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Structural investigation of a heteropolysaccharide isolated from the pods (fruits) of *Moringa oleifera* (Sajina)

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Abstract—A water-soluble polysaccharide was isolated from the aqueous extract of pods of *Moringa oleifera*. The polysaccharide contains D-galactose, 6-*O*-Me-D-galactose, D-galacturonic acid, L-arabinose, and L-rhamnose in a molar ratio of 1:1:1:1:1. On the basis of total hydrolysis, methylation analysis, periodate oxidation, and NMR (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HSQC, and HMBC) studies, the repeating unit of the *polysaccharide* is established as

E **D** **C** **B**

→3)-β-D-Gal*p*-(1→2)-β-L-Ara*p*-(1→2)-6-*O*-Me-α-D-Gal*p*-(1→4)-α-D-Gal*p*A-(1→
2
↑
1
α-L-Rha*p*
A

Keywords: *Moringa oleifera*; Polysaccharide; Structure; NMR analysis

1. Introduction

Moringa oleifera (Sajina) is an important medicinal plant distributed throughout India, and all parts of it are considered medicinal.[1,2] Its leaves[3] and fruits[4] are edible, rich in ascorbic acid, amino acids,[5] and the aqueous extract of leaves possesses pharmacological[6] activity toward blood pressure. The flowers contain both sucrose and free sugar, but the fruits show the presence of sucrose only.[7] Flowers are edible, stimulant, tonic, diuretic, and useful to increase the flow of bile.[8,9] A wax,[10] (mp 69–72 °C) was isolated from flowers. Several natural products of different classes such as terpenoids from stems,[11] and alkaloids and antibiotics[12] from seeds have been isolated. Juice from leaves and stem bark of *M. oleifera* exhibited antibacterial and antitubercular activity.[13,14] The root bark showed antiviral effect against vaccinia virus.[15] An ethanolic extract of the whole plant showed anticancer activity against human epidermoid carcinoma of nasopharynx in tissue culture and P_{388} lymphocytic leukemia in mice.[16] Detailed structural works on polysaccharides isolated from its gum[17] and flower[18] were carried out. Analysis of pods[19] (fruits) showed the presence of protein (2.5%), fat (0.1%), carbohydrate (3.7%), dietary fibers (4.8%), and minerals (2%). Moringa fruits are used by the local people as

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delicious vegetable, and available in the market of Midnapore city in large amount mostly in winter season (November–March every year). A (1→4)-linked glucan[20] from fruits has been reported by our group. Here, in this case a different polysaccharide from pods of *M. oleifera* was isolated from hot water extract followed by acetic acid treatment and gel filtration. Taking into account the usefulness of polysaccharides as immunomodulators,[21,22] and with a view to study the immunological parameters, a detailed structural study of this molecule was carried out and is reported herein.

2. Results and discussion

The fresh pods of *M. oleifera* (2 kg) were washed with water, and the inner part of them was boiled with distilled water for 10 h. The whole mixture was then kept overnight at 4 °C, and filtered through linen cloth. The filtrate was centrifuged at low temperature (8 °C), and the supernatant was precipitated with 1:5 water–EtOH. The precipitated material was collected through centrifugation, dissolved in water, and dialyzed through a cellulose bag to remove low molecular weight materials. The whole solution was then centrifuged at 8000 rpm at 6 °C. The residue was discarded, and the filtrate (water soluble part) was freeze dried, yielding 1.6 g of crude polysaccharide. The crude polysaccharide was then allowed to dissolve in 20% acetic acid, and the solution was heated in a water bath for 20 min to give two fractions, an acetic acid soluble fraction and an insoluble fraction. The whole solution was centrifuged at 8 °C, and the filtrate was then precipitated with 1:5 water–EtOH. The precipitated polysaccharide was collected through centrifugation, dialyzed through a cellulose bag and freeze dried. The acetic acid soluble fraction (30 mg) on fractionation through a Sepharose 6B column yielded only one homogeneous fraction (25 mg). This pure polysaccharide was identified as a heteropolysaccharide composed of D-galactose, 6-*O*-Me-D-galactose, D-galacturonic acid, L-rhamnose, and L-arabinose. The molecular weight[23] of this polysaccharide was estimated from a calibration curve prepared with standard dextrans as $\sim 1.96 \times 10^5$ Da. We report herein the structural characterization of this polysaccharide.

The pure polysaccharide showed a specific rotation of $[\alpha]_D^{25}$ +93.2 (*c* 0.86, water). It was hydrolyzed with 2 M CF_3COOH for 18 h at 100 °C. The GLC and GLC–MS analysis of the alditol acetates of the monosaccharides showed the presence of L-rhamnose, D-galactose, 6-*O*-Me-D-galactose, and L-arabinose in a molar ratio of 1:1:1:1 but the carboxyl-reduced polysaccharide on hydrolysis followed by GLC and GLC–MS examination of the corresponding alditol acetates showed the presence of L-rhamnose, D-galactose, 6-*O*-Me-D-galactose, and L-arabinose in a molar ratio of 1:2:1:1. This result confirms that D-galacturonic acid is present in the polysaccharide. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al.[24] The polysaccharide was methylated using the Ciucanu and Kerek method[25] and then by the Purdie method[26] followed by hydrolysis and alditol acetate conversion. The alditol acetates of the methylated material from the polysaccharide were analyzed by GLC using columns A and B as well as by GLC–MS using an HP-5 fused silica capillary column. The polysaccharide showed the presence of 1,2,3,5,-tetra-*O*-acetyl-4,6-di-*O*-methyl-D-galactitol; 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-arabinitol; 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-galactitol in a molar ratio of 1:1:1:1. These results indicate that nonreducing-end L-rhamnopyranosyl, (1→2,3)-linked D-galactopyranosyl, (1→2)-linked L-arabinopyranosyl and (1→2)-linked D-galactopyranosyl moieties are present in the polysaccharide. The carboxyl-reduced polysaccharide was methylated, and alditol acetates of the methylated sugars were identified by GLC analysis which showed the presence of the above peaks and a new peak of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol in a molar ratio of 1:1:1:1:1. This result indicates that (1→4)-linked D-Gal*p*A is also present in the polysaccharide.

Thereafter, a periodate oxidation experiment was carried out with the polysaccharide. The periodate-oxidized, reduced material upon hydrolysis with trifluoro acetic acid followed by GLC analysis showed the presence of D-galactose only. This indicates that L-rhamnose and L-arabinose are destroyed during oxidation. GLC analysis of the periodate-oxidized, reduced, and methylated polysaccharide showed that 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-D-galactitol was retained. This result indicates that the nonreducing-end L-rhamnopyranosyl, (1→2)-linked L-arabinopyranosyl, and (1→2)-linked D-galactopyranosyl moieties are destroyed during oxidation. A part of the periodate-oxidized polysaccharide on hydrolysis showed the absence of D-galacturonic acid in the paper chromatographic examination[27] indicating that it has been destroyed during oxidation, since D-galacturonic acid is present as (1→4)-linked residue in the repeating unit of the polysaccharide. Thus the periodate oxidation experiment confirms that D-galactose is only retained while the rest of the sugar moieties of the polysaccharide reacts with the diol oxidizing reagent.

The ^{1}H NMR spectrum (500 MHz) (Fig. 1) of this polysaccharide at 27 °C showed five anomeric proton signals at δ 5.12, 5.09, 5.07, 4.94, and 4.43 ppm in a molar ratio of 1:1:1:1:1. The methoxyl proton was observed at δ 3.78 ppm. The sugar residues were designated as **A**–**E** according to their decreasing anomeric proton chemical shifts (Table 1). In the ^{13}C NMR spectrum (125 MHz) (Fig. 2) at 27 °C five anomeric signals ap-

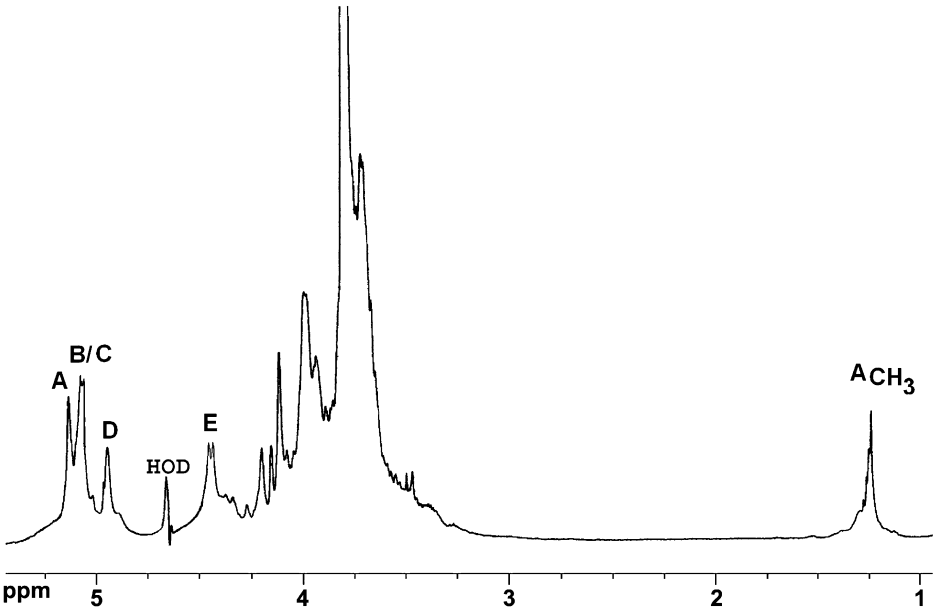


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the polysaccharide isolated from *Moringa oleifera*.

Table 1. The ^{1}H and ^{13}C NMR chemical shifts for the polysaccharide isolated from *Moringa oleifera*[a,b] in D_2O at 27 °C

Glycosyl residue	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6a,H-6b	OMe
A							
α-L-Rha*p*-(1→	103.0	71.2	71.6	73.3	69.5	19.0	
	5.12	4.11	3.98	3.64	3.93	1.25	
B							
→4)-α-D-Gal*p*A-(1→	103.0	69.9	70.1	81.0	71.4	173.5	
	5.09	3.70	4.11	4.19	4.42		
C							
→2)-6-*O*-Me-α-D-Gal*p*-(1→	101.9	80.8	70.0	70.3	71.4	72.9	55.0
	5.07	3.98	4.15	4.04	4.19	3.67,3.77	3.78
D							
→2)-β-L-Ara*p*-(1→	101.9	81.0	72.9	69.9	63.4		
	4.94	3.72	3.96	3.69	3.79,3.94		
E							
→2,3)-β-D-Gal*p*-(1→	103.0	81.0	79.9	70.0	75.1	63.6	
	4.43	3.98	3.71	3.89	3.85	3.76, 3.80	

[a] Values of the ^{13}C chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.
[b] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.67 ppm at 27 °C.

peared at δ 103 and δ 101.9 (Table 1) in a ratio of 3:2. It is worthy to note that the peak at 103 ppm corresponds to the anomeric carbon chemical shift for the α-L-rhamnosyl residue **A**, α-D-galacturonosyl residue **B** and β-D-galactosyl residue **E**, and the peak at 101.9 ppm corresponds to the anomeric carbon chemical shift of both the 6-*O*-Me-α-D-galactosyl residue **C** and β-L-arabinosyl residue **D** for which the two anomeric peaks appear in a molar ratio of 3:2. The methoxyl carbon can be assigned to the resonance at 55 ppm. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC, and HMBC NMR experiments.

Residue **A** has an anomeric proton chemical shift at 5.12 ppm, and $J_{H\text{-}1,H\text{-}2} \sim 1.9$ Hz, $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz indicating that it is an α-linked residue. Residue **A** was determined as Rha*p* due to signals for an exocyclic $-CH_3$ group and the weak coupling between H-1, H-2, and H-3. The anomeric carbon signal of residue **A** at 103 ppm was confirmed by the presence of cross peak **A** C-1, **E** H-2 in the HMBC experiment (Fig. 4, Table 3). Thus, considering the results of methylation analysis and NMR experiments, it may be concluded that **A** is an α-glycosidically-linked, nonreducing-end L-rhamnopyranosyl moiety.

Residue **B** was assigned as (1→4)-linked α-D-Gal*p*A as this spin system consisted of only five protons with a high chemical shift of the H-5 signal (4.42 ppm), weak coupling between H-3, H-4, and H-5 as well as a high

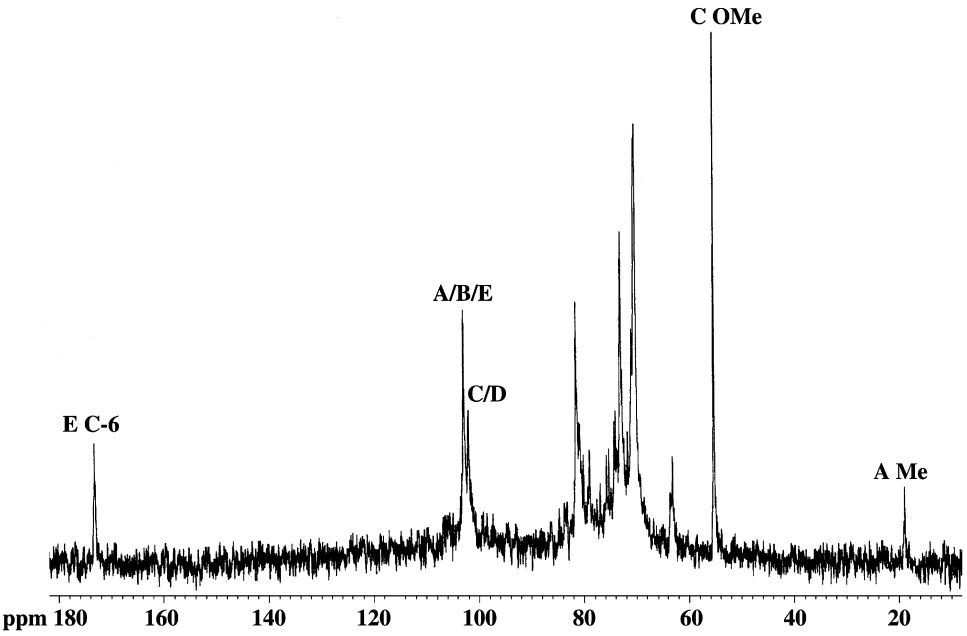


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the polysaccharide isolated from *Moringa oleifera*.

chemical shift of the C-4 signal (81.0 ppm). The anomeric proton chemical shift for moiety **B** at δ 5.09 ($J_{H\text{-}1,H\text{-}2} \sim 3.2$ Hz) and $J_{H\text{-}1,C\text{-}1} \sim 171$ Hz indicates that it is α-linked. The C-1 signal of residue **B** at 103 ppm was confirmed by the appearance of cross peak **B** C-1, **E** H-3 in the HMBC experiment (Fig. 4, Table 3). The carbon signal of residue **B** were at δ 69.9, δ 70.1, δ 71.4, and δ 173.5 correspond to C-2, C-3, C-5, and C-6 (carboxyl carbonyl) respectively.

The anomeric signals of residue **C** at δ 5.07 ($J_{H\text{-}1,H\text{-}2} \sim 3.2$ Hz) and $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz indicate that it is an α-linked residue. A large $J_{H\text{-}2,H\text{-}3}$ (~8.9 Hz) and relatively small coupling constant $J_{H\text{-}3,H\text{-}4}$ (~3.6 Hz) for residue **C** indicate that it is an α-D-galactosyl residue. The anomeric carbon chemical shift of moiety **C** at 101.9 ppm was confirmed by the presence of a cross peak **C** C-1, **B** H-4 in the HMBC experiment (Fig. 4, Table 3). The downfield shift of C-2 (80.8 ppm) and slightly upfield shift of C-3 (70.0 ppm) signals with respect to the standard value of methyl glycosides[28,29] indicate that (1→2)-linked D-galactose is present in the polysaccharide. The presence of methoxyl group at C-6 position of residue **C** was confirmed by the appearance of cross couplings between methoxyl proton (δ 3.78) and C-6 atom of D-galactose [**C** OCH_3 (H), **C** C-6] and between methoxyl carbon (δ 55) and its H-6a, H-6b atoms [**C** OCH_3 (C), **C** H-6a] and [**C** OCH_3 (C), **C** H-6b] in the HMBC experiment (Fig. 4, Table 3). These results indicate that residue **C** is (1→2)-α-D-6-*O*-Me-Gal*p*.

Residue **D** was assigned to Ara*p* as it showed a large coupling constant $J_{H\text{-}2,H\text{-}3}$ (~8.2 Hz) and $J_{H\text{-}3,H\text{-}4}$ (~5 Hz), relatively small coupling constant $J_{H\text{-}1,H\text{-}2}$, as well as it showed two H-5 signals (3.79, 3.94). The anomeric proton chemical shift for residue **D** at 4.94 ppm (unresolved), and carbon chemical shift of 101.9 ppm ($J_{H\text{-}1,C\text{-}1} \sim 170$ Hz) indicate that L-arabinose is β-linked. The C-1 signal of residue **D** at 101.9 ppm was confirmed by the presence of cross peak **D** C-1, **C** H-2 in HMBC experiment (Fig. 4, Table 3). Thus considering the results of methylation analysis and NMR experiments it may be concluded that **D** is 2-linked L-arabinose.

Residue **E** has an anomeric proton chemical shift at 4.43 ppm. A large coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~8.4 Hz) and $J_{H\text{-}1,C\text{-}1}$ value (162 Hz) indicate that it is a β-linked residue. The $J_{H\text{-}3,H\text{-}4}$ value (~3.5 Hz) and the $J_{H\text{-}2,H\text{-}3}$ value (~9.1 Hz) for residue **E** indicate that it is a β-D-galactosyl residue. The anomeric carbon chemical shift of moiety **E** at 103 ppm was confirmed by the presence of a cross peak **E** C-1, **D** H-2 in the HMBC experiment (Fig. 4, Table. 3). The downfield shift of C-2 (74.2 ppm), C-3 (79.9 ppm) signals, and a little upfield shift of C-4 (70.0 ppm) with respect to the standard value of methyl glycosides[28,29] indicate that residue **E** is 1,2,3-linked D-galactose.

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. Residue **A** has interresidue NOE contacts from H-1 to H-1, and H-2 of residue **E**. This indicates that residue **A** is linked at the 2-position of residue **E**, and the following sequence is established:

A **E**

α-L-Rha*p*-(1→2)-β-D-Gal*p*-(1→

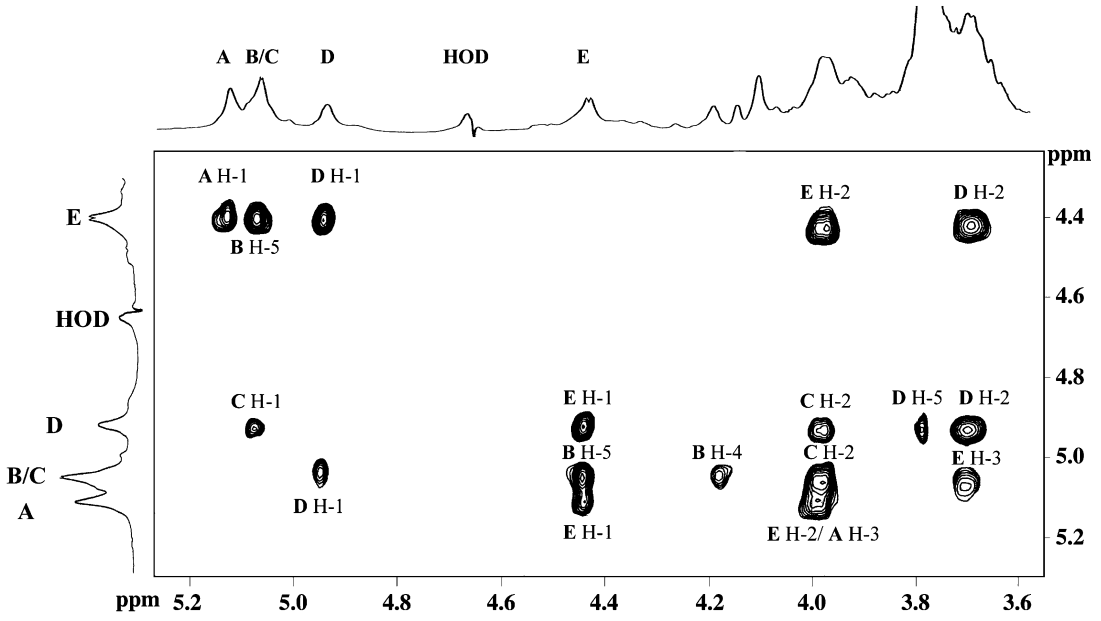


Figure 3. The NOESY spectra of the polysaccharide isolated from *Moringa oleifera*. The NOESY mixing time was 300 ms.

Table 2. NOE data for the polysaccharide isolated from *Moringa oleifera*

Anomeric proton		NOE contact protons	
Glycosyl residue	δ	δ	Residue, atom
α-L-Rha*p*(1→	5.12	4.43	**E** H-1
A		3.98	**E** H-2
→4)-α-D-Gal*p*A-(1→	5.09	3.71	**E** H-3
B		4.42	**B** H-5
→2)-6-*O*-Me-α-D-Gal*p*(1→	5.07	4.19	**B** H-4
C		3.98	**C** H-2
		4.94	**D** H-1
→2)-β-L-Ara*p*(1→	4.94	5.07	**C** H-1
D		3.98	**C** H-2
		3.72	**D** H-2
		3.79	**D** H-5
		4.42	**E** H-1
→2,3)-β-D-Gal*p*(1→	4.43	5.12	**A** H-1
E		4.94	**D** H-1
		3.72	**D** H-2
		3.98	**E** H-2

Residue **B** has an interresidue NOE contact from H-1 to H-3 of residue **E**. This indicates that residue **B** is linked at the 3-position of residue **E**, and the following sequence is established:

B **E**
α-D-Gal*p*A-(1→3)-β-D-Gal*p*-(1→
2
↑

Residue **C** has interresidue NOE contacts from H-1 to H-4 of residue **B** and H-1 of residue **D**. It is worth to note here that (**C** H-1, **B** H-4) coupling may also be due to intraresidual coupling of **B** itself, but the connectivity between **C** and **B** was confirmed through an HMBC experiment. Hence, it can be concluded that residue **C** is linked to the 4-position of residue **B**, and also **D** is glycosidically linked at the 2-position of **C**. Hence the following sequence is established:

D **C** **B**
β-L-Ara*p*-(1→2)-6-*O*-Me-α-D-Gal*p*-(1→4)-α-D-Gal*p*A6Me-(1→

Residue **D** has an interresidue NOE contact from H-1 to H-1, and H-2 of residue **C**. This indicates that residue **D** is linked at the 2-position of residue **C**. Furthermore, H-1 of residue **D** has NOE contact with H-1 of **E**, hence **E** is glycosidically-linked at the 2-position of residue **D**. Therefore, the following sequence is established:

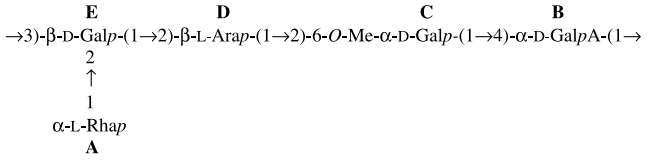
E **D** **C**
→ 3)-β-D-Gal*p*-(1→2)-β-L-Ara*p*-(1→2)-6-*O*-Me-α-D-Gal*p*-(1→
2
↑

Residue **E** has interresidue NOE contacts from H-1 to H-1 and H-2 of residue **D** and H-1 of residue **A** indicating that residue **E** is linked at the 2 position of residue **D**, and also **A** is glycosidically linked at the 2-position of residue **E**. Hence the sequence is as follows:

E **D**
→ 3)-β-D-Gal*p*-(1→2)-β-L-Ara*p*-(1→
2
↑
1
α-L-Rha*p*
A

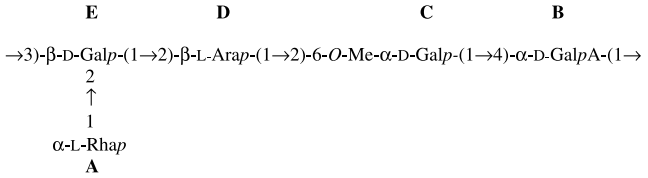
The above sequence of glycosyl residues of the polysaccharide was also determined from ROESY experiment.

Since **A**, **B**, **C**, **D**, **E** residues are present in a molar ratio of 1:1:1:1:1, therefore, the following pentasaccharide repeating unit for the polysaccharide is assigned as follows:

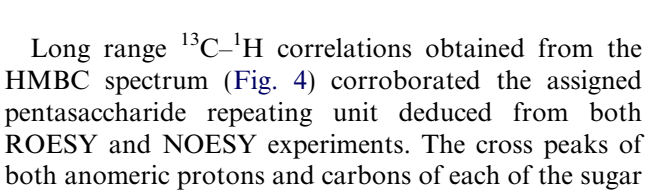


Long range ^{13}C–^{1}H correlations obtained from the HMBC spectrum (Fig. 4) corroborated the assigned pentasaccharide repeating unit deduced from both ROESY and NOESY experiments. The cross peaks of both anomeric protons and carbons of each of the sugar moieties were examined, and both inter and intraresidual connectivities were observed from the HMBC experiment (Table 3). Cross peaks were found between H-1 (5.09 ppm) of residue **B** and C-3 (79.9 ppm) of residue **E** (**B** H-1, **E** C-3); C-1 (103 ppm) of residue **B** and H-3 (3.71 ppm) of residue **E** (**B** C-1, **E** H-3). Similarly cross peaks between C-1 (101.9 ppm) of residue **C** and H-4 (4.19 ppm) of residue **B** (**C** C-1, **B** H-4); H-1 (5.07 ppm) of residue **C** and C-4 (81.0 ppm) of residue **B** (**C** H-1, **B** C-4) were observed. The cross peaks between C-1 (103 ppm) of residue **A** and H-2 (3.98 ppm) of residue **E** (**A** C-1, **E** H-2); H-1 (5.12 ppm) of residue **A** and C-2 (81.0 ppm) of residue **E** (**A** H-1, **E** C-2) were observed. The cross peaks between H-1 (4.94 ppm) of residue **D** with C-2 (80.8 ppm) of residue **C** (**D** H-1, **C** C-2) and C-1 (101.9 ppm) of residue **D** with H-2 (3.98 ppm) of residue **C** (**D** C-1, **C** H-2) was observed. The cross peaks between H-1 (4.43 ppm) of residue **E** and C-2 (81.0 ppm) of residue **D** (**E** H-1, **D** C-2); C-1 (103 ppm) of residue **E** and H-2 (3.72 ppm) of residue **D** (**E** C-1, **D** H-2) were observed.

Thus the appearance of these cross peaks clearly supports the presence of a pentasaccharide repeating unit in the polysaccharide isolated from the fruits of *M. oleifera*. Hence, the repeating unit is established as



In order to obtain information on the sequence of the sugar residues in the repeating unit, the polysaccharide was subjected to Smith degradation studies,[30,31] and the products were separated on a Sephadex G-25 column using water as the eluent, resulting in a fraction SDPS. GLC analysis of the alditol acetate of the acid hydrolyzed product from SDPS showed the presence of D-galactose only. The alditol acetate of the methylated product from SDPS analyzed by GLC using columns A and B showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol. The 125 MHz ^{13}C NMR experiment showed only one anomeric carbon signal at 103.0 ppm (Fig. 5) that corresponds to β-D-Gal*p*. Therefore, on the basis of the above results, it is concluded that the polysaccharide has the following structure:



3. Experimental

3.1. Isolation and purification of the polysaccharide

The fresh pods of *M. oleifera* were washed with water, and boiled with distilled water for 8 h. The whole mixture was then kept overnight at 4 °C, and filtered through linen cloth. The filtrate was centrifuged at low temperature. The supernatant was precipitated with 1:5 water–EtOH. The precipitated material was collected through centrifugation, and dissolved in water followed by dialysis through a cellulose bag to remove low molecular weight materials. The whole solution was then centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) at 6 °C. The residue was discarded, and the filtrate (water soluble part) was freeze dried, yielding 1.6 g of crude polysaccharide. The crude polysaccharide was then allowed to dissolve in 20% acetic acid, and the soln was heated in a water bath for 20 min to give two fractions; an acetic acid soluble fraction and an acetic acid insoluble fraction. The acetic acid soluble fraction was separated from the acetic acid insoluble fraction by centrifugation, and the supernatant was precipitated with 1:5 water–EtOH. The precipitated polysaccharide was collected through centrifugation, dialyzed through a cellulose bag and freeze dried. The acetic acid soluble fraction (30 mg) was purified by gel permeation chromatography on a column (90 × 2.1 cm) of Sepharose 6B in water as eluent (0.4 mL min^{-1}) using a Redifrac fraction collector. Ninety-five Test tubes (2 mL each) were collected, and monitored spectrophotometrically at 490 nm with the phenol–sulfuric acid reagent[32] using a Shimadzu UV–vis spectrophotometer, model-1601. One homogeneous fraction (test tubes 28–58) was collected and freeze dried, yielding 20 mg of material. The purification process was carried out in

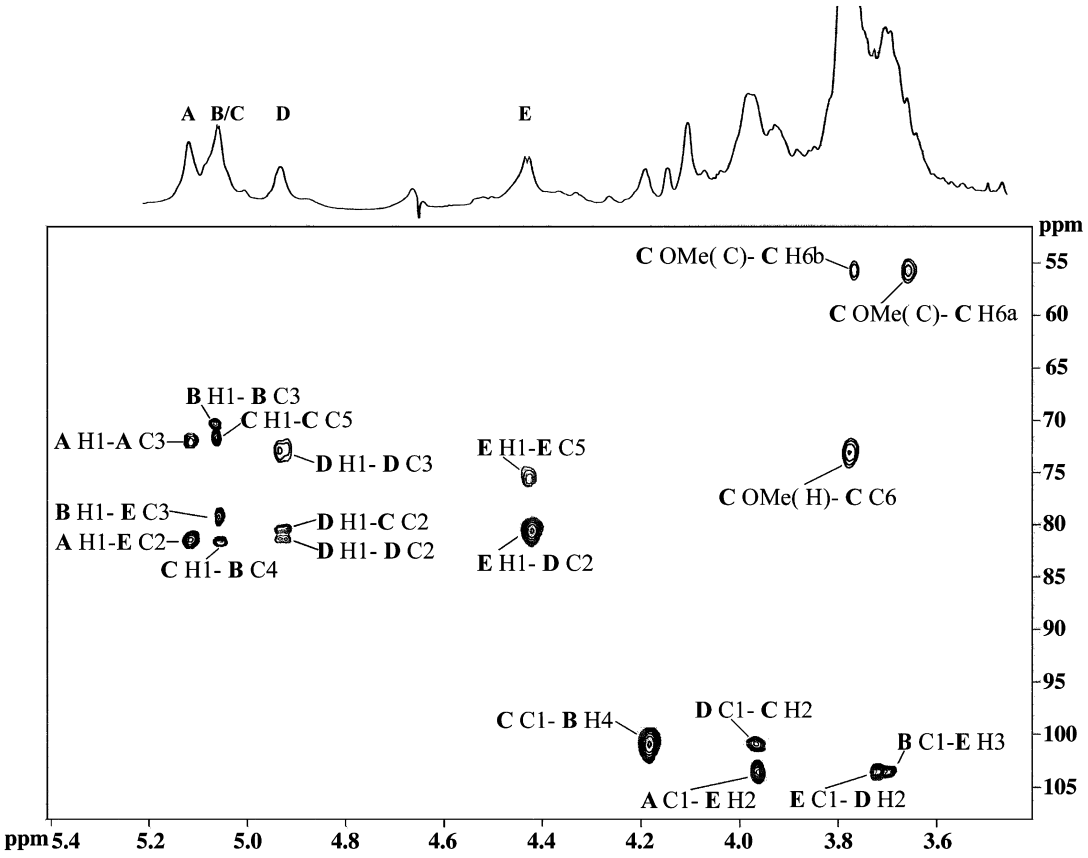


Figure 4. HMBC spectrum of polysaccharide, isolated from *Moringa oleifera*. The delay time in the HMBC experiment was 80 ms.

Table 3. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide of *Moringa oleifera*

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	α-L-Rha*p*-(1→	5.12	81.0	**E**	C-2
			71.6	**A**	C-3
		103.0	3.98	**E**	H-2
B	→4)-α-D-Gal*p*A-(1→	5.09	79.9	**E**	C-3
			70.1	**B**	C-3
		103.0	3.71	**E**	H-3
C	→2)-6-*O*-Me-α-D-Gal*p*-(1→	5.07	81.0	**B**	C-4
			71.4	**C**	C-5
		101.9	4.19	**B**	H-4
D	→2)-β-L-Ara*p*-(1→	4.94	80.8	**C**	C-2
			81.0	**D**	C-2
			72.9	**D**	C-3
		101.9	3.98	**C**	H-2
E	→2,3)-β-D-Gal*p*-(1→	4.43	81.0	**D**	C-2
			75.1	**E**	C-5
		103.0	3.72	**D**	H-2
		OCH_3 δ_H/δ_C			
C	→2)-6-*O*-Me-α-D-Gal*p*-(1→	3.78	72.9	**C**	C-6
		55.0	3.67	**C**	H-6a
			3.77		H-6b

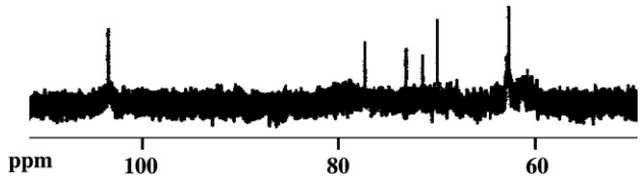


Figure 5. ^{13}C NMR spectrum (125 MHz, D2O, 27 °C) of the smith-degraded polysaccharide (SDPS) isolated from *Moringa oleifera*.

seven lots, and the collected polysaccharide overall recovery was 140 mg.

3.2a. Monosaccharide analysis

The polysaccharide sample (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °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 CH_3OH to remove excess boric acid. The reduced sugars (alditols) 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 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 spectrometry (GLC–MS) analysis was also performed on a Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 m). 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.

3.2b. Preparation of carboxyl-reduced polysaccharide[33]

The polysaccharide (3.5 mg) was dissolved in water (3.5 mL). Then 1-cyclohexyl-3- (2-morpholino-ethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC metho-*p*-toluenesulfonate) (94 mg) was added to the above solution with stirring, and the pH kept at ~4.75 during the reaction by addition of 0.01 M hydrochloric acid. After 2 h, 2 M aq sodium borohydride (2 mL) was added dropwise during 45 min, and the pH kept at ~7 by simultaneous addition of 4 M hydrochloric acid. After 1 h, the soln was dialyzed against distilled water and freeze dried. The procedure was repeated once again to ensure complete reduction. The carboxyl-reduced polysaccharide was hydrolyzed with 2 M CF_3COOH for 18 h at 100 °C, and after usual treatment the sugars were estimated by GLC.

3.3. Methylation analysis

The polysaccharide (4.0 mg) was methylated using the Ciucanu and Kerek method.[25] The methylated products were isolated by partition in 5:2 $CHCl_3$–water. 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 (1 mL) at 100 °C for 1 h, reduced with sodium borohydride, acetylated with 1:1 Ac_2O–pyridine and analyzed by GLC (using columns A and B) and GLC–MS (using HP-5 fused silica capillary column) using the same temperature-program indicated above. A portion of the methylated polysaccharide (2.0 mg) was dissolved in dry THF (2 mL), refluxed with lithium aluminum hydride[34] (40 mg) for 5 h, and kept overnight at room temperature. The excess of the reducing agent was decomposed by dropwise addition of EtOAc and aq THF. The inorganic materials were filtered off. The filtrate was evaporated to dryness giving the carboxyl-reduced product. The carboxyl-reduced methylated product was hydrolyzed with formic acid as before, and the alditol acetates of the reduced methylated sugars were prepared in the usual way and analyzed by GLC and GLC–MS experiments. The CMC reduced polysaccharide was also methylated following the same procedure and analyzed.

3.4. Periodate oxidation

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 soln was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h and neutralized with AcOH. The resulting material was obtained by co-distillation with MeOH. The periodate reduced material was divided into two portions. One portion was hydrolyzed with 2 M CF_3COOH for 18 h, and alditol acetates were prepared as usual. Another portion was methylated by the Ciucanu and Kerek method,[25] and alditol acetates of this methylated product were prepared. Alditol acetates were analyzed by GLC using columns A and B. Another portion of the periodate-oxidized, LAH-reduced polysaccharide was kept with 0.5 M CF_3COOH for 48 h at room temperature. The acid was removed, and the hydrolyzate was examined by paper chromatography. The hydrolyzate was also analyzed by GLC (as alditol acetates).

3.5. Absolute configuration of monosaccharides

The method used was based on Gerwig et al.[24] The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A soln 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 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) 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.

3.6. Optical rotation

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

3.7. Paper chromatographic studies

Paper chromatographic studies were performed on Whatman nos. 1 and 3 mm sheets. Solvent systems used were (X) 4:1:5 BuOH–AcOH–water (upper phase) and (Y) 8:2:1 EtOAc–pyridine–water. The spray reagent used was alkaline silver nitrate solution.[27]

3.8. Determination of molecular weight

The molecular weight of polysaccharides was determined by a gel-chromatography technique. Standard dextrans[23] 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 vol of the polysaccharide was then plotted in the same graph, and the molecular weight of the polysaccharide was determined.

3.9. NMR studies

The polysaccharide was kept over P_2O_5 in vacuum for several days, and then exchanged with deuterium[35] by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) four times. With a Bruker Avance DPX-500 spectrometer, ^1H, TOCSY, DQF-COSY, NOESY, ROESY, and HMBC NMR spectra were recorded in D_2O 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 4.67) using the WEFT pulse sequence.[36] The 2D-DQF-COSY experiment was carried out using standard Bruker software at 27 °C. 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 NMR spectrum of the polysaccharide, dissolved in D_2O was recorded at 27 °C using acetone as internal standard, fixing the methyl carbon signal at δ 31.05. The delay time in the HMBC experiment was 80 ms.

3.10. GLC experiments

All gas liquid chromatographies were performed on a Hewlett–Packard Model 5730 A gas chromatograph 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 GLC analyses were performed at 170 °C.

3.11. GLC–MS experiments

All the GLC–MS experiments were carried out in a Hewlett–Packard 5970 MSD instrument using HP-5 fused silica capillary column. 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.

3.12. Smith degradation[30,31]

The polysaccharide (50 mg) was oxidized with 0.1 M sodium metaperiodate (10 mL) at 27 °C in the dark during 48 h. The oxidation was stopped by the addition of 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h at 27 °C, neutralized with 50% AcOH followed by dialysis against distilled water, and freeze dried. This portion was subjected to mild hydrolysis with 0.5 M trifluoroacetic acid for 15 h at 25 °C to eliminate the residues of oxidized sugars attached to polysaccharide chain (Smith degradation). The excess acid was removed after repeated addition and evaporation of water. It was further purified by passing through a Sephadex G-25 column, and one fraction was obtained and freeze dried; yield, 10 mg. A part of this material (2 mg) was methylated and analyzed as usual by GLC and GLC–MS. The remainder was used for ^{13}C NMR studies.

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