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Structural analysis of an arabinan isolated from alkaline extract of the endosperm of seeds of *Caesalpinia bonduc* (Nata Karanja)

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ABSTRACT

A water-soluble arabinan (M_w ~2.0×10^2 kDa) isolated from the alkaline extract of the endosperm of *Caesalpinia bonduc* was found to consist of 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 3:2:1:1. On the basis of acid hydrolysis, methylation analysis, and NMR experiments (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC), the structure of the polysaccharide was established. The proposed repeating unit of the polysaccharide has a branched backbone composed of (1 → 5)-α-L-arabinofuranose residues where branching occurs at *O*-2, *O*-3 positions of one residue terminated with two arabinofuranosyl residues and *O*-2 of another one terminated with same residue situated at adjacent position.

1. Introduction

Caesalpinia bonduc (Nata Karanja) is an important medicinal plant widely distributed throughout the coastal region of India, Burma, Sri Lanka, and in other tropical and subtropical regions of the world (Anonymous, 1956; Gamble, 1967; Kapoor, 2005). It is an irregular thorny shrub with large bipinnate leaves. Its flowers are yellow and fruits are inflated pods having 1–2 seeds (Prajapati, Purohit, Sharma, & Kumar, 2006) known as nickernut. Seeds are used by local people as medicine and are available in the market of Midnapore city.

Anthelmintic activities of various extracts of leaf and fixed oil from seeds have been reported (Saravanan, Periyanayagam, Betanabhatla, Athimoolam, & Saraswathy, 2008). Seed extracts showed antimicrobial (Simin, Khaliq-uz-Zaman, & Ahmad, 2001), antidiabetic (Chakrabarti et al., 2005; Kannur, Hukkeri, & Akki, 2006a; Kannur, Hukkeri, & Akki, 2006b), antipyretic (Archana, Tandan, Chandra, & Lal, 2005), and adaptogenic (Kannur, Hukkeri, & Akki, 2006b) effects on animal models. The crude seed extracts, as well as a triglyceride of fatty acids isolated from seed kernel of *C. bonduc* were identified as potent antifilarial drug (Gaur et al., 2008; Rastogi, Shaw, & Kulshreshtha, 1996). Also, the methanolic extract of leaves shows antitumor activity against Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice (Gupta, Mazumder, Kumar, Sivakumar, & Vamsi, 2004).

As part of ongoing studies of *C. bonduc* seeds, a water-soluble arabinan has been isolated from the alkaline extract with a view to develop carbohydrate-based drugs. Detailed structural studies of this arabinan molecule show that it is similar to the molecule isolated from the nuts of *Prunus dulcis* (Dourado, Cardoso, Silva, Gama, & Coimbra, 2006), differing in only one linkage position of a non-reducing end sugar moiety.

2. Materials and methods

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

The endosperm (~500 g) of seeds of *C. bonduc* was boiled with 4% NaOH for 90 min. The whole mixture was kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) at 4 °C and the supernatant was precipitated by the addition of 5 vol EtOH. The precipitated material was collected through centrifugation, dissolved in water and dialysed through a cellulose bag (Sigma–Aldrich, retaining > M_w 12,400) to remove low molecular weight materials. The dialysate was then centrifuged at 4 °C and the water-soluble material was freeze dried, yielding 2.5 g of crude polysaccharide. The crude polysaccharide was then dissolved in 1% acetic acid and heated in a water bath at 100 °C for 90 min. This solution was centrifuged to give a soluble and

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an insoluble fraction. The soluble fraction was retained, and then precipitated by the addition of 5 vol EtOH. The precipitated polysaccharide was collected through centrifugation, redissolved in water, dialysed and freeze-dried to yield 1.0 g. A portion (30 mg) of this soluble fraction was purified by gel permeation chromatography on column (90 cm × 2.1 cm) of Sepharose 6B using water as eluant (0.4 mL min^{-1}). Fractions (2 mL) were collected and monitored for total carbohydrate as described below. Two homogeneous fractions, PS-I (8 mg) and PS-II (6.5 mg) were obtained. This chromatographic step was repeated seven times to yield 55 mg PS-I and 45 mg PS-II.

2.2. General analyses

Total carbohydrate was estimated by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using arabinose (20–200 μg) as the standard. Optical rotation was measured on a Jasco Polarimeter model P-1020 at 25 °C. 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 sugars 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 analyzes 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^{-1} 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 GLC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C min^{-1}) 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-I, 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 and GC–MS.

2.6. Methylation analysis

The polysaccharide (PS-I, 4.0 mg) was methylated using distilled DMSO and finely powdered dry NaOH (Ciucanu & Kerek, 1984). 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 sodium borohydride, acetylated with (1:1) acetic anhydride–pyridine, and analyzed by GC and GC–MS.

2.7. NMR studies

The pure polysaccharide (PS-I) 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 4.74) 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.

3. Results and discussion

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

The alkaline extract of the seeds endosperm of *C. bonduc* separated by gel-permeation chromatography yielded two fractions, PS-I and PS-II. Sugar analysis of PS-I by paper chromatography and GC of alditol acetates showed that it contained only arabinose (Ara). PS-II contained glucose (Glc) and Ara and was deduced to be glucoarabinan; it was not analyzed further.

Pure PS-I, estimated to be 98% carbohydrate by colorimetric analysis, had a specific rotation $[\alpha]_D^{25}$ −16.05 (c 0.074, water). The average molecular weight was determined as ~2.0×10^2 kDa compared with standard dextrans. Configuration analysis showed that the Ara was in the L form. Linkage analysis showed the presence of 2,3,5-Me_3-Ara; 2,3-Me_2-Ara; 3-Me-Ara; and Ara and thus the polysaccharide was deduced to comprise terminal non-reducing, (1 → 5)-linked, (1 → 2,5)-linked, and (1 → 2,3,5)-linked-L-arabinofuranosyl moieties in a molar ratio of nearly 3:2:1:1.

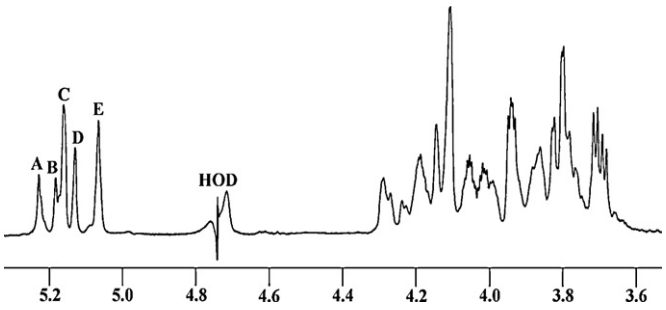


Fig. 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of polysaccharide, isolated from seeds of *Caesalpinia bonduc*.

3.2. NMR and structural analysis of polysaccharide

The 500 MHz ^{1}H NMR spectrum (Fig. 1, Table 1) of the polysaccharide showed five anomeric proton signals at δ 5.23, 5.18, 5.16, 5.13, and 5.07 in a molar ratio of nearly 1:1:2:1:2. Hence, the signals at δ 5.16 and 5.07 corresponded to two residues each while the other signals at δ 5.23, 5.18, and 5.13 indicated the presence of only one residue. The sugar residues were designated as **A**–**E** according to their decreasing anomeric proton chemical shifts (Table 1). In the 125 MHz ^{13}C NMR spectrum (Fig. 2a, Table 1) and DEPT-135 NMR spectrum (Fig. 2b, Table 1) four anomeric signals at δ 107.9, 107.5, 107.2, and 106.8 were present in a ratio of nearly 2:1:2:2. Signal at δ 106.8 was assigned to anomeric carbons of **A** and **B** residues, whereas signals at δ 107.2, 107.5, and 107.9 were assigned for anomeric carbons of **C**, **D**, and **E** residues, respectively. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, NOESY, ROESY (Fig. 3, Table 2), and HMQC (Fig. 4, Table 1) experiments. The proton coupling constants were measured from DQF-COSY experiments.

The very high anomeric carbon chemical shifts (δ 107.9–106.8) of all the arabinose moieties (**A**–**E**) indicated that these were present as furanose and not as a pyranose form. All of the residues present the $J_{C\text{-}1,\,H\text{-}1}$ values were found in the range of 169–171 Hz, which was not useful for the determination of anomeric configuration of L-arabinofuranosides because both α-L- and β-L-configuration gave $J_{C\text{-}1,\,H\text{-}1}$ values within the above range. The $J_{H\text{-}1,\,H\text{-}2}$ value of α-L-arabinofuranoside significantly differs from that of the β-anomer and can be used for the determination of the anomeric configuration (Mizutani, Kasai, Nakamura, Tanaka, & Matsuura, 1989). The coupling constants between H-1and H-2 ($J_{H\text{-}1,\,H\text{-}2}$) of all the present L-arabinofuranosyl residues were observed in the range of 1.4–1.8 Hz indicating these were present as the α-anomer.

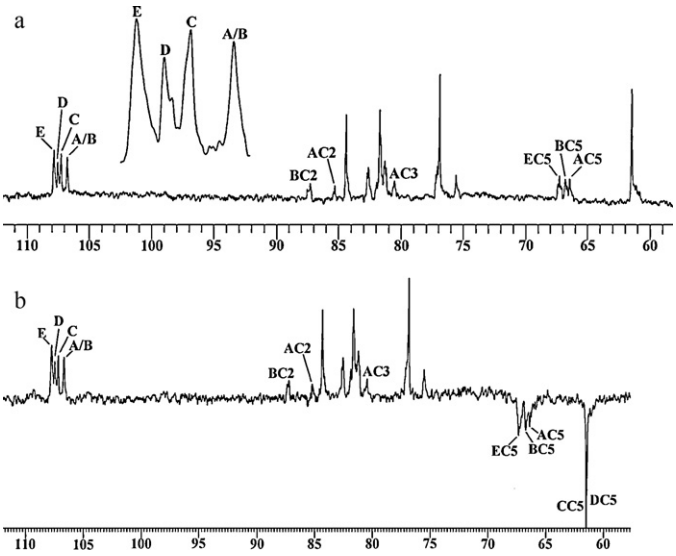


Fig. 2. (a) ^{13}C NMR (125 MHz, D_2O, 27 °C) spectrum of polysaccharide, isolated from seeds of *Caesalpinia bonduc*. (b) DEPT-135 NMR (D_2O, 27 °C) spectrum of polysaccharide, isolated from seeds of *Caesalpinia bonduc*.

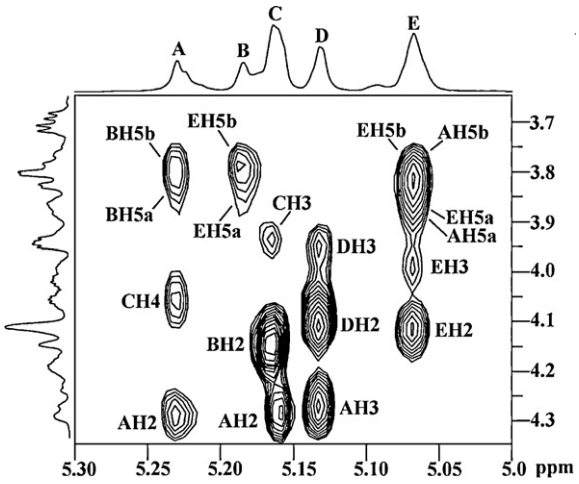


Fig. 3. The ROESY spectrum of polysaccharide, isolated from seeds of *Caesalpinia bonduc*. The ROESY mixing time was 300 ms.

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

Sugar residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5a, H-5b/C-5
→2,3,5)-α-L-Araf-(1→	5.23	4.29	4.27	4.24	3.95–3.92
A					3.87–3.84
	106.8	85.3	80.5	81.3	66.4
→2,5)-α-L-Araf-(1→	5.18	4.15	4.15	4.29	3.89–3.86
B					3.80–3.77
	106.8	87.3	75.6	81.7	66.8
α-L-Araf-(1→	5.16	4.11	3.93	4.06	3.82–3.79
C					3.71–3.68
	107.2	81.7	76.9	84.4	61.5
α-L-Araf-(1→	5.13	4.11	3.95	4.02	3.82–3.79
D					3.71–3.68
	107.5	81.7	76.9	84.4	61.5
→5)-α-L-Araf-(1→	5.07	4.11	3.99	4.19	3.88–3.85
E					3.79–3.76
	107.9	81.6	77.1	82.6	67.2

[a] The values of chemical shifts were recorded with respect to the HOD signal fixed at δ 4.74 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.

Table 2
The ROESY data for the polysaccharide isolated from seeds of *Caesalpinia bonduc*.

Anomeric proton		ROE contact protons	
Glycosyl residue	δ_H	δ_H	Residue, atom
→2,3,5)-α-L-Araf-(1→	5.23	4.29	**A** H-2
A		3.89–3.86	**B** H-5a
		3.80–3.77	**B** H-5b
→2,5)-α-L-Araf-(1→	5.18	3.88–3.85	**E** H-5a
B		3.79–3.76	**E** H-5b
α-L-Araf-(1→	5.16	4.29	**A** H-2
C		4.15	**B** H-2
		3.93	**C** H-3
α-L-Araf-(1→	5.13	4.27	**A** H-3
D		4.11	**D** H-2
		3.95	**D** H-3
→5)-α-L-Araf-(1→	5.07	3.95–3.92	**A** H-5a
E		3.87–3.84	**A** H-5b
		4.11	**E** H-2
		3.99	**E** H-3
		3.88–3.85	**E** H-5a
		3.79–3.76	**E** H-5b

Residue **A** has an anomeric carbon chemical shift at δ 106.8 with an anomeric proton signal at δ 5.23. The downfield shifts of C-2, C-3, and C-5 (Fig. 2a & b, Table 1) with respect to the standard methyl arabinofuranosides (Agrawal, 1992; Mizutani et al., 1989; Rinaudo & Vincendon, 1982) indicated that the residue **A** was (1 → 2,3,5)-linked arabinofuranose. The anomeric carbon chemical shift of residue **A** was confirmed by the cross-peaks (**A** C-1, **B** H-5a) and (**A** C-1, **B** H-5b) in the HMBC experiment (Fig. 5, Table 3).

Residue **B** has an anomeric proton signal at δ 5.18. The anomeric carbon chemical shift at δ 106.8 was confirmed by the cross-peaks (**B** C-1, **E** H-5a) and (**B** C-1, **E** H-5b) in the HMBC experiment. The downfield shifts of C-2 and C-5 (Fig. 2a & b, Table 1) with respect to the standard methyl arabinofuranosides indicated that the residue **B** was (1 → 2,5)-linked arabinofuranosyl moiety.

The anomeric proton signals at δ 5.16 and δ 5.13, and anomeric carbon chemical shifts at δ 107.2 and δ 107.5 have shown for **C** and **D** residues respectively. The carbon chemical shifts of residue **C** and **D** (Fig. 2a, Table 1) corresponded nearly to the value of standard methyl arabinofuranosides. The anomeric carbon chemical signal of residue **C** was confirmed by the cross-peaks (**C** C-1, **A** H-2) and (**C** C-1, **B** H-2), and for residue **D** was confirmed by the cross-peak (**D** C-1, **A** H-3), respectively, in the HMBC (Fig. 5, Table 3) experiment. Considering the results of methylation analysis and NMR

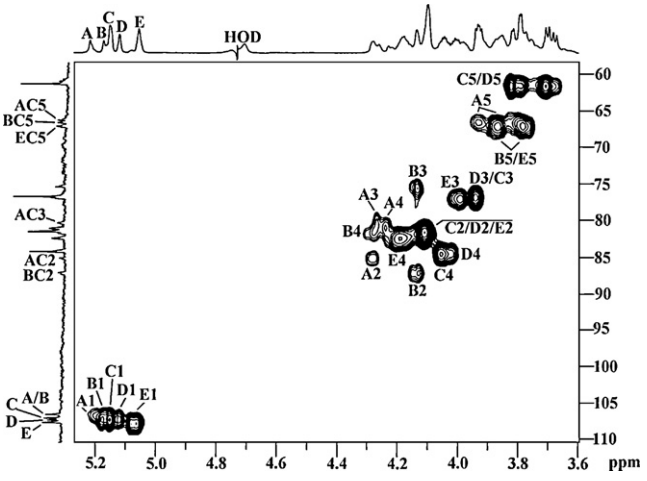


Fig. 4. HMQC spectrum of polysaccharide, isolated from seeds of *Caesalpinia bonduc*.

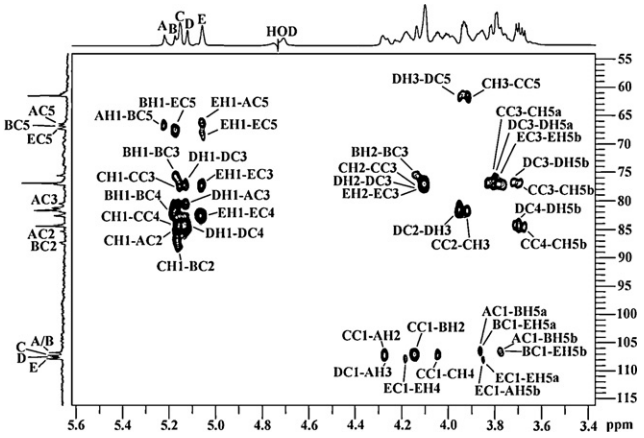


Fig. 5. HMBC spectrum of polysaccharide, isolated from seeds of *Caesalpinia bonduc*. The delay time in the HMBC experiment was 80 ms.

experiments, it was concluded that residue **C** and **D** were α-linked terminal L-arabinofuranosyl moieties.

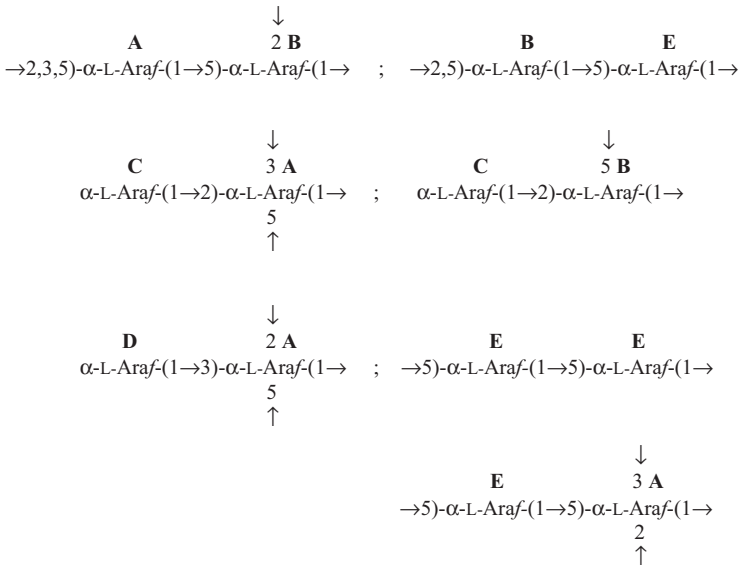
Residue **E** showed anomeric proton chemical signal at δ 5.07 and the anomeric carbon chemical signal at δ 107.9 was confirmed by the cross-peaks (**E** C-1, **A** H-5b) and (**E** C-1, **E** H-5a) in the HMBC experiment. The downfield shift of C-5 (Fig. 2a & b, Table 1) with respect to the standard methyl arabinofuranosides indicated that the residue **E** was (1 → 5)-linked arabinofuranosyl moiety.

Since **C** and **D** are present as non-reducing terminal ends, the backbone chain of the present arabinan would have (1 → 5)-linked residues consisting of **A**, **B**, and **E**, with (1 → 2,3,5), (1 → 2,5), and (1 → 5)-linked moieties, respectively. Residue **B** was more rigid part than **E**, and **A** was the most rigid part of the backbone. Hence, C-5 of **A** (δ 66.4) would be more shielded than C-5 of residue **B** (δ 66.8) and consequently C-5 of **E** (δ 67.2) would be less shielded in comparison to **A** and **B**. This observation was further confirmed by DEPT-135 (Fig. 2b) experiment.

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 *Caesalpinia bonduc*.

Residue	Sugar linkage	H-1/C-1 δ_H/δ_C	Observed connectivities		
			δ_H/δ_C	Residue	Atom
A	→2,3,5)-α-L-Araf-(1→	5.23	66.8	**B**	C-5
		106.8	3.89–3.86	**B**	H-5a
			3.80–3.77	**B**	H-5b
B	→2,5)-α-L-Araf-(1→	5.18	67.2	**E**	C-5
			75.6	**B**	C-3
			81.7	**B**	C-4
		106.8	3.88–3.85	**E**	H-5a
			3.79–3.76	**E**	H-5b
C	α-L-Araf-(1→	5.16	85.3	**A**	C-2
			87.3	**B**	C-2
			76.9	**C**	C-3
			84.4	**C**	C-4
		107.2	4.29	**A**	H-2
			4.15	**B**	H-2
			4.06	**C**	H-4
D	α-L-Araf-(1→	5.13	80.5	**A**	C-3
			76.9	**D**	C-3
			84.4	**D**	C-4
		107.5	4.27	**A**	H-3
E	→5)-α-L-Araf-(1→	5.07	66.4	**A**	C-5
			77.1	**E**	C-3
			82.6	**E**	C-4
			67.2	**E**	C-5
		107.9	3.87–3.84	**A**	H-5b
			4.19	**E**	H-4
			3.88–3.85	**E**	H-5a

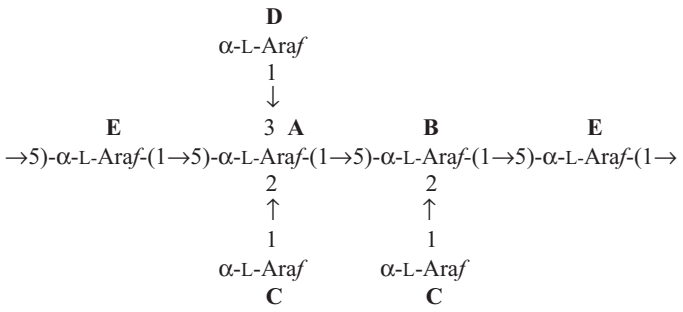
The sequence of glycosyl residues in the polysaccharide was determined from ROESY (Fig. 3, Table 2) and NOESY experiments, followed by confirmation with an HMBC experiment (Fig. 5, Table 3). The ROESY experiment showed inter-residual contacts from **A** H-1 to **B** H-5a and **B** H-5b, **B** H-1 to **E** H-5a and **E** H-5b, **C** H-1 to **A** H-2 and **B** H-2, **D** H-1 to **A** H-3, **E** H-1 to **A** H-5a and **A** H-5b, and **E** H-5a and **E** H-5b of another **E** residue along with



other intra-residual contacts. The ROESY spectrum showed no intra residual contacts of **C** and **D** residues from H-1 to its own H-5; hence the coupling from H-1 to H-5 for **A**, **B**, and **E** residues are their linking contacts. From these observations, the following sequences were established as

4. Conclusion

A water-soluble 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-Ara*f*, (1 → 5)-Ara*f*, (1 → 2,5)-Ara*f*, and (1 → 2,3,5)-Ara*f* in a relative proportion of approximately 3:2:1:1. On the basis of all the above chemical and spectroscopic evidences, the proposed structure of the repeating unit of the present arabinan molecule was established as



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