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Methylation studies of aldobiouronic acid from *Moringa oleifera* Lam. gum polysaccharide

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Abstract
Water soluble gum polysaccharide was extracted from *Moringa oleifera* Lam. plant as L-arabinose and D-galactose in 1:4 molar ratio with traces of L-fucose. Methylation of aldobiouronic acid was obtained from graded hydrolysis of degraded gum polysaccharide gave mixture of methyl sugars as: 2,3,4-tri-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-glucuronic acid.

Keywords: methyl sugars, aldobiouronic acid, *Moringa oleifera* gum polysaccharide

Introduction
Moringa oleifera Lam. Plant [1] belongs to Moringaceae family and commonly called as *Sainjana* upto 10 m in height. It occurs in Northern to Southern India, Thailand, Pakistan, Africa, Sri Lanka, Afghanistan, Nepal, Mexico, Philippines and America. Plants are used in indigenous system of medicine for the treatment of cardiovascular disease and also in gastrointestinal diseases. Gum are used for the treatment of dental infection and blood pressure. Pods are used as pickles and vegetable. Leaves are the rich source of Vitamin A & C, β-Carotine, protein, calcium and potassium which are used in scurby and natural antioxidant. Seeds are antipyretic, acrid, bitter and seed oils are used in rheumatism. Leaves extract are used for piles, fevers, bronchitis, eyes, ear infections, antitumor and anticancer. Leaves alkaloid Niazi Mian has been proposed to be a potent chemopreventive agent in carcinogenesis. Seeds extract have also been found to be effective on hepatic carcinogen metabolizing enzyme and antioxidant parameters and have specific protein fractions for skin and hair cure. Seeds peptide are also used to protect the human skin aging with dual activity as antipollution and hair conditioning. Gum contains a water soluble polysaccharide [2] for skin and hair cure. Seeds peptide are also used to protect the L-arabinose and D-galactose in 1:4 molar ratio with traces of L-fucose, methylation studies for gum polysaccharide structure [3] and periodate oxidation studies[4] for confirmation of polysaccharide structure obtained after methylation studies. Present manuscript mainly deals with the methylation studies of aldobiouronic acid which was obtained after gradual hydrolysis of degraded gum polysaccharide.

Materials and Methods
Unless otherwise stated that all evaporations were carried out under reduced pressure and melting points are uncorrected. The separation and identification of methyl sugars were carried out by descending technique of paper chromatography [5] on Whatman No. 1 and 3 MM filter paper sheet. The solvent mixture (v/v) were used for the detection of methyl sugars as (A) *n*-butanol-acetic acid-water (4:1:5, upper phase) [5] and (B) *n*-butanol-ethanol-water (4:1:5, upper phase) [6]. The *p*-anisidine phosphate reagent [7] (R) was used as a spray reagent for the appearance of methyl sugars present in the methylated hydrolysed *Moringa oleifera* Lam. gum product.

Methylation of aldobiouronic acid
The aldobiouronic acid (10gm) was methylated by Hakomari's method [8] with Barium aldobiouronic acid (10gm) by dissolving it in distilled water (50ml) and dimethyl sulphate (80ml) then added followed by drop wise addition of sodium hydroxide (40%, 250ml). It was non-reducing to Fehling's solution, filtered and neutralized with sulphuric acid and concentrated under reduced pressure at 45-50^{0}C and resulting methylated product was dissolved in sodium hydroxide (40%, 200ml) and dimethyl sulphate (105ml). The reaction mixture was hydrolysed with sulphuric acid (1N), filtered and filtrate extracted with chloroform in a liquid-liquid extractor to yielded a syrup (6gm). Syrup was then remethylated by Purdie's reagent [9] with methyl alcohol (40ml) methyl iodine (20ml) and silver oxide (10gm). The methylated product was then filtered and silver residue exhaustively extracted with hot methanol. Filtrate was evaporated under reduced pressure at 45-50^{0}C and resulting syrup was again methylated by dissolving it in methyl iodine (30ml) and added silver oxide (10gm) during a period of 8 hrs and obtained syrup, yield (4.6gm).
Methoxyl percentage in different fractions were determined by semi-micro method [10] and remaining residue after distillation was extracted with chloroform. The extract was evaporated under reduced pressure and syrup left behind was distilled in vacuo (0.1mm pressure) to give a syrup (0.750gm). Syrup was again methylated with Purdie's reagent as methyl iodide (15ml), methyl alcohol (30ml) and silver oxide (5gm). Methyl product was distilled in vacuum in a short neck distillation unit to give a syrup (1.8gm), B.P. 215-240^{0}C at 0.1mm pressure. Its methoxyl content was determined by semi-micro method and was found to be 48%, syrup which consisted of fully methylated aldobiouronic acid was further examined. Optical rotation of the compound measured in 1mm tube

($CHCl_3$, 2%), corresponded to $[\alpha]_D^{25}$, 19.8^{0}C. This value is in good agreement with the reported value for methyl-6-O-methyl (2,3,4-tri-O-methyl-β-D-glucopyranosyl uronic)-2,3,4-tri-O-methyl-D-galactopyranoside.

Hydrolysis of fully methylated aldobiouronic acid

Methylated compound (1.8gm) was refluxed with methanolic hydrogen chloride (4%, 80ml) for 45hrs. Methanol was then distilled off under reduced pressure at 40-50^{0}C and bulk reduced to 10ml then added hydrochloric acid (1N, 80ml) and solution was heated at 96-98^{0}C for 20hrs. It was then cooled, neutralized with freshly prepared silver carbonate and allowed to stand for 5hrs. The precipitated silver chloride and unreacted silver carbonate was removed by filtration. Hydrogen sulphide gas was passed in filtrate and precipitated silver sulphide was removed by filtration and filtrate evaporated to syrup under reduced pressure at 45-50^{0}C. It was obtained as brown powder which was exhaustively extracted with dry ether. The ether extract contained methylated D-galactose portion of syrup fully methylated product (0.58gm). The brown powder left after ether extraction was dissolved in distilled water (40ml), acidified with hydrochloric acid and extracted with chloroform in liquid-liquid extractor and evaporated to a syrup (0.60gm). This syrup was methylated glucuronic acid portion of fully methylated aldobiouronic acid.

Identification of methylated D-galactose portion

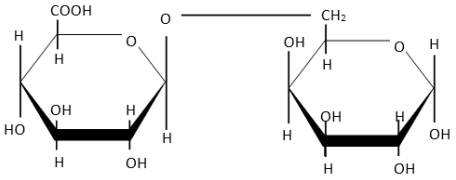
It contained 40% of methoxyl as determined by semi-micro method. The methoxyl value is in good agreement with the required value (41.8%) for a tri-O-methyl-D-galactose. Methoxyl compound (0.26gm) was refluxed for 2hrs with absolute alcohol (8 ml) and freshly distilled aniline (0.14gm). Most of the alcohol was removed by the distillation and on cooling crystals of aniline derivative was separated out. After recrystallisation from ethanol it had m.p. 164-166^{0}C. This value is in good agreement with the reported value for 2,3,4-tri-O-methyl-N-phenyl-D-galactopyranosyl amine [11].

Identification of methylated glucuronic acid portion

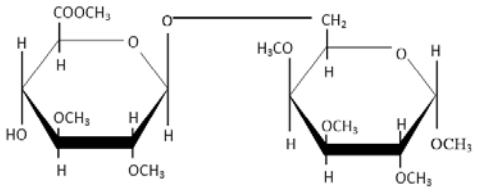
It was examined by partition paper chromatography on Whatman No. 1 filter paper sheet by descending technique with solvent mixture (B) as irrigating solvent and (R) used as spray reagent. It gave a pink spot whose *R*g value was 0.82, relative to 2,3,4,6-tetra-O-methyl-D-glucose. This value is in good agreement with the reported value for 2,3,4-tri-O-methyl-D-glucuronic acid[12]. The compound was taken in water (100ml) and bromine (1ml) then kept in dark for 3 days which was periodically shaken with mechanical stirrer. After filtration the excess bromine was removed by aeration. Solution was neutralized with freshly prepared silver carbonate and allowed to stand for 4hrs. The precipitated silver chloride and unreacted silver carbonate were removed by filtration and washed with distilled water then hydrogen sulphide gas was passed in filtrate, to remove the silver ions as silver sulphide. The precipitated silver sulphide was filtered off and filtrate was evaporated to dryness under reduced pressure at 45-50^{0}C to give a syrup (0.6gm). Distillation of the latter in vacuum (0.3mm pressure) and water-bath temperature at 160-180^{0}C to gave a syrup and its crystallization from petroleum ether afforded crystals which melted at 108^{0}C. The melting point is in good agreement with the reported m.p. of methyl 2,3,4-tri-O-methyl-D-glucurate-1-5-lactose.

Results and Discussion

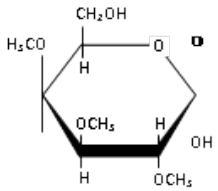
Moringa oleifera Lam. gum polysaccharide structure was determined for the nature and position of attachment of neutral sugars and uronic acid in adobe uronic acid were ascertained by the application of classical methylation techniques by Hakomari [8] and Purdie [9]. The aldobiouronic acid as shown in (Figure-1A) was exhaustively methylated first with dimethyl sulphate and sodium hydroxide then methyl alcohol, methyl iodide and silver oxide. The fully methylated aldobiouronic acid (Figure-1B) on hydrolysis which furnished equal amount of (I) : 2,3,4-tri-O-methyl-D-galactose (Figure-1C) and (II) ; 2,3,4-tri-O-methyl-D-glucuronic acid (Figure-1D). The compound was identified from its crystalline anilide derivative m.p. 165^{0}C. It was identify and established by converting into crystalline methyl pyranoside amino, m.p. 184^{0}C. The isolation of I & II suggested that the D-glucuronic acid is linked through its C_1 to C_6 position of D-galactose. The glycosidic linkages of uronic acid was assigned a β-configuration [13], because the fully methylated aldobiouronic acid was a laevorotatory, $[\alpha]_D^{25}$ -19.8^{0}C. Therefore, the aldobiouronic acid was obtained from *Moringa oleifera* Lam. gum polysaccharide was a 6-O-β-D-glucopyranosyl-uronic acid-D-galactose [14] structure as shown in Figure-1.



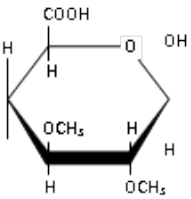
A. 6-O-β-D-glucopyranosyl-uronic acid-D-galactose.



B. Methylated aldobiouronic acid.



C. 2,3,4-tri-O-methyl-D-galactose.



D. 2,3,4-tri-O-methyl-D-glucuronic acid.

Fig 1: Structure of methylated aldobiouronic acid from *Moringa oleifera* Lam. gum polysaccharide

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