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Structural studies of an immunostimulating gluco-arabinan from seeds of *Caesalpinia bonduc*

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ABSTRACT

A water-soluble gluco-arabinan (PS-II, $M_W \sim 62$ kDa) isolated from the alkaline extract of the endosperm of *Caesalpinia bonduc* showed the presence of T-Glc*p*, (1→4)-Glc*p*, (1→2,3)-Glc*p*, T-Ara*f*, (1→5)-Ara*f*, (1→2,5)-Ara*f*, and (1→2,3,5)-Ara*f* in a relative proportion of approximately 2:2:2:3:2:1:1. The proposed repeating unit of the polysaccharide possessed a branched backbone of two (1→3)-α-D-glucopyranose followed by four (1→5)-α-L-arabinofuranose residues. In case of two (1→3)-α-D-glucopyranose, branching occurs at *O*-2 by a same residue terminated by another one at *O*-4 position. Out of four (1→5)-α-L-arabinofuranose residues, one residue is terminated at *O*-2 and *O*-3 by two arabinofuranose residues and another one situated at the adjacent position is terminated at *O*-2 with same residue, and two (1→5)-α-L-arabinofuranose residues are free from branching and located before and after the two branched arabinofuranose residues. This gluco-arabinan molecule and previously reported arabinan showed similar extent of splenocytes and thymocytes stimulation, but arabinan showed appreciable macrophage activations.

1. Introduction

Caesalpinia bonduc (Nata Karanja) is an important medicinal plant (Anonymous, 1956; Gamble, 1967; Kapoor, 2005) and the seeds (Prajapati et al., 2006) are well known as nickernut. Two water-soluble fractions (PS-I and PS-II) were isolated from alkaline extract of endosperm of seeds of *C. bonduc*. The first fraction, PS-I was identified as arabinan and reported in *Carbohydrate Polymers* (Mandal et al., 2011). The second fraction, PS-II was characterized as gluco-arabinan; detailed structural studies of which revealed that the arabinan part of the gluco-arabinan molecule is exactly the same with the repeating unit of the previously reported arabinan (PS-I) and the linkages of the other glucosyl moieties attached to it were assigned and the structure was established. With a view to develop carbohydrate-based drugs, some immunostimulating properties like splenocyte, thymocyte, and macrophage activations of both the molecules were studied and compared.

2. Materials and methods

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

The endosperm of seeds of *C. bonduc* was boiled with 4% NaOH for 90 min, kept overnight at 4 °C and filtered. The crude polysaccharide was isolated and purified by the method described earlier (Mandal et al., 2011). Crude material (210 mg) on fractionation through Sepharose 6B yielded two homogeneous PS-I, 55 mg and PS-II, 45 mg. The fractionation and purifications steps are shown in the following diagram.

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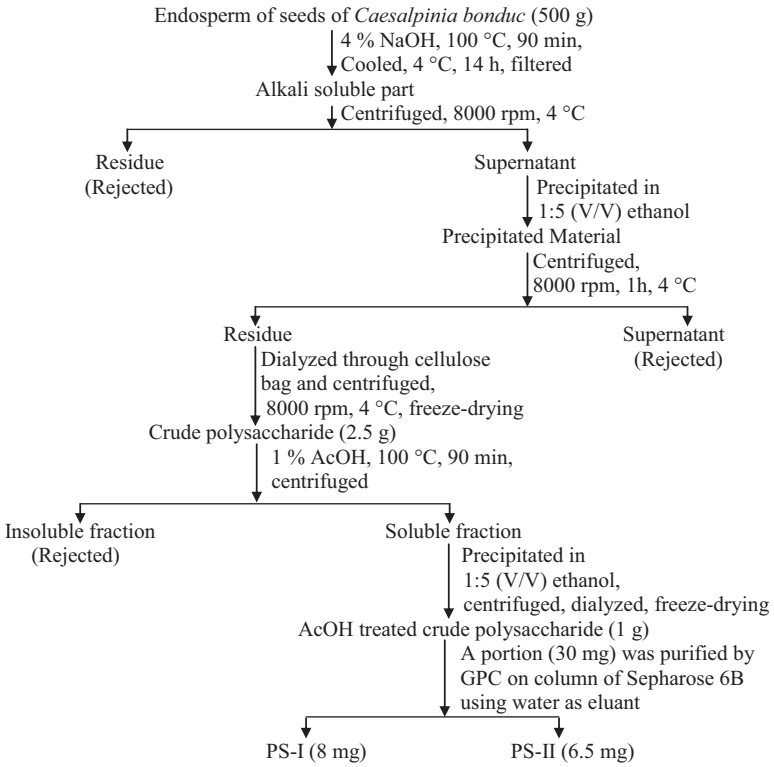


2.2. General analyses

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 28.6 °C. Total carbohydrate was estimated by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using arabinose (20–200 μg) as the standard. Paper 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). Silver nitrate in acetone (1.2%), methanol in sodium hydroxide solution, and 5% sodium thiosulphate solution were used as spray reagents (Hoffman, Lindberg, & Svensson, 1972). Alditol acetates of monosaccharides and the methyl sugar were analyzed by GC and GC–MS (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976). A gas–liquid chromatograph Hewlett-Packard 5730 A was used, having a flame ionization detector and glass columns (1.8 m × 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 GC analyses were performed at 170 °C. Gas–liquid chromatography–mass spectrometric (GC–MS) analysis was performed on Shimadzu GC–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 up to a final temperature of 200 °C.

2.3. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-permeation chromatography. 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 the polysaccharide was then plotted in the same graph, and molecular weight of polysaccharide was determined.

2.4. Absolute configuration of monosaccharides

The absolute sugar configuration was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. 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 *N,O*-bis(trimethylsilyl)trifluroacetamide (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.

2.5. Constituent sugar analysis

The polysaccharide (PS-II, 3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottomed flask at 100 °C for 4 h in a boiling water bath. The excess of acid was completely removed by co-distillation with water. 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 CH_3OH to remove excess boric acid. The reduced sugars (alditols) 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 GC.

2.6. Methylation analysis

The polysaccharide (PS-II) was methylated using Ciucanu and Kerek (1984). The polysaccharide (4.0 mg) was kept on P_2O_5 in a vacuum decicator for several days and then dissolved in 0.5 mL of distilled DMSO. Finely powdered anhydrous NaOH was added and stirring for 30 min. Then 1.0 mL CH_3I was added, stirring for 1.5 h.

The methylated products were isolated by partitioning 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 formolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, and excess formic acid was evaporated by co-distillation with distilled water, and then reduced with $NaBH_4$, acetylated with (1:1) acetic anhydride–pyridine, and analyzed by GC and GC–MS.

2.7. Periodate oxidation

The polysaccharide (PS-II, 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-oxidized-reduced (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965) material was divided into two portions. One portion was hydrolyzed with 2 M CF_3COOH and used for alditol acetates preparation, which were analyzed by GC. Another portion was methylated by Ciucanu and Kerek method (1984), and alditol acetates of the methylated products were prepared and analyzed by GC and GC–MS.

2.8. NMR studies

The pure polysaccharide (PS-II) was kept over P_2O_5 under vacuum for several days, and then exchanged with deuterium (Dueñas-Chaso et al., 1997) by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. Samples were dissolved in D_2O and NMR spectra were recorded on a Bruker Avance DPX-500 spectrometer at 27 °C. The ^1H and ^{13}C (both ^1H coupled and decoupled) NMR spectra were recorded at 27 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.76) using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992). The 2D-DQF-COSY experiment was carried out 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 was 300 ms. The ^{13}C chemical shifts were measured using acetone as internal standard, fixing the methyl carbon signal at δ 31.05. The delay time in the HMBC experiment was 80 ms.

2.9. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus was prepared from the normal mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells (RBC) were removed by hemolytic Gey's solution. After washing two times in HBSS the cells were resuspended in complete RPMI (Rose well Park Memorial Institute) medium. Cell concentration was adjusted to 1×10^6 cells/mL and the viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96-well flat-bottom tissue culture plates and incubated with 20 μL of various concentrations (5, only for splenocytes; 12.5; 25; 50; 100; and 200 μg/mL) of the polysaccharides (gluco-arabinan, PS-II and arabinan, PS-I). PBS (Phosphate Buffer Saline, 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 for splenocytes and thymocytes stimulations, respectively. All cultures were set up in triplicate for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes (% Splenocyte Proliferation Index or % SPI) and thymocytes (% Thymocyte Proliferation Index or % TPI) were checked by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay method (Mosmann, 1983; Ohno et al., 1993). The data are reported as the mean ± standard deviation of different observations and compared against PBS control (Maiti et al., 2008; Mallick, Maiti, Bhutia, & Maiti, 2010; Shah et al., 2007).

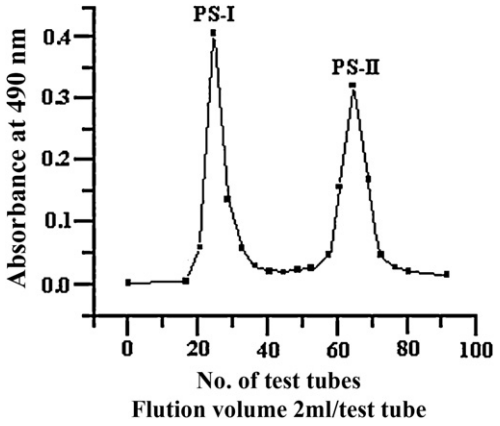


Fig. 1. Gel permeation chromatogram of crude polysaccharide, isolated from seeds of *Caesalpinia bonduc* using Sepharose 6B column.

2.10. In vitro activation of RAW cell (nitric oxide assay)

RAW 264.7 growing in complete Dulbecco's Modified Eagle's Medium (DMEM) was seeded in 96-well flat bottom tissue culture plates at 5×10^5 cells/mL concentration (180 μL) (Ohno, Hasimato, Adachi, & Yadomae, 1996; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006). Cells were left overnight for attachment and treatment of different concentrations (12.5, 25, 50, 100, 200, and 300 μg/mL) of the present polysaccharides (gluco-arabinan, PS-II and arabinan, PS-I) were given. The cells were cultured for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 incubator. The production of nitric oxide (NO) was estimated by measuring nitrite levels in the cell supernatant with Griess Reagent (1:1 of 1% sulfanilamide in 2.5% phosphoric acid, 0.1% napthyl ethyl diamine dihydrochloride in 2.5% phosphoric acid) (Green et al., 1982). Lipopolysaccharide (LPS, 4 μg/mL) was used as positive control. Absorbance was read at 550 nm.

3. Results and discussion

3.1. Isolation, purification, and chemical analysis of the polysaccharide

The crude polysaccharide obtained from alkaline extract of the seeds endosperm of *C. bonduc* separated by gel-permeation chromatography yielded two homogeneous fractions, PS-I and PS-II (Fig. 1). The PS-I was deduced to be an arabinan molecule (Mandal et al., 2011). The sugar analysis of PS-II by paper chromatography and GC of alditol acetates showed that it contained glucose (Glc) and arabinose (Ara) in a molar ratio of nearly 6:7, and was deduced to be a gluco-arabinan molecule. The periodate-oxidation experiment showed the presence of Glc and Ara in a molar ratio of nearly 1:1. The detailed structural characterization of PS-II was basically investigated by chemical and NMR analyses, and reported here in.

Pure PS-II had a specific rotation $[\alpha]_D^{28.6}$ +21.78 (c 0.15, water), and estimated to be 98.5% carbohydrate by colorimetric analysis. The average molecular weight was determined as

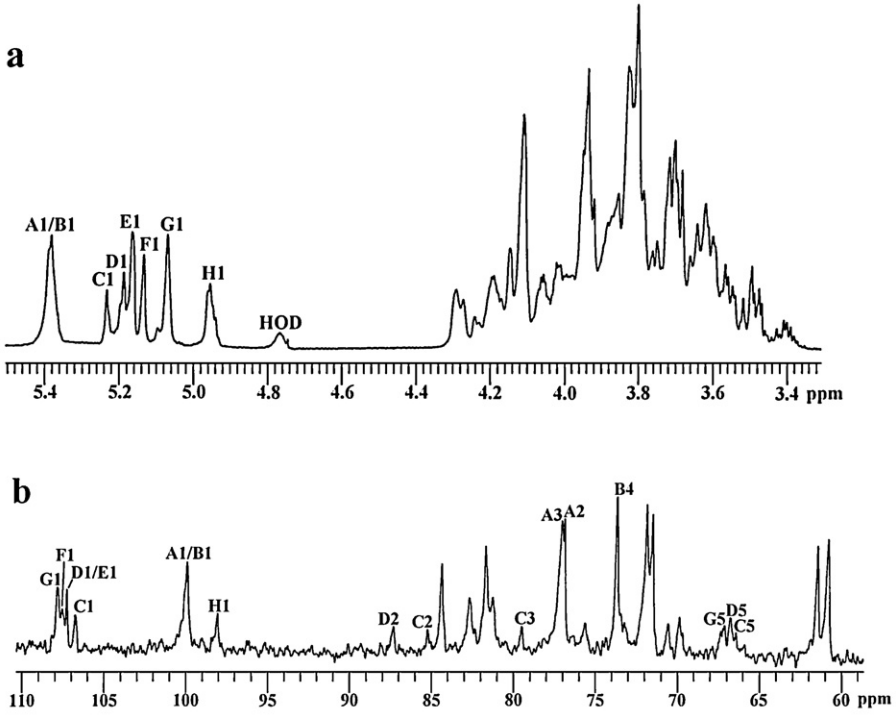


Fig. 2. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) and (b) ^{13}C NMR (125 MHz, D_2O, 27 °C) spectrum of polysaccharide (PS-II), isolated from seeds of *Caesalpinia bonduc*.

~62 kDa compared with standard dextrans. Configuration analysis showed that the Glc and Ara were in the D and L forms, respectively. Linkage analysis showed the presence of 2,3,4,6-Me_4-Glc, 2,3,6-Me_3-Glc, 4,6-Me_2-Glc, 2,3,5-Me_3-Ara, 2,3-Me_2-Ara, 3-Me-Ara, and Ara. Thus the polysaccharide was deduced to comprise terminal, (1→4)-linked, (1→2,3)-linked-D-glucopyranosyl; and terminal, (1→5)-linked, (1→2,5)-linked, and (1→2,3,5)-linked-L-arabinofuranosyl moieties in a molar ratio of nearly 2:2:2:3:2:1:1. These linkages were further confirmed by the periodate-oxidized (Goldstein et al., 1965; Hay et al., 1965), $NaBH_4$ reduced, and methylated polysaccharide, which showed the presence of 4,6-Me_2-Glc, 3-Me-Ara, and Ara in a molar ratio of nearly 2:1:1. These results clearly indicated that the terminal, (1→4)-linked-glucopyranosyl; and terminal, (1→5)-linked-arabinofuranosyl moieties are consumed during periodate-oxidation and agreed with the above result.

3.2. NMR and structural analysis of the polysaccharide (PS-II)

The 500 MHz ^{1}H NMR spectrum (Fig. 2a and Table 1) of the polysaccharide (PS-II) showed seven anomeric proton signals at δ 5.38, 5.23, 5.19, 5.17, 5.13, 5.07, and 4.95 in a molar ratio of nearly 4:1:1:2:1:2:2. Hence, the signals at δ 5.23, 5.19, and 5.13 indicated the presence of only one residue; the signals at δ 5.17, 5.07, and 4.95 corresponded to two residues each while the other signals at δ 5.38 corresponded for four sugar residues. The sugar residues were designated as **A–H** according to their decreasing anomeric proton chemical shifts. In the 125 MHz ^{13}C NMR spectrum (Fig. 2b and Table 1) six anomeric signals at δ 107.9, 107.6, 107.3, 106.8, 99.9, and 98.1 were present. Signal at δ 99.9 and 106.8 were assigned to the anomeric carbons of **A** and **B**, and **C** and **D** residues, respectively. Again, the signals at δ 107.3, 107.6, 107.9, and 98.1 were assigned for anomeric carbons of **E**, **F**, **G**, and **H** residues, respectively. All the assigning ^{1}H and ^{13}C signals were confirmed by HMQC experiment (Fig. 3 and Table 1). Also, the anomeric proton and carbon chemical signals were confirmed by the appearance of cross-peaks from NOESY (Fig. 4a and Table 2) and HMBC (Fig. 4b and Table 3) experiments, respectively. From DQF-COSY experiment the proton coupling constants and from proton-coupled ^{13}C NMR experiments the C-1, H-1 coupling constants were measured.

Residues **A**, **B**, and **H** were assigned as D-glucopyranosyl moieties due to showing of large coupling constants values $^{3}J_{H\text{-}2,\,H\text{-}3}$ and $^{3}J_{H\text{-}3,\,H\text{-}4}$ (~10 Hz). The anomeric signals and the coupling constants values $^{3}J_{H\text{-}1,\,H\text{-}2}$ (~3.5 Hz) and $^{1}J_{H\text{-}1,\,C\text{-}1}$ (~170 Hz) indicated that these were α-linked residues.

The very high anomeric carbon chemical shifts (δ 107.9–106.8) of all the arabinose moieties (**C–G**) indicated that these were present only as furanose form. The $^{3}J_{H\text{-}1,\,H\text{-}2}$ value can be significantly used for the determination of the anomeric configuration

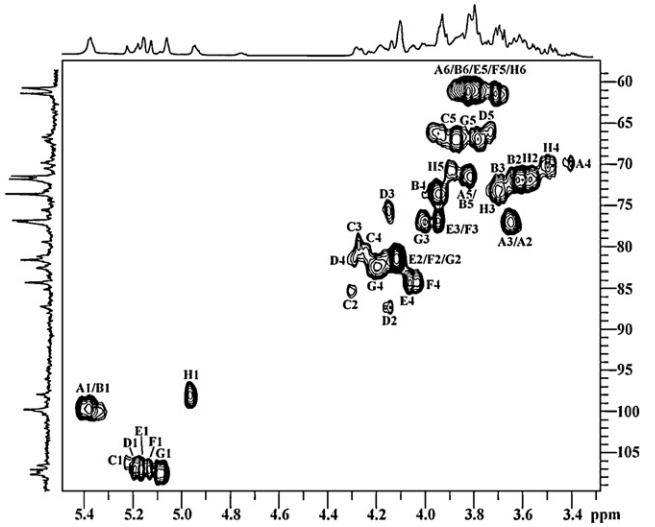


Fig. 3. HMQC spectrum of polysaccharide (PS-II), isolated from seeds of *Caesalpinia bonduc*.

Table 1
^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of the polysaccharide (PS-II) isolated from seeds of *Caesalpinia bonduc* recorded in D_2O at 27 °C.

Sugar residues	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
→2,3)-α-D-Glc*p*-(1→	5.38	3.64	3.66	3.41	3.83	3.83–3.81
A						3.72–3.70
	99.9	76.9	77.1	69.9	71.9	60.8
→4)-α-D-Glc*p*-(1→	5.38	3.62	3.70	3.94	3.81	3.83–3.81
B						3.72–3.70
	99.9	71.9	73.7	73.7	71.9	60.8
→2,3,5)-α-L-Ara*f*-(1→	5.23	4.29	4.27	4.24	3.96–3.92	
C					3.89–3.85	
	106.8	85.3	79.5	81.3	66.4	
→2,5)-α-L-Ara*f*-(1→	5.19	4.14	4.15	4.29	3.81–3.76	
D					3.75–3.71	
	107.3	87.3	75.6	81.8	66.8	
α-L-Ara*f*-(1→	5.17	4.11	3.96	4.06	3.88–3.78	
E					3.74–3.68	
	107.3	81.8	76.9	84.4	61.5	
α-L-Ara*f*-(1→	5.13	4.11	3.92	4.02	3.88–3.78	
F					3.74–3.68	
	107.6	81.8	76.9	84.4	61.5	
→5)-α-L-Ara*f*-(1→	5.07	4.11	3.99	4.19	3.89–3.85	
G					3.81–3.76	
	107.9	81.8	77.1	82.7	67.2	
α-D-Glc*p*-(1→	4.95	3.57	3.72	3.50	3.89	3.83–3.81
H						3.72–3.70
	98.1	71.9	73.7	70.6	71.6	60.8

[a] The values of chemical shifts were recorded with respect to the HOD signal fixed at δ 4.76 ppm at 27 °C.
[b] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.

of α- and β-anomer of L-arabinofuranoside (Mizutani, Kasai, Nakamura, Tanaka, & Matsuura, 1989). The $^{3}J_{H\text{-}1,\,H\text{-}2}$ (1.2–1.6 Hz) and $^{1}J_{C\text{-}1,\,H\text{-}1}$ (168–170 Hz) indicate that all of the present L-arabinofuranosyl residues were present as α-anomer.

For residues **A** and **B**, both residues showed the proton and carbon chemical shifts at δ 5.38 and 99.9 ppm. The downfield shifts (Fig. 2b and Table 1) of C-2 and C-3 of residues **A** and C-4 of residues **B** with respect to the standard values of methyl glycosides (Agarwal, 1992; Rinaudo & Vincendon, 1982) due to the effect of glycosylation indicated that residues **A** were (1→2,3)-linked and residues **B** were (1→4)-linked α-D-Glc*p* moieties.

Residue **C** had the anomeric proton and carbon chemical shifts at δ 5.23 and 106.8. The downfield shifts of C-2, C-3, and C-5 (Fig. 2b and Table 1) with respect to the standard methyl arabinofuranosides (Agarwal, 1992; Mizutani et al., 1989; Rinaudo & Vincendon, 1982) indicated that the it was (1→2,3,5)-linked α-L-arabinofuranosyl moiety.

Residue **D** had an anomeric proton shift at δ 5.19 with an anomeric carbon chemical signal at δ 107.3. The downfield shifts of C-2 and C-5 (Fig. 2b and Table 1) with respect to the standard methyl arabinofuranosides indicated that the residue was (1→2,5)-linked α-L-arabinofuranosyl moiety.

For residues **E** and **F**, the anomeric proton and carbon chemical signals were appeared at δ 5.17 and δ 5.13, and at δ 107.3 and δ 107.6, respectively. The carbon chemical shifts (from C-1 to C-5) of residues **E** and **F** (Fig. 2b and Table 1) corresponded nearly to the standard values (Agarwal, 1992; Rinaudo & Vincendon, 1982; Mizutani et al., 1989) of standard methyl arabinofuranosides. Considering the results of methylation analysis and NMR experiments, it was concluded that residues **E** and **F** were α-linked terminal L-arabinofuranosyl moieties.

Residue **G** showed anomeric proton chemical signal at δ 5.07 and the anomeric carbon chemical signal at δ 107.9. The downfield shift of C-5 (Fig. 2b and Table 1) with respect to the standard methyl arabinofuranosides indicated that the residue **G** was (1→5)-linked arabinofuranosyl moiety.

For residue **H**, the anomeric proton and carbon chemical shift appeared at (4.95 and 98.1 ppm, respectively. The carbon chemical signals from C-1 to C-6 were assigned from HMQC (Fig. 3 and Table 1) experiment and corresponded nearly to the standard methyl glycoside of glucose (Agarwal, 1992; Rinaudo & Vincendon, 1982). The methylation analysis and NMR spectroscopy results was concluded that it was an α-linked terminal D-glucopyranosyl moiety.

The sequences of glycosyl residues of the polysaccharide (PS-II) were determined from NOESY (Fig. 4a and Table 2) as well as ROESY experiments followed by confirmation with HMBC experiment (Fig. 4b and Table 3). The NOESY experiment showed inter-residual

Table 2
The NOESY data for the polysaccharide (PS-II) isolated from seeds of *Caesalpinia bonduc*.

Anomeric proton	NOE contact protons
A H-1	**G** H-5a
	G H-5b
	A H-2
	A H-3
	A H-5
B H-1	**A** H-2
	B H-2
	B H-4
	B H-5
C H-1	**D** H-5a
	D H-5b
	C H-2
D H-1	**G** H-5a
	G H-5b
E H-1	**C** H-2
	D H-2
F H-1	**C** H-3
	F H-2
G H-1	**A** H-3
	C H-5a
	C H-5b
	G H-2
H H-1	**B** H-4
	H H-2
	H H-3
	H H-5

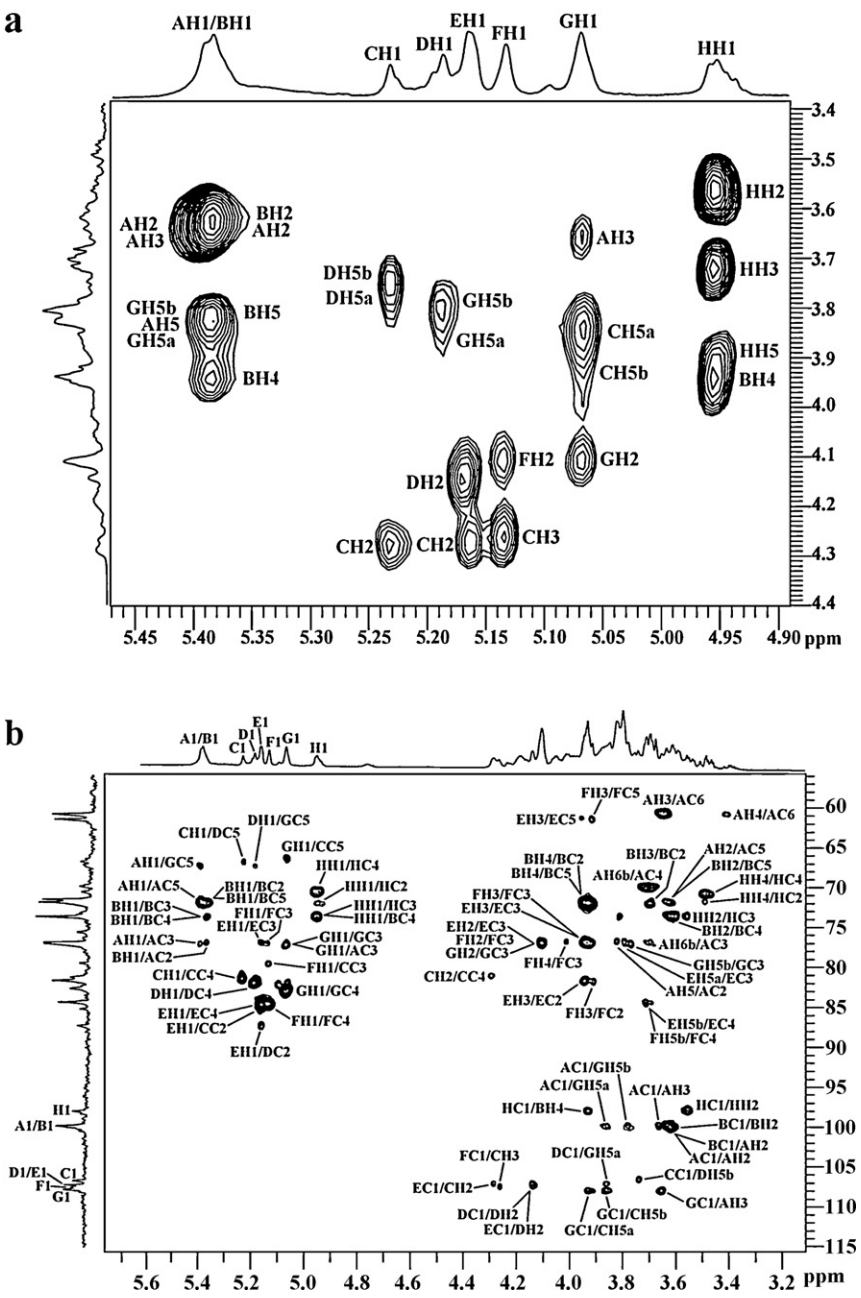


Fig. 4. (a) The NOESY spectrum of polysaccharide (PS-II), isolated from seeds of *Caesalpinia bonduc*. The NOESY mixing time was 300 ms. And (b) HMBC spectrum of polysaccharide (PS-II), isolated from seeds of *Caesalpinia bonduc*. The delay time in the HMBC experiment was 80 ms.

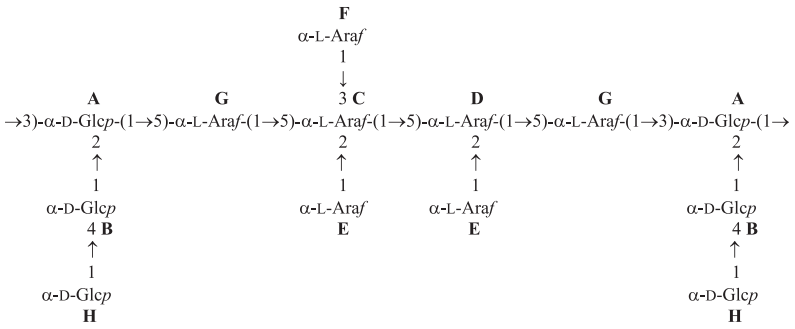
contacts: **A** H-1/**G** H-5a, **G** H-5b, and **A** H-3; **B** H-1/**A** H-2; **C** H-1/**D** H-5a and **D** H-5b; **D** H-1/**G** H-5a and **G** H-5b; **E** H-1/**C** H-2 and **D** H-2; **F** H-1/**C** H-3; **G** H-1/**A** H-3, **C** H-5a, and **C** H-5b; **H** H-1/**B** H-4. From these observations, the following sequences were established as

A-(1 → 3)-**A**; **A**-(1 → 5)-**G**; **B**-(1 → 2)-**A**;

C-(1 → 5)-**D**; **D**-(1 → 5)-**G**; **E**-(1 → 2)**C**; **E**-(1 → 2)-**D**;

F-(1 → 3)-**C**; **G**-(1 → 3)-**A**; **G**-(1 → 5)-**C**; **H**-(1 → 4)-**B**.

Residues **A** (4,6-Me_2-Glc) and **B** (2,3,6-Me_3-Glc) both may be present at the main chain of the repeating unit. If residue **H** (terminal) was connected to the (1→2,3)-linked **A** residue, there was a possibility of the (1→4)-linked **B** residue to be present in the main chain. But, the above connectivities observed from NOESY and HMBC clearly indicated that the terminal **H** was attached to the *O*-4-position of residue **B**. Hence, the only possibility that **B** was present in the side chain instead of the main chain and hence, the probable structural motif present in the gluco-arabinan molecule was proposed as



3.3. Immunological studies of gluco-arabinan (PS-II)

Immunostimulating activities (splenocyte, thymocyte, and macrophage activation) of the polysaccharide (gluco-arabinan, PS-II) on primary cells of murine immune system were studied *in vitro*. Proliferation of splenocytes and thymocytes is an indicator of immunostimulation, and these stimulation tests were carried out with these polysaccharides in mouse cell culture medium by the MTT method (Mosmann, 1983; Ohno et al., 1993). The splenocyte and thymocyte proliferation index as compared to PBS control closer to 100 or below indicates low stimulatory effect on immune system. The present polysaccharide, PS-II showed the maximum splenocyte proliferation indexes (% SPI, Fig. 5a) at 12.5 μg/mL, 1.38 fold higher than control PBS, whereas thymocyte proliferation indexes (% TPI, Fig. 5b) showed maximum effect at 25 μg/mL (1.41 fold higher than PBS). The macrophage activation of the molecule (PS-II) showed no significant NO production at any of the concentration (Fig. 5c).

Table 3
The significant $J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (PS-II) isolated from seeds of *Caesalpinia bonduc*.

Anomeric proton	HMBC contact carbons	Anomeric carbon	HMBC contact protons
A H-1	**G** C-5 **A** C-3 **A** C-5	**A** C-1	**G** H-5a **G** H-5b **A** H-2 **A** H-3
B H-1	**A** C-2 **B** C-2 **B** C-3 **B** C-4 **B** C-5	**B** C-1	**A** H-2 **B** H-2
C H-1	**D** C-5 **C** C-4	**C** C-1	**D** H-5b
D H-1	**G** C-5 **D** C-4	**D** C-1	**G** H-5a **D** H-2
E H-1	**C** C-2 **D** C-2 **E** C-3 **E** C-4	**E** C-1	**C** H-2 **D** H-2
F H-1	**C** C-3 **F** C-3 **F** C-4	**F** C-1	**C** H-3
G H-1	**A** C-3 **C** C-5 **G** C-3 **G** C-4	**G** C-1	**A** H-3 **C** H-5a **C** H-5b
H H-1	**B** C-4 **H** C-2 **H** C-3 **H** C-4	**H** C-1	**B** H-4 **H** H-2

Since the structure of the arabinan part in gluco-arabinan is similar to the previously reported arabinan molecule, PS-I, hence for comparison immunological parameters of this molecule were

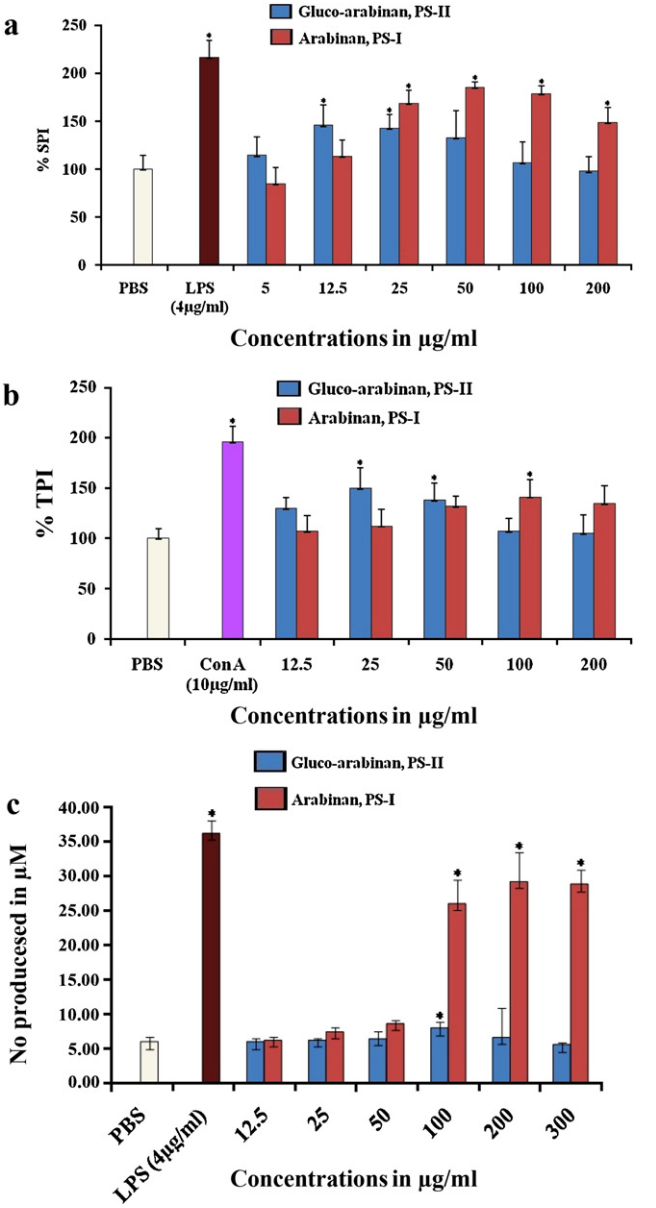


Fig. 5. *In vitro* study of immunostimulation by the polysaccharides (gluco-arabinan, PS-II and arabinan, PS-I) isolated from seeds of *Caesalpinia bonduc* through (a) splenocyte, (b) thymocyte proliferation assay, and (c) NO production by RAW 264.7 cells (significant compared to the PBS control, corresponds to $P \leq 0.05$, and marked with "*").

also investigated. The arabinan, PS-I showed splenocyte activation (Fig. 5a) maximum at 50 μg/mL (1.76 fold higher than PBS) and thymocyte (Fig. 5b) at 100 μg/mL (1.33 fold higher than control PBS) indicating PS-II activates both splenocytes and thymocytes better at lower concentrations in comparison to previously reported arabinan. It is noteworthy to mention that the gluco-arabinan does not exhibit appreciable macrophage activation (Fig. 5c), whereas the arabinan exhibits optimum macrophage activation at 200 μg/mL (4.9 fold higher than the PBS). The comparison of these activations was shown in Fig. 5a (% SPI), Fig. 5b (% TPI), and Fig. 5c (macrophage activation).

Cellular responses like proliferation and NO production by polysaccharides depend on the molecular mass, the degree of branching, conformational structure (Bohn & BeMiller, 1995; Ohno, Miura, Nakajima, & Yadomae, 2000; Okazaki, Adachi, Ohno, & Yadomae, 1995), in addition to the cell type, the abundance of cell surface receptors like dectin-1, CR3, and TLRs (Gantner, Simmons, Canavera, Akira, & Underhill, 2003). In present case, the arabinan, PS-I possess different structure with respect to gluco-arabinan, PS-II where the glucose residues are present; hence their internalization and downstream signaling may vary in different immune cells which is also reflected in our findings. Preliminary studies regarding cell surface binding and internalization studies of fluorescent labeled PS-I and PS-II in RAW cell line, showed higher rate of binding and uptake by arabinan (PS-I) than gluco-arabinan (PS-II). Further studies regarding these are in progress.

4. Conclusion

An immunostimulating water-soluble gluco-arabinan was isolated from alkaline extract of the endosperm of seeds of *C. bonduc* and purified by gel-permeation chromatography. This polymer was composed of T-Glc*p*, (1→4)-Glc*p*, (1→2,3)-Glc*p*, T-Ara*f*, (1→5)-Ara*f*, (1→2,5)-Ara*f*, and (1→2,3,5)-Ara*f* in a relative proportion of approximately 2:2:2:3:2:1:1 and showed splenocytes and thymocytes activations. A comparison of immunostimulation between gluco-arabinan and arabinan revealed that both the molecules are efficient splenocytes and thymocytes stimulators but the latter molecule exhibits better macrophage activations than the former.

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