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Structure Determination of *Rauwolfia Serpentina* Benth Water Soluble Seed Polysaccharide by Methylation Studies

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Abstract

Seed polysaccharide was subjected to methylation by Haworths & Hakomaris method. The partially methylated polysaccharide having-OCH_3, 41.8%. The purified methylated polysaccharide hydrolysed with sulphuric acid was found to contain a mixture of 6 methylated sugars. The mixture of hydrolysed methylated sugars was separated on Whatman No.3 MM filter paper and purified by fractional dissolution. Metyl sugars were identified by determining their specific rotations, methoxyl contents, demethylation analysis and preparing their suitable crystalline derivatives and their molar ratio of the methylated sugars.

Keywords: *Rauwolfia Serpentina Benth. seed* polysaccharide, dimethyl sulphate, sodium hydride, sulphuric acid.

Introduction

Rauwolfia serpentina Benth. plant belongs to family Apocynaceae and called as Sarpagandha. It is an evergreen plant, perennial, glabrous and erect under shrub grows to the height of 15-45 cm. It is widely distributed in sub-Himalayan tract from Punjab eastwards to Nepal, Sikkim, Bhutan, Assam, in the lower hills of the Gangetic plains, Eastern and Western ghats, in central India and in Andamans.

Rauwolfia serpentina Benth. is a medicinal plant and is gifted with unique alkaloids that have remarkable medicinal properties. Reserpine alkaloids are present in root, stem, leaves and seeds of plant and percentage of alkaloids depends on the geographical place from where the plant is collected. Rauwolfia alkaloids extracted

from the roots, leaves and seeds, medically it is important therapeutic agent both as antihypertensives and as sedatives. It is also used for relief of various nervous system disorders both psychic and molar including anxiety status, excitement and maniacal behaviors. Roots extract are employed for the treatment of intestinal disorders, diarrhoea, dysentery and anthelmintic. It is believed to stimulate uterine contraction and recommended for the uses in child birth. Juice of leaves has been used as remedy for corneal opacity.

Rauwolfia alkaloids, pharmacologically can be divided into reserpine and ajmaline groups, while reserpine is used for sedative action in mild anxiety states chronic psychoses and in treatment of chronic schizophernic patients. Seeds also contains alkaloids, fresh seeds are heavier than water, contain 0.2-0.3% alkaloids. Hindus used this plant for centuries as febrifuge and as an antidote to the bite of poisonous snakes. It is used to treat dysentery and other painful intestinal infections. In ayurvedic system of medicine product ranges as confide (Speman Forte), Lukol, Serpina etc.

Material & Methods

Isolation of polysaccharide

Seeds (250gm) of *Rauwolfia serpentina* Benth. were collected from F.R.I. Dehradun, then washed the seeds with water dried, crushed to a greyish powder. Powdered seeds (100gm) were dissolved in distilled water (800ml) for 24 hrs. The contents were stirred by mechanical stirrer then the viscous solution was filtered by muslin cloth, it was again filtered by sharpel's super centrifuge to remove all suspended particles. Filtrate was precipitated with ethanol to precipitate out all polysaccharide in light brown form. At the time of precipitation of polysaccharide was stirred by mechanical stirrer. The precipitate of polysaccharide was filtered through sintered funnel G-3 under suction and dried in vaccum at 60°c after washing with acetone and petroleum ether. It was obtained as a greyish powder (8.67 gm) had sulphated ash 1.84%, optical $\{\alpha\}^{25}{}_{D}$ + 31.2°c H_2O.

Methylation of Polysaccharide

The methylation studies of purified polysaccharide (6gm) from *Rauwolfia Serpentina* Benth. seeds (Haworth, 1915) was done by dissolving in 50 ml water and dimethyl sulphate (50ml) and sodium hydroxide solution (40%, 140ml) were added in a small quantity during a period of 8 hrs duration with constant mechanical stirring at 4-5°c make up the volume to 250ml with distilled water. The reaction mixture was kept in dark at 4-8°c in an atmosphere of nitrogen. After treating the reaction mixture, was kept on stirring overnight and excess of dimethyl sulphate was decomposed by heating over boiling water-bath for 2 hrs. The resultant mixture was filtered and obtained filtrate was diluted with water to dissolve unreacted sodium hydroxide and partially neutralized with sulphuric acid (1N) in freezing mixture. The precipitated sodium sulphate was filtered and aqueous filtrate was extracted with chloroform. The complete neutrilization was done by dilute acetic acid, the solution was dialysed in running water for 6 days to remove the dimethyl sulphoxide and other inorganic salts.

The resultant solution was concentrated to 100 ml and extracted with chloroform in a liquid-liquid extractor for 28 hrs. The chloroform extract was dried with anhydrous sodium sulphate, filtered, concentrated then dried under high vaccum to light yellow glassy mass (5.125gm). The depleted water portion was filtered, concentrated to a small volume and again extracted with chloroform for 22 hrs. The chloroform extract was dried with anhydrous sodium sulphate and concentrated to a glassy brownish yellow solid. The yield of total methylated product was obtained (5.120gm) and found-OCH_3, 44.56%, which exhibited hydroxyl group(OH) on absorption band at 3500-3600 cm^{-1} in I-R spectra (KBr).

The partially methylated coumpound was further remethylated by Hakomari's method(1964). The methylated product was dissolved in distilled dimethyl sulphoxide (100 ml) by mechanical stirrer in an inert atmosphere of nitrogen for 3 hrs. Sodium hydroxide (2 gm) was added to the solution in a small portion during a period of 24 hrs then the contents were stirred at room temperature for further four hours till the evolution of hydrogen gas ceased. Methyl iodide solution (10ml) was added drop-wise to the reaction mixture in a period of 1 hr and the stirring continued for 10 hrs more. For further addition of sodium hydroxide (2gm) in 20 ml dimethyl sulphoxide and methyl iodide (5ml) were made on successive days. Chloroform (300ml) was added to abstract the reaction mixture. A drop of this extract gave neutral test when added to water and it spotted on a pH paper. The chloroform solution was filtered to remove the precipitated sodium iodide and the filtrate was washed thoroughly with distilled water and concentrated to syrup (20 ml). This syrup was dialyzed against running water and concentrated to a syrup (20 ml).This syrup was dialyzed against running water for 40 hrs to remove dimethyl sulphoxide and inorganic ions. The dialysed solution was concentrate (30 ml) and extracted with chloroform. The solvent layer was dried over anhydrous sodium sulphate and concentrated under high vaccum to yield a glassy yellow mass (4.86 gm). Found-OCH_3, 41.8%, showing a slight hydroxyl peak at absorption band at 3500-3600cm^{-1} region in I-R spectra (Barker et. al., 1956).

Above partially methylated polysaccharide was further remethylated three times with Purdie's reagent (Purdie & Irvine, 1903) with methyl alcohol, metyl iodide and silver-oxide which gave fully methylated seeds polysaccharide (4.56gm). Found-OCH_3, 42.82%. This methylated product did not show any hydroxyl peak at 3500-3600 cm^{-1} region in I-R spectra (KBr).

Fractionation of Methylated polysaccharide

Methylated *Rauwolfia serpentina* Benth. seeds polysaccharide (5gm) was fractionated by fractional dissolution method (Chanda et.al.1950) with a mixture of petroleum ether (40-60°) and chloroform mixture with increasing amount of latter solvent being increased from 0-25%. The mixture was refluxed on boiling water-bath (3 hrs) for each fraction. The solution was filtered and residue completely dissolved in solvent mixture containing petroleum ether (75%) and chloroform (25%). The obtained solution of each fraction was evaporated and residue dried under high vaccum (15 mm) over phosphorous pentaoxide (P2O5) to a constant weight. The specific rotation

of each fraction were taken in chloroform and methoxyl contents of individual methyl sugar fractions were determined by usual manner are given in Table – 1.

Table 1: Fraction of Methylated *Rauwolfia Serpentina* Benth. Seed Polysaccharide.

Fraction No.	State of methyl sugar fraction	Solvent composition(%)		Yield (gm)	-OCH_3 (%)	$\{\alpha\}^{25}_D$ ($CHCl_3$)
		Pet. Ether (40^0-60^0C)	$CHCl_3$			
		1	Oily liquid	100	0	0.35
2	Oily liquid	95	5	0.36	-	-
3	Crispy solid	90	10	0.56	51.6	$+72.8^0$C
4	Crispy solid	85	15	0.83	49.4	$+62.4^0$C
5	Crispy solid	80	20	0.74	43.4	$+58.2^0$C
6	Crispy solid	75	25	0.94	45	$+70.0^0$C
7	Crispy solid	70	30	0.77	30.2	$+68.2^0$C
8	Crispy solid	65	35	0.64	47.2	$+39.0^0$C

The fraction 3-8 were analytically similar and formed major component were combined and further study was carried out only on this material. The I-R spectra of this product showed complete absence of hydroxyl peak at 3500-3600 cm^{-1} absorbance region.

Hydrolysis of Methylated Polysaccharide

Hydrolysis of fully methylated seeds polysaccharide (1.76gm) was carried out in two stage (Croon, et.al., 1960). The methylated polysaccharide was obtained from fraction (3-8) were hydrolyzed (Whistler, 1965) with sulphuric acid (72%, 20ml) then mixture was kept at 20^0C for 2 hrs. The content were diluted with water to have the concentration of the solution 12% with respect to sulphuric acid and left overnight at room temperature. The reaction mixture was heated over boiling water-bath for 6 hrs and hydrolysate was neutralized with barium carbonate slurry with constant mechanical stirring for 10 hrs then it left for overnight. The neutral hydrolysate was filtered and to remove the barium sulphate and unreacted barium carbonate. The traces of inorganic impurities were removed by passing the hydrolysate through ion-exchange resins then the solution concentrated to syrup which consisting the mixture of neutral methylated sugars.

Separation of Methylated Polysaccharide

The methylated hydrolysate was separated by descending techniques of paper chromatography on whatman No. 3MM filter paper in the solvent mixture(D) and used a spray reagent to reveal the presence of six spots of methyl sugars. The methyl sugars were cut out with the help of guide spots and strips were eluted with water

according to (Dent, 1947). The eluted methyl sugars fractions were co-concentrated separately and dried under high vaccum, which were characterized, identified and results are given in Table–2. The tetra and tri methyl sugars were dissolved in chloroform, filtered and co-concentrated to syrup then it dried under high vaccum. The dimethyl sugars were not completely soluble in chloroform.

Table 2: Hydrolysis Product from Methylated *Rauwolfia Serpentina* Benth. Seed Polysaccharide

Fr.No.	Rf. Values in solbent (D)	Methylated sugars fraction
1	0.83	2, 3, 4, 6-tetra-O-methyl-D-glucose
2	0.8	2, 3, 4, 6-tetra-O-methyl-D-mannose
3	0.84	2, 3, 6-tri-O-methyl-D-glucose
4	0.64	2, 3, 6-tri-O-methyl-D-mannose
5	0.46	2, 3-di-O-methyl-D-mannose
6	0.74	2, 3-di-O-methyl-D-glucose

These sugars were purified by absolute alcohol and finally each fraction was decolorized by animal charcoal. The yield of different methylated sugar fraction were found as follows:

- fraction I : 250 mg,
- fraction II : 260 mg,
- fraction III : 250 mg,
- fraction IV : 600 mg,
- fraction V : 280 mg,
- fraction VI : 220 mg

Characterization of Methylated Polysaccharide

The resolution of neutral methylated sugars mixture were first attempted on cellulose column chromatography by elution with the petroleum ether ($60\text{-}80^0$) and n-butyl alcohol (1:1) successively but no homogenous fractions could be obtained. Preparative partition paper chromatographic technique on whatman No. 3MM filter paper with solvent mixture (A). The paper strip corresponding to the individual methyl sugars were eluted with water according to the Dent's method. This furnished the six methyl sugar fractions which were characterized and identified as follows :

Fraction – I : 2, 3, 4, 6 – Tetra-O-Methyl-D – Glucose

The methyl sugar syrup (250 mg) moved as a single spot corresponding to D-glucose on paper chromatogram in solvent mixture (A). It had Rf 0.83 in solvent mixture (D) and Rg 1.00 in solvent (A), m.p. 87^0C, $\{\alpha\}^{25}_D$ $+72.8^0$C(H2O).Found:-OCH3, 51.4%. Calculated for (C10H20O6) requires,-OCH_3, 51.6%.

Fraction-II: 2, 3, 4, 6-Tetra-O-Methyl-D-Mannose
Methyl sugar syrup (260 mg) gave D-mannose on paper chromatogram in solvent mixture (D). It had Rf 0.80 in solvent (D) and Rg 0.97 in solvent (A) optical rotation $\{\alpha\}^{25}_{D}$ +62.4^{0}C (CHCl3). Dimethylation of sugars (50 mg) with usual manner gave only one hexose sugar parallel to the D-mannose was non-reducing sugar. Found :-OCH3, 48.8%. calculated for C10H20O6 required 49.4%.

Fraction-III: 2, 3, 6-Tri-O-Methyl-D-Glucose
The methyl sugar syrup (250mg) gave single spot parallel to D-glucose on paper chromatogram in solvent mixture (D). It had Rf 0.84 in solvent (D) and Rg 1.06 in solvent (A), optical rotation $\{\alpha\}^{25}_{D}$ + 58.2^{0}C (CHCl3). Found-OCH_3, 42.8%, calculated for C9H18O6 requires-OCH3, 43.4%.

Fraction-IV: 2, 3, 6-Tri-O-Methyl-D-Mannose
Methyl sugar syrup (600 mg), had Rf 0.64 in solvent mixture (D) and Rg 0.92 in solvent (A), m.p. 107-108^{0}C, optical rotation $\{\alpha\}^{25}_{D}$ +70.0^{0}C ($CHCl_3$). It was pure chomatographically on paper chromatogram in solvent on demethylation, it gave a single elongated spot parallel to D-mannose on paper chromatographic examination.

Fraction-V: 2, 3-Di-O-Methyl-D-Mannose
Methyl sugar syrup (280 mg) gave a single spot of D-mannose on paper chromatogram in solvent (D). It had Rf 0.46 solvent mixture (D) and Rg 0.61 in solvent (A), m.p. 107-108^{0}C, optical rotation $\{\alpha\}^{25}_{D}$ +68.2^{0}C (CHCl3).

Fraction-VI: 2, 3-Di-O-Methyl-D-Glucose
Methyl sugar syrup (220 mg) gave a single spot parallel to D-glucose on paper chromatogram in solvent mixture (D). It had Rf 0.74 in solvent (D) and Rg 0.50 in solvent (A), optical rotation $\{\alpha\}^{25}_{D}$ +39^{0}C (CHCl3) by Bell (1948). It gave D-glucose on dimethylation by usual manner. Found :-OCH3, 46.4%, calculated for C8H16O6 required-OCH3, 47.2%.

Quantitative Estimation of Methylated Sugars
Methylated sugar mixture (1.5gm) was quantitatively estimated by alkaline hypodite method (Hamilton and Smith, 1956) and separated by paper chromatography in solvent on whatman No. 3 MM filter paper.

All six methylated sugars fractions (5 ml) were collected separately in ground stopper (Dent, 1950) flasks and iodine solution (0.1 N, 1 ml) was added. A solution (2 ml) containing sodium bicarbonate (0.2M) at pH 10.6 was pipetted into each sugar solution flask and they were stoppered. To prevent the loss of iodine due to the evaporation the stoppers were moistened with the drop of potassium iodide solution (10%) and kept for 3 hours. The reaction mixtures were diluted with water (25 ml) and then acidified with sulphuric acid (2N) carefully to avoid the vigorous evolutions of carbon dioxide. The liberated iodine was titrated against sodium thiosulphate

(hypo) solution (0.1N) using starch as an indicator. A blank reading was also carried out simultaneously under similar conditions. The Molar ratio of the Rauwolfia serpentina Benth. seeds methylated sugars are in Table-3.

Table 3

Fr.No.	Methylated sugars fraction	Molar Ratio
1	2, 3, 4, 6-tetra-O-methyl-D-glucose	1
2	2, 3, 4, 6-tetra-O-methyl-D-mannose	1
3	2, 3, 6-tri-O-methyl-D-glucose	1
4	2, 3, 6-tri-O-methyl-D-mannose	4
5	2, 3-di-O-methyl-D-mannose	1
6	2, 3-di-O-methyl-D-glucose	1

Result & Discussion

Seed polysaccharide was subjected to methylation by Haworth (1915) and Hakomari's method (1964) by three times successively with dimethyl sulphate, sodium hydride, dimethyl sulphoxide and sodium hydroxide (45%), yielded a light yellow product. The partially methylated polysaccharide having –OCH3, 41.8%, exhibited a slight absorption band of hydroxyl group at 3500-3600 cm^{-1} region in I-R spectra (KBr).

The above partially methylated product was further remethylated by purdie's reagent with methyl alcohol, methyl iodide & silver oxide with three times to get a fully methylated product. The fully methylated polysaccharide having,-OCH3, 42.82% which gave no hydroxyl peak at 3500-3600cm^{-1} region in I-R spectra (KBr). The fully methylated polysaccharide was fractionated with petroleum ether (40-60^{0}C) and chloroform mixture containing increasing proportion of latter, obtained 8 methyl sugar fractions out of them 2 are in oily form while 6 are as crispy solids.

The purified methylated polysaccharide was hydrolysed with sulphuric acid (72%) was found to contain a mixture of 6 methylated sugars (Whistler, 1965). The mixture of hydrolysed methylated sugars was separated on Whatman No.3MM filter paper, purified and identified by fractional dissolution method (Chanda, et.al., 1950), also identified by determining their specific rotation, methoxyl contents, demethylation analysis and preparing their suitable crystalline derivatives and molar ratio of the methylated sugars as determined by the alkaline hypoiodite method (Hamilton & Smith, 1950).

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