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Structural assignment of a heteropolysaccharide isolated from the gum of *Cochlospermum religiosum* (Katira gum)

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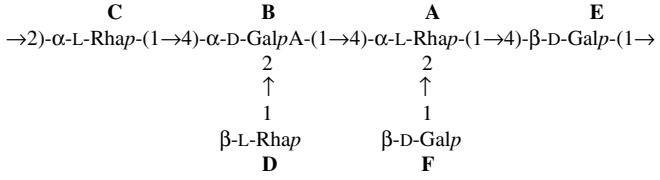
Abstract—A heteropolysaccharide isolated from the gum (Katira) of *Cochlospermum religiosum* was found to consist of D-galactose, D-galacturonic acid and L-rhamnose in a molar ratio 2:1:3. Structural assignment of the polysaccharide was carried out using total acid hydrolysis, methylation analysis, periodate oxidation, Smith degradation and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, HMBC and HSQC) and the repeating unit of the polysaccharide was established as



Keywords: Katira gum; *Cochlospermum religiosum*; Polysaccharide; Structure; NMR spectroscopy

1. Introduction

Cochlospermum religiosum[1,2] is a small or medium sized, deciduous, soft wooded tree. The tree occurs all over India from Garhwal via the west sub-Himalayan tracts to West-Bengal, Bihar, Orissa and the Decan peninsula. The tree yields a gum that exudes from the fibrous, deeply furrowed bark, which is known as Katira gum. Exudate gums[3] are polysaccharides produced by plants as a result of stress, including physical injury and fungal attack. Gum Arabic[4–6] (*Acacia Senegal*), gum Tragacanth[7–9] (*Astagalus gsmnnmifer*), gum Karaya[10,11] (*Sterculia urens*), gum Ghatti[12] (*Anogeissus latifolia*) and gum katira[13,14] (*C. religiosum*) have been used by humans for thousands of years in various food and pharmaceutical applications.[15]

Katira gum is pale and semi-transparent, insoluble in water, but swells into a pasty transparent mass with water. This gum has assumed great importance in recent years and exported annually from India for use in the cigar paste and ice-cream industry.[1] The gum is sweet, thermogenic, anodyne, sedative and useful[16] in cough, diarrhoea, dysentery, pharyngitis, gonorrhoea, syphilis and trachoma. In 1953, Hirst and Dustan[13] for the first time attempted to establish the structure of the polysaccharide of this gum and showed that it consists of equimolecular proportion of L-rhamnose, D-galactose and D-galacturonic acid, together with traces of a ketohexose. By methylation analysis, they established the presence of the fragments: terminal L-rhamnose, terminal D-galactose, 1→2 linked L-rhamnose, 1→4-linked D-galactose, 1→2,4-linked L-rhamnose and a mixture

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of methylated uronic acid, but no detailed structure was established. In 1962, Aspinall et al.[14] attempted to establish the detailed structure. They observed the presence of 1→2,4-linked D-galacturonic acid in the inner chain of this polysaccharide with similar residues of neutral sugars. However, the detailed structure of this polysaccharide was not established. The present investigation shows the presence of similar neutral sugar residues along with D-galacturonic acid in the inner chain as reported earlier.[13,14] In addition, we have established the structure of the polysaccharide of Katira gum using total acid hydrolysis, methylation analysis, periodate oxidation, Smith degradation and NMR studies.

2. Results

2.1. Isolation and purification of polysaccharide

The gum was successively purified using CH_3OH, soxhlet extraction, dialysis and finally Sepharose gel permeation chromatography to yield a pure polysaccharide (PS). The total carbohydrate of this fraction was estimated to be 95% using the phenol–sulfuric acid method,[17] and the uronic acid content was estimated as 16% by the carbazole method. The molecular weight[18] of this fraction was estimated from a calibration curve prepared with standard dextans as $\sim 1.56 \times 10^5$ Da. The PS showed specific rotation of $[\alpha]_D^{28.2}$ +44.8 (*c* 0.6, water).

2.2. Analysis of sugar residues in PS

The hydrolysis of PS (3 mg) was carried out with 2 M CF_3COOH for 18 h By paper chromatographic analysis,[19] the hydrolyzed product showed the presence of D-galactose, D-galacturonic acid and L-rhamnose. GLC analysis of the alditol acetates of the sugars showed the presence of L-rhamnose and D-galactose in a molar ratio of 3:2, but carboxyl-reduced polysaccharide on hydrolysis followed by GLC examination showed the presence of L-rhamnose and D-galactose in a molar ratio of 3:3. This result confirms that D-galacturonic acid is present in this polysaccharide. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al.[20]

2.3. Methylation analysis

The polysaccharide was methylated using the Ciucanu and Kerek method[21] and then by the Purdie method[22] followed by hydrolysis and alditol acetate preparation. The alditol acetates of the methylated material from the polysaccharide were analyzed by GLC and by GLC–MS using an HP-5 fused silica capillary column. The polysaccharide showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactiol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol, 1,2,5-tri-*O*-acetyl-6-deoxy-3,4-di-*O*-methyl-L-rhamnitol, 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methyl-L-rhamnitol and 1,2,4,5-tetra-*O*-acetyl-6-deoxy-3-*O*-methyl-L-rhmnitol in a ratio of nearly 1:1:1:1:1. The carboxyl-reduced polysaccharide was methylated, and alditol acetates of the methylated sugars were identified by GLC–MS analysis, which showed the presence of the above peaks along a new peak 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol in a ratio of nearly 1:1:1:1:1:1.

2.4. Periodate oxidation analysis

Periodate oxidation was carried out with the polysaccharide. The periodate-oxidized, reduced material obtained from the polysaccharide upon hydrolysis with CF_3COOH followed by GLC analysis showed the presence of only L-rhamnose. GLC analysis of periodate-oxidized, reduced ($LiAlH_4$) and methylated polysaccharide showed that the 1,2,4,5-tetra-*O*-acetyl-6-deoxy-3-*O*-methyl-L-rhamnitol and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol were retained. Upon hydrolysis, the periodate-oxidized polysaccharide showed the presence of L-rhamnose and D-galacturonic acid by paper chromatography.[19]

2.5. NMR studies

2.5.1. ^{1}H and ^{13}C experiments. The ^{1}H NMR spectrum (500 MHz) (Fig. 1, Table 1) of this polysaccharide at 27 °C showed six signals in the anomeric region at δ 5.23, 5.17, 5.13, 4.63, 4.57 and 4.52 ppm in a ratio of nearly 1:1:1:1:1:1. In the ^{13}C NMR spectrum (125 MHz) (Fig. 2, Table 1) at 27 °C, five anomeric signals appeared at δ 105.0, 104.5, 104.0, 101.0 and 99.0 ppm in a ratio of 1:1:1:2:1. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC and HMBC NMR experiments. The six sugar residues were designated as **A–F**, according to their decreasing anomeric chemical shifts in ^{1}H NMR spectrum. The presence of three L-rhamnosyl moieties is confirmed because three different CH_3(C) signals are observed at δ 17.2, 17.2 and 17.0 ppm (Fig. 2).

2.5.2. NOESY experiment. NOESY spectra of this PS were acquired with a 300 ms mixing time to provide both inter- and intra-residual coupling (Fig. 3, Table 2). The sequence of glycosyl residues of the PS was determined from this experiment. Inter-residue NOE contacts were observed from H-1 of residue **A** with H-4 of residue **E**, H-1 of residue **B** with H-4 of residue **A**, H-1 of residue **C** with H-4 of residue **B**, H-1 of residue **D** with H-2 of residue **B**, H-1 of residue **E** with H-2 of residue **C** and H-1 of residue **F** with H-2 of residue **A** along with other intra-residual couplings.

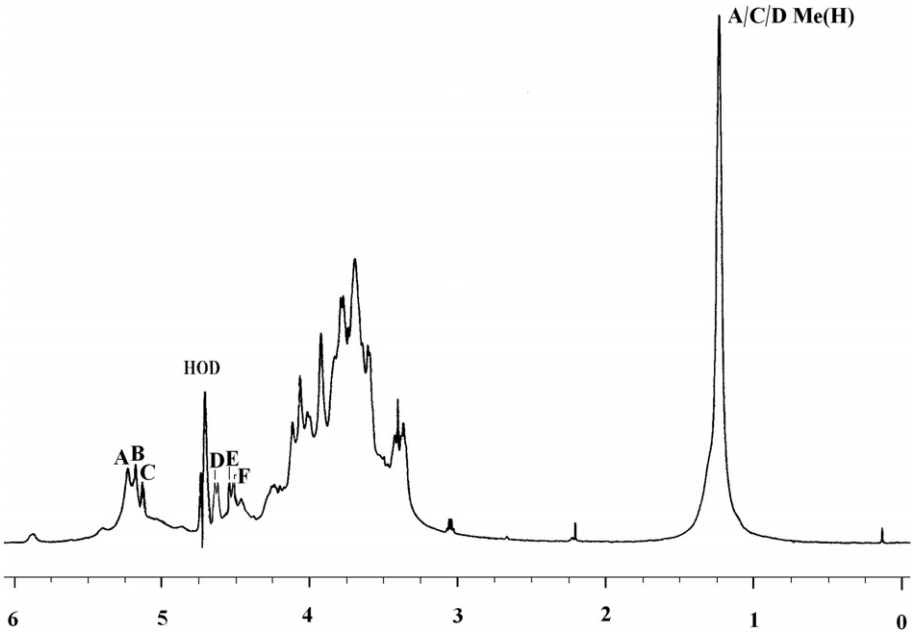


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of polysaccharide isolated from *Cochlospermum religiosum*.

Table 1. The ^{1}H and ^{13}C NMR chemical shifts of the polysaccharide isolated from *Cochlospermum religiosum* 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-5/C-5	H-6a/C-6	H-6b
→2,4)-α-L-Rha*p*-(1→ **A**	5.23 101.0	4.05 76.4	3.92 68.5	3.69 78.2	3.78 69.4	1.21 17.2	
→2,4)-α-D-Gal*p*A-→ **B**	5.17 99.0	4.00 76.8	4.10 70.2	4.24 77.8	4.43 69.2	 174.0	
→2)-α-L-Rha*p*-(1→ **C**	5.13 101.0	4.06 77.8	3.78 68.9	3.36 72.5	3.69 70.2	1.21 17.2	
β-L-Rha*p*-(1→ **D**	4.63 104.0	3.92 68.7	3.69 75.4	3.59 72.5	3.64 72.2	1.21 17.0	
→4)-α-D-Gal*p*(-1→ **E**	4.57 104.5	3.59 72.5	3.77 69.9	4.13 76.8	3.78 70.2	3.69 61.0	3.73
β-D-Gal*p*-(1→ **F**	4.52 105.0	3.60 71.2	3.69 72.5	3.92 68.9	3.73 75.4	3.82 61.5	3.78

2.5.3. HMBC experiment. Long-range ^{13}C–^{1}H correlations were obtained from the HMBC spectrum (Fig. 4a–c, Table 3). The cross-peaks of both anomeric protons and carbons of each of the sugar moieties were examined, and both inter and intra-residual connectivities were observed in this experiment.

2.5.4. Smith degradation studies.[23,24] From the results described above, four out of six sugar moieties in the PS should be sensitive to Smith degradation. Therefore, the PS was subjected to Smith degradation as described in Section 4, and the products were purified by gel-filtration chromatography using Sephadex G-25. The ^{13}C NMR spectrum (Fig. 5, Table 4) of purified SDPS in D_2O showed two anomeric signals at δ 103.0 and δ 99.0 ppm along with other signals.

3. Discussion

The methylation results of the polysaccharide indicated the presence of terminal D-galactopyranose, (1→4)-linked D-galactopyranose, (1→2)-linked L-rhamnopyranose, terminal L-rhamnopyranose and (1→2,4)-linked L-rhamnopyranose. The carboxyl-reduced methylated PS indicated that (1→2,4)-linked D-Gal*p*A was also present. From the result of periodate-oxidation, it was observed that the terminal D-galactopyranosyl, terminal

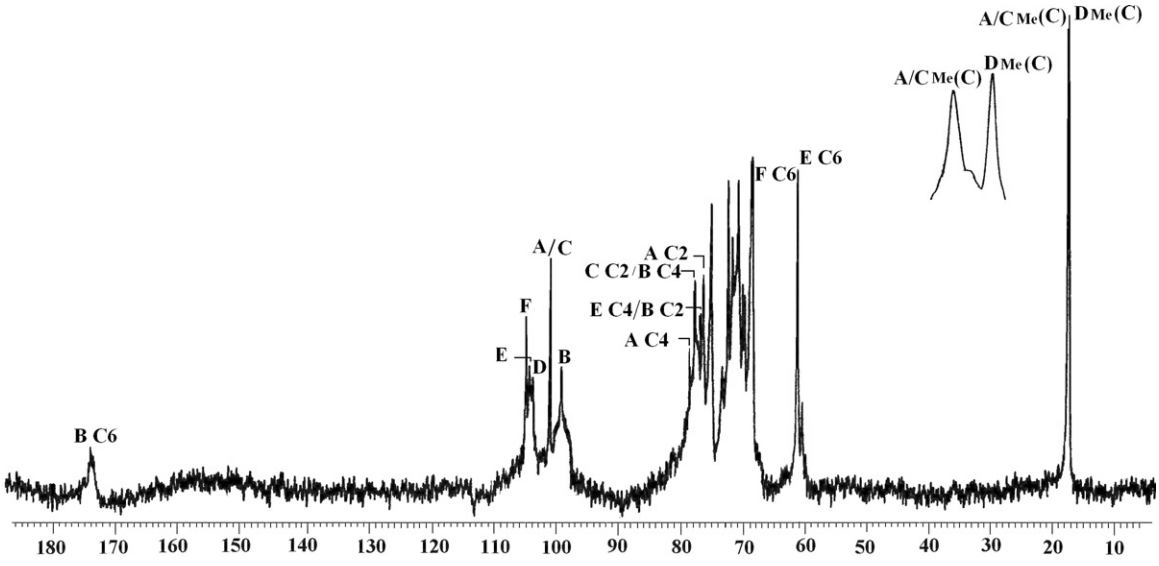


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of polysaccharide isolated from *Cochlospermum religiosum*.

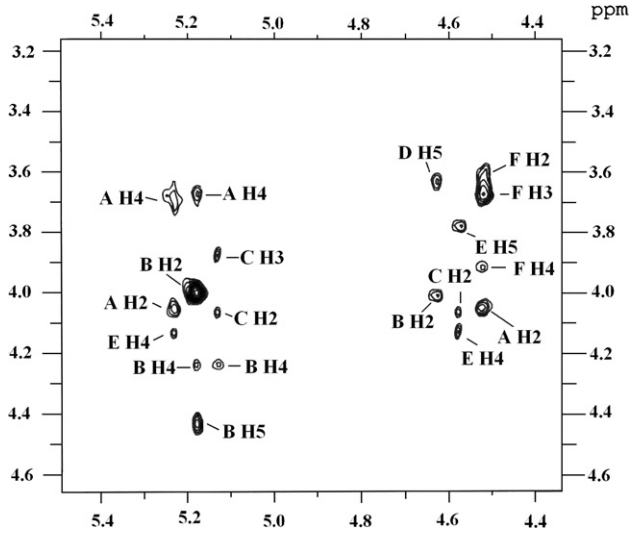


Figure 3. NOESY spectrum of polysaccharide isolated from *Cochlospermum religiosum* (mixing time was 300 ms).

Table 2. NOE effect of polysaccharide isolated from *Cochlospermum religiosum*, observed in the NOESY spectrum recorded in D_2O at 27 °C

Sugar residue Glycosyl residue	δ	δ	NOE contact Protons residue
→2,4)-α-L-Rha*p*(1→ **A**	5.23	3.69	AH-4
		4.05	AH-2
		4.13	EH-4
→2,4)-α-D-Gal*p*A (1→ **B**	5.17	4.00	BH-2
		4.24	BH-4
		4.43	BH-5
		3.69	AH-4
→2)-α-L-Rha*p* (1→ **C**	5.13	3.78	CH-3
		4.06	CH-2
		4.24	BH-4
β-L-Rhap(1→ **D**	4.63	3.64	DH-5
		4.00	BH-2
→4)-α-D-Galp(1→ **E**	4.57	3.78	EH-5
		4.13	EH-4
		4.06	CH-2
β-D-Galp(1→ **F**	4.52	3.60	FH-2
		3.69	FH-3
		3.92	FH-4
		4.05	AH-2

L-rhamnopyranosyl, (1→2)-linked L-rhamnopyranosyl and (1→4)-linked D-galactopyranosyl moieties were consumed during oxidation. The ^1H and ^{13}C NMR experiments indicated the presence of six sugar residues, designated as **A**–**F**, as indicated earlier.

Residue **A** has an anomeric chemical shift at δ 5.23, with coupling constant values of $J_{H\text{-}1,H\text{-}2} = \sim 2.0$ Hz, $J_{H\text{-}1,C\text{-}1} = \sim 170$ Hz indicating that it is α-linked. Residue **A** was determined to be an Rha*p* due to the signals for an exocyclic $-CH_3$ group. The anomeric carbon signal of residue **A** at 101 ppm was confirmed by the presence of a cross-peak **A** C-1, **E** H-4 in the HMBC experiment (Fig. 4c, Table 3). The downfield shift of C-2 (δ 76.4 ppm), C-4 (δ 78.2 ppm) carbon signals with respect to standard values of methyl glycosides[25,26] indicates that residue **A** is (1→2,4)-linked.

Residue **B** has an anomeric proton signal at δ 5.17 ppm and $J_{H\text{-}1,H\text{-}2} = \sim 3.2$ Hz and $J_{H\text{-}1,C\text{-}1} = \sim 171$ Hz, which indicates that it is α-linked. This residue showed only five protons signals and a high chemical shift of H-5 (δ 4.43) was observed. Thus, residue **B** was determined to be Gal*p*A due to the signal for a carboxylic acid –COOH group. The anomeric carbon sig-

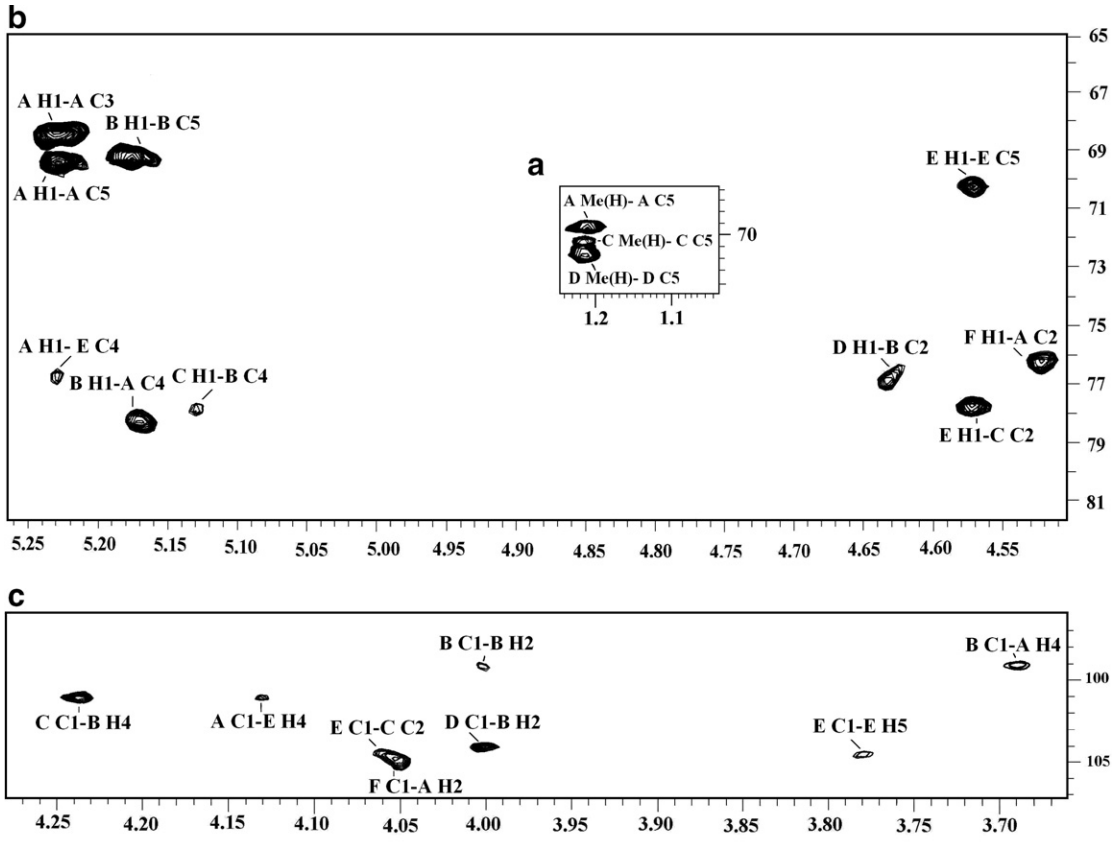


Figure 4. (a) Coupling between methyl proton and adjacent carbon in HMBC spectrum. (b) Anomeric proton region of HMBC spectrum. (c) Anomeric carbon region of HMBC spectrum.

Table 3. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the polysaccharide isolated from *Cochlospermum religiosum*

Residue	Sugar linkage	H-1/C-1		Observed connection	
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→2,4)-α-L-Rha*p*(1→	5.23	77.8	**E**	C-4
		101.0	4.13	**E**	H-4
B	→2,4)-α-D-Gal*p*A(1→	5.17	78.2	**A**	C-4
		99.0	3.69	**A**	H-4
C	→2)-α-L-Rha*p* (1→	5.13	77.8	**B**	C-4
		101.0	4.24	**B**	H-4
D	β-L-Rha*p* (1→	4.63	76.8	**B**	C-2
		104.0	4.00	**B**	H-2
E	→4)-β-D-Gal*p*(1→	4.57	77.8	**C**	C-2
		104.5	4.06	**C**	H-2
F	β-D-Gal*p*(1→	4.52	76.4	**A**	C-2
		105.0	4.05	**A**	H-2

nals of residue **B** at δ 99.0 ppm were confirmed by the presence of a cross-peak **B** C-1, **A** H-4 in the HMBC experiment (Fig. 4c, Table 3). The downfield shift of C-2 (δ 76.8 ppm), C-4 (δ 77.8 ppm) carbon signals with respect to standard values of methyl glycosides[25,26] indicates that residue **B** is (1→2,4)-linked.

Residue **C** has an anomeric chemical shift at δ 5.13; coupling constant values of $J_{H\text{-}1,H\text{-}2} = \sim 1.9$ Hz, $J_{H\text{-}1,C\text{-}1} = \sim 171$ Hz indicate that it is an α-linked residue. Residue **C** was determined as Rha*p* due to the signals for an exocyclic $-CH_3$ group. The anomeric carbon signal of residue **A** at 101 ppm were confirmed by

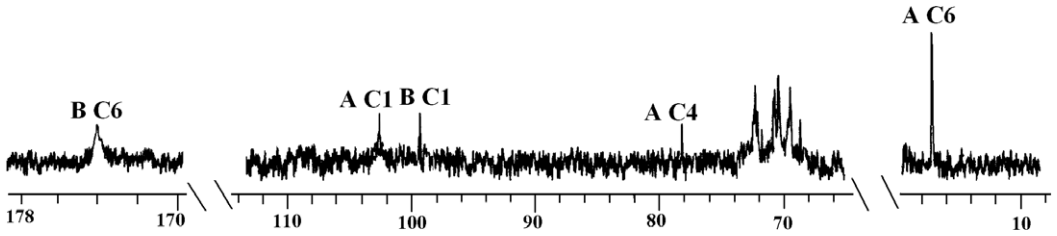


Figure 5. ^{13}C NMR (125 MHz, D_2O, 27 °C) spectrum of the Smith-degraded polysaccharide (SDPS), isolated from *Cochlospermum religiosum*.

Table 4. ^{13}C NMR data for Smith-degraded polysaccharide

Atoms	Residue	
	→4)-α-L-Rha*p*(1→	α-D-Gal*p*A(1→
C-1	103.0	99.0
C-2	70.7	68.9
C-3	71.0	70.7
C-4	78.2	71.9
C-5	69.7	72.5
C-6	17.2	174.0

the presence of a cross-peak **C** C-1, **B** H-4 in the HMBC experiment (Fig. 4c, Table 3). The downfield shift of C-2 (δ 77.8 ppm) carbon signal with respect to standard value of methyl glycosides[25,26] indicates that residue **C** is (1→2)-linked.

Residue **D** has an anomeric proton signal at δ 4.63 ppm and $J_{H\text{-}1,H\text{-}2} = \sim 1.6$ Hz and $J_{H\text{-}1,C\text{-}1} = \sim 168$ Hz indicating that it is β-linked sugar moiety. Residue **D** was determined to be Rha*p* due to the signals for an exocyclic $-CH_3$ group. The anomeric carbon signal of residue **D** at δ 104.0 ppm was confirmed by the presence of cross-peak **D** C-1, **B** H-2 in the HMBC experiment (Fig. 4c, Table 3). Thus, considering the result of methylation analysis and NMR experiments, it may be concluded that residue **D** is a terminal β-L-rhamnosyl moiety.

Residue **E** has an anomeric proton signal at δ 4.57 ppm and $J_{H\text{-}1,H\text{-}2} = \sim 7.9$ Hz, $J_{H\text{-}1,C\text{-}1} = \sim 162$ Hz, which demonstrates that it is β-linked. The $J_{H\text{-}3,H\text{-}4}$ value (~3.4 Hz) and the $J_{H\text{-}2,H\text{-}3}$ value (~9.2 Hz) for residue **E** indicate that it is a β-D-galactosyl residue. The anomeric carbon signal of residue **E** at δ 104.5 ppm was confirmed by the presence appearance of a cross-peak **E** C-1, **C** H-2 in the HMBC experiment (Fig. 4c, Table 3). The downfield shift of C-4 (δ 76.8 ppm) carbon signal with respect to standard values of methyl glycosides[25,26] indicates that residue **E** is (1→4)-linked.

Residue **F** has an anomeric proton chemical shift at 4.52 ppm. A large coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~7.5 Hz) and $J_{H\text{-}1,C\text{-}1} = \sim 162$ 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 (~9.0 Hz) for residue **F** indicate that it is a β-D-galactosyl residue. The C-1 signal of residue **F** at δ 105.0 ppm was confirmed by the appearance of a cross-peak **F** C-1 to **A** H-2 in the HMBC experiment (Fig. 4c, Table 3). Thus, considering the results of methylation analysis and NMR experiments it may be concluded that **F** is a β-glycosidically linked, terminal D-galactopyranosyl moiety.

From the results of interresidual NOE contacts (Fig. 3, Table 2), the sequences of glycosyl moieties were determined and the following connectivities are observed:

A **E** **B** **A**

α-L-Rha*p*-(1→4)-β-D-Gal*p*-(1→ ; α-D-Gal*p*A-(1→4) - α-L-Rha*p*-(1→

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

α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→ ; β-L-Rha*p*-(1→2)-α-D-Gal*p*A-(1→
4
↑

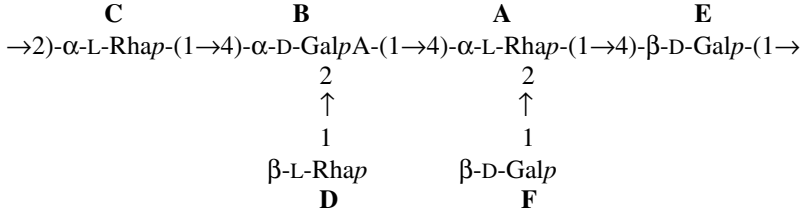
E **C** **F** **A**

→4)-β-D-Gal*p*-(1→2)-α-L-Rha*p*-(1→ ; β-D-Gal*p*-(1→2)-α-L-Rha*p*-(1→
4
↑

These linkages were further confirmed by an HMBC experiment (Fig. 4a–c, Table 3). Cross-peaks were found

between H-1 (δ 5.23 ppm) of residue **A** and C-4 (δ 76.8 ppm) of residue **E** (**A** H-1, **E** C-4); C-1 (δ 101.0 ppm) of residue **A** and H-4 (δ 4.13 ppm) of residue **E** (**A** C-1, **E** H-4) and between **A** Me-H and C-5 of **A** (**A** Me-H, **A** C-5). The cross-peaks between H-1 (δ 5.17 ppm) of residue **B** and C-4 (δ 78.2 ppm) of residue **A** (**B** H-1, **A** C-4); C-1 (δ 99.0 ppm) of residue **B** and H-4 (δ 3.69 ppm) of residue **A** (**B** C-1, **A** H-4) were observed. The cross-peaks between H-1 (δ 5.13 ppm) of residue **C** and C-4 (δ 77.8 ppm) of residue **B** (**C** H-1, **B** C-4); C-1 (δ 101.0 ppm) of residue **C** and H-4 (δ 4.24 ppm) of residue **B** (**C** C-1, **B** H-4) and between **C** Me-H and C-5 of **C** (**C** Me-H, **C** C-5) were observed. The cross-peaks between H-1 (δ 4.63) of residue **D** and C-2 (δ 76.8 ppm) of residue **B** (**D** H-1, **B** C-2); C-1 (δ 104.0 ppm) of residue **D** and H-2 (δ 4.00 ppm) of residue **B** (**D** C-1, **B** H-2) and between **D** Me-H and C-5 of **D** (**D** Me-H, **D** C-5) were observed. The cross-peaks between H-1 (δ 4.57 ppm) of residue **E** and C-2 (δ 77.8 ppm) of residue **C** (**E** H-1, **C** C-2) and C-1 (δ 104.5 ppm) of residue **E** and H-2 (δ 4.06 ppm) of residue **C** (**E** C-1, **C** H-2) were observed. The cross-peaks between H-1 (δ 4.52 ppm) of residue **F** and C-2 (δ 76.4 ppm) of residue **A** (**F** H-1, **A** C-2) and C-1 (δ 105.0 ppm) of residue **F** and H-2 (δ 4.05 ppm) of residue **A** (**F** C-1, **A** H-2) were observed.

From the NOESY as well as HMBC correlations, the hexasaccharide repeating unit in the polysaccharide isolated from the gum of *C. religiosum* is assigned as



To confirm further the sequence of the sugar residues in the repeating unit, the PS was subjected to Smith degradation[23,24] and the products were separated on a Sephadex G-25 column using water as the eluent, resulting in one fraction (SDPS) only. The ^{13}C NMR spectrum (Fig. 5, Table 4) of SDPS in D_2O showed two anomeric signals at δ 103.0 for an α-L-rhamnosyl moiety (**A**) and at δ 99.0 ppm for an α-D-galacturonic acid moiety (**B**) indicating a disaccharide. No signals at δ 76.4 ppm and δ 76.8 ppm were observed in the ^{13}C NMR spectrum of the Smith-degraded polysaccharide, which confirms that the terminal β-D-galactopyranosyl unit (**F**) and terminal β-L- rhamnopyranosyl unit (**D**) attached to C-2 of **A** and **B**, respectively, were consumed during oxidation. Two other signals, appearing at δ 71.9 and δ 68.9 ppm (Fig. 5), were due to the loss of **C** and **D** at C-4 and C-2 position of **B**, respectively. Furthermore, the ^{13}C NMR spectrum of the Smith-degraded polysaccharide showed no anomeric signals at δ 105.0, δ 104.5, δ 104.0 and δ 101.0 ppm, which clearly indicate that the terminal D-galactose, (1→4)-linked β-D-Gal*p*, terminal L-rhamnose and (1→2)-linked α-L-Rha*p* units were consumed during oxidation. The ^{13}C signals of α-L-rhamnosyl moiety, C-1 to C-6 appeared at δ 103.0, 70.7, 71.0, 78.2, 69.7 and δ 17.2 ppm and of α-D-galacturonic acid moiety, C-1 to C-6 appeared at δ 99.0, 68.9, 70.7, 71.9, 72.5 and 174.0 ppm. Hence, from all these data the structure of the Smith-degraded polysaccharide is established as

α-D-Gal*p*A-(1→4)-α-L-Rha*p*

4. Experimental

4.1. Isolation and purification of the polysaccharide

The gum was collected from the local merchant shop, Midnapore town, and was washed with CH_3OH several times and then dried. The dried material was ground to a powder and then submitted to Soxhlet extraction with petroleum ether at 60–80 °C for 60 h. Subsequent Soxhlet extractions with a mixture of petroleum ether–benzene (1:1) and benzene–chloroform (1:1) in succession for 72 h yielded a white material. This solid was dissolved in distilled water and dialyzed through a cellulose membrane (Sigma–Aldrich, retaining MW > 12,400) against distilled water to remove low molecular weight materials. The whole solution was then centrifuged, the residue was discarded and the filtrate was freeze-dried.

The purity of the polysaccharide was determined by gel-permeation chromatography on a Sepharose-6B column (65 × 2 cm) by loading 25–30 mg crude polysaccharide for each run. The column was eluted with distilled water with a flow rate of 11 s/drop. A total of 95 test tubes containing 3 mL eluent were collected using a Redifrac fraction collector. These were monitored by phenol–H_2SO_4 procedure[17] at 490 nm on a Shimadzu UV–vis spectrophotometer, model 1601. Fractions (test tube nos. 26–52) corresponding to a single peak were pooled and freeze-dried; yield ~ 22 mg. The same procedure was followed several times to collect the purified materials.

4.2. Molecular weight determination

The average molecular weight of the polysaccharide was determined by gel-chromatography on a Sepharose-6B column (65 × 2 cm) by eluting with distilled water at a flow rate of 11 s/drop. The elution volume of this fraction was plotted in a standard calibration curve prepared by plotting the elution volume of standard dextrans[18] (T-10, T-40, T-200) against the logarithm of their respective molecular weights. The total carbohydrate of the polysaccharide was determined using the phenol–H_2SO_4 reagent.

4.3. 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 $NaBH_4$ (about 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 2 h to give the alditol acetates, which were analyzed by GLC performed with a Hewlett-Packard-5810 gas chromatograph equipped with a flame ionization detector. The instrument was fitted with a glass column (1.8 m × 6 mm) packed with 3% ECNSS-M on Gas chrom Q (100–120 mesh) at 170 °C and 1% OV-225 on Gas chrom Q (100–120 mesh) at 170 °C.

4.4. Preparation of Carboxyl reduced polysaccharide[27]

The polysaccharide (3.5 mg) was dissolved in water (3.5 mL) and then 1-cyclohexyl-3-(2-morpholino-ethyl)-carbo-di-imide-*p*-toluene sulfonate (CMC) (94 mg) was added to with stirring and pH maintained at ~4.75 by the addition of 0.01 M hydrochloric acid. After 2 h, 2 M aqueous sodium borohydride (2 mL) was added dropwise during 45 min, and the pH maintained at ~7 by simultaneous addition of 4 M hydrochloric acid. After 1 h, the solution 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.

4.5. Methylation analysis

The polysaccharide (4.0 mg) was methylated using Ciucanu and Kerek method[21] and then by the Purdie method.[22] The methylated products were isolated by partition between $CHCl_3$ and H_2O (5:2, v/v). The organic layer containing products was washed with 3 mL water for three times and dried. The methylated products were then hydrolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, reduced with $NaBH_4$, acetylated with (1:1) Ac_2O–pyridine and GLC–MS (using a HP-5 fused silica capillary column) using the same temperature program as indicated above. A portion of the polysaccharide (2.0 mg) was dissolved in dry THF (2 mL), heated at reflux with $LiAlH_4$[28] (40 mg) for 5 h and kept overnight at room temperature. The excess reductant was decomposed by dropwise addition of ethyl acetate and aqueous THF. The inorganic materials were filtered and the filtrate was evaporated to dryness giving the carboxyl-reduced product. The carboxyl-reduced material was methylated and then 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–MS. The CMC reduced polysaccharide was also methylated following the same procedure and analyzed.

4.6. 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 oxidation process 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 and neutralized with CH_3COOH. The resulting residual material was obtained by repeated addition of CH_3OH, followed by distillation. The residue was subjected to both hydrolysis and methylation by the usual procedure described above, and the products were analyzed by GLC–MS. Another portion of the periodate oxidized, $LiAlH_4$-reduced polysaccharide was methylated by the usual procedure and analyzed by GLC–MS. Again, the periodate oxidized material was hydrolyzed with CF_3COOH. The excess acid was removed, and the hydrolyzate was examined by paper chromatography. The hydrolyzate was also analyzed by GLC (as alditol acetates).

4.7. Absolute configuration of monosaccharides

The method used was based on Gerwig et al.[20] 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. The reactants were then evaporated and TMSi-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-

TMSi-(+)-2-butyl glycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharide.

4.8. Optical rotation

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

4.9. Paper chromatographic studies

Paper chromatographic studies were performed on Whatman number 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.[19]

4.10. Smith degradation experiment[23,24]

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 the addition of 1,2-ethanediol, and the solution was dialyzed through a cellulose bag against distilled water and the dialyzed material was freeze-dried. Then, the freeze-dried material was dissolved in a minimum volume of water and was then reduced with $NaBH_4$ for 15 h at 25 °C, before being 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. The material was collected and kept over P_2O_5 in vacuum for several days and then deuterium exchange followed by lyophilization with D_2O. Then, ^{13}C NMR spectra of Smith-degraded material were determined.

4.11. NMR studies

The polysaccharide was kept over P_2O_5 in vacuum for several days and then exchanged with deuterium[29] by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) four times. With a Bruker Avance DPX-500 spectrometer, ^1H, TOCSY, DQF-COSY, NOESY, HSQC and HMBC NMR spectra were recorded in D_2O at 27 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.67) using the WEFT pulse sequence.[30] 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 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 an internal standard, fixing the methyl carbon signal at δ 31.05. The delay time in the HMBC experiment was 80 ms.

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References

1. *Wealth of India*; CSIR: New Delhi, 1962; Vol. 2, p 261.
2. Prajapati, N.; Purohit, S.; Sharma, A.; Kumar, T. *A Hand Book of Medicinal Plant*; Agrobios: Jodhpur, 2003.
3. Izydorczyk, M.; Cui, S. W.; Wang, Q. In *Food Carbohydrates; Polysaccharide Gums Structures Functional Properties and Applications*; Cui, S. W., Ed.; Taylor & Francis: New York, 2005; pp 281–282.
4. Anderson, D. M. W.; Stoddart, J. F. *Carbohydr. Res.* **1966**, *2*, 104–114.
5. Islam, A. M.; Phillips, G. O.; Sljivo, M. J.; Williams, P. A. *Food Hydrocolloids* **1997**, *11*, 493–505.
6. Idris, O. H. M.; Williams, P. A.; Phillips, G. O. *Food Hydrocolloids* **1998**, *12*, 379–388.
7. Anderson, D. M. W.; Bridgeman, M. M. E. *Phytochemistry* **1985**, *24*, 2301–2304.
8. Anderson, D. M. W.; Grant, D. A. D. *Food Hydrocolloids* **1988**, *2*, 417–423.
9. Gecgil, A. S.; Yalabik, H. S.; Groves, M. J. *Plant Med.* **1975**, *27*, 284–286.
10. Le Cerf, D.; Irinei, F.; Muller, G. *Carbohydr. Polym.* **1990**, *13*, 375–386.
11. Anderson, D. M. W.; McNab, C. G. A.; Anderson, C. G.; Braown, P. M.; Pringuer, M. A. *Int. Tree Crops J.* **1982**, *2*, 147–154.
12. Tischer, C. A.; Iacomini, M.; Wagner, R.; Gorin, P. A. *Carbohydr. Res.* **2002**, *337*, 2205–2210.
13. Hirst, E. L.; Dustan, S. *J. Chem. Soc.* **1953**, 2332–2337.
14. Aspinall, G. O.; Hirst, E. L.; Johnston, J. M. *J. Chem. Soc.* **1962**, 2785–2789.
15. Phillips, G. O.; Williams, P. A. *Food Ingred. Anal. Int.* **2001**, *23*, 26–28.
16. Kirtikar, K. R.; Basu, B. D. In *Indian Medicinal Plant Cochlospermaceae*; Blatter, E., Caius, J. F., Mhaskar, K. S., Eds.; Bishen Singh Mahendra Pal Singh: Dehradun, India, 1998; Vol. 1, pp 214–215.
17. York, W. S.; Darvill, A. K.; McNeil, M.; Stevenson, T. T.; Albersheim, P. *Methods Enzymol.* **1985**, *118*, 33–40.
18. Hara, C.; Kiho, T.; Tanaka, Y.; Ukai, S. *Carbohydr. Res.* **1982**, *110*, 77–87.
19. Hoffman, J.; Lindberg, B.; Svensson, S. *Acta Chem. Scand.* **1972**, *26*, 661–666.

20. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349–357.
21. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
22. Purdie, T.; Irvine, J. C. R. *J. Chem. Soc.* **1904**, *85*, 1049–1070.
23. Abdel-Akher, M.; Hamilton, J. K.; Montgomery, R.; Smith, F. *J. Am. Chem. Soc.* **1952**, *74*, 4970–4971.
24. Datta, A. K.; Basu, S.; Roy, N. *Carbohydr. Res.* **1999**, *322*, 219–227.
25. Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307–3330.
26. Rinaudo, R.; Vincendon, M. *Carbohydr. Polym.* **1982**, *2*, 135–144.
27. Taylor, R. L.; Lindberg, B. *Carbohydr. Res.* **1969**, *10*, 77–85.
28. Abdel-Akher, M.; Smith, F. *Nature (London)* **1950**, *166*, 1037–1038.
29. Dueñas Chasco, M. T.; Rodriguez-Carvajal, M. A.; Mateo, P. T.; Franko-Rodriguez, G.; Espartero, J. L.; Iribas, A. I.; Gil-Serrano, A. M. *Carbohydr. Res.* **1997**, *303*, 453–458.
30. Hård, K.; Zadelhoff, G. V.; Moonen, P.; Kamerling, J. P.; Vliegenthart, J. F. G. *Eur. J. Biochem.* **1992**, *209*, 895–915.