Determination of Sugar Composition of Polysaccharides in Caesalpinia pulcherrima Galactomannan Solution Using HPTLC

Nilima A. THOMBRE*, Paraag S. GIDE

M.E.T.’s Institute of Pharmacy, Bhujbal Knowledge City, Adgaon, Nasik 422 003, Maharashtra, INDIA

Galactomannans (GM), a group of neutral nontoxic polysaccharides naturally occurring in the seeds of some legumes, are used in the textile, pharmaceutical, biomedical, cosmetics and food industries. As to date there are no documented reports on determination of the sugar composition in polysaccharides by HPTLC and the following study is an attempt in this direction. GM from seeds of Caesalpinia pulcherrima L. (CP) was isolated and purified by precipitation method using alcohol. A new, simple, sensitive, selective, precise, and robust HPTLC method for analysis of monosaccharides such as galactose, mannose, xylose and glucose in isolated CP GM has been developed and validated. Chromatograms were developed using a mobile phase of acetone: water (9:1 v/v) on pre-coated plate of silica gel GF TLC plate and quantified by UV spectrophotometer at 254nm. The Rf values were 0.45, 0.34, 0.40 and 0.60 for mannose, galactose, xylose and glucose, respectively. The linearity of method was found to be within the concentration range of 2000-10000 ng/spot for all above monosaccharides. The limit of detection for mannose, galactose, xylose and glucose was found to be 0.14986 ng/spot, 0.31973 ng/spot, 0.27569 ng/spot, and 0.36808 ng/spot, respectively. The limit of quantification for mannose, galactose, xylose and glucose was found to be 1.498 ng/spot, 3.197 ng/spot, 2.756 ng/spot, and 3.680 ng/spot, respectively. The method was also validated for precision, specificity and recovery. This developed method was used to analyze CP GM for monosaccharides compositions.

Key words: HPTLC, Galactomannans, Monosaccharides, Caesalpinia pulcherrima

*Correspondence: nilimathombre@gmail.com, Tel: +91 253 2303515
INTRODUCTION

*Caesalpinia pulcherrima* (CP) belongs to Leguminosae, (Family-Fabaceae and Subfamily- Caesalpinioideae) is a traditional medicine plant with thorny bushy legume locally known as Dwarf Poinciana, Dwarf Flamboyan, Pride of Barbados, Barbados Pride, Barbados Flower-fence, Peacock Flower, Paradise Poinciana, Red Bird-of-Paradise is widely distributed in tropical and sub-tropical regions like India, Myanmar, Vietnam, Sri Lanka, and Malay Peninsula. Various pharmacological activities of *C. pulcherrima* L. have been reported such as analgesic, antimicrobial activity, anti-ulcer and anti-inflammatory (1-4), antibacterial and antifungal activity (5), antitumor (6), cytotoxic activity (7), astringent, abortifacient, emmenagogue (8), selective activity against DNA Repair-Deficient Yeast Mutants (9).

Galactomannans (GM) are polysaccharides with (1→4)-β-D-mannopyranosyl residues decorated with (1→6) linked α-D-galactopyranosyl residues (10, 11). GM (often called "Pharaoh's Polysaccharides") is water soluble hydrocolloids, high molar mass, water-soluble non-ionic polysaccharides forming highly viscous, stable aqueous solutions (12, 13). GM are widely useful in the industry mainly as thickening, stabilizing agents in a range of applications (14), in the development of edible films or coatings for food applications (15), gelling agents (16), excellent thickeners and stabilizers of emulsions, and the nontoxic nature allows their use in the textile, pharmaceutical, biomedical, cosmetics, food industries (17, 18) mass-efficient aqueous thickeners, gellants, and nutritional supplements (19). The purity of the polysaccharides was evaluated both by the total amount of monosaccharides obtained in the monosaccharide composition and by the amount of mannose and galactose present per mg of sample. Some of the analytical methods reported for the qualitative and quantitative analysis of galactomannan from *C. pulcherrima* are discussed herein. Cerqueira et al. and Andrade et al. reported the results of polysaccharide analyses by gas chromatography with a split injector and a flame ionization detector which confirmed that mannose (M) and galactose (G) are the major monosaccharides present in the polysaccharide material extracted from *C. pulcherrima* with M/G ratio about 2.8 to 2.83 (10, 20) (Fig.1). The purity of the polysaccharides was evaluated both by the total amount of monosaccharides obtained in the monosaccharide composition and by the amount of mannose and galactose present per mg of sample. Some of the analytical methods reported for the qualitative and quantitative analysis of galactomannan from *C. pulcherrima* are discussed herein. Cerqueira et al. and Andrade et al. reported the results of polysaccharide analyses by gas chromatography with a split injector and a flame ionization detector which confirmed that mannose (M) and galactose (G) are the major monosaccharides present in the polysaccharide material extracted from *C. pulcherrima* with M/G ratio about 2.8 to 2.83 (10, 20) (Fig.1). There are a few reports of quantification of polysaccharide composition from *C. pulcherrima* using Size exclusion chromatography method and TLC method only (13).

![Figure 1 Chemical structure of Mannose (a), Xylose (b), Galactose (c) and Glucose (d)](image-url)
In the present work we quantified the composition of monosaccharides such as mannose, galactose, glucose, and xylose from the isolated galactomannan from *C. pulcherrima* by HPTLC at the UV-Visible wavelength (254 nm). Monosaccharides were quantified densitometrically from the methanolic extract. The present paper describes the precise, accurate, sensitive HPTLC method for determination of monosaccharides from the galactomannan extract. Also to our knowledge, no information related to selective (HPTLC) determination of composition of monosaccharides from the isolated galactomannan from *C. pulcherrima* has ever been mentioned in literature.

**EXPERIMENTAL**

**Materials**

All reagents were of analytical grade unless indicated otherwise. D(+) -glucose, D(+) -galactose, D(+) -mannose, D(+) -xylose (all with purities ≥98%), were of analytical grade (E. Merck, India). Deionized water was obtained in the laboratory, using ionic interchanged columns Milli-Q (Millipore). All the other chemicals used were also of analytical grade (E. Merck, India).

**Extraction of galactomannan**

Pods of *C. pulcherrima* were collected from selected shrubs, growing in Nasik, Maharashtra, India. The seeds were isolated from the pods, cleaned and suspended in 99% ethanol in a proportion 1:3 (seeds: ethanol in volume) at 60 to 80 °C for 30 min to inactivate enzymes and eliminate low-molecular-weight compounds. The ethanol was decanted. The treated seeds were grinded mechanically. The endosperm was manually separated from the germ and the husk followed by addition of distilled water in a proportion of 1:5 (endosperm: water in terms of weight) and kept for approximately 24 h. Further distilled water was added in a double proportion and continued to mix in a blender for 5 min. The suspension was filtered through nylon net and centrifuged at 25,000 g (Remi Labs, India) for 30 min at 25± 0.5 °C to remove insoluble matter. Galactomannan was precipitated by addition of 99% ethanol. The ethanol was decanted and the precipitated galactomannan was lyophilized and kept in a dry place (desiccators) till further use (10, 21).

**Instrumentation**

HPTLC plates pre-coated with Si-gel Si60F254 aluminium back sheet TLC plates (20cm x 10 cm) were purchased from Merck. All Samples were applied on HPTLC plates by spray-on technique with Linomat V applicator using Hamilton (Reno, Nevada, USA) syringe of capacity 100 µL. Further the plates were developed in 20 × 10 cm CAMAG twin trough TLC chambers. Developed HPTLC plates were scanned using CAMAG TLC Scanner 4 (ATS4), fitted with winCATS1.4.0 planar chromatography manager software.

**Preparation of standard stock solution and calibration curve standards**

Standard stock solutions of galactose, mannose, xylose and glucose (5 mg each) were prepared by accurately weighing 5 mg of each monosaccharides in four separate 10 mL volumetric flasks and dissolved in deionised water. All solutions were further diluted with deionised water to get a concentration of 50 µg/mL, each. Further, the stock solutions were diluted with deionised water to obtain calibration curve standards with concentrations of 2000, 4000, 6000, 8000 and 10000 ng/µL, respectively.

**Preparation of sample solution**

Monosaccharide components were determined according to Shirakawa et al. (22). Briefly, exact 50mg crude CP was hydrolyzed in 2N HCl at 100 °C for 3 h at 0.1MPa. The hydrolysates were converted to the corresponding aditol chlorides. The solution was sonicated for 30 min, filtered through the Whatman filter paper No. 41 and the residue was washed and volume was adjusted to 10 mL with same solvent. This solution was further diluted with acetone: water (9:1, v/v) to get concentrations of 2000, 4000, 6000, 8000 and 10000 ng/µL, respectively.
**Chromatographic conditions**

Chromatography was performed on 20×10 cm aluminium backed sheet silica gel 60 F254 HPLTLC plates stored in desiccator (prewashed with 0.02 M sodium acetate and activated at 110 °C for 5 min prior to chromatography). Samples were applied to plates as 6 mm bands, band width: 15 mm and 20 mm from the bottom of the plates by means of Linomat V applicator (Camag, Muttenz, Switzerland) equipped with 100 µL Hamilton syringe (Reno, Nevada, USA). The rate of application was 15 µL/S, ascending development of the plates to the distance of 9 mm was performed at 25±2°C with Acetone : water (9:1, v/v) as mobile phase in 20 ×10 cm twin trough glass chamber (Camag, Muttenz, Switzerland), previously saturated for 30 min with mobile phase. The average development time was 20 min. After development, the plates were sprayed with phosphomolybdic acid reagent (0.5 % w/v) and dried at 105 -110 °C for 5 min. Densitometric scanning was performed on Camag TLC scanner 4 in the reflectance absorbance mode at 254 nm for all measurements and operated by win-CATS software (1.4.0) supplied by Anchrom Technologies (Mumbai). The source of radiation was deuterium lamp, continues emit UV spectrum between 400 nm to 200 nm. The slit dimensions were 6.00 mm × 0.45 mm. Evaluation was performed via peak area with linear regression.

10 µL of the CP galactomannan sample solution was spotted in duplicate on precoated HPTLC plates with 200 µm layers of Si-gel Si 60 F254 using an automatic applicator Linomat-5. Chromatograms were developed, scanned and the peak areas recorded. The quantitative estimation of monosaccharide components like galactose, mannose, xylose, glucose in the sample was detected by using the calibration curve of monosaccharides like galactose, mannose, xylose, glucose from 2000-10000 ng/µL.

**Method validation**

The developed method was validated as per the International Conference on Harmonization (ICH) guidelines with respect to linearity and range, specificity, precision, accuracy, limit of detection and limit of quantification (23). The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. LOD was calculated using the following formula:

\[
\text{LOD} = \frac{3.3 \times \text{Standard deviation of the Y-intercept}}{\text{slope of calibration curve}}
\]

The quantification limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy (24). LOQ was calculated using the following formula:

\[
\text{LOQ} = \frac{10 \times \text{Standard deviation of the Y-intercept}}{\text{slope of calibration curve}}
\]

**RESULTS AND DISCUSSION**

Sensitivity and specificity are the customary measures of the detection limit and the susceptibility to endogenous interferences, respectively, of an analytical method. GC-MS is widely regarded as the most specific analytical method available for drug detection and quantitation, although there were no falsely positive or negative results. GC-MS provides the highest degree of specificity. However, the application of GC-MS is limited to volatile analytes, and some amount of pre-analytical derivatization is frequently required. Polar, typically non-volatile compounds such as sugars or hydroxycarboxylic acids are easier to analyse by HPTLC. Non-polar, volatile compounds such as terpenes are ideally analysed by GC-MS (25-27).

However, in quantitative applications where the need for spectral data on individual analytes is less intense, HPTLC with multi-wavelength detection can provide comparable sensitivity and precision with limited specificity via UV and derivative spectra. An advantage of the HPTLC method described here is that less sample pre-treatment is required where derivatisation may required prior to analysis by GC-MS (25-27).

The GC-MS method is precise owing to the good resolution of the capillary column as compared to HPTLC method. HPTLC method is most widely used for the separation and
isolation of components of plant extracts, food stuffs, drinks and drugs. In addition to this it can be utilised for the rapid quantitative evaluation of monosaccharides from herbal extracts, polysaccharides, galactomannans etc. (25-27).

A number of experimental parameters, such as mobile phase composition, scan modes and detection wavelengths, were optimized during method development in order to obtain accurate, precise and reproducible results for the determination of mannose, galactose, xylose and glucose in Caesalpinia pulcherrima galactomannan, respectively. The mobile phase consisting of acetone: water (9:1, v/v) gave optimum separation among monosaccharides with minimum tailing. After development, the plates were sprayed with phosphomolybdic acid reagent (0.5% w/v) and colour differentiation of the sugars was observed (Fig. 2). The Rf values for mannose, galactose, xylose and glucose were found at 0.45, 0.34, 0.40 and 0.60, respectively. Fig. 3 depicts the chromatogram for the separated monosaccharides from galactomannan sample.

**Linearity and range**

The calibration standards in the range 2000-10000 ng/spot were analyzed in triplicate and plot of peak area vs. concentration was subjected to least square regression. The respective linear equations obtained were $y = 2608.4x + 30$, $R^2 = 0.9998$ for mannose, $y = 1234.4x + 30$, $R^2 = 0.9999$ for galactose, $y = 1472.x + 30.31$, $R^2 = 0.9992$ for xylose and $y = 718.4x - 100$, $R^2 = 0.9987$ for glucose, respectively.

**Accuracy**

The accuracy was assessed by the methodological recovery. The recovery of the method was calculated by comparing established concentration of spiked samples to the theoretical concentrations. The mean percentage recovery for each compound was calculated at each concentration level and reported with its standard deviation. The intra-day and inter-day percentages of accuracy obtained for mannose, galactose, xylose and glucose at the concentrations of 4000, 5000 and 6000 ng/spot are respectively shown in Table 1. The % recoveries of intra-day for mannose were 100.000 ± 0.0022%, 100.012 ± 0.00 51% and 100.004 ±0.0043%, respectively, the mean recovery for all the concentration levels was 100.0053±0.00611%. For galactose, the % recoveries of intra-day were 100.000± 0.0036%, 100.004 ± 0.0085% and 99.997 ± 0.0064%, respectively (Table 1). The mean value covering all the concentration levels was 100.0003±0.003512%. For xylose, the %
recoveries of intra-day were 100.001 ± 0.0059%, 99.998 ± 0.0063% and 99.993 ± 0.0015%, respectively (Table 1). The mean value covering all the concentration levels was 99.99733±0.004041%. For glucose, the

% recoveries of intra-day were 100±0.0036%, 99.997±0.0057% and 100.008±0.0021%, respectively (Table 1). The mean value covering all the concentration levels was 100.0016667±0.0057%.

**Precision**

The repeatability (intra-days precision) is expressed as percentage relative standard deviations (% RSD) for the mannose at the concentrations of 4000, 5000 and 6000 ng/spot, their % RSD values were 0.0060, 0.0045 and 0.0032, respectively, and for the time-different intermediate precision (inter-days precision) the % RSD values were 0.0020, 0.0044 and 0.0063, respectively. The % RSD values of intra-days precision for Galactose at the concentrations of 4000, 5000 and 6000 ng/spot were 0.0032, 0.0015, 0.0025, and for inter-days precision the % RSD levels were 0.0062, 0.0096 and 0.0057, respectively. The % RSD values of intra-days precision for xylene at the concentrations of 4000, 5000 and 6000 ng/spot were 0.0065, 0.0068, 0.0047, and for inter-days precision the % RSD levels were 0.0075, 0.0029 and 0.0037, respectively. The % RSD values of intra-days precision for glucose at the concentrations of 4000, 5000 and 6000 ng/spot were 0.0052, 0.0062, 0.0011, and for inter-days precision the % RSD levels were 0.0035, 0.0068 and 0.0037, respectively. The

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**Figure 3. 3D HPTLC Chromatogram of monosaccharides in *Caesalpinia pulcherima* galactomannan solution**
% RSD levels of intra-day and inter-day precision were less than 1.0 in all cases, which indicated that there were no significant variations in the analysis of mannose, galactose, xylose and glucose at the concentrations, which are shown in Table 1. The low % RSD value indicated the suitability of this method for routine analysis of monosaccharides in galactomannan solutions.

**Limits of detection and quantification**

The limit of detection for mannose, galactose, xylose and glucose was found to be 0.14986 ng/spot, 0.31973 ng/spot, 0.27569 ng/spot, and 0.36808 ng/spot, respectively. The limit of quantification for mannose, galactose, xylose and glucose was found to be 1.498 ng/spot, 3.197 ng/spot, 2.756 ng/spot, and 3.680 ng/spot, respectively.

**Specificity**

The densitometric method is a simple and fast analytical method for determination of monosaccharides in galactomannans. In our study an isocratic procedure was developed for the study of densitometric determination of monosaccharides present in galactomannans. The chromatogram of the samples showed a clear and compact peak of pure monosaccharides and no additional peaks at different Rf values were found (Fig.3).

### Table 1. Results of Accuracy and Precision studies for CP galactomannan

<table>
<thead>
<tr>
<th>Monosaccharides</th>
<th>Concentration (ng/mL)</th>
<th>Intra-day (n=3)</th>
<th>Inter-day (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Accuracy (%)</td>
<td>Precision (%) RSD</td>
</tr>
<tr>
<td>Mannose</td>
<td>4000</td>
<td>100.000 ± 0.0022</td>
<td>0.0060</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>100.012 ± 0.0051</td>
<td>0.0045</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td>100.004 ± 0.0043</td>
<td>0.0032</td>
</tr>
<tr>
<td>Galactose</td>
<td>4000</td>
<td>100.000 ± 0.0036</td>
<td>0.0032</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>100.004 ± 0.0085</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td>99.997 ± 0.0064</td>
<td>0.0025</td>
</tr>
<tr>
<td>Xylose</td>
<td>4000</td>
<td>100.001 ± 0.0059</td>
<td>0.0065</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>99.998 ± 0.0063</td>
<td>0.0068</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td>99.993 ± 0.0015</td>
<td>0.0047</td>
</tr>
<tr>
<td>Glucose</td>
<td>4000</td>
<td>100 ± 0.0036</td>
<td>0.0052</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>99.997 ± 0.0057</td>
<td>0.0062</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td>100.008 ± 0.0021</td>
<td>0.0011</td>
</tr>
</tbody>
</table>

**Results of sample solution**

The monosaccharides such as mannose, galactose, xylose and glucose from CP galactomannan solution showed Rf 0.45, 0.34, 0.40 and 0.60 in the chromatogram of CP galactomannan solution (25). The variation in the Rf values was observed due to polar nature of the mobile phase towards the monosaccharides present in the CP galactomannan solution (Table 2). The low % RSD value indicated the suitability of this method for routine analysis of monosaccharides in pharmaceutical and herbal drug samples. Thus, monosaccharide composition of CP galactomannan was found to contain mannose: galactose: glucose: xylose in a proportion of 2.8:1:0.1:0.08, with M/G ratio 2.80. The value of M/G obtained
for *C. pulcherrima* is close to the value obtained by Andrade et al (1999) and by Cerqueira et al (2009).

**Table 2.** Quantification of monosaccharides in CP galactomannan as per HPTLC-UV Analysis

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Rf value of monosaccharides</th>
<th>Area at Rf</th>
<th>Monosaccharide content in the CP galactomannan (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose</td>
<td>0.45</td>
<td>9180.885</td>
<td>2.8</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.34</td>
<td>1562.062</td>
<td>1.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.40</td>
<td>185.98</td>
<td>0.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.60</td>
<td>70.84</td>
<td>0.08</td>
</tr>
</tbody>
</table>

**CONCLUSION**

The developed HPTLC technique is precise, specific, accurate, and based on normal-phase. The proposed chromatographic method was successfully applied for quantitative determination of carbohydrates from various sources. The method can be used to determine the purity of the sugars available from various sources by detecting the related impurities. The data's and HPTLC fingerprint profile of monosaccharides from galactomannan could be used as a valuable analytical tool in the routine analysis of sugars in food, herbal drugs, phytochemicals and products made there from. When compared with the reported polysaccharide analyses method, the developed HPTLC method is both time and cost effective for the determination of polysaccharide analyses.

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