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## **Methylation technique used for the identification of seed polysaccharide structure from *Cassia hirsuta* Linn. plant**

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### **ABSTRACT**

Water soluble seeds polysaccharide was extracted from *Cassia hirsuta* Linn. plant by acid hydrolysis with sulphuric acid and identification by column and paper chromatographic analysis to be composed of D-galactose and D-mannose in 1:4 molar ratio. Methylation studies of seeds polysaccharide were carried out by Hakomari's and Purdie's method and its Infrared spectroscopy indicated that the polysaccharide was a galactomannan. D-mannose residues linked with (1→4)-β type on main polymer chain while D-galactose and D-mannose residues with (1→6)-α type linkages at non-reducing end. Methylated galactomannan on acid hydrolysis with sulphuric acid gave certain medicinal chemicals like methyl sugars as: 2, 3, 4, 6- tetra-O-methyl-D-galactose; 2,3,6-tri-O-methyl-D-mannose and 2,3-di-O-methyl-D-mannose is 1:3:1 molar ratio. On the basis of above finding methylation results a tentative polysaccharide structure has been proposed for the water soluble *Cassia hirsuta* Linn. seeds.

**Key words:** Methyl sugars, monosaccharides, structure of *Cassia hirsute*, seeds polysaccharide.

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### **INTRODUCTION**

*Cassia hirsuta* Linn. plant<sup>[1,2]</sup> belongs to the Family- Caesalpineaceae and commonly called as *Stinking Cassia and Hairy Senna*. It is a terrestrial perennial, erect shrub up to 150 cm in tall, stem rounded, solid glabrous, flowering period from September - December and fruiting in November - January. It is a native of Tropical America and now distributed in Malaysia, Indo-China, Thailand, Asian & African Tropics, Laos, Java, Brazil, California, New Mexico and India<sup>[2]</sup>. It is used as a green manure and forage plant. In Africa, it planted as a shade plant in young coffee plantation. Leaves and young pods are eaten, usually steamed or cooked in vegetable or in salads. In Java, the leaves are use medicinally for treating herpes. A decoction of leaves is used against irritation of skin in Thailand. In Laos, the seeds are used as a substitute for

coffee. Phytomedicinally the plant parts or extracts are used to healing illness in man<sup>[3]</sup>. Plants as gifts of nature have many therapeutic properties combined with much nutritive value, which have made their use in chemotherapy as valuable as the synthetic drugs. Herbal organ<sup>5</sup> of the body are used to feed and restore to health those parts, which have become weakened. It is a medicinal plant widely used for stomach troubles, dysentery, abscesses, rheumatism, fever and other diseases. Seeds contain a phytotoxin, tannins and 0.25% chrysoarobin. Seeds contain a water soluble sugar extract as D-galactose and D-mannose in 1:4 molar ratio from hydrolysed compound on paper chromatogram. Present manuscript mainly deals with the methylation studies for polysaccharide structure of *Cassia hirsuta* Linn. seeds galactomannan.

## MATERIALS AND METHODS

Optical rotation of methylated polysaccharides are found in equilibrium values and melting points are uncorrected, while evaporations were carried out at 45 – 50°C under reduced pressure. Paper chromatographic analysis of methylated compounds were examined by descending technique<sup>[4]</sup> on Whatman No. 3 MM filter paper sheet with upper phase of the solvent mixtures (V/V), (A) *n*-butanol, ethanol, water, (4:1:5)<sup>[5]</sup>; (B) *n*-butanol, acetic acid, water (4:1:5)<sup>[6]</sup>, (C) benzene, ethyl alcohol, water (169:47:15)<sup>[7]</sup> and (D) butanone, water (azeotropic mixture)<sup>[8]</sup>. Spray reagents were used for the detection of methyl sugars as (R<sub>1</sub>) *p*-anisidine phosphate<sup>[9]</sup> and (R<sub>2</sub>) acetonical silver nitrate, alcoholic sodium hydroxide<sup>[10]</sup>. Derivatives of methyl sugars were prepared by refluxing an ethanolic solution of sugars with freshly distilled aniline solution for 1 hr. on boiling water-bath.

### Methylation of galactomannan

Polysaccharide (10 gm) was partially methylated by Srivastava's method<sup>[11]</sup> with water (50 ml), dimethyl sulphoxide (180 ml), sodium hydroxide (45% 100 ml) and dimethyl sulphate solution (75 ml) in an atmosphere of nitrogen thrice time. Resultant product was heated carefully on water-bath (2 hrs) to decompose the excess of dimethyl sulphate, Mixture was filtered and filtrate neutralized with sulphuric acid (12 N). Precipitate of sodium sulphate was filtered off and filtrate extracted with chloroform in a liquid-liquid extractor. Solvent layer was worked up to yield a glassy yellow compound (9.24 gm).

Above partially methylated compound was again remethylated by Hakomari's method<sup>[12]</sup> in dimethyl sulphoxide (100 ml) with mechanical stirring in an atmosphere of nitrogen (5 hrs). Content was further stirred at room temperature (5 hrs) till the evolution of hydrogen gas was ceased. Methyl iodide solution (10 ml) was added dropwise to the reaction mixture to a period of 2 hrs. with stirring (10 hrs). Further addition of sodium hydride (2 gm), dimethyl sulphoxide (25 ml) and methyl iodide (5 ml) were made on successive days then added chloroform (400 ml) to reaction mixture. Chloroform mixture was filtered to remove the sodium iodide precipitate and filtrate washed with distilled water and concentrated to syrup (20 ml). This syrup was dialysed against running water for 48 hrs. to remove dimethyl sulphoxide and inorganic ions and concentrated up to 30ml. Chloroform layer was dried over anhydrous sodium sulphate and concentrated under high vacuum to yield a glassy yellow product (7.98 gm). Found:- OCH<sub>3</sub>, 39.2% showed a hydroxyl peak of absorption band at 3500-3600 cm<sup>-1</sup> region in IR-spectroscopy (KBr)<sup>[13]</sup>. Finally the partially methylated galactomannan was further

remethylated thrice times by Purdie's reagents<sup>[14]</sup> with methyl alcohol, methyl iodide and silver oxide to gave fully methylated product, yield (7.38 gm) Found : - OCH<sub>3</sub>, 40.01% , This methylated galactomannan did not show any hydroxyl peak at 3500-3600 cm<sup>-1</sup> absorption band in IR-spectra (KBr).

### Hydrolysis of methylated galactomannan

Methylated galactomannan (2.64 gm) was hydrolysed<sup>[15]</sup> with sulphuric acid (72%, 50 ml) and reaction mixture was kept at 0°C in ice-bath for 2 hrs. Contents were diluted with water to have 12% concentration with respect to sulphuric acid and left over night. Reaction mixture was then heated on boiling water-bath for 4 hrs. at 100°C. Hydrolysate was neutralized with barium carbonate, filtered and filtrate concentrated to a thin syrup which consisting a mixture of neutral methylated sugars.

### Fractionation of methylated galactomannan

Methylated galactomannan (6 gm) was fractionated by fractional dissolution method<sup>[16]</sup> with pet. ether (40-60 °C) and chloroform mixture with increasing amount of latter solvent being increased in stages on water-bath for 2 hrs. Solution obtained from each fraction was evaporated and residue dried under high vacuum (15 mm over P<sub>2</sub>O<sub>5</sub>) to a constant weight. The specific rotation of the each methyl sugar fractions were taken in chloroform and methoxyl content of the individual fraction were determined by usual manners and results of each fractions are given in table -1.

**Table.1. Fractionation of methylated sugars of *Cassia hirsuta* Linn. seeds galactomannan**

S. No	State of methyl sugars	Solvent composition (%)		Yield (gm)	-OCH <sub>3</sub> (%)	[α] <sup>25</sup> <sub>D</sub> (CHCl <sub>3</sub> )
		Pet- ether (40-60°C)	CHCl <sub>3</sub>			
1	Oil liquid	100	00	0.5424	-	-
2	Oil liquid	95	05	0.6592	-	-
3	Oil liquid	90	10	0.6242	-	-
4	Cryspy solid	85	15	1.8964	52.3	73.4 °C
5	Cryspy solid	80	20	2.5448	41.9	15.4 °C
6	Cryspy solid	75	25	0.9268	29.7	66.4 °C

### Characterisation of methylated galactomannan

Resolution of neutral methylated sugars mixture were first attempted on cellulose column chromatographic analysis with pet. ether (60 -80 °C) and *n*-butyl alcohol in 7:3 and 1:1 molar ratio but no homogenous methyl sugar fractions could be formed. Paper chromatographic technique was carried out on Whatman No. 3 MM filter paper sheet with solvent mixture (A) and used (R<sub>1</sub>) as spray reagent. Strips of methyl sugars were cut out with the help of guide spots and diluted with water according to Dent's method<sup>[17]</sup>. Methyl sugars fraction were evaporated separately which were characterised and identified as follows:

**I: 2,3,4,6-tetra-O-methyl-D-galactose:** Methyl sugar syrup (550 mg) gave single spot corresponding to D-galactose on paper chromatogram in solvent (A). Found : -OCH<sub>3</sub>, 51.93%, calculated for C<sub>10</sub>H<sub>20</sub>O<sub>6</sub> requires -OCH<sub>3</sub>, 52.3%. It gave D-galactose on demethylation<sup>[18]</sup> with hydrobromic acid (40%). It was identified as: 2,3,4,6-tetra-O-methyl-D- galactose by conversion into anilide derivatives which was prepared by usual manner as: 2,3,4,6 -tetra-O-methyl-N-

phenyl -D-galactopyranosyl amine, had m.p. 189 -190 °C, Lit m.p. 190-191 °C<sup>[19]</sup>. It had Rf 0.72 in solvent (D) and Rg 0.88 in solvent (A), optical rotation  $[\alpha]_D^{25} + 73.4^0$  (CHCl<sub>3</sub>) and  $+ 103^0$  (H<sub>2</sub>O), Lit  $[\alpha]_D + 75.8^0$  (CHCl<sub>3</sub>) and  $+ 110^0$  (H<sub>2</sub>O)<sup>[20]</sup>  $+ 106^0$  (H<sub>2</sub>O)<sup>[21]</sup>.

**II: 2,3,6-tri-O-methyl-D-mannose:** Sugar syrup (800 mg) gave single spot of D-mannose on paper chromatogram in solvent (A). It had Rf 0.47 in solvent (D) and Rg 0.81 in solvent (A), optical rotation  $[\alpha]_D^{25} + 15.4^0$  (CHCl<sub>3</sub>) and  $-12.6^0$  (H<sub>2</sub>O), Lit  $[\alpha]_D + 15.7^0$  (CHCl<sub>3</sub>) and  $-11.6^0$  (H<sub>2</sub>O)<sup>[22]</sup>. It gave D-mannose on demethylation, Found : -OCH<sub>3</sub>, 41.02 %, calculated for C<sub>9</sub>H<sub>18</sub>O<sub>6</sub> requires, OCH<sub>3</sub>, 41.9 %. Derivative was prepared by usual manners as 2,3,6- tri-O-methyl-D-mannonic acid phenyl hydrazide, had m.p. 126-127 °C, Lit m.p. 130-132 °C<sup>[23]</sup>.

**III: 2,3-di-O-methyl-D-mannose:** Syrup (750 mg) gave a single spot of D-mannose on paper chromatogram in solvent (A). It had Rf 0.42 in solvent (D) and Rg 0.66 in solvent (A), optical rotation  $[\alpha]_D^{25} + 66.4^0$  (CHCl<sub>3</sub>) and  $+4.56^0$  (MeOH) and  $-15.2^0$  (H<sub>2</sub>O), Lit  $[\alpha]_D + 65.4^0$  (CHCl<sub>3</sub>) and  $+4.8^0$  (MeOH) and  $- 15.8^0$  (H<sub>2</sub>O)<sup>[20]</sup>. It gave D-mannose on demethylation, Found:- OCH<sub>3</sub>, 28.21%, calculated for C<sub>8</sub>H<sub>16</sub>O<sub>6</sub> requires. -OCH<sub>3</sub> 29.7%, Derivative was prepared by usual manner as: 2,3-di-O-methyl -  $\gamma$  -D- mannolactone, had m.p. 105-106 °C, Lit m.p. 106-108 °C<sup>[21]</sup>.

#### Quantitative estimation of methylated sugars

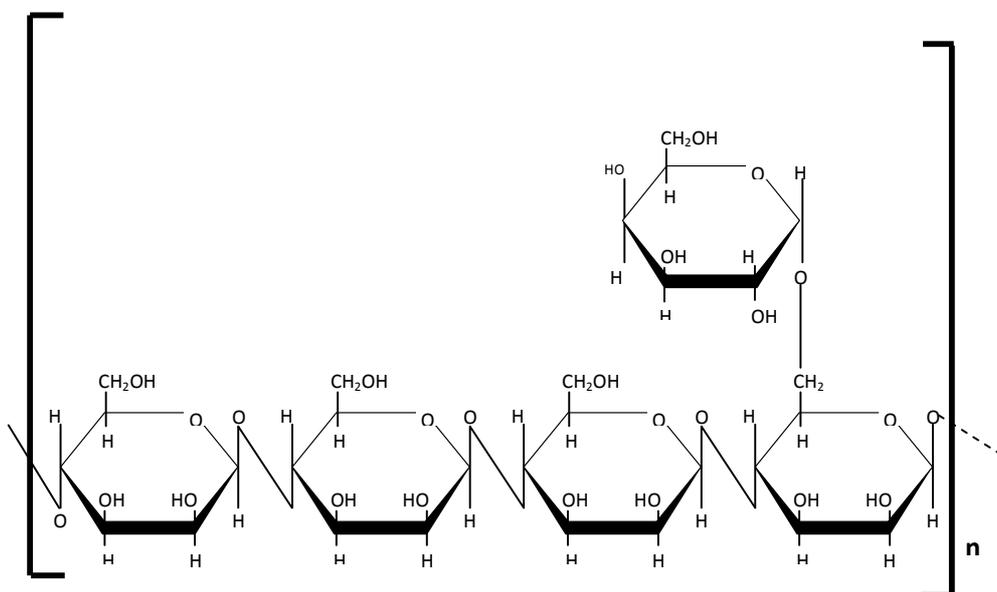
Methyl sugars (1.5 gm) was quantitatively estimated by alkaline hypiodite method<sup>[24]</sup> and separated by paper chromatographic analysis on Whatman No. 3 MM filter paper sheet in solvent mixture (B) and used (R<sub>2</sub>) as spray reagent for the detection of methyl sugars. The different methyl sugar zones were cut out with the help of guide spots and eluted with water according to the Dent's method<sup>[17]</sup>. It was found that the methyl sugars were identified as : 2,3,4,6-tetra-O-methyl-D-galactose; 2,3,6- tri-O-methyl-D-mannose and 2,3-di -O- methyl- D-mannose in 1:3:1 molar ratio.

## RESULTS AND DISCUSSION

Water soluble seeds polysaccharide from *Cassia hirsuta* Linn was methylated by Shrivastava is and Hakomari's method using as sodium hydroxide, sodium hydride, dimethyl sulphate, and dimethyl sulphoxide then Purdie's reagent with methyl alcohol, methyl iodide and silver oxide to give fully methylated product. It showed the hydroxyl peaks at 3500-3600 cm<sup>-1</sup> region absorption band in I.R.- spectroscopy (KBr). Acid hydrolysis of fully methylated sugars with H<sub>2</sub>SO<sub>4</sub> (1 N) afforded three methyl sugar spots on Whatman No. 3 MM filter paper sheet by paper chromatography. Methylated sugars fraction were identified as : 2,3,4,6 - tetra-O-methyl-D-galactose; 2,3,6 -tri-O-methyl-D-mannose and 2,3 -di-O-methyl -D-mannose was found in 1:3:1 molar ratio and quantitatively determined by alkaline hypiodite method. Formation of 2,3,4,6-tetra-O-methyl-D-galactose indicates that the D-galactose is at the non- reducing end of repeating unit in polymer chain and it is glycosidically attached through (1→6)-  $\alpha$ -type linkages with 2,3 -di-O -methyl-D-mannose. Isolation of 2,3,6- tri-O-methyl-D-mannose indicated that the main chain or backbone of polysaccharide polymer is composed of D-mannose unit which are joined by (1→4)- $\beta$ -type glycosidic linkages. Formation of 2,3-di-O-methyl-D-mannose reveals the branching point in the main polymer chain are constituted with C<sub>1</sub>, C<sub>4</sub>, & C<sub>6</sub> position are attached through (1→4)- $\beta$  - type and (1→6)- $\alpha$  - type linkages. Methyl sugars 2,3- di-O-

methyl -D- mannose is attached glycosidically through (1→4)-β-type linkages with 2,3,6-tri-O-methyl-D-mannose while (1→6)-α-type linkages with 2,3,4,6-tetra-O-methyl-D-galactose in back bone of polymer chain.

Molar ratio between tetra, tri and di methyl hexoses were found to be 0.95%, 3.02%, & 1.05% which clearly indicates that there is one branch point in the repeating unit of the polymer chain of polysaccharide structure. Since the molar ratio of D-galactose and D-mannose was found to be 1:4 moles. Therefore, it indicated that the every five sugar hexoses repeating unit of polymer chain consists of one unit of D-galactose and four units of D-mannose sugars. On the basis of above finding methylation results a polysaccharide structure of water soluble *Cassia hirsuta* Linn. seeds polysaccharide as shown in Figure-1 has been proposed for the galactomannan. Seeds galactomannan are commercially and medically used in sugars, textiles, pharmaceutically, ice cream, pudding, cosmetic, bakery, industry and also in air pollution to minimize the air pollutant in the environment.



**Fig.1. Polysaccharide structure of *Cassia hirsuta* Linn seeds polysaccharide.**

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