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# Linkage Analysis in an Nonasaccharide from Prosopis juliflora by Methylation, Periodate Oxidation and NMR studies.

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**Abstract:** *Prosopis juliflora* (Sw.) DC. commonly known as mesquite, vilayti babul, vilaytikikar belongs to family leguminosae sub family (mimosoidae). *P. juliflora* is often quoted as native to the Caribbean where it is found often in coastal areas.It's a thorny ever-green to semi-evergreen fast growing tree capable of growing in arid and semi-arid regions and attaining heights up to 10m. A polysaccharide was isolated from *Prosopis juliflora* seed and purified. Purified *juliflora* polysaccharide has been partially hydrolysed to get structural oligosaccharides. An oligosaccharide has been isolated from the hydrolysed product of *Prosopis juliflora* seed polysaccharide. On complete hydrolysis it furnished two monosugars, galactose and mannose in the molar ratio of 5:4. Methylation,$^{1}$HNMR,$^{13}$C NMR studies and periodate oxidation indicated the presence of (1→4) and (1→6) linkages among structural monosaccharide units.

**Keywords:** Prosopis, Nonasaccharide, Methylation, NMR, Periodate Oxidation, D- Galactose and D- Mannose.

## Introduction

*P. juliflora* commonly known as mesquite, vilayti babul, vilaytikikar belongs to family leguminosae sub family (mimosoidae). *P. juliflora*is often quoted as native to the Caribbean where it is found often in coastal areas, but several authors have suggested that it was introduced possibly with the arrival of the first human settlers from Venezuela (circa 0-1000 AD). It is a large shrub to a small evergreen tree. Usually tree attains a height of 9-12m and a girth of 90cm. Under favourable conditions the tree attains a height of 18m. If repeatedly coppiced from ground level it maintains as a multistemmed shrub of 3-4m in height. Plant thrives well under hot dry climate with mild winter and scanty rainfall. The optimum absolute maximum shade temperature required by the plant is between 40.5-45.5˚C and the mean annual rainfall requirement is 150-750mm. It can tolerate pH upto 11.5 [1]. Prosopis wood is moderately heavy with specific gravity 0.83 to 0.88 air dry and 0.781 oven dry. The wood weighs about 800kg/m$^3$ and is chunked or chipped and sold as a special wood or charcoal for barbeques in the USA and in Australia and found to have strong resistance, considerably stronger than that of teak (*Tectonagrandis*), particularly when dry [2].Bark of the tree exudes gum that forms adhesive mucilage and can be used as an emulsifying agent. The gum is also used as an adulterant and substitute of gum arabic and proves to be an excellent source of sugar. From ethno-botanic viewpoint, it shows medicinal properties in particular for treating pharyngeal ailments, dysentery and to strength teeth. The average annual gum yield from a mature *P. juliflora* tree was estimated to be 1 kg/tree/yr, with a range of 0.25-2.5 kg/ tree/yr[1].

Fruit is indehiscent pods that are of high nutritive value as a fodder and food crop. Dry P. juliflora pods 82.7% dry matter, 9.91%crude protein, 0.83%ether extract, 54.16% soluble carbohydrates, 25.9%cell wall constituents, 37.6%ash (mineral residue), 0.16%calcium and 0.13%phosphorus [3]. It provides good fodder for livestock and in some places more important is the usefulness of its pods as a fodder and food crop. This plant may be associated with fodder palm (Opuntiaficusindica) and buffel grass (Cenchrusciliaris), increasing considerably the fodder production per unit. The protein replenishing efficiency obtained with *P. juliflora* pod flour did not differ significantly with the type of malnutrition provoked and correspond to 68% of casein repletion efficiency the reference protein recommended by FAO (WHTR-3/UNUP-129.1980). The leaves are rich in plant nutrients especially nitrogen and are therefore useful green manure. They contain phosphorus, nitrogen, potassium and calcium. The leaves contain 0.8% of a dark green wax (m.p.74.5). Leaves possess many antibacterial and medicinal properties [1].

There are number of reports on chemical constituents isolated from the different parts of *Prosopisjuliflora* viz. root, stem, leaf, bark, pods and flowers which have been reported in literature [4]. *P. julifora* seed gum solution in water is used as viscosity improver for fracturing geological formations, stable to bacteria, with low residue content and high viscosity was reported [5]. The use of polysaccharide from endosperm of *P. juliflora* as a thickening agent was reported [6].

*P. juliflora* is a widely distributed weed, and is a cheap and promising source of galactomannan polysaccharides, but not being utilized presently. So, further research on detailed knowledge of the chemical nature of this type of polysaccharide is of interest to the plant chemist for its further utilization.

*P.juliflora* polysaccharide produces one hepta-, one octa-, and one nonasaccharide on partial hydrolysis. In the present paper methylation, $^{1}$H NMR, $^{13}$C NMR studies and periodate oxidation results of Nonasaccharide has been discussed. The present paper is a part of complete structure elucidation of *P.juliflora* polysaccharide.

## Experimental and General Methods of analysis

Solutions were concentrated at or below 40$^{0}$C in a rotary evaporator under reduced pressure. All melting points are uncorrected. Optical rotation was determined on Autopol-II, automatic polarimeter (Rudolph Research, Flanders, New Jersey) at 589nm, D-lines of sodium. Paper chromatography was carried on Whatmann 1 and 3mm filter paper sheets using the following solvent systems; n-Butanol: Ethanol: Water (4:1:5, $S_1$) upper layer; n-Butanol: Pyridine: Water (6:4:3, $S_2$) and Ethyl acetate: acetic acid: n-Butanol: Water (4:3:2:2, $S_3$). Detection was effected with acetonical silver nitrate ($R_1$) and Aniline phthalate spray ($R_2$). Detection with acetonical silver nitrate (solution of $AgNO_3$, water and acetone) was done by treatment with following agents: (a) To silver nitrate solution (12.5 g in 10ml water), one litre acetone was added with continuous shaking. Distilled water was added dropwise with stirring until the white precipitate completely dissolved to form a clear solution. (b) Sodium hydroxide (20g) was dissolved in 400ml of ethanol. (c) Aqueous ammonia solution.

The dried chromatograms were dipped and passed through reagent solution (a) for about 5min, dried at room temperature and passed through reagent (b); when the dark brown spots were visualized the paper was dipped in reagent (c) for some time with shaking (5-10min). Finally the chromatograms were washed with water and dried in air [7].

Gas liquid chromatography of the sugar mixtures was carried out on a Shimadzu Gas Chromatograph GC-9A fitted with flame ionization detector (FID). The samples were analyzed in the form of their alditol acetates on ECNSS-M (3%) on Gas chrom Q (100-200mesh) packed into 5’×1/8” stainless steel column under the following operating conditions. (Column temperature 170$^{o}$C, nitrogen flow rate 35-40ml/min ($C_1$).

### Isolation of polysaccharide

The seeds of *Prosopisjuliflora* were collected from Jodhpur, Rajasthan. The moisture content of seeds was found to be 11%. The seeds were air dried in shade to moisture content of 0.09%. Endosperm (20 g) was isolated by removing seed coat and germ by impact grinding using high speed domestic grinder. Seed coat was soaked in cold water for 3 hrs and endosperm was isolated from swollen seed coat by separating the seed coat with the help of forceps which was stirred vigorously in distilled water (1000 ml) for 5h at room temperature and centrifuged to remove water insoluble impurities. The supernatant solution was poured into three times its volume of ethanol with constant stirring. The polysaccharide was precipitated out in the form of a fluffy precipitate. The precipitate was again dissolved in water and added to ethanol. Precipitate was treated successively with dry solvent ether and acetone. It was filtered under vacuum and dried in vacuum desiccator at room temperature. The polysaccharide (18.0g) so obtained was deionised by passing the aqueous solution successively through the columns of freshly regenerated cation [Dowex-50W-X8] and anion [Seralite-SRA-400] exchange resins. The columns were washed with distilled water until the washings showed a negative Molisch test for carbohydrates. The combined eluents were concentrated to small volume [1/4th] and subjected to further purification by dialysis. In the process, the concentrated product was transferred into a cellophane bag and dialyzed for 72h in running water. The dialyzed product was concentrated and re-precipitated with a large volume of ethanol to obtain finally the pure polysaccharide. It was kept overnight, alcohol was decanted off, and the precipitated polysaccharide was dried by treating with solvent ether, acetone and absolute ethanol. It was filtered and lyophilised at -40$^{o}$C to obtain finally the pure polysaccharide in the form of a white amorphous powder (16.1g).

**Partial hydrolysis**

Polysaccharide (5.0g) was heated with sulphuric acid (0.05 N, 100 ml) on steam bath at 100$^{o}$C for 3 h. The hydrolysate was cooled, neutralized with saturated solution of barium carbonate till neutral pH and filtered. The combined filtrate was concentrated to syrup (4.73g). The mixture of oligosaccharides and monosaccharide was resolved into its components by preparative chromatography on whatmann No.3 mm filter paper sheets using solvent system $S_2$. The strips corresponding to individual oligosaccharides were eluted with water, elutes were concentrated separately, to obtain the three oligosaccharides. Homogeneity of the oligosaccharides was checked by paper chromatography in solvent system $S_1$, $S_2$, $S_3$ using $R_1$ and $R_2$ as spray reagent.

**Methylation of oligosaccharide**

Oligosaccharide (0.0321g) was methylated completely according to the method of Hakomari [8] using sodium hydride-dimethylsulphoxide followed by Purdie [9]. Each methylated oligosaccharide was recovered by chloroform extraction. After evaporation of the chloroform extracts to dryness, the residues were hydrolyzed with formic acid (90%, 10ml) for 1h on steam bath at 100$^{o}$C, the solutions evaporated and treated with aqueous sulphuric acid (0.13 M, 15ml) for 18h on a steam bath. The partially methylated compound was subjected to Purdie’s methylation by dissolving it in methyl iodide (5 ml) with stirring under inert atmosphere and silver oxide (0.50 g) was added periodically in 4 h and reaction was allowed for 6 h. This process was repeated two times on the successive days. The methylated oligosaccharide processed further as above, and obtained in the syrup form with the yield (0.0171g)

**Preparation of alditol acetates**

Alditol acetates of the hydrolyzed material were prepared by the method as described [10]. Sodium borohydride (0.020g) was added to hydrolyzates, and the mixture was kept for 18h at room temperature. The mixture was neutralized by slow addition of dilute acetic acid (6ml), and concentrated to dryness in vacuum rotator at 40$^{o}$C. Sodium was removed by passing it through cation exchange resin (Dowex-50 W-X8). Boric acid was removed by co distillations, in the vacuum rotator with methanol (3 × 5ml). The residue was treated with redistilled acetic anhydride and pyridine, 1:1 (4ml) and refluxed for 6h. Toluene (6ml), which gave an azeotrope with acetic anhydride, was added and the mixture was distilled as above, until the rate of distillation decreases. A new portion of toluene (6ml) was added and the solution was concentrated to dryness. It was dissolved in water (10ml) and the acetylated sugars separated by shaking with dichloromethane (4 × 25ml). Traces of water present in dichloromethane were removed by adding anhydrous sodium sulphate followed by filtration and washing with dichloromethane before concentration.

**Periodate oxidation**

To a solution of oligosaccharide (0.0510 g in 25 ml) in water an aqueous solution of sodium metaperiodate (0.2 g in 50 ml) was added and the volume of the resultant solution was made upto 100ml. A blank solution of sodium metaperiodate (0.2 g in 100 ml) was also prepared. These were kept in dark at room temperature (25$^{o}$C) for 192 h. To determine periodate consumed, an aliquot (5 ml) of the periodate reaction mixture was added to a solution containing distilled water (20 ml), potassium iodide (20%, 2 ml) and sulphuric acid (0.5N, 3 ml). The liberated iodine was immediately titrated with 0.1N sodium thiosulphate solution using starch as an indicator [11-13].

Liberation of formic acid was determined by the methods reported earlier [11, 14-16]. To an aliquot (5 ml) of the periodate reaction mixture was added acid free ethylene glycol (0.5 ml), followed by an excess of potassium iodide (20%, 5 ml) after 10min. To the above solution an excess of 0.01 N sodium thiosulphate was back titrated with 0.01 N iodine solution using starch as an indicator. A blank solution was titrated concurrently.

## Result and discussion

The polysaccharide was isolated from the endosperm of seeds in 81% yield as detailed in experimental section.

### Partial hydrolysis

*Prosopisjuliflora* seed endosperm polysaccharide upon hydrolysis with dilute sulphuric acid (0.05N, 3h) furnished a mixture of oligosaccharides along with monosaccharides. Preliminary paper chromatographic examination of the hydrolyzates revealed the presence of three oligosaccharides along with 2 monosaccharides, D-galactose and D-mannose. The Rgal values of oligosaccharides were 0.31, 0.19 and 0.11 respectively in the solvent system $S_2$. From this mixture, the oligosaccharides were separated by preparative chromatography on whatmann No.3 mm sheets and each oligosaccharide eluted separately and the elutes combined to isolate pure polysaccharide. The homogeneity of the oligosaccharides was checked by paper chromatography using organic solvent systems $S_1$, $S_2$ and $S_3$ and spray reagents $R_1$ and $R_2$. The degree of polymerization of three oligosaccharides corresponds to one hepta, one octa and one nonasaccharides. The nature and sequence of glycosidic linkages was determined by methylation analysis, periodate oxidation of the oligosaccharide.

### Linkage analysis of Nonasaccharide

Thisnonasaccharide [α] + 129$^{0}$ (c 0.05%, $H_2O$), m.p. 240-242$^{0}$C (d), was found homogenous and gave single spot [Rgal 0.11] using organic solvent $S_2$ and spray reagents $R_1$ and $R_2$ upon chromatographic examination. Upon complete acid hydrolysis, it gave D- galactose and D- mannose on paper chromatogram in solvent systems $S_1$, $S_2$ and $S_3$. The alditol acetates of the hydrolysate of oligosaccharide on GLC analysis under conditions C-1 showed two peaks corresponding to D-galactose and D- mannose in the molar ratio of 5:4.

The nonasaccharide was completely methylated by Hakomari method [8] followed by Purdie [9]. Complete methylation was confirmed by IR spectrum of the methylated octasaccharide which showed complete absence of –OH band (3590-3225cm-1). It was hydrolysed and transformed into its alditol acetates according to the method ofJannson [10]. GLC of the resulting alditol acetate under conditions C1, furnished 2, 3, 4, 6- tetra-O-methyl-D-galactose, 2, 3-di-O-methyl-D-mannose, 2, 3, 4, 6-tetra-O-methyl-D-mannose in the molar ratio of 4:4:1 respectively.

On the basis of methylation study it was found that 2, 3, 4, 6- tetra-O-methyl-D-galactose(4 moles) and 2, 3, 4, 6-tetra-O-methyl-D-mannose (1mole) showedfive non reducing end chain groups. Presence of four units of 2, 3- di-O-methyl-D-mannose indicated 4, 6-di linked mannose and branched structure of the oligosaccharide in which non-reducing galactose unit is attached by$1\rightarrow6$ linkages to$1\rightarrow4$ linked mannose units of the backbone.

Evidence supporting the presence of $1\rightarrow4$ and $1\rightarrow6$ linkages in the framework of oligosaccharide has been obtained from the$^{1}$H NMR and $^{13}$C NMR spectroscopy. Resonances of the anomeric protons in $^{1}$H NMR spectroscopy are well separated and identified. The doublet at δ5.20 (J1, 2 ~3.3Hz) and the singlet at δ5.05 indicate that these signals have arisen from anomeric α-D-galactopyranose and β-D-mannopyranose units respectively (Fig.1).[17].

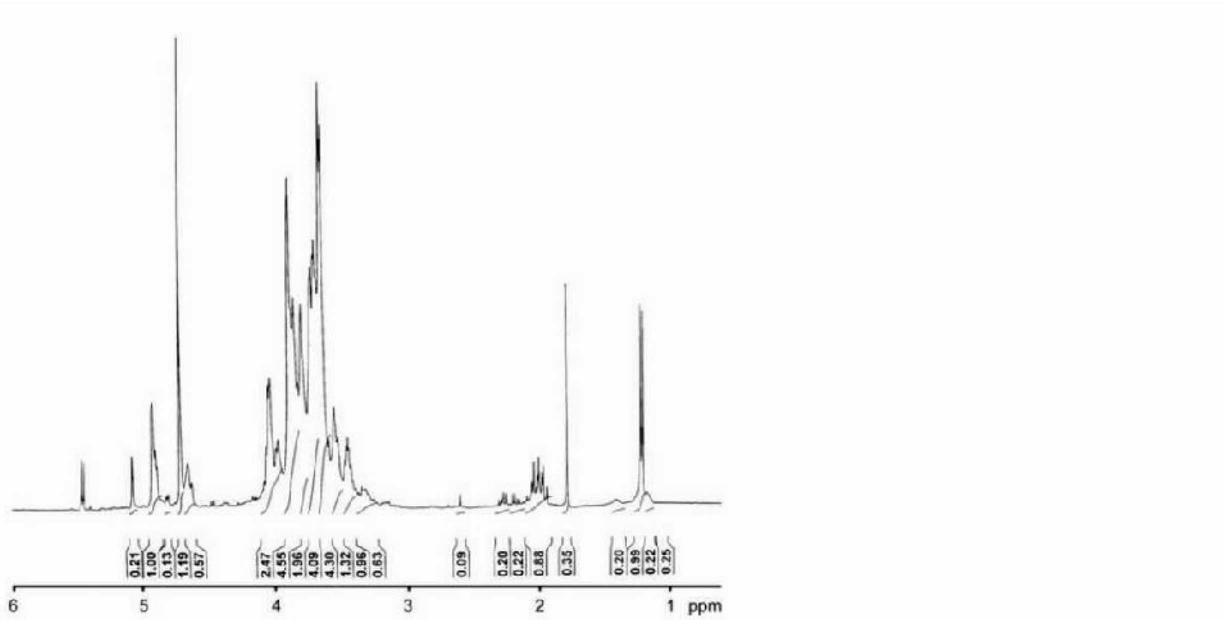

**Fig.1. $^{1}$H NMR Spectrum of nonasaccharide**

The $^{13}$C NMR spectrum showed fully resolved resonances of all carbon atoms (Table-1). The spectrum showed a signal at δ 98.6 for anomeric carbon of D-galactose residues [18]. A signal of anomeric carbon of D-mannose units at δ 99.6 appeared, which represents non-substituted and non-reducing end residues Further a signal was also observed in the anomeric region at δ 100.2 which corresponds to the substituted mannosyl residues and reducing end of the mannan backbone[19].

**Table-1: Assignments of Peaks in the $^{13}$C-NMR spectrum of the Nonasaccharide**

| Type of unit | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|
| α-D-Galactopyranosyl | 98.6 | 68.4 | 69.3 | 69.5 | 71.7 | 61.2 |
| β-D-Mannopyranosyl residue, unbranched at O-6 | 99.6 | 69.9 | 71.3 | 76.8 | 74.9 | 60.8 |
| β-D-Mannopyranosyl residue, branched at O-6 | 100.2 | 69.9 | 71.3 | 76.8<br>77.1 | 73.3 | 66.9<br>66.4 |
| β-D-Mannopyranosyl (nonreducing) end-chain group | 99.6 | 69.9 | 71.3 | 76.8 | 74.9 | 60.8 |
| β-D-Mannopyranosyl (reducing) end-chain residue | 100.2 | 69.9 | 71.3 | 77.1 | 73.3 | 66.4 |

The resonances of C-2, C-3 and C-5 of galactopyranosyl and mannopyranosyl units are well resolved lines and readily identified. C-4 of mannopyranosyl units produced two signals (at δ 76.8, 77.1) corresponding to branching patterns A and B. Branching pattern indicates the distribution of D-galactose on the mannan backbone [20-21].

Signals in the doublet result from the two nearest neighbor probabilities of mannopyranosyl units in the main chain [20]. In the spectrum, peak B is more prominent in comparison to A (~60% of B) (Fig.2). It clearly indicates the larger proportion of the galactopyrnosyl units in comparison to the mannopyranosyl units[20]. A signal at δ 76.8 (C-4) also corresponds to the β-D-mannopyranosyl non-reducing end chain group[19].

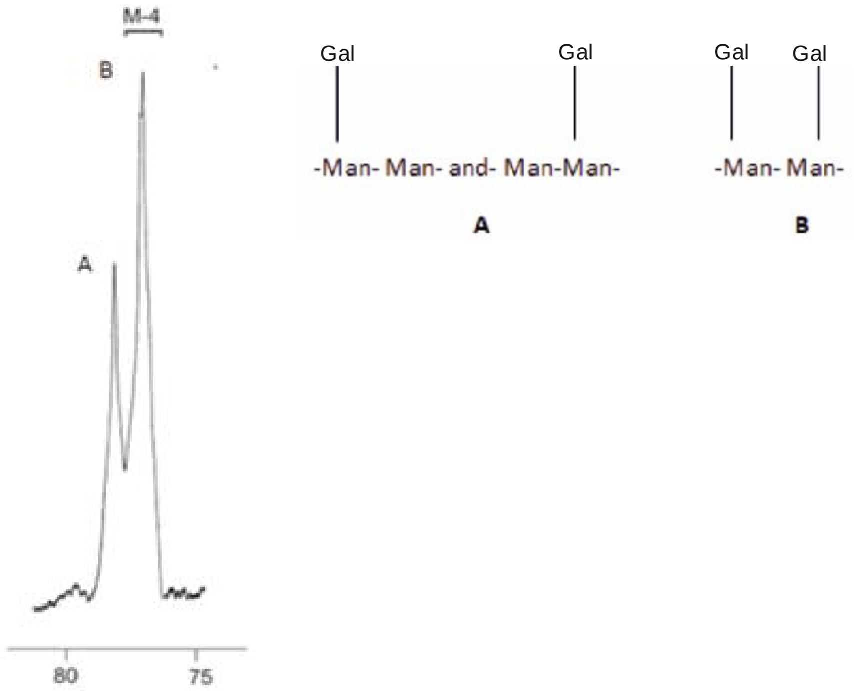


**Fig.2. $^{13}$C NMR Spectral region of C-4 (mannose); A and B are probable diad interpretation**

It was observed that substituted mannose units are branched at O-6, the resonances of their C-6 were readily identified suggesting that peak at lowest field (δ 66.9 ppm) originates from the C-6 resonance of the intermediate unit from groups of three contiguous, substituted mannosyl residues (Triad I)and peak at (δ 66.4 ppm) originates due to superposition of signals from triads wherein two contiguous mannosyl units are substituted [22] (Triad II) (Fig.3).

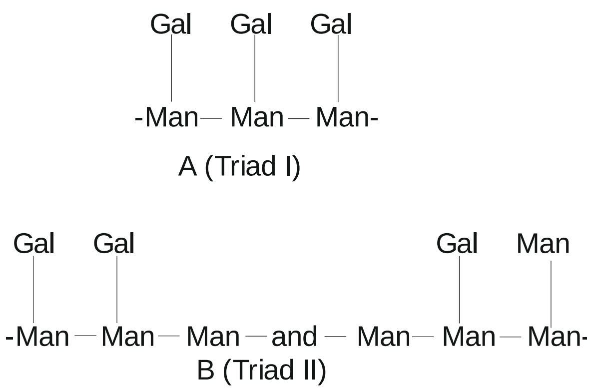


**Fig.3. $^{13}$C NMR Spectral region of C-6 (mannose); A and B are probable triad interpretation**

These results suggest that the oligosaccharide has the fundamental structural pattern having β-(1→4) main chain of D-mannose units and side chain of single α-(1→6)-D-galactose residues.

Evidence supporting the presence of 1→4 and 1→6 linkages in the framework of oligosaccharide has been obtained from the results of periodate oxidation [11- 16]. The oligosaccharide when subjected to oxidation with sodium metaperiodate, consumed 1.64 mol of periodate per anhydrohexose unit and released 0.61 mol of formic acid per anhydrohexose unit. As the oligosaccharide structure consists of 9 sugar residues, the above results can be interpreted in terms of the consumption of 14.76 mol of periodate with simultaneous liberation of

5.49 mol of formic acid by the repeating unit of oligosaccharide. Thus, periodate oxidation data of oligosaccharide as calculated on the basis of proposed structure and obtained experimentally are found to be in close agreement with each other. Therefore, above structure proposed on the basis of methylation analysis and NMR spectroscopic studies gets corroborated by the oxidation studies.

## Conclusion

From the results of methylation, periodate oxidation and NMR studies of the oligosaccharide obtained from *P. juliflora* seed polysaccharide, it can be concluded that it contains two type of linkage i.e. $1\rightarrow4$ and $1\rightarrow6$ in its framework. The linkage sequence also indicated that in the nonasaccharide, non reducinggalactose units is attached by ($1\rightarrow6$) linkages to ($1\rightarrow4$) linked mannose units of the backbone. On the basis of the results of methylation and periodate oxidation study, structure represented in fig.4 justify the linkage position in Prosopis polysaccharide.

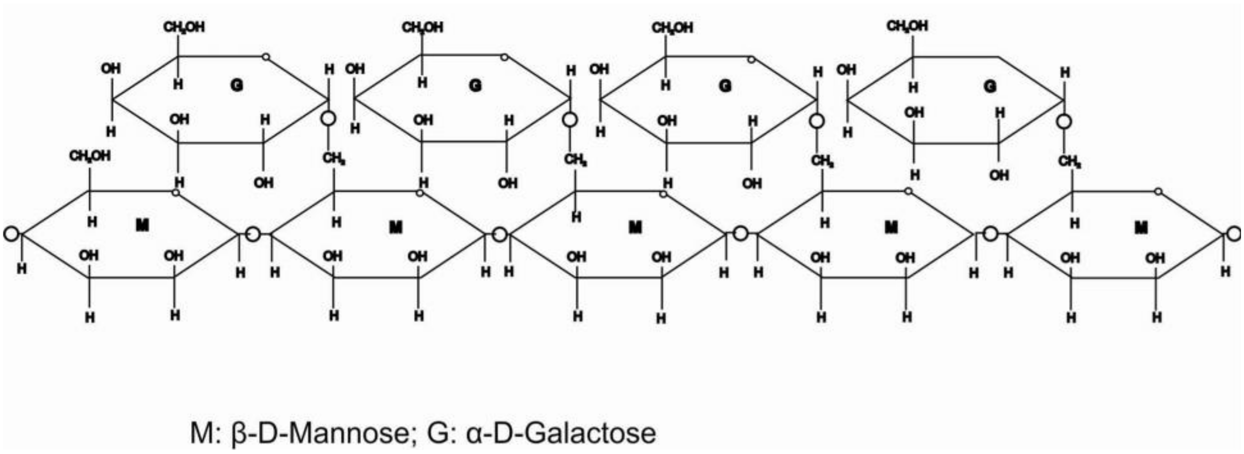


**Fig.4. Structure of nonasaccharide showing $1\rightarrow4$ and $1\rightarrow6$ linkages**

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