Structure and rheological properties of a xyloliglucan extracted from *Hymenaea courbaril* var. *courbaril* seeds


Instituto de Bioquímica, Universidade Federal de Pernambuco – UFPE, Av. Prof. Moraes Rego s/n, CEP: 50.670-420, Recife, PE, Brasil.

**ABSTRACT**

*Hymenaea courbaril* var. *courbaril* seed xyloliglucan was efficiently extracted with 0.1 M NaCl, followed by ethanol precipitation (yield = 72 ± 5% w/w). Its amorphous structure was identified by the pattern of X-ray diffraction. The monosaccharide composition was determined by GC/MS analysis of the aldol acetates and showed the occurrence of glucose:xylose:galactose:arabinose (4:0:34:20:6). One-(1D) and two-dimensional-(2D) NMR spectra confirmed a central backbone composed by 4-linked β-glucose units partially branched at position 6 with non-reducing terminal units of α-xylose or β-galactose-(1→2)-α-xylose disaccharides. The xyloligucan solution was evaluated by dynamic light scattering and presents a polydisperse and practically neutral profile, and at 0.5 and 1.0% (w/v) the solutions behave as a viscoelastic fluid. The polysaccharide did not show significant antibacterial or hemolytic activities. Overall our results indicate that xyloligucan from *H. courbaril* is a promising polysaccharide for food and pharmaceutical industries.

**1. Introduction**

Polysaccharides are polymers widespread in nature and synthesized in high amounts by plants and microorganisms. They are a source of biopolymers generally non-toxic, biodegradable, and biocompatible, sometimes exhibiting properties of biorecognition [1]. The polysaccharides have a wide range of chemical structure and physiochemical properties that cannot be easily reproduced synthetically. Thus, the possibility to obtain these compounds from natural sources renders numerous polysaccharides, which often present a lower cost than synthetic polymers [2].

Xyloligucans are branched polysaccharides usually found in the primary cell walls and in the higher plants seeds with structural and storage functions, respectively. They have a 4-linked β-D-glucan backbone, substituted at position 0-6 by branches of α-D-xylose or of β-D-galactose-(1→2)-α-D-xylose disaccharide [3-5]. Xyloligucans can be extracted from seeds of different species such as *Copaifera langsdorffii*, *Hymenaea courbaril* and *Tamarindus indica* that are combined with the same structural groups, but the proportion and arrangement of these groups give a fine structure that varies according to the species and even within the same species [6-8].

Xyloligucans can be used in food, medical and pharmaceutical industries, even to biotechnological processes [9], due to its characteristics, such as Newtonian flow behavior in the wide range of shear rate, water-holding ability and their resistance against heat, salt and pH regimes [10].

*H. courbaril* var. *courbaril* belongs to Caesalpiniaaceae family that occurs abundantly throughout Brazilian forests [11]. It is undemanding regarding fertility and soil moisture, besides it is important for the recovery of deforested areas and afforestation of large parks and gardens [12]. Each tree produces an average of 10 kg of seeds/year that are a source of xyloligucan [11-13].

This work aims to extract and purify xyloligucan from seeds of *H. courbaril*. Furthermore, we intend to determine the structure of the polysaccharide and its rheological properties, complement by evaluation of their antimicrobial and hemolytic activities, in order to determine the potential biotechnological applications of the polysaccharide.
2. Materials and methods

2.1. Materials

The pods of H. courbaril were collected in September 2010 at the Parque Nacional do Catimbau, Buíque-Pernambuco, Brazil. Botanical identification was made by depositing testimony specimens in the Herbarium of the Instituto Agronômico de Pernambuco (IPA 84893), Brazil. All the chemicals were of analytical grade.

2.2. Extraction and purification of xyloglucan

The xyloglucan from the seeds of H. courbaril was obtained by the method of Albuquerque et al. (2014) [14]. The seeds were manually removed from dry pods, washed and boiled in distilled water at 100 °C for 30 min for enzyme inactivation and softening of the hulls. After this period, the hull was removed and seeds were dried at 60 °C to constant weight (mᵢ). The dry seeds were triturated with 0.1 mol L⁻¹ NaCl [15% (w/v)] at 25 °C in an industrial blender (Model TR-02, Metcalúrgica Siemens Ltda, Santa Catarina, Brazil) and this crude extract obtained was subsequently centrifuged (Thermo Fisher Scientific Sorvall RC6, USA) for 20 min at 1500 × g. The supernatant was filtered through a voil tissue, followed by a new filtration using screen printing cloth (90 thread type), and precipitated with 46% ethanol [1:3 (v/v)] for 16 h. The precipitate was filtered on screen printing cloth (110 thread type), washed with 100% ethanol [1:3 (v/v)] for 30 min and twice with acetone PA [1:3 (w/v)] for 30 min, filtered on screen printing cloth (110 thread type) between each washing and finally dried at 60 °C until constant weight (mₒ). The dry precipitate was milled and stored in amber glass bottles.

The yield of the extraction in mass was determined as the ratio of the final mass of the precipitate powder (mₒ) by the initial mass of the dry seeds (mᵢ) and expressed as % (w/w). The total content of carbohydrate and protein in the precipitated powder was determined by the phenol sulfuric acid method [15] and by Bradford method [16] respectively.

2.3. Structural characterization

2.3.1. Gel filtration chromatography

Xyloglucan (1 mg) was applied to a Superose 6 column (30 × 1 cm) from Pharmacia, linked to a HPLC (high performance liquid chromatography) system, equilibrated with 0.5 mol L⁻¹ ammonium acetate (pH 5). The column was eluted with the same solution at a flow rate of 0.5 mL min⁻¹. The eluent was monitored simultaneously by absorbance at 280 nm and by differential refractive index. The column was calibrated with dextrins with average Mr of 500 and 50 kDa.

2.3.2. X-ray patterns

X-ray diffraction pattern of the xyloglucan was obtained using a Bruker D8 Advance (Germany) diffractometer (30 kV, 30 mA) equipped with CuKα radiation at wavelength 0.154 nm. The measurements were carried out for an angular interval varying from 5–60° (2θ range), scanning rate of 5° min⁻¹, step of 0.02° and 2 s per step. The total diffracted area and the area under the crystallinity peaks were evaluated by integration after correcting the data for absorption in order to determine the polysaccharide crystallinity.

2.3.3. Monosaccharide composition

After acid hydrolysis of the xyloglucan (5 mol L⁻¹ trifluoroacetic acid for 2 h at 100 °C), the alditol acetate derivatives were analyzed by gas-liquid chromatography/mass spectrometry (GCMS–QP2010 Shimadzu, Japan) [17].

2.3.4. Methylation analysis

Xyloglucan (5 mg) was subjected to two rounds of methylation [18]. The methylated polysaccharide was hydrolyzed with 5 mol L⁻¹ trifluoroacetic acid for 2 h at 100 °C, reduced with borohydride, and the alditols were acetylated with acetic anhydride/pyridine (1:1 v/v). The alditol acetates of the methylated sugars were dissolved in chloroform and analyzed in a gas chromatography/mass spectrometry unit (GCMS-QP2010 Shimadzu, Japan) with a Restek column RTX-5MS. The sample was analyzed using a split ratio of 30. The carrier gas was helium and a temperature gradient of 110 °C to 250 °C, with variation of 2 °C min⁻¹. The temperatures of the injector, ion source and interface were 260 °C, 200 °C and 230 °C, respectively.

Different periods of time for hydrolysis of the methylated polysaccharide were tested (from 1 up to 4 h) in order to assure total hydrolysis of the constitutive units but without significant lost of xyllose residues. Two hours were chosen as the appropriated time for the hydrolysis.

2.3.5. Nuclear magnetic resonance spectroscopy

One (1D) and two-dimensional (2D) spectra of the xyloglucan were recorded using a Bruker DRX 400 MHz apparatus with a triple resonance probe, as described previously [11,19]. Approximately 5 mg of each sample was dissolved in 0.5 mL of 99.9% deuterium oxide (Cambridge Isotope Laboratory, Cambridge, MA, USA). All spectra were recorded at 50 °C with HOD (deuterated water exhibiting a peak due to exchange with residual H₂O) suppression by presaturation. For 1D ¹H NMR spectra, 32 scans were recorded, using an inter-scan delay equal to 1 s. For 2D ¹H/¹H TOCSY (total correlated spectroscopy) and ¹H/¹³C HSQC (heteronuclear single quantum coherence) experiments, spectra were recorded using states TPI (time proportion phase incrementation) for quadrature detection in the indirect dimension. TOCSY spectra were run with 4046 × 400 points with a spinlock field of 10 kHz and a mixing time of 80 ms. Two-dimensional ¹H/¹³C Multiplicity-Edited HSQC spectra were recorded at 50 °C with HOD suppression by presaturation, with 256 scans. The increment number setup was set to 64, and states-TPPI were used for quadrature detection in the indirect dimension and run with 1024 × 256 points with globally optimized alternating phase rectangular pulses (GARP) for decoupling. Chemical shifts were displayed relative to external trimethyl-silylpropionic acid at 0 ppm for ¹H and relative to methanol for ¹³C.

2.4. ζ-potential and dynamic light scattering (DLS)

DLS and ζ-potential (ζ) measurements were carried out with the xyloglucan solution 0.5% (w/v) in water at pH 5.8 adjusted with 0.1 M NaOH. The measurements were carried out on a ZetaSizer Nano ZS90 (Malvern Instruments, U.K.). The DLS cumulants analysis (30 scans) provides the characterization of a sample through the mean apparent Z-average hydrodynamic diameter (Rh) for the particle or molecule size (nm). The width parameter, known as the polydispersity index (PDI), was determined from the intensity of scattered light (fixed angle of 90°) at 25 °C. The Z-average diameter is the mean hydrodynamic diameter (molecule size), determined from the intensity of scattered light. This measuring was carried out in triplicate with samples analyzed in a period of 144 h, with intervals at 48 h for each analysis. The ζ-potential values were calculated using the Smoluchowski equation [20]. Each sample was analyzed in a folded capillary cell. The results are given as average ± standard deviation.
2.5. Rheological measurements

The rheological properties of the xyloglucan aqueous solutions at 0.5 and 1.0% (w/v) were conducted in a stress controlled rheometer (Anton Paar MCR 301, Austria) equipped with two concentric cylinder geometry cell with outer diameter (o.d.) = 28 mm and internal diameter (i.d.) = 24 mm, with temperature controlled at 25°C. Measurements were performed and 50 points were acquired.

2.5.1. Rotational flow

Continuous shear tests were carried out over a shear rate (γ) range of 1–1000 s\(^{-1}\) to measure the apparent viscosity (η). The points were acquired while simultaneously collecting viscosity data.

2.5.2. Oscillatory flow

Dynamic oscillatory mode measurements were conducted varying the applied torque/deformation angular frequency (ω) from 1 to 500 rad s\(^{-1}\). Storage (G\(^{′}\)) and loss (G\(^{″}\)) moduli sample responses were recorded during the frequency sweep at a rate of one acquisition every 5 s. The strain deformation amplitude (γ\(′\)) was fixed at 0.2%.

2.5.3. Stress-strain

The rheometer was operated in oscillatory mode. For a fixed frequency ω≡2π – rad s\(^{-1}\) an externally applied shear stress (τ) was varied from 1 to 300Pa and the (G\(^{′}\)) and (G\(^{″}\)) moduli sample responses were recorded. One acquisition was carried out every 5 s.

2.6. Biological activities

2.6.1. Antibacterial activity

Gram-negative strains [Escherichia coli (UFPEDA 224), Klebsiella pneumoniae (UFPEDA 396), Salmonella enteritidis (UFPEDA 414), Pseudomonas aeruginosa (UFPEDA 416), Proteus vulgaris (UFPEDA 740)] and gram-positive strains [Staphylococcus aureus (UFPEDA 02), Bacillus subtilis (UFPEDA 86), Micrococcus luteus (UFPEDA 100), Enterococcus faecalis (UFPEDA 138)] were provided by Culture Collection of Microorganisms of Department of Antibiotics, Universidade Federal de Pernambuco (UFPEDA). Bacteria strains were grown in shaker flasks (250mL) containing Nutrient Broth and incubated overnight in an orbital shaker at 100 rpm and 37°C. The biomass concentration was determined measuring the suspension turbidity at 600 nm and then converted to colony forming units (10\(^5\)–10\(^6\) CFU mL\(^{-1}\)) using appropriate calibration curves (turbidity equivalent to 0.5 in the McFarland scale). Xyloglucan antibacterial activity was investigated by the disc diffusion method [21], using 20μL of xyloglucan solution (0.5% w/v) and positive controls containing neomycin and clindamycin (both with 10 μg/disc). All tests were carried out in triplicate.

2.6.2. Hemolytic activity for toxicity evaluation

The hemolytic activity was determined according to a literature method [22]. The red blood cells were diluted in saline solution to obtain a 1% suspension (v/v) and 1.1 mL was mixed with 0.4 mL of xyloglucan solution (0.25–2.0 mg mL\(^{-1}\) in saline). The minimum and maximum hemolytic controls were suspensions of red blood cells containing saline (hemolysis 0%) and Triton X-100 2.0 mg mL\(^{-1}\) (hemolysis 100%), respectively. All experiments were carried out in triplicate and the results expressed as average ± standard deviation. The hemolytic activity was expressed in relation to Triton X-100 and calculated by the following equation: Hemolytic activity (%) = (As – Ab)/(Ac – Ab) × 100, where Ac is the absorbance of the control, As is the absorbance in the presence of the xyloglucan solution and Ab is the absorbance of Triton X-100 solution.

Fig. 1. Gel filtration chromatography (numbers 1 and 2 indicate the elution volumes of dextrans with 500 and 50kDa, respectively).

3. Results and discussion

3.1. Extraction and yield

Most methods for extraction of xyloglucan from seeds described in the literature consist of two basic stages, where the triturated seeds pass by aqueous extraction, followed by purification using alcoholic precipitation [8,23,24].

In the present work extraction with NaCl was used because it increases the solubility of the contaminating free proteins (salting in effect). The crude extract obtained was subsequently centrifuged to remove the residue, in agreement with the literature [5]. Washing with ethanol removed contaminant, while washing with acetone permitted further removal of proteins. These washes with ethanol and acetone also promote dehydration of the polysaccharide, thus contributing to preparation of a complete dried sample. The extraction yield was 72 ± 5% (w/w) related to dry weight of the seeds, being 81 ± 7% of this mass of polysaccharide content and by Bradford method [16] no protein was detected.

We believe that our methodology allowed us to achieve a high yield which is far above those of previous works, such as 15.5% [23] and 38% [5]. Different extraction times for xyloglucan were compared and demonstrated a variation of yield from 5.4 to 40.8% [25]. However, these authors did not mention clearly if your yield are related to moist or dry weight of the seeds.

3.2. Gel filtration chromatography

Gel filtration chromatography reveals that xyloglucan is a polydisperse polysaccharide with average molecular mass ≥500 kDa (Fig. 1). Clearly no degradation occurred during extraction and purification of the polysaccharide. The absence of fractions with the absorbance at 280 nm confirms that the xyloglucan does not contain contaminant proteins.

3.3. Patterns of X-ray diffraction

X-ray diffraction profile (Fig. 2) shows characteristics of amorphous structures with no sharp peaks. X-ray diffractogram
presented a typical peak at approximately $2\theta = 20^\circ$, similar to those found for a xyloglucan from Goko Food and Chemical Co. Ltd. [26] and a galactoxyloglucan extracted from Tamarind seed [27].

It is known that crystalline structures generate sharp narrow diffraction peaks while amorphous components generate a broad one. The interpretation of broad amorphous peaks of several amorphous structures in X-ray scattering profile is difficult and hence the ratio between these intensities is used to calculate the index of crystallinity in the material [28]. The crystallinity index, related with the alignment of molecules in a particle structure [29], was calculated from X-ray diffraction profile for the xyloglucan, giving a value of 0.37. Furthermore, the crystallinity depends on how the material is dried or prepared, and that the drying process can lead to a better organization of the chain, thus increasing the crystallinity of the material [30].

3.4. Monosaccharide composition

The monosaccharide composition of the xyloglucan was determined based on gas-liquid chromatography/mass spectrometry analysis of the alditol acetates formed after acid hydrolysis, yielding (in %w/w): glucose (40%), xylose (34%), galactose (20%) and arabinose (6%).

Glucose, xylose and galactose were detected with a molar ratio of $\approx 4:3:2$ respectively. Minor amount of arabinose was also detected ($\approx 6\%$). No other sugar was detected up to a limit of <2% as% of dry weight. The branching pattern of xyloglucan from different plant species was evaluated and demonstrated that some xyloglucans have an additional arabinose unit linked to the galactose residues at the non-reducing end of the side chain [31]. Kay & Petkowicz [25] suggest that the presence of arabinose is due to co-purified arabinan, which corroborates our result. They also confirmed the absence of arabinose residues in shorter extraction processes, while others workers [3,11] described arabinose traces in xyloglucans from seeds.

3.5. Methylation analysis

The substitution of hydroxyl free radical by methyl by methyl is accomplished in methylation analysis, where different O-methyl derivatives are obtained after hydrolysis and acetylation. The methylation of this polysaccharide, followed by acid hydrolysis and acetylation of the alditols, yields the derivatives units shown in Table 1.

The structure proposed for the polysaccharide was based on our experimental data (methylation and NMR analysis) and also on literature information [5,11,32] as summarized in Fig. 3.

The types of units found in the polysaccharide are shown in Fig. 3 and confirmed in Table 1. Clearly the preponderance of 2,3,6-tri-O-methyl and 2,3-di-O-methyl derivatives from glucose indicates that the preponderant structure is composed of 4-linked (unit II) and 4,6-substituted units (unit I) of this sugar. The presence of these two derivatives was also observed in a very similar proportion in the xyloglucan from Guibourtia hymenifolia (Moric.) J. Leonard [9] and in the xyloglucan from H. courbaril seeds [13], confirming 4-linked glucose residues as typical of xyloglucan cellulose backbones.

Galactose occurs as 2,3,4,6-tetra-O-methyl derivative, indicating that this sugar is at non-reducing terminal (unit IV). Two third of the xylose units occur as 2,3,4-tri-O-methyl derivative, indicating non-reducing terminal branches (unit V), as galactose. One third of xylose residues occur as 3,4-di-O-methyl derivatives, meaning that the structure presents 2-linked xylisyl units (unit III). Of course, the alditol acetate derivatives from 2,3-di-O- and 3,4-di-O-methyl xyloses are the same compound. We attributed our results to the 2,3-di-O-methyl xylose based on literature data for similar compounds [5,11,32] and also on our own NMR data (see next section).

The detection of 2,3-di-O-methyl glucose in approximately the same proportion as the sum of tetra-methyl derivative from galactose + tri-methyl derivatives from xylose, agrees with the structure proposed for the polysaccharide, contain a central core of 4-linked glucose units (unit II), partially branched at position 6 (unit I). Two third of the xylose units (unit V) and the totality of the galactose units (unit IV) occur as non-reducing terminal. One third of the xylose units (unit III) are 2-linked intermediate residues on the branches.

The overall recoveries of methylated alditol acetates were in good agreement with the monosaccharide composition obtained for the native polysaccharide after hydrolysis, reduction and acetylation (Section 3.4). Arabinose, which is present in small amount in the polysaccharide, was not detected even when we employed different periods of hydrolysis (from 1 up to 4 h).

3.6. Nuclear magnetic resonance spectroscopy

The structure of the xyloglucan from H. courbaril was investigated using one-dimensional (1D) and two-dimensional (2D) NMR spectra. The $^1$H-NMR spectrum (Fig. 4A) showed three signals in the anomeric region: one at 5.17 ppm, ascribed to substituted $\alpha$-xylose units (Xyl') (unit III in Fig. 3), another at 4.98 ppm to terminal non-reducing $\alpha$-xylose (Xyl) (unit V in Fig. 3), and finally the signal at 4.58 ppm, which is an overlapped of $\beta$-glucose + $\beta$-galactose anomeric protons (units I, II+IV in Fig. 3). No signals assigned to
contaminant proteins were observed in the 1D $^1$H-NMR spectrum, such as signals from aromatic amino acids at 6.0–7.0 ppm. The edited $^{13}$C-$^1$H HSQC spectrum (Fig. 4C) allowed us to distinguish the anomeric signals from β-galactose (105.13 and 4.59 $^{13}$C/$^1$H ppm) (unit IV) and β-glucose (103.02 and 4.58 $^{13}$C/$^1$H ppm) (units I and II), which were coincident in the proton spectra. Signals from CH occur in phase (positive peaks in blue) while those from CH$_2$ occur as anti-phase (negative peaks in green). This spectrum allowed us to identify signals from C6/H6 (67.30/3.90–4.04 ppm), ascribed to β-glucose and β-galactose units, and those from C5/H5 (62.4/3.58–3.76 ppm) of α-xylene. Signals from C4 (79.75 ppm) and some of C6 (67.30 ppm) from β-glucose, as well as signals from C2 of some α-xylene units (80.81 ppm, Xyl), were shifted downfield compared with non-glycosylated units (compared chemical shifts from units III vs V, in Table 2 and also literature data [32]. No signals ascribed to non-reducing β-arabinofuranosyl units were observed at 109/5.3 ppm. This type of residue was reported in other plant polysaccharide [25]. They may occur only in minor amounts in our xyloligoclan preparation and not detected on the NMR spectrum.

The $^1$H-$^1$H COSY and $^1$H-$^1$H TOCSY spectra (Fig. 5) allowed us to identify the chemical shifts by scalar coupling of the spin systems. The COSY spectrum (Fig. 5A) confirmed the overlapping of the anomeric signals from β-galactose and β-glucose units at 4.58 ppm. The TOCSY spectrum (Fig. 5B) allowed us to identify the four spin systems and to determine their proton chemical shifts (Table 2). However some signals from β-glucose and β-galactose are coincident. Furthermore the chemical shifts of H2 and H3 from β-galactose units are very close as well as the chemical shifts of H3 and H4 from β-glucose.

The backbone formed by 4-linked β-D-glucan is common to all xyloligucans, but considerable differences are observed in the branches of the polysaccharide, such as arrangements in number, position and type of residues. This variation is a consequence of interspecific variations [3] and also of the extraction methods [25].

In conclusion, the $^1$H and $^{13}$C chemical shifts shown in Table 2 were similar to those reported for a plant polysaccharide composed of a central backbone, containing 4-linked β-glucose units (unit II), partially branched at position 6 (unit I) [32]. The polysaccharide also is highly branched, containing β-galactose and α-xylene as non-reducing terminal units (unit IV and V respectively). Furthermore, residues of 2-linked α-xylene are also found as intermediated residues in the branches (unit III).

3.7. ζ-potential and dynamic light scattering (DLS)

The information obtained by ζ-potential and Dynamic Light Scattering (DLS) are crucial to indicate the occurrence of stable, functional nanostructures [33]. PDI, obtained by DLS, is a measure of the size distribution width. When polydispersity equals zero, the sample is monodisperse. Values of PDI close to or above 0.5 represent heterogeneous solutions in relation to the particle size and are characteristic of samples outside the standards [34]. The term “particle” represents the molecule of polysaccharide, which stay disperses into diluted solution. This term has been used in the literature for different polysaccharides [35]. For a xyloligoclan solution 0.5% (w/v) at pH 5.8, Z-average and PDI were 296.60 ± 12.40 nm and 0.32 ± 0.02, respectively, indicating a reasonably polydisperse solution. Z-averages values of 170 nm [24] and 80 nm [25] were reported for xyloligoclan from H. courbaril seeds at 1 mg mL$^{-1}$, which are lower concentrations than ours (0.5% w/v), as well as the different method for extraction.

ζ-potential of xyloligoclan was evaluated in order to determine the charges of the polysaccharide; a ζ-potential value of $-11.3±0.27$ mV was found, thus showing that xyloligoclan is a practically neutral polysaccharide. The polysaccharides may be constituted either by polyoncations or by polyanions, depending on their functional group, and may also be neutral, which is the case of different types of polysaccharides with a higher content of mannose and galactose units, with ζ-potential values between $-13.7$ and $-2.1$ mV [33]. In this work the obtained xyloligoclan shows a structure composed by glucose (40%), xylose (34%), galactose (20%) and arabinose (6%), typical of a neutral polysaccharide.

### Table 2

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Retention time (nL - min)</th>
<th>Relative retention time</th>
<th>Proportion ( % of total)</th>
<th>Methyl derivative</th>
<th>Units$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.33</td>
<td>1.00</td>
<td>22</td>
<td>2,3,4,5-tri-Me-</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Xyl</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>29.46</td>
<td>1.21</td>
<td>11</td>
<td>3,4-di-Me-Xyl</td>
<td>III</td>
</tr>
<tr>
<td>3</td>
<td>32.57</td>
<td>1.33</td>
<td>15</td>
<td>2,3,4,5-tetra-</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Me-Gal</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>36.69</td>
<td>1.50</td>
<td>19</td>
<td>2,3,5,6-tri-Me-</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glc</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>41.90</td>
<td>1.72</td>
<td>33</td>
<td>2,3-di-Me-Glc</td>
<td>I</td>
</tr>
</tbody>
</table>

$^a$ The type of methyl derivative was identified by the typical fragmentation spectrum.

$^b$ See Fig. 3.
Table 2

<table>
<thead>
<tr>
<th>Structure/Chemical shifts</th>
<th>Units*</th>
<th>C1/H1</th>
<th>C2/H2</th>
<th>C3/H3</th>
<th>C4/H4</th>
<th>C5/H5</th>
<th>C6/H6</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-Glcp</td>
<td>I/II</td>
<td>103.02/4.58</td>
<td>73.65/3.43</td>
<td>74.93/3.70</td>
<td>79.75/3.73</td>
<td>74.14/3.84</td>
<td>67.30/3.90–4.04</td>
</tr>
<tr>
<td>β-D-Galp</td>
<td>IV</td>
<td>105.13/4.59</td>
<td>72/3.66</td>
<td>73.9/3.68</td>
<td>69.4/3.96</td>
<td>75.8/3.7</td>
<td>61.84/3.79–3.88</td>
</tr>
<tr>
<td>α-D-Xylopy</td>
<td>III</td>
<td>99.69/4.97</td>
<td>72.3/3.57</td>
<td>73.93/3.75</td>
<td>70.2/3.65</td>
<td>62.4/3.58–3.76</td>
<td></td>
</tr>
<tr>
<td>α-D-Xylopy</td>
<td>V</td>
<td>99.5/5.17</td>
<td>80.8/3.7</td>
<td>72.8/3.96</td>
<td>70.2/3.65</td>
<td>62.4/3.58–3.76</td>
<td></td>
</tr>
<tr>
<td>β-D-Glcp</td>
<td>I/II</td>
<td>103.02/4.58</td>
<td>73.65/3.43</td>
<td>74.93/3.70</td>
<td>79.75/3.73</td>
<td>74.14/3.84</td>
<td>67.30/3.90–4.04</td>
</tr>
<tr>
<td>β-D-Galp</td>
<td>IV</td>
<td>105.13/4.59</td>
<td>72/3.66</td>
<td>73.9/3.68</td>
<td>69.4/3.96</td>
<td>75.8/3.7</td>
<td>61.84/3.79–3.88</td>
</tr>
<tr>
<td>α-D-Xylopy</td>
<td>III</td>
<td>99.69/4.97</td>
<td>72.3/3.57</td>
<td>73.93/3.75</td>
<td>70.2/3.65</td>
<td>62.4/3.58–3.76</td>
<td></td>
</tr>
<tr>
<td>α-D-Xylopy</td>
<td>V</td>
<td>99.5/5.17</td>
<td>80.8/3.7</td>
<td>72.8/3.96</td>
<td>70.2/3.65</td>
<td>62.4/3.58–3.76</td>
<td></td>
</tr>
</tbody>
</table>

* See Fig. 3.

Fig. 5. 1H-1H COSY (A) and 1H-1H TOCSY (B) spectra of the xylolucan from H. courbaril seeds. Xyl and Xyl represent glycosylated (Xyl) and non-glycosylated (Xyl) xylose (spin systems traced in pink and green, respectively). The coincident spin system of glucose and galactose is traced in red.

The oscillatory behavior of the xylolucan at concentrations of 0.5 and 1.0% (w/v) was investigated by rotational, non-destructive oscillatory and stress-strain studies in order to evaluate its performance and potential as a biotechnological product.

Most part of the rheological characterization of xylolucans from different sources investigates flow properties of this polysaccharide by mixing with other components [37–39] or chemical modification [23,24,40].

The rotational flow studies were performed on the xylolucan with the apparent viscosity as a function of shear rate (Fig. 6A). For both concentrations, the system behaves as a Newtonian fluid for most part of the shear-rate interval in agreement with data from the literature [41–43]. These authors show xylolucan aqueous solutions exhibiting typical Newtonian behavior at low concentrations (≤0.5% w/w) and shear-thinning behavior for concentrations usually higher than 1% (w/v).

In addition to the properties of stability against heat, pH and shear, xylolucan cannot form gel simply by changes in its concentration. Although there is an increase in its viscosity, xylolucan gels are formed only in the presence of alcohol or high levels of sugar [10,42,44].

Furthermore, the properties of xylolucans in water, such as solubility, viscosity and gelling ability, are closely related to their chemical structures. This type of behavior is expected for most polymer solutions and was already observed for other polysaccharides composed essentially of glucose, xylose and galactose [23,40].

The oscillatory studies measured G’ and G” as a function of the oscillatory angular frequency (Fig. 6B) are similar to the behavior observed for others native xylolucan solutions [23,41,45,46]. For the two concentrations tested, G’ was higher than G” for low frequencies, indicating that the system has a liquid behavior which dissipates most of the energy externally applied. G’ and G” continuously increase as a function of oscillating frequency, so it is possible to observe a cross-over between the moduli; above the cross-over, an elastic behavior sets in G’ > G”, typical for a concentrated polymer solution [47].

The ability to provide both behaviors between liquid and solid states suggests the use of our native xylolucan with 0.5 and 1.0% (w/v) concentrations as films/coatings widely useful in many industries as a biodegradable material.
Fig. 6. Rheological analysis of the solutions obtained from the xylolucan of the H. courbaril seeds at 0.5 (ball symbols) and 1.0% (square symbols) \([w/v] \). (A) rotational flow studies of apparent viscosity as a function of shear-rate, (B) oscillatory studies measuring \(G'' \) (filled symbols) and \(G' \) (open symbols) as a function of frequency and (C) stress-strain experiment measuring \(G' \) and \(G'' \) as a function of shear stress.

Our results showed that solutions Triton X-100 at concentration ranging from 0.25 to 2.00 \( \text{mg mL}^{-1} \) presented \( \text{HC}_{50} \) from 50.70 ± 0.04 to 100%, based on the highest hemolytic effect observed. Significant hemolysis was not detected at these concentrations of xylolucan, with \( \text{HC}_{50} \) of 5.40 ± 0.32 to 6.10 ± 0.08%, thus leading to the conclusion that the polysaccharide is devoid of hemolytic activity and therefore shows potential for application in the health industry without apparent toxicity.

Acknowledgments

The authors Isabel R.S. Arruda, Priscilla B.S. de Albuquerque and Alexandre G. Silva are recipient of a scholarship from Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE). Gustavo R.C. Santos is recipient of a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors express their gratitude to the CNPq for research grants and fellowship (MTSC, PASM, MGCC). The authors acknowledge Centro de Tecnologias Estratégicas do Nordeste (CETENE) by analytical support and CNPq and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) by financial support. Author Antonio A. Vicente wishes to thank the Fundação para a Ciência e a Tecnologia (FCT) Strategic Project PEst-OE/EBB/LA0023/2013 and the Project “BioInd: Biotechnology and Bioengineering for improved Industrial and Agro-Food processes”.

3.9. Biological activities: antibacterial and hemolytic

Antibacterial activity of xylolucans was not tested previously. We now tested this possible effect but did not observe any action of the xylolucan as inhibitor of bacterial growth. Perhaps the monosaccharides from xylolucan could serve as carbon source for microbial growth, differently from that observed e.g. for chitosan [48], that exhibits antimicrobial activity.

The hemolytic activity of xylolucan was performed to rule out a possible mechanism of toxicity and to check the safety of the polysaccharide, thus indicating if it is suitability for pharmaceutical preparations [49]. In hemolytic tests the compounds are considered toxic when the hemolysis contents equal or surpass 50%, the so-called \( \text{HC}_{50} \) [50].
NORTE-07-0124-FEDER-000028, co-funded by the Programa Operacional Regional do Norte (ON.2-O Novo Norte), QREN and FEDER (Portugal).

References