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Structural and Phytochemical Investigation of Water Soluble Polysaccharide Isolated from *Cassia Senna Tora* (L.) Seeds

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Abstract – Senna tora (L.) (Family: Fabaceae) is a fast growing and spreading Indian shrub. Senna tora produces in rural area of Bastar, Bilaspur, Durg, and Sarguja district of Chhattisgarh, India The Sarguja District accounts for more than 60 % of the Charota production of the State. The seeds have been found to be an alternative source of commercial gums and are used as an anthelmintic, digestive, and to treat piles, skin diseases, and abdominal troubles. The present investigation deals with isolation, purification and characterization of galactomannans from the seeds of Senna tora. Gum obtained from the seeds of S. tora is known as 'Panwar gum'. Chemically it is neutral heteropolysaccharide of D-galactose and D-mannose with molar ratio 3:4. The galactomannan extraction was based on mechanical separation of the endosperm, water dissolution, centrifugation and precipitation with acetone. The characterized polysaccrides from the gum has the basic structure of galactomannans with a main chain of (1→4)-linked β-D-mannopyranosyl units to which single α-(1→6)-D-linked galactopyranosyl units.

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1. INTRODUCTION:

Senna tora (L.) belonging to the *family Fabaceae* is an annual under shrub which grows all over the tropical countries (India, Pakistan, Bangladesh, and west China). *Cassia Senna tora* is a local annual weed found all over in Chhattisgarh, Maharashtra and Madhya Pradesh during rainy season. It is commonly known as Ponwar (Hindi), Tarota (Marathi) and Charota (Chhattisgarhi). It is also known as "Chakramard" in Ayurveda [51, 52]. In view of the easy availability of the plant, the medicinal value of the seeds and the high demand of seed gums throughout the world, the proper- ties of the seed polysaccharide (gum) obtained from *S. tora* were investigated. The present investigation was aimed at isolation and characterization of purified polysaccharide from the seeds of *S. tora* in order to survey its potential applications as an additive/excipient to nutraceuticals and pharmaceuticals. objective of the present research work was extraction of polysaccharide from *Senna tora* seeds and further characterization The S. tora seed is composed of hull, endosperm, and germ. Seeds of *S. tora* contain about 23.2% of proteins, rich in all essential amino acids, particularly, methionine and tryptophan. Several compounds belonging to anthraquinone and naphthopyrone groups have been isolated from seeds of this plant

The current investigation found that galactomannan consisting of D-galactose and D-mannose in the molar ratio 3 : 4 has been isolated from the seeds of Cassia S. tora Hydrolysis of the methylated polysaccharide resulted in three methylated sugars: (a) 2,4-di-O-methyl-D-mannose, (b) 2,4,6-tri-O-methyl-D-mannose, and (c) 2,3,4,6-tetra-O-methyl-D-galactose in the molar ratio 2: 3: 2.

2. MATERIALS AND METHODS:

A. Collection of plant material:

The pods of *S. tora* were collected from Sarguja, district of Chhattisgarh in the month of October 2019, The seeds were manually separated dried and kept in a cool, dry place until further use.

B. Isolation of galactomannans:

The seed consists of an outer husk, an endosperm and the ovary or germ. Only the endosperm, which contains mainly polysaccharides, is used for the production of the Cassia gum. The splitting procedure starts with roasting of the seeds. During the roasting process the endosperm (split) remains intact and flexible, while husk and germ, which are more sensitive to heat and finally powdering to 100 mesh sizes. The powder was soaked in petroleum ether-ethanol solution (1: 1) overnight to remove lipids and then it washed with ethanol and dried in

vacuum oven. The endosperm powder of *Cassia tora* seeds (30 g) was soaked in 500 ml of distilled water and stirred under overhead stirrer for 3-4 hrs. The obtained viscous material was passed though the cloth. The marc obtained was pressed to remove the mucilage and boiled with 400 ml of water for 2-3 hrs. Viscous solution obtained was filtered through muslin cloth. The marc obtained was not discarded but it was sent for multiple extractions with decreasing quantity of extracting solvent, that is, water with the increase of number of extractions. The Extraction was continued until the material becomes free from mucilage. All the viscous solutions obtained were mixed together. An equal quantity of 15% trichloroacetic acid was added to the mixture to precipitate protein. The solution was centrifuged and the supernatant was precipitated out by addition of acetone in the ratio 1:0.5 with continuous stirring. The coagulated mucilage, which formed as a white mass, was transferred to an evaporating dish and dried in hot air oven at 40ºC, powdered, and stored in airtight containers. The extracted *Cassia tora* gum was subjected in mini grinder to grind fine *Cassia tora* gum powder.

C. Purification of gum:

Crude and impure polysaccharides were purified by the following method.

a) Repeated precipitation:

The above obtained crude gum was dissolved in warm water, re-precipitated using ethanol (1:1), dried at 40°C, powdered and stored in airtight container at room temperature. The process of dis-solution in water and precipitation with alcohol was repeated until an almost white precipitate was obtained. The dried polysaccharide was milled and sifted with a 60 mesh for further use.

b) Deproteinization:

After precipitation the mucilage was deproteinized by shaking well its aqueous solution with chloroform, and then centrifuged, when the denatured protein formed gel at interface. For removing most of the proteins used this method for several times.

c) Complexation with Fehling's solution:

After the deproteinized polysaccharide was dissolved in water and treated with Fehling's solution. The precipitated complex was centrifuged and washes with Fehling's solution. It was decomposed with 1N HCl solution and the polysaccharides regenerated by the addition of the ethyl alcohol. After dissolution and re-precipitation obtained off white coloured materials.

When pure galactomannan dispersed in cold water formed a viscous solution which failed to reduce Fehling's solution. The galactomannan shows the minimum sulphated ash 0.179% and optical rotation $[\alpha]_D^{25}$: +67.5°(water), methoxy and acetyl contents were found to be negligible and yield 25 gm.

D. Homogeneity:

The homogeneity of galactomannan was approved by the following basis.

a) Fractional precipitation:

A purified galactomannan was separated into two fractions by fractional precipitation with different volume of ethyl alcohol. The results obtained such as ratio of the building sugars and optical rotation $[\alpha]_D^{25}$: +67.5°in water were identical to that of identical to that of original polysaccharides showing it to be homogeneous.

b) Zone electrophoresis:

A part of polysaccharides was subjected to conventional zone electrophoresis [04, 05] on whatman no.-1 paper in borate buffer pH 9.2. The intensity of the characteristic yellow orange colour developed into the phenol-sulphuric acid regent, was measured in Klett-Summerson photoelectric colorimeter. A single sharp peak indicating the homogeneous nature of galactomannan by plot of absorbance against segment number.

c) Acetylation and deacetylation:

For the acetylation of polysaccharides used acetic anhydride and sodium acetate. The acetylated polysaccharides optical rotation shows $[\alpha]_D^{25}$: +27.4° in chloroform. Deacetylation regenerated with $[\alpha]_D^{25}$: +67.4° in water confirming the homogeneity of galactomannans.

d) Paper chromatographic examination in different mobile phase:

500g of pure polysaccharide dissolved in 50 ml of 2N sulphuric acid reflux for 40 hrs. on water bath. The following mobile phase used for identification of the hydrolysate polysaccharide

S. No.	Mobile Phase	Ratio of mobile phase
1	Water: 1-Butanol: Ethanol	5 : 6 : 2
2	Water : 1-Butanol : Acetic Acid	6 : 5 : 2
3	Water :1-Butanol : 2-Propanol	4 : 11 : 7

In all above three mobile phases the result was essentially the same showing homogeneity of the polysaccharide.

E. Investigation of polysaccharides:

By the complete hydrolysis of polysaccharides indicate the presence of D-galactose and D-mannose and they are confirmed by their specific optical rotation $[\alpha]_D^{25}$ in water is + 78.6° and +12.6° respectively and by melting point of D-galactose is 165-166°C and also of their derivatives penta-acetate and D-galactaric acid. And also confirmed by co-chromatography with their standard samples. After the quantitative estimation of sugar moieties by periodate oxidation showed that the ratio of the galactose and mannose was 3:4 in the polysaccharide. After monitoring by paper chromatography of graded hydrolysis 08 with 0.5 N sulphuric acids the galactose was found at 16 min. followed by mannose at 52 min.

As per result found that the presence of α-linkage galactose units on periphery as terminal groups. The way in which this building unit forms the polysaccharides has been ascertained by methylation studies. It was methylated by Haworth and Purdie [09, 10] methods to afford a semi solid yellowish mass having $[\alpha]_D^{25}$: + 47° in chloroform.

F. Component identification of sugars:

2.5g of pure polysaccharides dissolved in 2N sulphuric acid and reflux for 40 hrs. on water bath and hydrolysate substance neutralized with barium carbonate, filtered, diluted and analyzed with chromatographically.

a) Paper chromatography:

Paper chromatographic spotting of hydrolysate were applied on two sheets of whatman no.-1 filter paper, and developed mobile phase water: 1-butanol: ethanol (5:6:2) and water: 1-butanol: 2-propanol (4:11:7)

The above chromatogram dried in air and sprayed with aniline hydrogen phthalate and heated in oven at 105°C for 30 min. each chromatogram showed two brownish spot.

The RF and RG values of the two spots corresponded to D-galactose and D-mannose as given bellow in table no.-1

Table No.-1

Sugar identification	Rf in mobile phase 7		RG in mobile phase 5	
	Observed	reported	observed	Reported
D-galactose	0.19	0.18	0.08	0.08
D-mannose	0.25	0.24	0.13	0.12

The identity of D-galactose and D-mannose was confirmed by co- chromatography with authentic samples of D-galactose and D-mannose.

b) Analysis with column chromatography:

The syrupy hydrolysate was fractionated by elution from cellulose column, small sample of hydrolysate dissolved in methanol: water mix solution and examine and on the column 2x25 cm. The column was left overnight for separation with mobile phase no.5 and fraction of elute were collected in tubes. Fractions were examined by paper chromatography with standard samples of D-galactose and D-mannose. The fractions containing same sugars combined together, concentrated and recrystallized, so obtained two fractions examined as bellow.

i. Observation of first fraction:

The first fraction was solid, it was recrystallized from aqueous methanol had m.p.-131°-132°C, $[\alpha]_D^{25}$: +12.6° (water), it formed the derivative D-mannose Phenylhydrazone and m.p. - 195°-196°C (literature-199°-201 °C), so this fraction was identified D-mannose

ii. Observation of second fraction:

After recrystallization of second fraction from aqueous methanol found m.p. 165^0-166^0C, $[\alpha]_D^{25}$: +78.6° (water) and derivative D-galactose phenylhydrazone m.p.-153^0-155 ^{0}C (literature-155^0C), so above result identified that fraction to be D- galactose.

F. Structural studied of polysaccharide:

a) Methylation:

Methylation of the pure polysaccharide done by Haworth's method by using sodium hydroxide and dimethyl sulphate and then by Purdie's method using silver oxide and methyl iodide. 10 g of pure polysaccharide taken in 500 ml of round bottom flask and filtered with ground glass joint dissolved it in 150 ml of 10% sodium hydroxide solution under stirring. The mixed solution of (50% solution of NaOH) sodium hydroxide and dimethyl sulphate in the 2:1 ratio also under stirring and maintaining the temperature 38 to 42^0C. This process repeated and

then the solution was concentrated with acetone under the reduce pressure and extracted for the removing of sodium sulphate, this extraction repeated four times for the complete methylation of the process. Final extraction done by chloroform and dried over anhydrous sodium sulphate,

The partly methylated product was 9.7 g and colour was whitish brown. This product was further methylated by the Purdie's method. The partly methylated polysaccharide was dissolved in moisture free methanol in round bottom flask. The temperature of the reaction mixture maintaining at the 38° to 42°C on the water bath. A calcium chloride tube was placed at the top of the condenser to prevent the entry of the moisture at the time of reaction. Add silver oxide 10 g and methyl iodide 12 ml by the addition in 10 hrs. vise-versa of about similar quantity of methyl iodide and silver oxide. The contents were stirred continuously during the reaction after the completion of addition; reaction mixture was heated on a water bath under stirring for reflux using the calcium chloride guard tube and nitrogen gas. The total filtrate and extracts were evaporated under reduced pressure and Resulting thick material was methylated two times under the same condition. The methylated product was obtained as brownish masses.

b) hydrolysis of methylated polysaccharide:

150 mg of methylated polysaccharide was dissolved in 30 ml of 85% of formic acid and the solution was reflux for 8 hrs. on the water bath. The solution was cooled and concentrated under reduced pressure to a thick from which acid was removed under vacuum and it dissolved in 20 ml of 2N sulphuric acid and hydrolyzed for 15 hrs. on the water bath, cooled and neutralized with barium carbonate and filtered with filter paper. The precipitate washed with distilled water. The total filtrates concentrated under reduced pressure yellowish brown colour thick material obtained.

c) Identification of methylated compound:

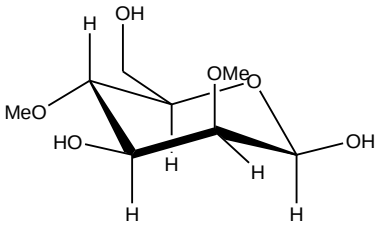
The methylated sugar was separated on whatman no.1 chromatography paper, by using mobile phase water :1-butanol : ethanol, (4:5:1) and water: 1-butanol : 2-propanol, (3:11:6). The chromatography shows only three spot after spraying aniline phthalate and drying at 110°C. The R_{TMG} value were calculated in each case and compared those reported in giving in table no.-2

Table no.-2

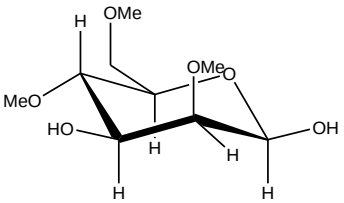
S. No.	Methylated sugars identity	Mobile phase no.-5		Mobile phase no.-7	
		R_{TMG} Found	R_{TMG} Reported	R_{TMG} Found	R_{TMG} Reported
01	2,4-di-O-methyl-D-mannose	0.62	0.63	0.68	0.69
02	2,4,6-tri-O-methyl-D-mannose	0.89	0.88	0.90	0.90
03	2,3,4,6-tetra-O-methyl-D-galactose	0.91	0.90	0.98	0.98

The methylated monosaccharide quantitative estimation done by the method of Hirst, Hough, and Jones indicated that the methylated sugars [A] structure 01, [B] structure 02 and [C] structure 03 were present in molar ratio 2:3:2 respectively in the methylated galactomannan.

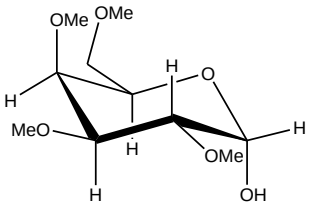
Glucose was used as reference sugar and the percentage of anhydrohexose residues calculated from methylation studies was 30.1%. Hence all the galactose units were of pyranose form as end groups from which 2, 4, 6-tetra-O-methyl-D-galactose arises, after methylation and subsequent hydrolysis of the methylated galactomannan. The formation of 3, 4-di-O-methyl-D-mannose indicated that this must be appeared from the mannose units which constitute the branch points in the polysaccharides.



Structure No. -01



Structure No.-02



Structure No.-03

d) Structure of methylated sugars:

The identification of 2,4,6-tri-O-methyl-D-mannose as one of the hydrolysis product of the methylated polysaccharide indicates its appearance from monoterminal, unbranched D-mannopyranosyl residues joined by (1→4) glycosidic bonds in the backbone of the galactomannan. Thus methylation analysis showed that the galactomannan consists of main chain of (1→4) linked D-mannopyranosyl units to which are attached at 6-position as side chain of (1→6) linked D-galactopyranosyl unit.

The foregoing evidences indicate that the simplest repeating unit of the galactomannan consists of 7 sugar moieties, out of which 3 are galactose which are present as non-reducing end groups while rest of them are 4 mannose units which form (1→4) linked main chain.

Determination of terminal group by periodate oxidation [14,15] and subsequent titration of liberated formic acid corresponds to 0.225 mole of formic acid per 100 gram of the polysaccharides after composition of 1.135 mole of metaperiodate. On the basis of these results of terminal groups were found to be 45% per repeat ion unit which is in close resemblance with that of by methylation studies 45%.

After ox polysaccharides examination in 4 hours showed the absence of D-galactose while a small amount D-mannose was detected. However prolonged oxidation (104 hours) destroyed both the hexodes. The considerable difference in rates of oxidation of galactose and mannose is probably attributable to a steric effect resolution from a ramified structure of the galactomannan. On the other hand, galactose is more accessible to the periodate regent since it is present as terminal unit only.

The result may be explained on the basis of mannose residues linked throughC1, C4 and C6, a conclusion either drawn from methylation studies. Further information regarding anomeric nature of the linkage the and sequential arrangement of hexagon have been derived from studies of various oligosaccharides obtained by acid catalysed partial fragmentation of the galactomannan.

The hydrolysate upon paper chromatographic separation on preparative scale afforded 4 oligosaccharides along with the building sugars, D-galactose and D-mannose.

The following oligosaccharides were identified.

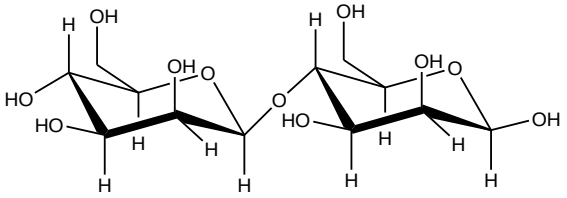
Mannobiose; β-D-mannopyranosyl (1→4)-D-mannopyranose.

Epimelibiose; α-D-galactopyranosyl (1→6)-D-mannopyranose.

Mannotriose; β-D-mannopyranosyl (1→4) - β-D-mannopyranosyl (1→4)-D-mannopyranose.

Galactosyl mannobiose; α-D-galactopyranosyl (1→6)- β-D-mannopyranosyl (1→4)-D-mannopyranose.

Oligosaccharide {1} Mannobiose; was obtained in crystalline form m.p.203-204°C, $[\alpha]_D^{25}$: - 9° (water) and to be reduced sugar. Its complete hydrolysis gave only mannose. The equivalent weight 174.5 by hypo iodide method corresponded to a hexose disaccharide. Emulsion hydrolysis showed presence of β-glycosidic linkage and osazone derivative formed m.p.203-205°C, Identical with mannobiosazone. Periodate oxidation study showed the liberation of 2.03 moles of formic acid with consumption of 4.11 moles of sodium metaperiodate per mole of oligosaccharide indicating the presence of (1→4) linkage between mannose units. On the basis of above fact, the oligosaccharide was identified as β-D-mannopyranosyl (1→4)-D-mannopyranose, (Structure No.-04).

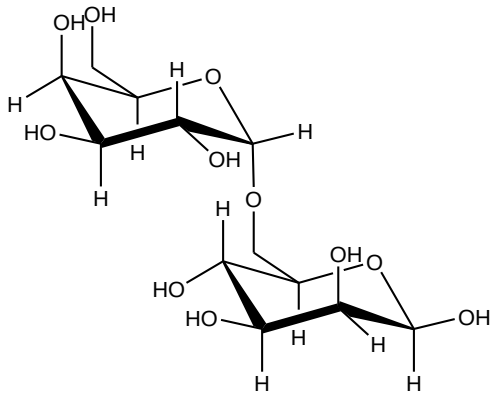


Structure No.-04

The oligosaccharides {2} Epimelibiose; α-D-galactopyranosyl (1→6)-D-mannopyranose. A crystalline sugar, m.p.-200-202°C $[\alpha]_D^{30}$: +120.5° (water). Its reduced Fehling's solutions. Paper chromatography in mobile phase pyridine: water : ethyl acetate (1:2:2), water : 1-butanol : ethanol (4:5:1) and water :1-butanol : 2-propanol (3:11:6) proved to be a single identity. Complete hydrolysis and subsequent paper chromatographic examination of hydrolysate indicated the presence of D-galactose and D-mannose in oligosaccharides.

Quantitative estimation of Hirst & Jones method showed molar ratio of two as 1:1. The equivalent weight 171.1 corresponded to hexose disaccharides and periodate oxidation showed that one mole of oligosaccharide consumed 5.5 moles of sodium metaperiodate and yielded 3.2 moles of formic acid. Emulsion failed to hydrolyse the oligosaccharide, indicating the presence of (1→6)-α-linkage. It gives osazone derivatives which was identified with epimelliboisazone

and the oligosaccharide was confirmed to be epimelibiose; α-D-galactopyranosyl (1→6)-D-mannopyranose (Structure No.-5).



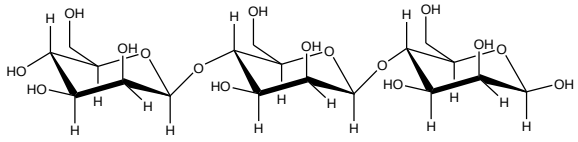
Structure No.-05

Oligosaccharides {3} Mannotriose; β-D-mannopyranosyl (1→4) - β-D-mannopyranosyl (1→4)-D-mannopyranose was obtained as solid, m.p. 165-167°C.

$[\alpha]_D^{25}$: -19° (water) was found chromatographically pure in mobile phase pyridine: water : ethyl Acetate (1:2:2), pyridine: water : ethyl acetate (5:4:11) and water :1-butanol : 2-propanol (3:11:6)

Equivalent weight 265 determined by hypoiodite method showed it to be monohydrate of trisaccharide. Emulsion hydrolysis gives the indication the presence of β-anomeric linkage among the mannose unit.

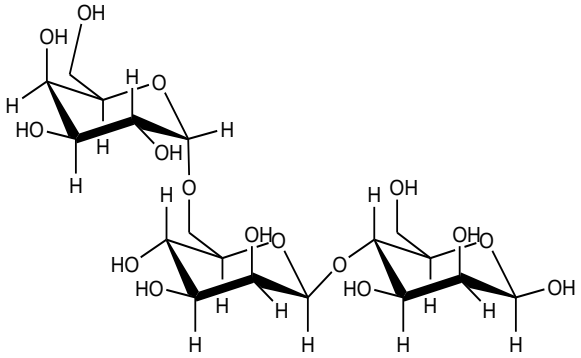
By periodate study indicates the consumption of 5.21 moles of metaperiodate with liberation of 3.03 moles of formic acid per mole of sugar. Partial hydrolysis gave the mannose and mannobiose. Thus the above result showed the presence of (1→4) linkage among mannose units. So the oligosaccharide identified as mannotriose; β-D-mannopyranosyl (1→4) -β-D-mannopyranosyl (1→4)-D-mannopyranose (Structure No, -6).



Structure No.-06

Oligosaccharides(4) Galactosyl mannobiose; α-D-galactopyranosyl (1→6)-β-D-mannopyranosyl (1→4)-D-mannopyranose also obtained in crystalline form m.p.226-228°C $[\alpha]_D^{25}$: +92° (water) and was found pure in mobile phase pyridine: water : ethyl acetate (1:2:2) and water :1-butanol : 2-propanol (3:11:6)

The complete hydrolysis of sugar yielded galactose and mannose in molar ratio 1:2. Its equivalent weight 265.7 corresponded to a monohydrate of trisaccharide. Acid catalysed partial hydrolysis produced epimelibiose, mannobiose, galactose and mannose. By the periodate oxidation it liberated 3.04 mole of formic acid with the consumption of 6.12 mole of metaperiodate. When emulsion hydrolysis for 6 days gave epimelibiose and mannose only. This sugar was identified as α-D-galactopyranosyl (1→6) - β-D-mannopyranosyl (1→4)-D-mannopyranose (Structure No.-7).



Structure No.-07

2. CONCLUSION:

The plants of the genera cassia generally possess considerable medicinal value and are also a good source of mucilages. Owing to the high medicinal value and increasing industrial demand of plant mucilages, we were prompted to undertake a structural study of the polysaccharides obtained from the seeds of *Cassia Senna tora*. The current investigation found that galactomannan consisting of D-galactose and D-mannose in the molar ratio 3: 4 has been isolated from the seeds of Cassia S. tora Hydrolysis of the methylated polysaccharide resulted in three methylated sugars: (a) 2,4-di-O-methyl-D-mannose, (b) 2,4,6-tri-O-methyl-D-mannose, and (c) 2,3,4,6-tetra-O-methyl-D-galactose in the molar ratio 2: 3: 2.

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