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Ram Babu Singh
Prof. Department of Zoology, School of Life Sciences, Dr. B. R. Ambedkar University, Khandari Campus, Agra, U.P. India

Correspondence
Ram Babu Singh
Prof. Department of Zoology, School of Life Sciences, Dr. B. R. Ambedkar University, Khandari Campus, Agra, U.P. India

Seeds cake polysaccharide structure from *Madhuca longifolia* Linn. Plant by methylation studies

Ram Babu Singh

Abstract
Acid hydrolysis of fully methylated water soluble seeds cake polysaccharide from *Madhuca longifolia* Linn. was carried out by Hakomari and Purdie's method to produced certain important medicinal chemicals like methyl sugars. It was identified by usual manner and methyl sugars were obtained as : 2,3,4,6-tetra-O-methyl-D-glucose; 2,3,6-tri-O-methyl-D-glucose; 2,3,4-tri-O-methyl-D-glucose; 2,4,6-tri-O-methyl-D-glucose; 2,3,4-tri-O-methyl-L-rhamnose; 3,4-di-O-methyl-L-rhamnose and 4-O-methyl-L-rhamnose were present in the molar ratio of 1:4:1:1:1:1:2 respectively. On the basis of methylation results a tentative polysaccharide structure has been assigned to the *Madhuca longifolia* Linn. seeds cake polysaccharide.

Keywords: Methyl sugars, methylation of *Madhuca longifolia* seeds polysaccharide

1. Introduction
Madhuca longifolia Linn. Plant [1] belongs to the family-Sapotacceae and commonly called as *Mahua* and occurs in all over India. Plants and seeds are used in Ayurvedic system of medicine. Mahua seed oil is used in skin diseases, rheumatism and headache. Seed oils is also used in the manufacturing of soaps, laundry soap, cooking purposes, for making candles, etc. Mahua flowers are also used in the preparation of distilled liquors. Wood is used for building purposes as beams, door, window, frames, furnitures, sports goods, musical instruments, ship building, boats, bridges and railway sleepers. Seeds cake contains a water soluble sugars [2] as D-glucose and L-rhamnose in the molar ratio of 7:4 by column and paper chromatographic analysis from hydrolysed product. Present manuscript mainly deals with the methylation studies of the purified *Madhuca longifolia* Linn. seeds cake polysaccharide for proposing a possible polysaccharide structure.

2. Materials and Methods
Unless otherwise stated that all evaporations were carried out at 40-45^{0}C under reduced pressure. Through specific rotations of methylated seeds polysaccharides were found in equilibrium values and melting points are uncorrected. Paper chromatographic analysis were carried out by descending technique [3] on Whatman No. 3 MM filter paper sheets with upper phase of the following solvent mixtures (v/v) : (A) *n*-butyl alcohol-ethyl alcohol-water (4:1:5) [4], (B) ethyl acetate-acetic acid-water (9:2:2) [5], (C) *n*-butyl alcohol-acetic acid-water (4:1:5) [4], (D) ethyl acetate-pyridine-water (2:2:7) [6], (E) benzene-ethyl alcohol-water (169:47:15) [7]. The following spray reagents were used for the detection of methyl sugars from methylated hydrolysed compound as (R_1) *p*-anisidine phosphate [8] and (R_2) acetonical silver nitrate-alcoholic sodium hydroxide [9]. Derivatives of methylated sugars were prepared by refluxing an ethanolic solution of methyl sugars with freshly distilled aniline solution for 2 hrs on a boiling water-bath at 100^{0}C.

3. Results
Methylation of seeds polysaccharide
Purified seeds cake polysaccharide (10 gm) was partially methylated by Hakomari's method [10] with dimethyl sulphoxide (100 ml) by stirring with mechanical stirrer in an inert atmosphere of nitrogen for 3 hrs. Sodium hydroxide (2 gm) was added to the extraction mixture during a period of 12 hrs then the contains were strirred for further 4 hrs till the

evolution of hydrogen gas were ceased out. Methyl iodide solution (10 ml) was added to the reaction mixture and stirring for 10 hrs more. Four further addition of sodium hydride (2 gm) in dimethyl sulphoxide (20 ml) and methyl iodide (5 ml) then added chloroform (300 ml) to the reaction mixture which gave neutral test on pH paper. Solution was filtered to remove the precipitate of sodium iodide and filtrate washed with water then concentrated to a syrup (20 ml). This syrup was dialysed against running water for 36 hrs to remove dimethyl sulphoxide and inorganic ions then concentrated to syrup (30 ml) and extracted with chloroform. It was dried over anhydrous sodium sulphate and concentrated under high vacuum to yield a yellow product (6.58 gm), Found : -OCH_3, 36.25%, which showed a slight hydroxyl peak at absorption band at 3500-3600 cm^{-1} in IR-Spectra (KBr).

The above partially methylated seeds cake polysaccharide was further remethylated by Purdie's reagent [11] with methyl alcohol, methyl iodide and silver oxide gave fully methylated product yield (5.8 gm). Found: –OCH_3, 40.8%, this methylated product did not show any hydroxyl peak at absorption band in IR-Spectra (KBr) at 3500-3600 cm^{-1} region [12].

Hydrolysis of methylated polysaccharide

The fully methylated seeds polysaccharide (1.56 gm) was hydrolysed [13] with sulphuric acid (72%, 25 ml) and reaction mixture kept in ice-bath for 2 hrs then heated on steam-bath for 4 hrs. after proper dilution with water to bring down the acid concentration upto 12% to a syrup. Hydrolysate was neutralized with barium carbonate slurry, filtered and filtrate concentrated to a thin syrup, which consisting a mixture of neutral methylated sugars.

Fractionation of methylated polysaccharide

Methylated polysaccharide (5 gm) was purified by fractional dissolution method [14] with a mixture of petroleum ether (40-60^{0}C) and chloroform with the increasing amount of the later solvent on a water-bath for 2 hrs. Solution of each fraction was evaporated and residue dried under high vacuum (15 mm, over P_2O_5) to a constant weight. The specific rotation of each fraction of methyl sugars were taken in chloroform and methoxyl contents of the individual methyl sugars fraction were determined as usual manner and results are shown in Table-1.

Table 1: Fractionation of methylated *Madhuca longifolia* Linn. seeds cake polysaccharide.

Fr. No.	Methyl sugars (State)	Solvent Composition (%)		Yield (gm)	-OCH_3 (%)	$[\alpha]_D^{20}$ ($CHCl_3$)
		Pet. ether (40-60^{0}C)	$CHCl_3$			
1.	Oily liquid	100	00	0.3428	-	-
2.	Oily liquid	95	05	0.3216	-	-
3.	Oily liquid	90	10	0.3024	-	-
4.	Cryspy solid	85	15	0.6280	49.8	+73^0
5.	Cryspy solid	80	20	0.4608	41.3	+69^0
6.	Cryspy solid	75	25	0.5240	42.4	+70^0
7.	Cryspy solid	70	30	0.5120	43.2	+68^0
8.	Cryspy solid	65	35	0.7240	45.6	+28^0
9.	Cryspy solid	60	40	0.4522	31.8	+19^0
10.	Cryspy solid	55	45	0.5628	16.7	+22^0

Characterization of methylated polysaccharide

Methylated sugars were charecterized [15] by column chromatography with petroleum ether (60-80^{0}C) and *n*-butyl alcohol in 7:3 and 1:1 ratio, but no homogeneous sugar fractions could be obtained. Hydrolysed methyl sugars was separated by paper chromatography on Whatman No. 3 MM filter paper sheet in solvent mixture (A) and used (R_1) as spray reagent for the detection of methyl sugars. The individual methyl sugar strips were cut out with the help of guide spots and eluted with water [16]. The eluted methyl sugars component were evaporated separately, to furnish 10 sugar fraction, out of them 3 fractions were obtained as oily liquid form and 7 fractions as a cryspy solid form. These methyl sugar fractions were characterized and identified as follows:

Fraction-I: 2, 3, 4, 6-tetra-O-methyl-D-glucose

Sugar syrup (260 mg) on paper chromatographic analysis in solvent mixture (A) gave a single spot parallel to the authentic sample of D-glucose, had *R*f 0.82 (D) and *R*g 1.00 (A), $[\alpha]_D^{29}$ +73^{0}C ($CHCl_3$). Found: –OCH_3, 49.6%, calculated for $C_{10}H_{20}O_6$ required, –OCH_3, 51.5%. Demethyaltion [17] of methyl sugars (50 mg) with hydrobromic acid (48% w/w, 10 ml) at 100^{0}C for 10 min. on water-bath, indicated that this D-glucose was non-reducing sugar, had m.p. & mixed m.p. 85-87^{0}C. Derivatives was prepared with alcoholic solution of aniline on water-bath, yielded 2,3,4,6-tetra-O-methyl-N-phenyl-D-glucopyranosyl amine after recrystallisation with ethanol, had m.p. & mixed m.p. 137-138^{0}C [18].

Fraction-II 2, 3, 6-tri-O-methyl-D-glucose

Sugar syrup (460 mg) was moved parallel to D-glucose on paper chromatogram in solvent mixture (A), had *R*f 0.60 (D) and *R*g 0.89 (A), $[\alpha]_D^{29}$ +69^{0}C ($CHCl_3$). Found: –OCH_3, 41.3%, calculated for $C_9H_{10}O_6$, required, –OCH_3, 41.9%. Product showed D-glucose on paper chromatography by demethylation which indicated that this sugar was in backbone of the polymer chain. Derivative was prepared by boiling with ethanol and phenylhydrazine, gave crystals of 2, 3, 6-tri-O-methyl-D-glucuronic acid phenylhydrazide after recrystallisation with ethanol, had m.p. & mixed m.p. 145-146^{0}C [18].

Fraction-III: 2, 3, 4-tri-O-methyl-D-glucose

Sugar syrup (510 mg) gave a single spot parallel to the authentic sample of D-glucose by paper chromatography in solvent mixture (A). On demethylation, it had m.p. & mixed m.p. 123^{0}C and having *R*f 0.58 (D) and *R*g 0.90 (A), $[\alpha]_D^{29}$ +68^{0}C ($CHCl_3$). Found: –OCH_3, 43.2%, calculated for $C_9H_{18}O_6$ required, –OCH_3, 44%. Derivative of this sugar was prepared with phenyl hydrazine and ethanol gave crystals of 2, 3, 4-tri-O-methyl-D-glucophenylhydrazide after recrystallisation with ethanol, had m.p. & mixed m.p. 138-139^{0}C.

Fraction-IV: 2, 4, 6-tri-O-methyl-D-glucose

Sugar syrup (520 mg) gave a single spot parallel to the D-glucose on paper chromatogram in solvent mixture (A). On demethylation, it had m.p. & mixed m.p. 125^{0}C and having *R*f 0.62 (D) and *R*g 0.85 (A), $[\alpha]_D^{29}$ +70^{0}C ($CHCl_3$). Found: –OCH_3, 42.4%, calculated for $C_9H_{18}O_6$ required, –OCH_3, 42.9%. Derivative was prepared with phenyl hydrazine, after recrystallisation with ethanol, had m.p. & mixed m.p. 140-142^{0}C.

Fraction-V: 2,3,4-tri-O-methyl-L-rhamnose

Sugar syrup (720 mg) by demethylation gave a spot corresponding to the L-rhamnose on paper chromatogram having m.p. & mixed m.p. 100^{0}C, had *R*f 0.87 (D) and *R*g 1.04 (A), $[\alpha]_D^{29}$ +28^{0}C ($CHCl_3$). Found: –OCH_3, 45.9%, calculated for $C_9H_{18}O_6$ required, –OCH_3 45.9%. Derivative of this sugar was prepared by dissolving it in ethanol and aniline. Solution on evaporation then keeping the resulting syrup in refrigerator which furnished the crystals of 2, 3, 4-tri-O-methyl-L-rhamnosyl amine after recrystallisation with ethanol, had m.p. & mixed m.p. 108-110^{0}C [19].

Fraction-VI: 3, 4-di-O-methyl-L-rhamnose

Sugar syrup (450 mg) was moved parallel to the L-rhamnose on paper chromatogram having m.p. & mixed m.p. 90^{0}C, $[\alpha]_D^{29}$ +19^{0}C ($CHCl_3$). Found: –OCH_3, 31.8%, calculated for $C_8H_{16}O_6$ required, –OCH_3, 32.2%. Sugar was converted into 3,4-di-O-methyl-L-rhamnolactone, by the same method of oxidation and lactonization. It was on recrystallisation with petroleum ether gave long needles of 3,4-di-O-methyl-L-rhamnolacetone, had m.p. & mixed m.p. 76^{0}C [19].

Fraction-VII: 4-O-methyl-L-rhamnose

Sugar syrup (560 mg) gave a spot on paper chromatographic analysis with solvent mixture (A), had *R*f 0.23 (D) and *R*g 0.68 (A). Demethylation of the methylated sugar fraction gave a spot corresponding to the L-rhamnose on paper chromatogram, having m.p. & mixed m.p.85^{0}C. It had $[\alpha]_D^{29}$ +22^{0}C ($CHCl_3$). Found: –OCH_3, 16.9%, calculated for $C_7H_{14}O_5$ required, –OCH_3, 17.4%. The methylated sugar was identified as 4-O-methyl-L-rhamnose by converting into □-lactone. The sugar was dissolved in water and oxidized with bromine for 20 hrs. The resulting solution was aerated and neutralized with silver carbonate and then silver ions was removed by passing through hydrogen sulphide and worked up as usual to a syrup. This on distillation (140^{0}C, 0.002mm pressure) gave a semi solid mass, which crystallized on cooling. After recrystallisation with acetone and petroleum ether 40-60^{0}C gave fine needles shape crystals of 4-O-methyl-L-rhamnose-□-lactone, had m.p. & mixed m.p. 81-82^{0}C, Lit. m.p. 82^{0}C [20].

Quantitatively estimation of methylated sugar

The methylated sugar (600 mg) was quantitatively estimated by paper chromatographic analysis on Whatman No. 3MM filter paper sheet in solvent mixture (B) and zones containing methyl sugars were cut out with the help of guide spot and eluted with water according to the Dent's method [16]. The obtained methylated sugars were estimated by alkaline hypoiodite method [21]. It was found that the methyl sugars from *Madhuca longifolia* Linn. seeds cake polysaccharide were identified as : 2,3,4,6-tetra-O-methyl-D-glucose; 2,3,6-tri-O-methyl-D-glucose; 2,3,4-tri-O-methyl-D-glucose; 2,4,6-tri-O-methyl-D-glucose; 2,3,4-tri-O-methyl-L-rhamnose; 3,4-di-O-methyl-L-rhamnose and 4-O-methyl-L-rhamnose were present in the molar ratio of 1:4:1:1:1:1:2 respectively.

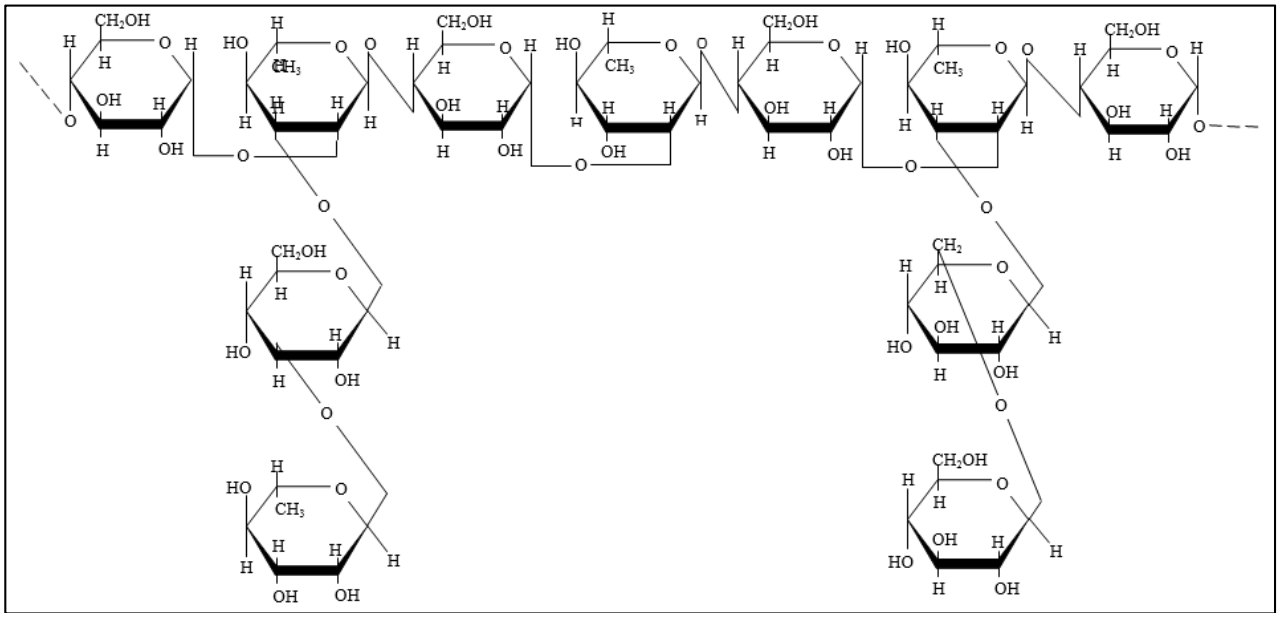

Fig 1: Seeds cake polysaccharide structure from *Madhuca longifolia* Linn. plant.

4. Discussion

The water soluble seeds cake polysaccharide was obtained from *Madhuca longifolia* Linn. by usual manner as D-glucose and L-rhamnose in the molar ratio of 7:4. Seeds cake polysaccharide was subjected to the structural investigation upon methylation studies by Hakomari and Purdie's method using sodium hydroxide, sodium hydride, dimethyl sulphate, dimethyl sulphoxide, methyl alcohol, methyl iodide and silver oxide to give the fully methylated product. It was hydrolysed with sulphuric acid (1 N) afforded seven methyl sugar fractions as : 2,3,4,6-tetra-O-methyl-D-glucose; 2,3,6-tri-O-methyl-D-glucose; 2,3,4-tri-O-methyl-D-glucose;2,4,6-tri-O-methyl-D-glucose; 2,3,4-tri-O-methyl-L-rhamnose; 3,4-di-O-methyl-L-rhamnose and 4-O-methyl-L-rhmanose were present in 1:4:1:1:1:1:2 molar ratio. Thus the formation of 2, 3, 4, 6-tetra-O-methyl-D-glucose and 2, 3, 4-tri-O-methyl-L-rhamnose residues indicated the two non-reducing terminal end in the average repeating unit of the polymer chain. The

linkages of 2,3,4,6-tetra-O-methyl-D-glucose is attached through (1→6)-β-type with 2,3,4-tri-O-methyl-D-glucose while 2,3,4-tri-O-methyl-L-rhamnose is attached through (1→3)-α-type linkages with 2,4,6-tri-O-methyl-D-glucose similarly the isolation of 2,4,6-tri-O-methyl-D-glucose is linked through C_1 and C_3 i.e. (1→3)-β-type linkages with 4-O-methyl-L-rhamnose and (1→3)-α-type with 2,3,4-tri-O-methyl-L-rhamnose. The attachment of 2,3,4-tri-O-methyl-D-glucose is linked through C_1 and C_6 position i.e. (1→3)-β-type linkages with 4-O-methyl-L-rhamnose and (1→6)-β-type linkages with 2,3,4,6-tetra-O-methyl-D-glucose. Formation of 2, 3, 6-tri-O-methyl-D-glucose and 4-O-methyl-L-rhamnose indicated that the main chain of the polysaccharide polymer, which is composed of D-glucose and L-rhamnose sugar units. These sugar residues are attached through (1→2)-α-type and (1→4)-α-type interglycosidic linkages. The formation of 4-O-methyl-L-rhamnose is a product of methylation, further point out that the repeating unit carries several L-rhamnose residues moieties triply linked through C_1, C_2 and C_3, reveals the branching point in the polymer chain. Since the molar ratio between D-glucose and L-rhamnose was found to be 7:4, therefore every 2 groups of the repeating unit indicated the 11 sugar units in the polysaccharide structure. On the basis of above methylation results a tentative polysaccharide structure has been proposed for the *Madhuca longifolia* Linn. seeds cake polysaccharide as shown in Figure-1.

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