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Seeds polysaccharide structure by methylation studies from *Cassia glauca* Lam. plant

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Abstract
Fully methylated water soluble seeds polysaccharide with sulphuric acid from *Cassia glauca* Lam. seeds and precipitated with ethanol then hydrolysed by sulphuric acid. After analysis with Gas Liquid Chromatography, Column and Paper chromatographic analysis of hydrolysate was to be composed of D-galactose and D-mannose in the molar ratio of 1:4. Studies of methylation of polysaccharide by Haworth's, Hakomari's and Purdie's method and its IR-spectroscopy and ^{1}H and ^{13}NMR Spectroscopy indicated that the polysaccharide was a galactomannan with a chain of D-mannopyranose and D-galactopyranose residue linked β-type with (1→4), which carried alternatively α-type with (1→6)-D-galactopyranose residue. Methylated galactomannan on acid hydrolysis (H_2SO_4) gave important 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 in 1:3:1 molar ratio. On the basis of above finding methylation results a tentative water soluble seeds polysaccharide structure has been proposed for *Cassia glauca* Lam. Plant.

Keywords: methyl sugars, methylation, *Cassia glauca* Lam. seeds polysaccharide

Introduction
Cassia glauca Lam. Plant [1, 2] belongs to the family-Caesalpiniaceae is an evergreen, deciduous tree usually 10m in height. It occurs in all over India, Myanmar, Sri Lanka, Malaysia, Australia, Thailand, Pakistan, China, South America and Nepal. According to Ayurvedic system of medicine, the bark and leaves are used in the treatment of diabetes and gonorrhea. Its seeds oil are used in indigenous system of medicine for the treatment of skin and leucoderma diseases. Preliminary analysis were carried out the nature of the constituent water soluble polysaccharide [3] as D-galactose and D-mannose in 1:4 molar ratio from hydrolysed compound by GLC, column and paper chromatographic analysis, periodate oxidation studies [4] with tentative polysaccharide structure have already been studies. Present investigation mainly deals with the methylation studies of purified *Cassia glauca* Lam. seeds polysaccharide for proposing a possible polysaccharide structure. The commercial uses of the seeds polysaccharide are in the various industries linked with food items are in sugar, textile, pudding, pastry, ice-cream, printing, dyeing, bakery, cosmetics, pharmaceutical etc. Polysaccharide will also be explored for their air pollution minimizing capacity in the environment. Recently the polysaccharide structure has also been isolated from the seeds of *Withania somnifera* Dunal [5], *Wrightia tinctoria* R. Br. (Roxb.) [6], *Moringa oleifera* Lam. Gum [7], *Madhuca longifera* Linn [8].

Materials and Methods
Unless otherwise stated that all evaporations were carried out at 45-50 ^{0}C under reduced pressure. Through specific rotations *Cassia glauca* Lam. methylated polysaccharides were found in the equilibrium values and melting points are uncorrected. Paper chromatographic analysis of methylated sugars mixture were carried out by descending technique paper Chromatography [9] on Whatmann No. 3MM filter paper sheet with upper phase of the following solvent mixtures (v/v). were used for the identification of methyl sugar (A) *n*-butyl alcohol- ethyl alcohol- water (4:1:5) [10], (B) *n*-butyl alcohol- acetic acid- water (4:1:5) [10], (C) ethyl acetate- acetic acid- water (9:2:2) [11] and (D) ethyl acetate-pyridine- water [12]. The following spray reagents were used for the detection of methyl sugars as: (R_1) *p*-anisidine phosphate [13] and (R_2) acetonical silver nitrate- alcoholic sodium hydroxide [14]. Derivatives of methyl sugars were prepared by refluxing on ethanolic solution of sugars with freshly distilled aniline solution for 1 hr on a boiling water-bath at 100 ^{0}C.

Methylation of Seeds Polysaccharide
Purified seeds polysaccharide (10 gm) was partially methylated by Haworth's method [15] with dissolving it in distilled water (50 ml) then sodium hydroxide solution (45%, 150 ml) and dimethyl sulphate solution (70 ml) were added in a small quantities during a period of 8 hrs with a constant stirring at 5-8 ^{0}C in an atmosphere of nitrogen for three times. Resultant product was then heated carefully on a steam-bah for 2 hrs to Decomposed the excess of dimethyl sulphate present in the reaction mixture. It was filtered and obtained filtrate, neutralized with cold sulphuric acid. The precipitate of sodium sulphate was filtered off and aqueous filtrate was extracted with chloroform in a liquid-liquid extractor. The solvent layer was worked upto yield a glassy yellow mass (8.24 gm).
Above partially methylated compound was further remethylated by Hakomari's Method [16] with distilled dimethyl sulphoxide (100 ml) with mechanical stirrer in an inert atmosphere of nitrogen for 5 hrs. Contents were stirred at room temperature for 6 hrs till the evolution of hydrogen gas were ceased. The methyl iodide solution (10 ml) was added dropwise to the reaction mixture to a period of 2 hrs and stirring was continued for 10 hrs more. Five further

addition of sodium hydride (2 gm in 20 ml dimethyl sulphoxide) and methyl iodide (5 ml) were made on the successive days. Chloroform (400 ml) was then added to the extract of reaction mixture. A drop of this extract gave neutral test when added to water and then it spotted on a pH paper. Chloroform reaction mixture was filtered, to remove the precipitated sodium iodide and the filtrate was washed thoroughly with distilled water and concentrated about 20 ml. This syrup was dialyzed against running water for 48 hrs to remove the dimethyl sulphoxide and inorganic ions. Dialyzed solution was concentrated upto 30 ml and then it extracted with chloroform. The solvent layer was dried over anhydrous sodium sulphate and concentrated under high vaccum to yield a glassy yellow product (6.80 gm). Found: -OCH_3, 39.2%, which showed a slight hydroxyl peak of absorption band at 3500-3600 cm^{-1} region in IR-spectra (KBr) [17]. The above partially methylated polysaccharide was further remethylated three times by Purdie's reagent [18] with methyl alcohol, methyl iodide and silver oxide which gave fully methylated product, yield (6.42 gm), Found: -OCH_3, 40.01%. This methylated product did not show any hydroxyl peak at absorption band in IR-spectra (KBr) at 3500-3600 cm^{-1}.

Hydrolysis of methylated polysaccharide

Fully methylated seeds polysaccharide (1.80 gm) was hydrolysed [19] with sulphuric acid (72%, 25ml). Reaction mixture was kept in ice-bath for 2 hrs at 0 ^{0}C and then it heated on a steam-bath for 6 hrs at 100 ^{0}C, after proper dilution, it bring down the acid concentration upto 12% to a syrup. Hydrolysate was neutralized with barium carbonate slurry, filtered and filtrate finally concentrated to a thin syrup which consisting the mixture of neutral methylated sugars.

Fractionation of methylated polysaccharide

Methylated polysaccharide (5 gm) was fractionated by fractional dissolution method [20] with pet. ether (40-60 ^{0}C) and chloroform mixture with the increasing amounts to latter solvent being increased in stages on a steam-bath for 3 hrs at 100 ^{0}C. Solution obtained from the each fraction was evaporated and residue dried under high vacuum (15mm over P_2O_5) to a constant weight. Specific rotations of the each methyl sugar fractions were taken in chloroform and methoxyl contents of individual methyl sugar fractions were determined by usual manner and obtained results are given in Table-1.

Table 1: Fractionation of methylated *Cassia glauca* Lam. seeds polysaccharide.

S.No.	State of methyl sugars	Solvent composition (%)		Yield (gm)	-OCH_3 (%)	$[\alpha]^{24}_D$ ($CHCl_3$)
		Pet. ether (40-60 ^{0}C)	Chloroform			
1	Oily liquid	100	00	0.6224	-	-
2	Oily liquid	95	05	0.6592	-	-
3	Oily liquid	90	10	0.7324	-	-
4	Oily liquid	85	15	0.6542	-	-
5	Crispy solid	80	20	1.9200	50.6	+18.6^0
6	Crispy solid	75	25	0.6124	40.2	+20.4^0
7	Crispy solid	70	30	1.5448	42.6	+16.2^0

Characterization of methylated polysaccharide

The resolution of neutral methylated sugars mixture were first attempted on cellulose column chromatography with pet. ether (60-80 ^{0}C) and *n*-butyl alcohol in 7:3 and 1:1 molar ratio, but no homogenous methyl sugar fractions could be obtained. Partition paper chromatographic technique was carried out on Whatmann No. 3MM filter paper sheet with solvent mixture (A) and used (R_1) as spray reagent which was next adopted for the resolution of the neutral methylated sugars mixture. Paper strips corresponding to the individual methyl sugars were cut out with the help of guide spots and diluted with water according to the Dent's method [21]. This furnished the three methyl sugars fraction were evaporated separately which were characterized and identified as follows:

1. 2, 3, 4, 6-tetra-O-methyl-D-galactose

Methyl sugar syrup (500gm) gave a single spot corresponding to D-galactose on paper chromatogram in solvent mixture (A), Found: -OCH_3, 54.6%, calculated for $C_{10}H_{20}O_6$ required–OCH_3, 55.4%. It gave D-galactose on demethylation with hydrobromic acid [22]. It (175 mg) was identified as 2, 3, 4, 6-tetra-O-methyl-D-galactose by conversion into anilide derivative was prepared by usual manner as: 2, 3, 4, 6-tetra-O-methyl-N-phenyl-D-galactopryanosyl amine, having m.p. & mixed m.p. 191-193 ^{0}C, Lit. m.p. 190-191 ^{0}C [23]. It had R_f 0.72 in solvent (D) and R_g 0.92 in solvent (A), optical rotation $[\alpha]^{24}_D$ +73.6 ^{0}C ($CHCl_3$) and +110 ^{0}C (H_2O), Lit. $[\alpha]_D$, +75.0 ($CHCl_3$) and +110-111 ^{0}C (H_2O) [24].

2. 2, 3, 6-tri-O-methyl-D-mannose

Methyl sugar syrup (900mg) gave a single elongated spot on paper chromatogram parallel to the D-mannose in solvent mixture (A). It had R_f 0.59 in solvent (D) and R_g 0.82 in solvent (A), m.p. & mixed m.p. 106-108 ^{0}C, $[\alpha]^{24}_D$ +15.6 ^{0}C ($CHCl_3$) and -12 ^{0}C (H_2O), Lit. $[\alpha]_D$ +15.7 ^{0}C ($CHCl_3$) and -10 ^{0}C (H_2O) [25]. It gave D-mannose on demethylation. Found: -OCH_3, 41.4 ^{0}C%, calculated for $C_9H_{18}O_6$ requires –OCH_3, 42.8%. The derivative was prepared by usual manner as 2, 3, 6-tri-O-methyl-D-mannonic acid phenyl hydrazide, having m.p. & mixed m.p. 130-131 ^{0}C, Lit. m.p. 130-132 ^{0}C [26].

3. 2, 3-di-O-methyl-D-mannose

Methyl sugar syrup (850mg) gave a single spot of D-mannose on paper chromatogram in solvent system (A). It had R_f 0.43 in solvent (D) and R_g 0.56 in solvent (A), m.p. & mixed m.p. 108-109 ^{0}C, $[\alpha]^{24}_D$ +65.2 ^{0}C ($CHCl_3$) +4.4^0 (MeOH) and -15.4^0 (H_2O), Lit. $[\alpha]_D$ +65.4^0 ($CHCl_3$), -15.8^0 (H_2O) and +4.8^0 (MeOH). It gave D-mannose on demethylation. Found: -OCH_3, 29.6%, calculated $C_8H_{16}O_6$ requires, 29.2%. Derivative was prepared by usual manner as 2, 3-di-O-methyl-γ-D-mannolactone, having m.p. & mixed m.p. 106-107 ^{0}C, Lit. m. p. 107-108 ^{0}C [27].

Quantitative estimation of methylated sugars

Methyl sugar mixture (2gm) was quantitatively estimated by

alkaline hypoiodite method [28] and separated by paper chromatography on Whatmann No. 3MM filter paper sheet in solvent mixture (B) and used (R_2) as spray reagent for the detection of methyl sugars. The zones containing methyl sugars were cut out with the help of guide spots and eluted with water according to the Dent's method [21]. 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 the molar ratio of 1:3:1 respectively. The structure of methylated sugars fraction from *Cassia glauca* Lam. seed polysaccharide are shown in Figure-1.

Results and Discussion

Water soluble *Cassia glauca* Lam. seeds polysaccharide was methylated by Haworth's and Hakomari's method using sodium hydroxide, sodium hydride, dimethyl sulphate and dimethyl sulphoxide and then Purdie's reagent with methyl alcohol, methyl iodide and silver oxide to give fully methylated product. It did not showed the hydroxyl peaks at 3500-3600 cm^{-1} absorption band in IR-spectroscopy (KBr). The acid hydrolysis of fully methylated polysaccharide with sulphuric acid (1N) afforded 4 methyl sugars spot on Whatman No. 3MM filter paper sheet by paper chromatography. Methylated sugars fraction were identified as: (I) 2, 3, 4, 6-tetra-O-methyl-D-galactose; (II) 2, 3, 6-tri-O-methyl-D-mannose and (III) 2, 3-di-O-methyl-D-mannose in molar ratio of 1:3:1 respectively and quantitatively determined by alkaline hypoiodite method. Formation of 2, 3, 4, 6-tetra-O-methyl-D-galactose indicates that the D-galactose is at the non-reducing end of the polymer chain and is glycosidically attached through (1→6)-α-type linkages with 2, 3-di-O-methyl-D-mannose, since the polysaccharide is non-reducing to the Fehling's solution. Isolation of 2, 3, 6-tri-O-methyl-D-mannose indicated that the main polymer chain or backbone of the polysaccharide polymer which is composed of D-mannopyranose units are attached glycosidically through (1→4)-β-type glycosidic linkages. Methyl sugar 2, 3-di-O-methyl-D-mannose reveals that the branching point in the main polymer chain constitute with C_1, C_4 and C_6 position are attached (1→4)-β-type and (1→6)-α-type linkages. The 2, 3-di-O-methyl-D-mannose is attached glycosidically, (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. It is clearly indicates that there is one branch point in the repeating unit of the main polymer chain of the seeds 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 5 sugar hexoses repeating unit of the polymer chain consists of one hexose units of D-galactose and four hexose units of D-mannose. On the basis of above finding methylation results a polysaccharide structure of water soluble *Cassia glauca* Lam. seeds (Figure-1) has been proposed for the galactomannan.

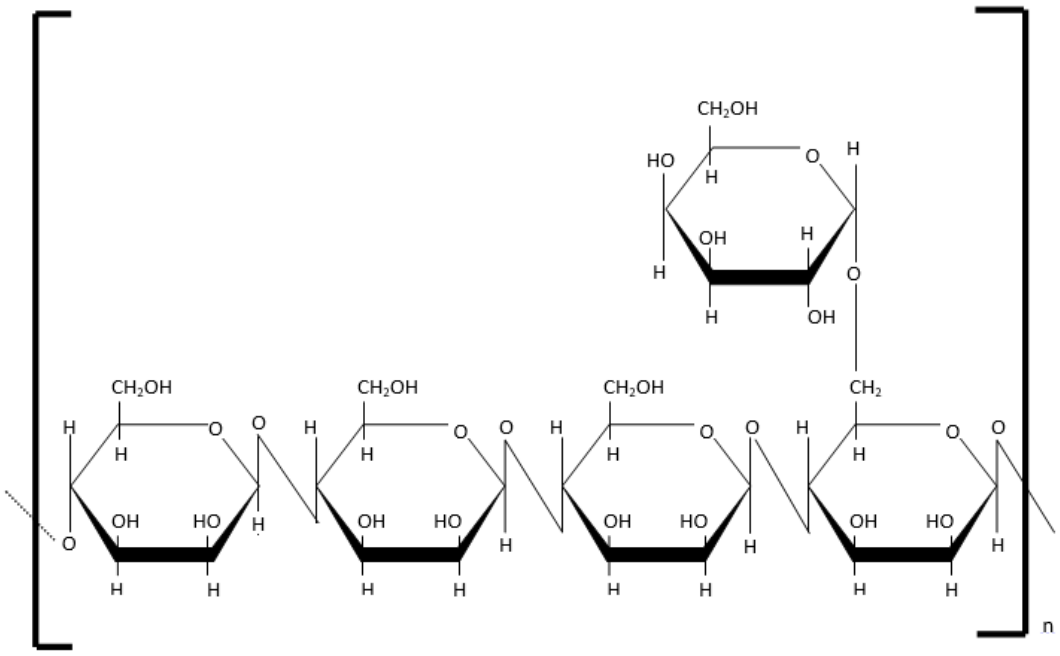

Fig 1: Seed Polysaccharide Struture from *Cassia Glauca* lam. Plant

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