were carried out in mouse cell culture medium with PS by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Ohno et al. 1993). The stimulation of the splenocyte (Fig. 4b) and thymocyte (Fig. 4c) were examined with this PS and the asterisks on the columns indicated the statistically significant differences compared to PBS (Phosphate Buffered Saline) control. The splenocyte and thymocyte proliferation index as compared to PBS control if closer to one or below indicated the low stimulatory effect on immune system. Both splenocyte and thymocyte proliferation index was found to be maximum at 50 µg/mL of the PS as compared to other concentrations. Hence, 50 µg/mL of the PS can be considered as the optimum concentration for both the splenocyte and thymocyte proliferation. Again the polysaccharide was nontoxic up to the concentration 500 µg/mL. Therefore, the

biological immunoenhancing effect of the PS was nontoxic and highest at 50 µg/mL concentration. From the above observations it was clear that this PS can act as efficient immunostimulating agent. Some mushroom (Maity et al., 2011; Patra et al., 2011; Dey et al., 2013) and plant (Patra et al., 2010; Patra et al., 2013) polysaccharides had also shown similar type of splenocyte, thymocyte as well as macrophage activations as reported earlier by our group.

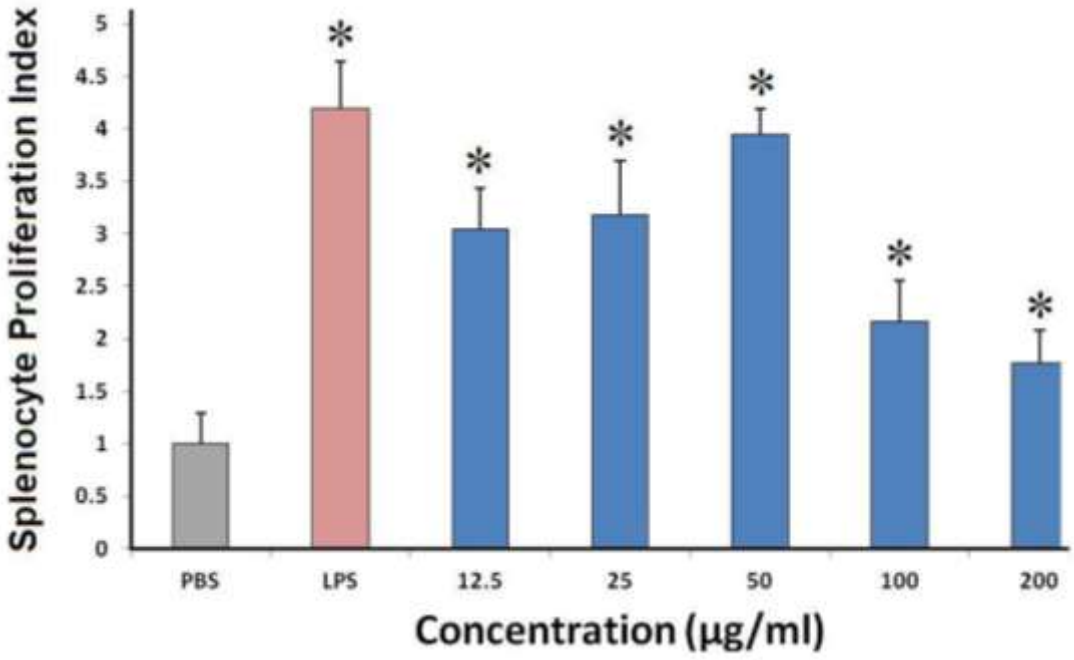


Fig. 4b. Effect of different concentrations of the polysaccharide on splenocyte proliferation.

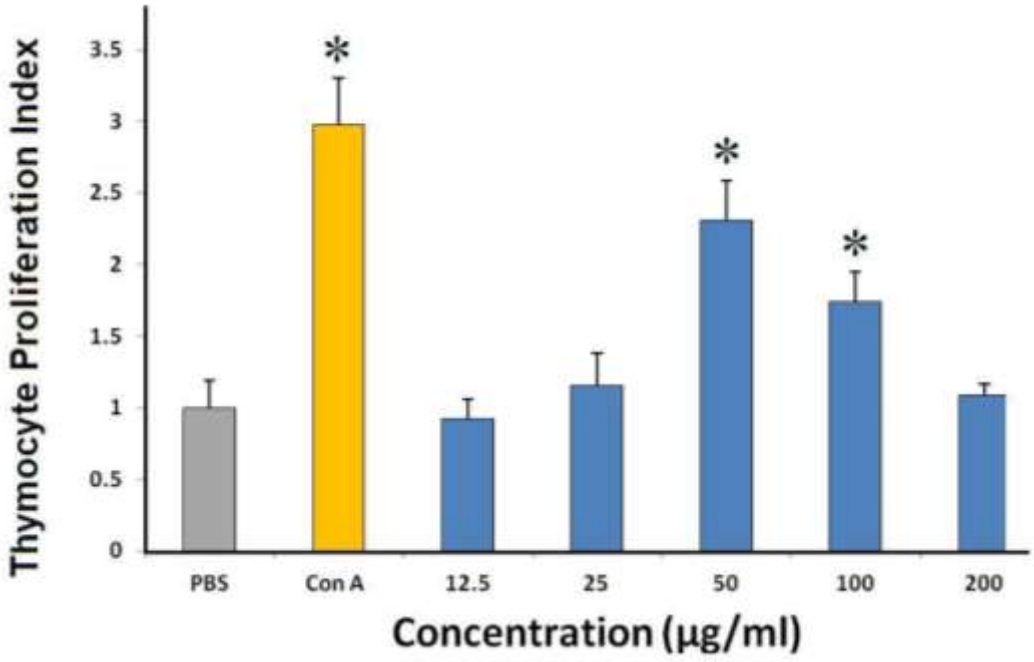
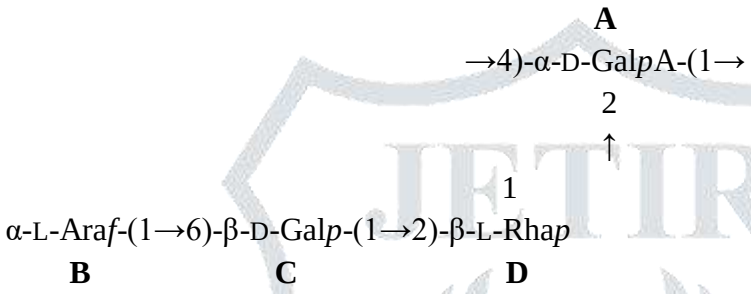


Fig. 4c. Effect of different concentrations of the polysaccharide on thymocyte proliferation.

4. Conclusion

A water soluble heteropolysaccharide was isolated from aqueous extract of the leaves of *Azadirachta indica* and purified by gel-filtration chromatography. This molecule showed splenocyte and thymocyte activation and phagocytic response of macrophages in a dose dependent manner. On the basis of chemical analysis and NMR studies the structure of the repeating unit of the polysaccharide was established as



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Conflicts of interest

The author declares no conflict of interest

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