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Extract Galactomannan from Leucaena Leucocephala Plant Seeds

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Abstract
This research paper describes how to extract galactomannan from Leucaena leucocephala plant seeds. A process for extracting the seed extract from the Leucaena leucocephala plant involves crushing five seeds, followed by solvent extraction.
Leucaena leucocephala was used to isolate a water-soluble galactomannan that contains D-galactose (1 part) and D-mannose (3 parts). Three methylated sugars, in the molar ratios of 1.00:1.07:2.19, were produced by hydrolysis of methylated seed gum: 2, 3, 4, 6, tetra-o-methyl-D-galactose, 2, 3, 6, tri-o-methyl-D-mannose, and 2, 3 di-o-methyl-D-mannose. The seed gum was partially hydrolyzed by acid, releasing four oligosaccharides: mannotriose, galactosyl mannobiose, epimelebiose, and mannobiose. Studies on periodate methylation and oxidation both revealed 25% of end groups. Recent investigations have shown that galactose units exclusively occur in terminal positions in galactomannans.

Key words: Acidic hydrolysis, Methylation, periodate oxidation enzymatic hydrolysis, mucilage, end group analysis.

INTRODUCTION

Phytochemical refers to the extraction, screening and identification of the medicinally active substances found in plants. Some of the bioactive substances that can be derived from plants are flavonoids, alkaloids, carotenoids, tannin, antioxidants and phenolic compounds.

In single word “Biologically active compound found in plants.

The plants of Leucaena leucocephala (Family Leguminasae) have an evergreen shrub or tree with a fairly open, rounded crown; it can grow from 5 - 20 meters tall. The bole(trunk of tree) is generally short and can be 10 - 50cm in diameter.

Image and seeds of Plant Leucaena leucocephala

Known Hazards

The plant is classified as 'Least Concern' in the IUCN Red List of Threatened Species. The leaves of most forms of this plant contain the unusual amino acid mimosene. In large quantities this can be harmful. There are low-mimosene cultivars.

Its range from Central America - northern Panama, north to central Mexico and found in Dry coastal regions, waste ground.

There are some more properties, due to these reasons; it is very much using i.e.

Weed Potential – Yes	Conservation Status- Least Concern
Edibility Rating ****	Medicinal Rating - **
Other Uses Rating - ****	Habit-Evergreen Tree Height-10.00m
Growth Rate – Fast	Pollinators-Insects, Self
Self-fertile – Yes	Cultivation Status - Cultivated, Ornamental, Wild

That's why it is very much uses in different areas i.e.

Medicinal used

The roasted seeds are emollien. A decoction of the root and bark is abortifacien. Different parts of Leucaena leucocephala plants have been found efficacious in different types of diseases [(1)], but sometimes plants as a whole has been used and recommended for treatment [(2)].

Seeds of Leucaena leucocephala were subjected to phytochemical investigations and were shown to yield a substantial quantity of galactomannan. Galactomannans are frequently used in pharmaceutical [(3, 4)].

Gum arises from the stems under ill-defined conditions of injury and disease or from sterile hybrids, especially Leucaena leucocephala x Leucaena esculenta. The gum has been analyzed and found similar to gum arabic, and of potential commercial value.

Plants are sometimes used in re-reforestation projects.

The dried seeds are widely used for ornamentation.

Red, brown and black dyes are extracted from the pods, leaves and bark.

Agro forestry use

Leucaena leucocephala is an aggressive colonizer of disturbed ground and ruderal sites, and thus an excellent pioneer species for restoring woodland cover.

leucocephala is used for a variety of purposes, such as fencing, soil fertility , firewood , fiber livestock fodder.

During the 1970s and 1980s, it was promoted as a "miracle tree" for its multiple uses. It has also been described as a "conflict tree" because it is used for forage production but spreads like a weed in some places. Ideally used multipurpose tree in Mexico, where it provides food, medicines and a range of commodities for the local population, and is also commonly sold as a food in local and national markets, Vigorous and fast-growing, it is often cultivated in many areas of the tropics as an ornamental and is also used in reforestation and soil stabilization projects, as a shade plant for coffee.

Widespread and locally abundant in Central America, this species is not believed to be under any threat.

The galactomannans reported here is of potential medicinal use[(2)], hence a phytochemical investigation of structure for repeating unit of the galactomannan was undertaken and forms part of this present communication.

METHOD FOR EXTRACTING GALACTOMANNAN FROM SEED OF MEDICINAL PLANT LEUCAENA LEUCOCEPHALA

The present invention provides a method for extracting a Leucaena leucocephala plant seed extract galactomannan. A method for extracting a Leucaena leucocephala plant seed extract, which includes crushing seeds, followed by extraction with a solvent.

Galactomannan are polysaccharides consisting of a mannose backbone with galactose side groups (more specifically, a (1-4)-linked beta-D-mannopyranose backbone with branch points from their 6-positions linked to alpha-D-galactose, (i.e. 1-6-linked alpha-D-galactopyranose).

They are effective viscosifiers and thickeners, and are also excellent stiffeners and stabilizers of emulsions. These are also used in the textile, pharmaceutical, biomedical, cosmetics, and food industries.

This biopolymer, endosperm cell wall storage in the seeds of several botanical families.

Fenugreek galactomannan is unique compared to other galactomannans in that the ratio of mannose to galactose is 1:1.

The yields of these galactomannan extracts are limited to 15-20%. U.S. Pat. No. 5,847,109 describes a composition comprising fenugreek-derived galactomannan having 50 or more repeat units. The molecular weight of the composition according to this patent is ˜18,000 daltons.

Other sources of Galactomannan include guar gum (mannose: galactose ∼2:1), tara gum (mannose: galactose ∼3:1), locust bean gum or carob gum, (mannose: galactose ∼4:1), and cassia gum, (mannose: galactose ∼5:1).

Galactomannan is a component of the cell wall of the mold Aspergillus and is released during growth. Detection of galactomannan in blood is used to diagnose invasive aspergillosis infections in humans.

The plants of Leucaena Leucocephala (Family Leguminosae/Fabaceae), commonly known as ‘wild tamarind’, are low scrubby trees of tropical and sub tropical region.

Chinese Patent Application CN103030702 describes processing extracellular galactomannan secreted by Aspergillus fumigatus through alcohol to obtain a precipitate which is a crude galactomannan extract.

SUMMARY OF INVENTION

In our Paper we Obtain an extract from plant seeds of Leucaena leucocephala having a higher yield.

Method for producing an extract of polysaccharide Galactomannan from seeds Leucaena leucocephala plant, comprising crushing of seeds, followed by extraction with a solvent.

The process of extraction includes deproteinization and defatting of seeds using petroleum products such as petroleum benzene and petroleum ether.

These treated seeds are further suspended in acetic acid before pressing to obtain mucilage. The mucilage/gum obtained is further precipitated in ethyl alcohol to obtain purified polysaccharide Galactomannan.

Product yield of at least 70-80% wherein the product Galactomannan has structure as below.

a-D-Galp
1
↓
6

4→[b-D-Manp (1→4 –b–D-Manp (1→4)–b–D-Manp (1→4) -1→]n

Purified galactomannan structure contains seven units of monosaccharides, two galactose units, and five mannose units.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG-1 Trees of Leucaena leucocephala in India, and (on right) seeds of the plant.
FIG.2 Step by step method for extracting polysaccharide galactomannan from seeds of the plant Leucaena leucocephala and its purification.
FIG. 3 Step by step methods for checking the homogeneity of the extracted polysaccharide galactomannan.
FIG. 4 Structural studies of the purified polysaccharide Galactomannan.
FIG. 5 Structural studies of the purified polysaccharide by peridative oxidation.

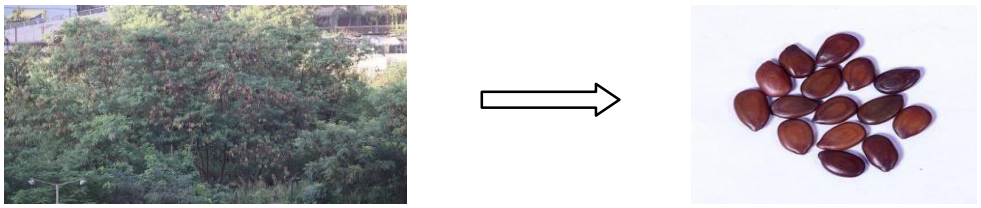

Fig-1 Trees of Leucaena leucocephala in India, and (on right) seeds of the plant.

DETAILED DESCRIPTION

Leucaena seeds are potential sources of protein and energy. The protein content was 31.1% and the calculated metabolizable energy of the seeds was 2573.26 kcal/kg. Amino acids profile of Leucaena seeds were 1.39% lysine%, 0.36% methionine, 0.35% cystine, 2.62% arginine, 4.63% glutamic acid, 0.87% threonine, 1.38% glycine, 1.11% alanine, 1.11% valine, 0.93% isoleucine, 1.81% leucine and 0.71% methionine + cystine. Antinutritional Factors (ANFs) were 0.75% , tannin and 697.50 mg/100g phytate.

Present inventors have now found that an extract of Galactomannan obtained from seeds of plants Leucaena leucocephala has a higher yield of up to 70-80% compared to the various sources available in the previous research Paper. The isolated Galactomannan has the following structure containing seven units of monosaccharides, two galactose units, and five mannose units.

Pre-Extraction Processing of plant material Leucaena leucocephala

The Seeds of Leucaena leucocephala were isolated from these pods and stored in 2-4^{0}C storage. the seeds were crushed followed by--→ deproteinization --→defatting using petroleum ether and petroleum benzene at 60-80^{0}C temperature.
(500gm of the defatted and deproteinized seeds were suspended in 1% aqueous acetic acid overnight (12hours)

The term 'Deproteinization' refers to the removal of protein from the crude plant extract, while, the term 'Defatted' refers to the removal of steroidal compounds, oily substances, fats from the crude plant extract.

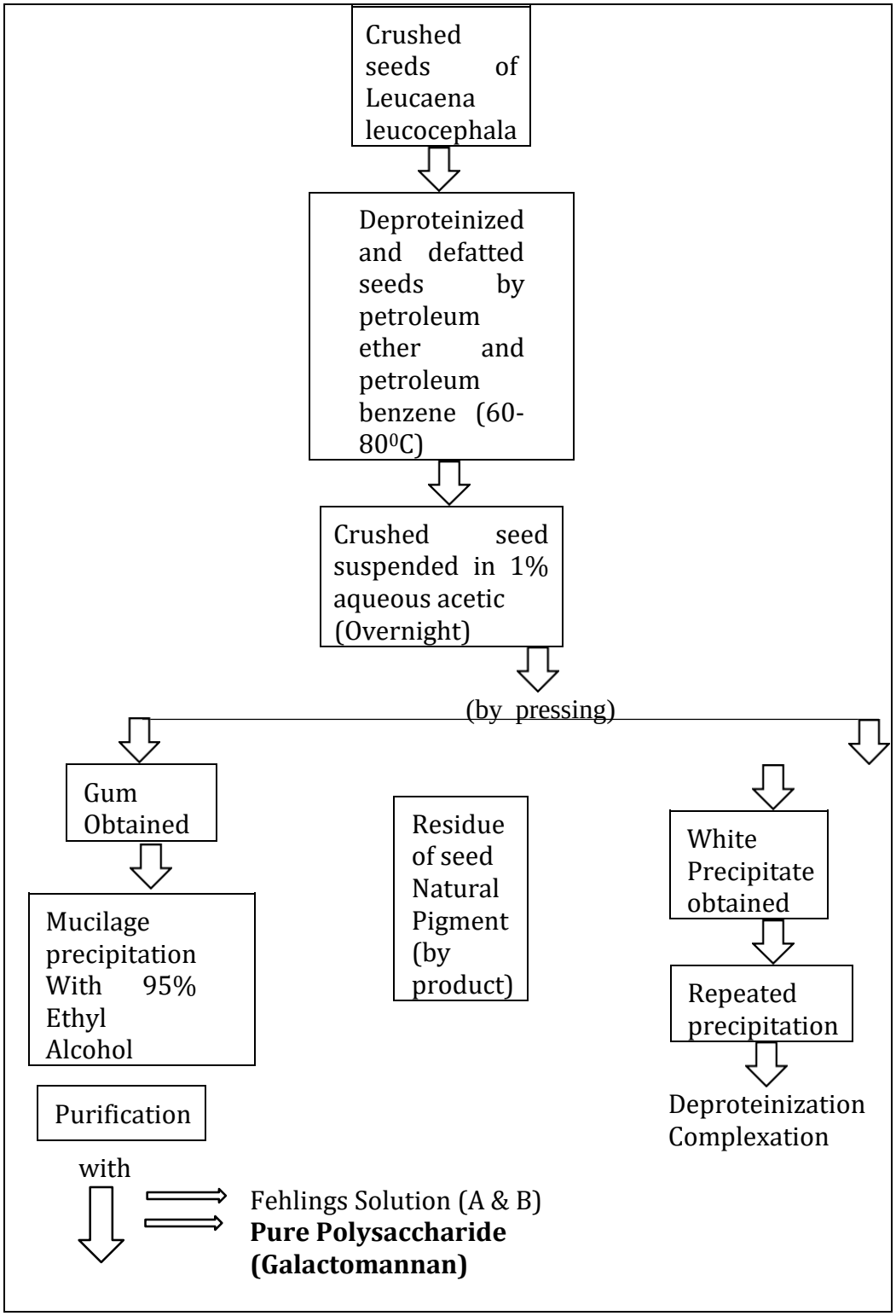

FIG.2 Step by step method for extracting polysaccharide galactomannan from seeds of the plant Leucaena leucocephala and its purification.

Supercritical Extraction Process

Overnight suspended seeds were filtered and mucilage was obtained by pressing the seeds. The mucilage is gum obtained from seeds.

The mucilage is precipitated in 95% ethyl alcohol and repeated precipitation for six times to obtain a white fibrous product (22gm). This white fibrous product was crude plant extract Galactomannan.

The precipitate was dried in the oven and has minimum ash content (0.3%). The dried precipitate was further treated to obtain the purified product. Deproteinization and complexation with Fehling's Solution (A & B) were performed to obtain purified polysaccharide Galactomannan. The purified polysaccharide from this process was 22 gm showing a yield of 70-80%.

Examples:1: Homogeneity of polysaccharide

Three homogeneity processes performed such as fractional precipitation, Zone Electrophoresis, Acetylation & deacetylation.

(a) Fractional Precipitation

A technique that separates ions from solutions based on their different solubilities. Purified polysaccharide Galactomannan (2.0 g) was dissolved in distilled water (H_2O) (200 ml) at 60^0C. The solution was treated with

1. 100 ml ethanol (C_2H_5OH) to obtain fraction (A)
2. 1000 ml of ethanol C_2H_4O) to obtain fraction (B)

(b) Zone – Electrophoresis

Electrophoretic separation technique typically used for analyzing proteins, nucleic acids, and biopolymers.

Purified polysaccharide Galactomannan was performed in 0.05 M Sodium Tetrahydroxyborate (Na_2BO_4)(pH 9.2) for 6 hours at 320 volts and 3.7 mA^0. A plot of absorbance against segment numbers showed a sharp peak confirming the homogeneous nature of purified polysaccharide Galactomannan.

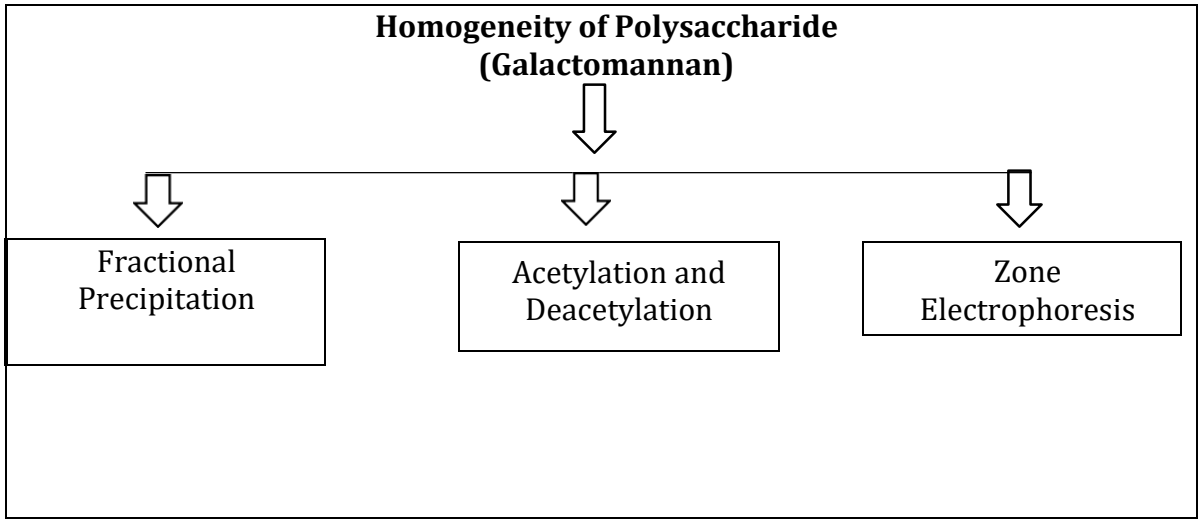

FIG. 3 Step by step methods for checking the homogeneity of the extracted polysaccharide galactomannan.

Structural studies of Polysaccharide Galactomannan

Paper chromatography (PC) are performed at room temperature by the descending technique on Whatman No.1 paper with the following four main solvents –

A	Butanol: Ethanol	BuOH: Et0H	H_2O (5:1:4) (5)
B	Ethyl Acetate: Pyridine	Et0AC: Pry	H_2O (5:2:7) (6)
C	Ethyl Acetate	Et0Ac	Pry: H_2O (2:1:2) (7)
D	Ethyl Acetate	Et0AC	Pry: H_2O (10:4:3)

FIG. shows the step-by-step process of structural studies performed on the purified polysaccharide galactomannan.

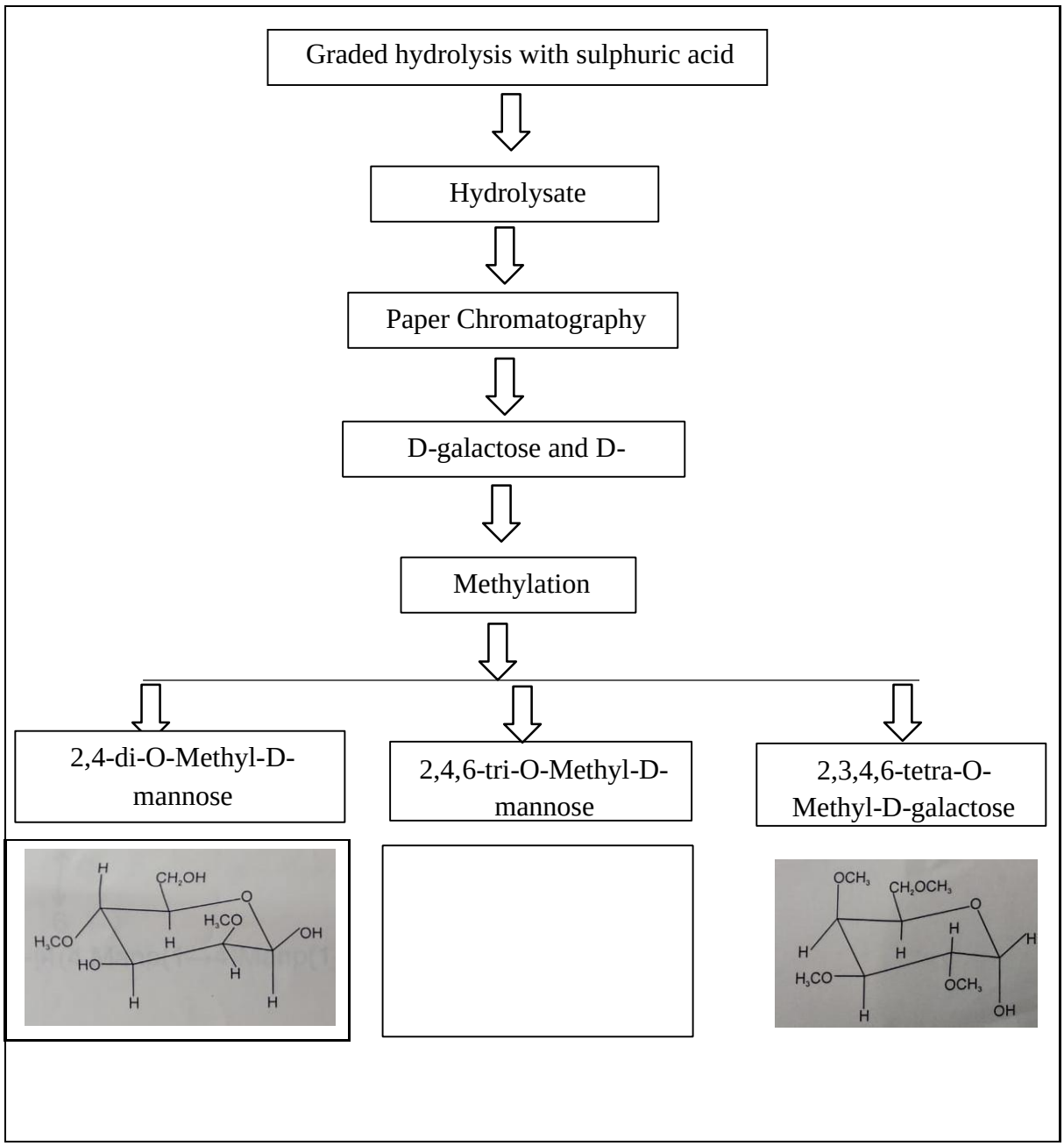

FIG. 4 Structural studies of the purified polysaccharide Galactomannan.

The purified polysaccharide Galactomannan was subjected to graded hydrolysis with 1M sulphuric acid (H_2SO_4) at 100^0 C for 24 hours. The Paper Chromatography of hydrolysate in solvent (C)

revealed the substructure galactose (R_f 0.15) and mannose (R_f 0.21). Upon graded hydrolysis with 0.05 M sulphuric acid (H_2SO_4) galactose was liberated first, followed by mannose.

The complete hydrolysis yielded D-galactose and D-mannose in molar ratio 1:3 respectively.

The polysaccharide Galactomannan (0.3 g) together with ribose (30 mg) as reference was subjected to Paper Chromatography with solvent (B) and individual monosaccharides were determined by periodate oxidation.

Periodate oxidation used to split bonds between vicinal carbons bearing unsubstituted hydroxyl or amino groups. The Periodate oxidation of the polysaccharide liberated 0.178 mole of Formic Acid of polysaccharide indicating 25% end groups, the galactose, and mannose residues were completely oxidized in 48 hours.

The polysaccharide (8 g) was subjected to Haworth methylation followed by Purdie's methylation. Completely methylated polysaccharide. The methylated derivative (50 mg) was hydrolyzed with 85% HCO_2H for 6 hours at 100^0C and with 0.75 M sulphuric acid (H_2SO_4) for 12 hours at 100^0C. The hydrolyzed polysaccharide was subjected to preparative Paper Chromatography with Solvent A.

The following product were isolated and characterized by anilide/hydride derivatives.

1. 2, 3, di – 0 – methy1- D – mannose
2. 2, 3, 6 tri – O – methy1- D – mannose
3. 2, 3, 4 tetra – O – methy1- D – galactose

The methylated polysaccharide 2 g together with D-glucose as reference was treated with 0.75 M sulphuric acid (H_2SO_4) for 18 hours at 100^0C. The resulting methylated sugars were separated by Paper Chromatography with solvent A and determined by alkaline hypoiodite. The molar ratio of fractions 1 to 3 was 1.00:1.07:2.19. Polysaccharide 4 g was partially hydrolyzed with 0.1 M sulphuric acid (H_2SO_4) for 12 hours. The hydrolysate on paper chromatography gave six spots in solvent C and was separated from the paper by elution with distilled water.

The following oligosaccharides were obtained and identified:

(1) Epimellibiose (6-0-a-D- galactosyl- D-mannose), Acid hydrolysis gave galactose and mannose.

(2) Mannobiose: Acid hydrolysis showed the presence of mannose units only.

(3) Mannotriose: Complete anhydrous hydrolysis indicated the presence of mannose units only and partial hydrolysis resulted in the formation of mannose mannobiose.

(4) Galactosyl mannobiose: Hydrolysis yielded galactose and mannose 1:3, its equivalent weight 265.0.

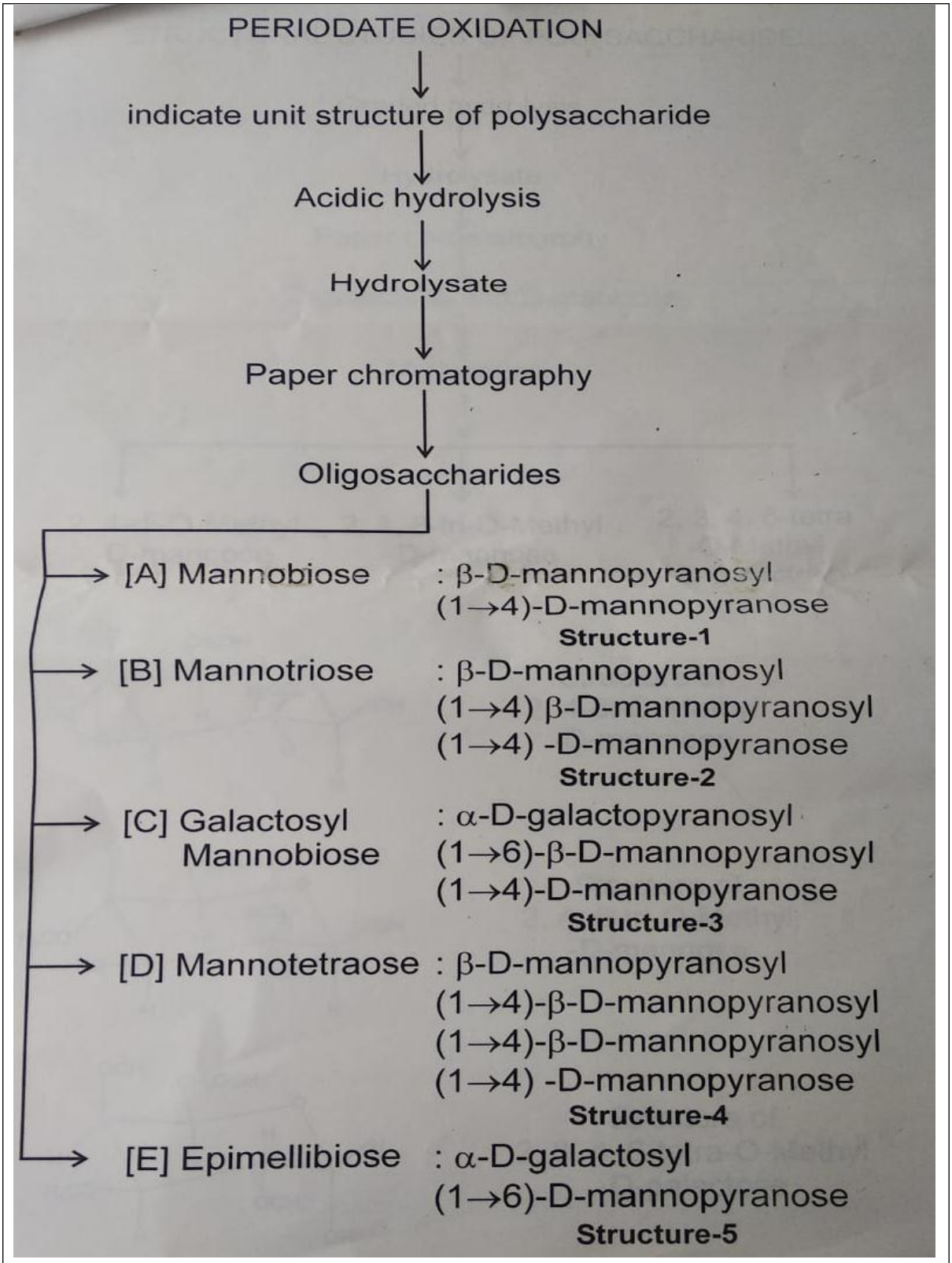

FIG. 5 Structural studies of the purified polysaccharide by peridative oxidation.

In addition to the two-component monosaccharides D-galactose, and D-mannose, it was found to give four oligosaccharides wherein Two were homogeneous and two were heterogeneous. The homogeneous Oligosaccharides were found to have (1 → 4) linkage between mannose units but the heterogeneous members had (1 → 6) linkages between galactose and mannose units.

These structural studies of the polysaccharide and of different oligosaccharides obtained by partial acid hydrolysis suggest the following structure of galactomannan which contains seven units of monosaccharides, two galactose units, and five mannose units.

No difference was observed between the purified polysaccharide Galactomannan obtained from the present invention upon comparison with purified Galactomannan obtained from Fenugreek seed

CLAIMS

1. A method of extracting a Leucaena leucocephala plant seed extract, comprising the steps of:

a. crushing seeds of Leucaena leucocephala plant to provide crushed seeds;

b. deproteinizing and defatting of seeds;

c. pressing deproteinized and defatted seeds to obtain mucilage; and

d. extracting the remaining part of the crushed seeds with precipitation, to obtain Leucaena leucocephala
plant seed extract.

2. Leucaena leucocephala plant seed extract is Galactomannan.
3. solvent in step (b) is petroleum ether and petroleum benzene.
4. Deproteinizing and defatting in step (b) occurs at a temperature of 60° C. to 80° C.
5. Defatted seeds suspended in acetic acid for up to 12 hours.
6. Solvent for precipitation in step (d) is ethyl alcohol.
7. Product obtained from step (d) is deproteinized to obtain purified seed extract.
8. Product obtained from step (d) is treated with Fehling's solution to obtain purified seed extract.
9. The method as claimed in any of the claims 1 to 8, wherein, the Leucaena leucocephala plant seed extract is Galactomannan of structure:

a-D-Galp
1
↓
6

4→[b-D-Manp (1→4 –b–D-Manp (1→4)–b–D-Manp (1→4) -1→]n

10. Purified galactomannan structure contains seven units of monosaccharides, two galactose units, and five mannose units.

QUESTIONNAIRES ANALYSIS AND CONCLUSION:

1. Difference between galactomannan extracted from Aspergillus (as per state of art) and galactomannan of present invention? We just carried out a very quick search and identified that galactomannan may also be extracted from Aspergillus.

- There is no difference between galactomannan of both but they differ in the % of yield and also differ in byproducts. The % yield of aspergillus is 40% and the Lucenea lucocephala is 84%.Due to large difference in yield we prefer to choose Lucenea lucocephala

2. If it is different, we need more structural details. If it is the same, why it is better to extract from plant than from Aspergillus (fungus). Furthermore, why only seeds of the plant and not other organs.

   -Due to its ease availability in local area
3. Show the steps in a flow chart (s) to avoid clarity objections in the future.
4. Alternatives for the chemicals used in the experiments. It will help to broaden the scope.

   -The other chemical used H_2SO_4 with different molarity 11M/1M reduced with N_6BH_4 acetylated with acetic anhydride using methyl imidazole as catalyst Alditolacetate formed were analyzed by Gas Chromatography and flame ionization detection 3-phenyl colorimetric method.
5. Any other technique used apart from electrophoresis and paper chromatography

   -Other Method which characterized galactomannan are NMR, GPC, HPLC, FTIR, SEM elemental analysis etc.
6. Paper chromatography is only possible for lab level extraction, what are possible methods for scale-up of the process.

   -In Place of paper chromatography we can used column chromatography (Both are available automatic and manual) used in industrial scale, HPLC, and Gas chromatography can also used.
7. The purity of extracted galactomannan and impurities present in final product is not mentioned.

   - A galactomannan hydrocolloids exhibiting about 50% to about 90% by weight anhydromannose and about 10% to about 50% by weight anhydroglactose residue. Less than 3% by weight other non-aqueous im purity less than 1% by weight of protein molecules and about 5-15% by weight water.
8. If any experiments conducted to compare the galactomannan from Aspergillus and galactomannan from the present invention, kindly provide those.- NO,
9. Full forms of all abbreviations used in the disclosure are:-

BuOH – Butanol (CH3CH2CH2CH2OH)	EtOH – Ethenol (CH3CH2OH)
EtOAC- Ethyl Acetate (CH3COOC2H5)	Pry – Pyridine (C5H5N)
PC – Paper Chromatography	Rf – Retardation Factor
$(alfa)^{25}D$ – Specific retention	R_{fac} – Retardation Factor
Galp – Galactose	Manp – Mannose
(1-4) and (1-6) – Glycosidic linkage	(1-4) – are present b/w mannose
(1-6) – are present between Mnap and Galp unit.	

9. Leucaena leucocephala is Widely distributed in which place of India ?

   Leucaena leucocephala is widely distributed in India especially in South India and some part of Middle India and also in Gorakhpur Region.

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