Xylans from the Medicinal Herb *Phyllanthus niruri*

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Phyllanthus niruri is a well-known medicinal herb that is widely used in Asia, Africa, and South America. The characterization of two purified polysaccharides from the whole plant has been investigated. Methylation analysis and ^{13}C NMR spectroscopy showed the chemical structure of two xylans. A hot 15% aqueous KOH fraction yielded a linear β-(1→4)-linked xylan, and 2% aqueous KOH afforded a complex acidic heteroxylan, with a (1→4)-linked β-Xyl*p* main chain, substituted by rhamnose, arabinose, and 4-*O*-methylglucuronic acid side chains. These contained nonreducing end-units of arabinose, xylose, galactose, glucose, and nonmethylated glucuronic acid.

Plants of the genus *Phyllanthus* are part of one of the largest families of higher plants, the Euphorbiaceae. *Phyllanthus* contains more than 600 species, widely distributed throughout South America, Asia, and Africa, of which *Phyllanthus niruri* L. is one of the most common species found in Brazil.[1] The plant has been examined in terms of the identification and characterization of its secondary metabolites, as well as their diverse biological activities. Such metabolites include many alkaloids, flavonoids, lignans, polyphenols, triterpenoids, and vitamin C.[2] Some of the secondary metabolites of the genus *Phyllanthus* occur as glycosides, such as quercetin, kaempferol,[3] and the phyllanthostatins,[4] among others. Several biological investigations on these metabolites have demonstrated significant results, such as on the inhibition of the hepatitis B virus,[5] hypoglycemic, hypotensive, and diuretic effects,[6] and antinociceptive,[7] antitumor,[4] antioxidative,[8] and anti-inflammatory[9] properties.

Santos and co-workers[7] showed an increased antinociceptive activity of crude extracts of *Phyllanthus corcovadensis*, when compared to their isolated steroids. Thus, the presence of a variety of compounds in an extract may enhance biological activity. Since many of the secondary metabolites present in the plant are well known, and as there is a lack of information on the primary components of this medicinal herb, we have now characterized two xylans obtained via alkaline extraction.

Studies on xylans from other medicinal plants revealed this class of polysaccharides to have biological activities, as observed for a glucuronoxylan from *Mahonia aquifolium*, which had immunomodulatory and antitussive properties.[10]

Lipid extraction of the dried plant was carried out prior to aqueous extraction and resulted in a 9.0% yield of total lipids. After defatting, aqueous extraction was carried out to yield 9.8% of extract. The alkaline extractions were carried out, sequentially, from the residual material of aqueous extractions and yielded 7.3%, 3.7%, and 5.5% polysaccharide, respectively, from 2, 5, and 15% aqueous KOH, at 100 °C. The alkaline extractions gave a higher total yield (16.5%) when compared to that of aqueous extractions, as the alkali disrupts the cell wall ultrastructure, while water weakens it. The yield from 5 to 15% aqueous KOH increased substantially, indicating that the increase of alkali concentration favors the release of hemicelluloses from the wall, as can be predicted from data obtained by Sun and co-workers.[11]

Table 1. Monosaccharide Composition of Aqueous and Alkaline Extracts of *P. niruri*[a]

	monosaccharide composition (mol %)						
	Rha	Ara	Xyl	Man	Gal	Glc	UA
AE	5	11	13	9	20	42	nd
CE2		41	32		14	9	4
CE5		25	54		8	6	7
CE15	1	3	73	7	6	3	7

[a] UA, uronic acid; nd, not determined; AE, aqueous extract; CE2, CE5, and CE15, alkaline extractions using 2%, 5%, and 15% aqueous KOH, respectively.

Table 1 shows some differences in terms of the monosaccharide composition of the polysaccharides, obtained from aqueous and sequential alkaline extractions. The arabinose, galactose, and glucose content decreased with each extraction, while that of xylose increased significantly. These data suggest that polymers bound to the cell wall to a lesser degree, such as pectins and arabinogalactans, were released under aqueous or mild alkaline extractions, while the less branched xylans, which are highly associated with the cell wall structure, are preferably released under stronger alkaline conditions. The presence of uronic acid was also observed in all crude fractions (CE2, CE5, CE15) from the alkaline extracts.

The crude 2% aqueous KOH extract (CE2) was submitted to purification procedures such as freeze−thawing, Fehling precipitation, membrane dialysis, and ultrafiltration afforded a purified polysaccharide (AX, 6.2% yield) that was water soluble and homogeneous (HPSEC; Figure 1). The molar mass of the polysaccharide was 1.6×10^5 g mol^{-1}. It contained Rha, Ara, Xyl, Gal, Glc, and uronic acid in a molar ratio of 8:9:58:7:1:17. The polysaccharide was carboxy-reduced to give CR-AX with Rha, Ara, Xyl, 4-*O*-methylglucose, Gal, and Glc in a molar ratio of 5:6:59:16:7:7. The fragments characterizing the derived 4-*O*-methylglucitol acetate had *m/z* 87, 99, 129, 159, 189, confirmed by its retention time (Figure 2). Besides the large amount of natural 4-*O*-methylglucuronic acid, nonmethylated glucuronic acid was also found as the relative molar percentage of glucose increased from 1% to 7% after carboxyreduction of the polysaccharide.

Methylation analysis was carried out on CR-AX, which provided more significant GC-MS data on resulting *O*-methyl alditol acetates, not complicated by the presence

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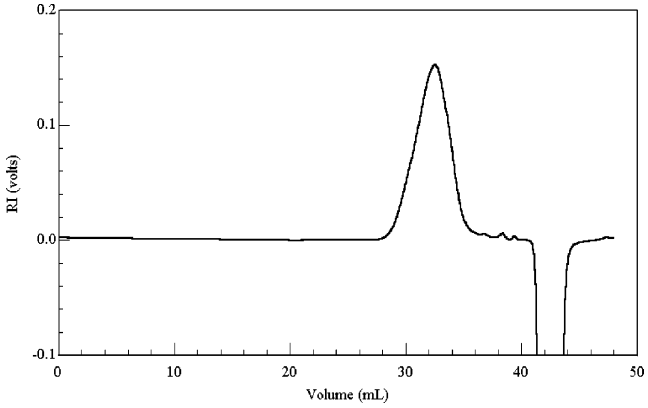

Figure 1. Elution profile of AX with HPSEC, using a refractive index detector.

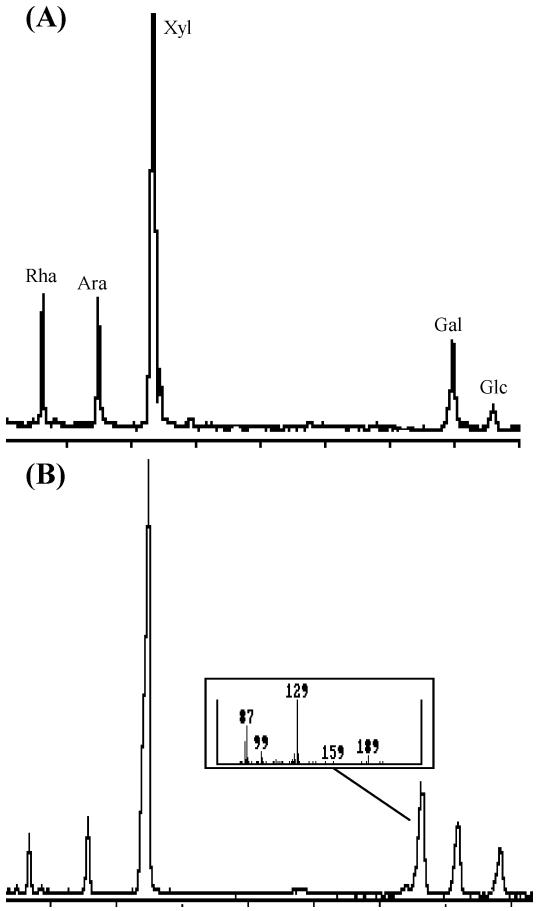

Figure 2. Elution profile of (A) AX and (B) CR-AX with GC-MS. Inset: Fragmentation of the 4-*O*-methylglucuronic acid, using $NaBD_4$ as the reducing agent.

of uronic acid (Table 2). CR-AX contained nonreducing ends of Ara*f* (2%), Xyl*p* (2%), Gal*p* (6%), Glc*p* (1%), and Glc*p*A (6%). The side chains were composed of 2-*O*- (3%) and 2,4-di-*O*- (3%) substituted Rha*p*, and 5-*O*- (2%) and 3,5-di-*O*- (1%) substituted Ara*f*. 2,3-Me_2-Xyl (46%) arose from (1→4)-linked Xyl*p* units from the main chain or even units from the side chains. 2-*O*- and 3-*O*-Me-Xyl (7%) and Xyl (4%), from fully substituted units, were also detected. CR-AX, different from the native sample, revealed the presence of glucuronic acid as nonreducing end-units of Glc*p* (6%) and a high proportion of 3,4,6-Me_3-Glc (17%) from the 4-*O*-Me-glucuronic acid. The large amount of the latter and that the units are not present as nonreducing end are of interest. We thus suppose that nonreducing ends of arabinose, xylose, galactose, glucose, and even glucuronic acid are *O*-2 substituents of 4-*O*-methylglucuronic acid.

Table 2. Partially *O*-Methylalditol Acetates Formed on Methylation Analysis of Carboxy-Reduced (CR-AX) Heteroxylan from *P. niruri*

alditol acetates	fragments (*m/z*)[a]	CR-AX (fragment area %)[b]
2,3,5-Me_3-Ara	87,101,102,118,129,161	2
2,3,4- Me_3-Xyl	88,101,102,117,118,161,162	2
3,4-Me_2-Rha	88,89,115,130,131,190	3
2,3,4,6-Me_4-Glc	87,102,118,129,145,161,162,205	7
2,3-Me_2-Ara	87,102,118,129,189	2
2,3,4,6-Me_4-Gal	87,102,118,129,145,161,162,205	6
2,3-Me_2-Xyl	87,102,118,129,189	46
3-Me-Rha	88,101,117,130,143,160,190,203	3
2-Me-Ara	85,99,118,127,159,201	1
2- Me-Xyl[c]	85,99,118,127,159,201	7
3-Me-Xyl[c]	87,88,129,130,189,190	
3,4,6-Me_3-Glc	87,88,101,129,130,145,161,190	17
Xyl	86,103,115,128,145,188	4

[a] Fragments obtained after using $NaBD_4$ as the reducing agent. [b] Results obtained using a DB-225 column. [c] Both derivatives were quantified together due to coelution in a DB-225 column.

The ^{13}C NMR spectrum (Figure 3) showed AX to be a complex polysaccharide with nine signals in the anomeric region. Those at δ 107.3, 106.9, and 106.2 were from the *O*-substituted and nonreducing ends of α-Ara*f*, respectively.[12] Those at δ 104.2 and 103.3 were from β-Gal*p*.[12] The main signals at δ 101.5 and 101.2 were from 4-*O*- and 2-*O*- and/or 3-*O*-substituted β-Xyl*p* units, respectively.[13] Signals at δ 98.3 and 97.4 corresponded to α-Rha*p* and nonreducing end-units of α-Glc*p*A, respectively.[13] The signals at δ 76.2, 73.6, 72.6, and 63.0 were from C-4, C-3, C-2, and C-5 of the (1→4)-linked-Xyl*p* units.[14,15] Those at δ 82.3 and 59.7 corresponded to C-4 and CH_3 from the 4-*O*-methylglucuronic acid units.[16] The signal at δ 61.0 was from C-6 of nonreducing end-units of Gal*p*.

The crude 15% aqueous KOH extract (CE15) was also submitted to purification by freeze−thawing followed by Fehling precipitation. The precipitate of both sequential treatments afforded, at the end, a linear xylan (HX, 2.3% yield). The ^{13}C NMR spectrum of HX (Figure 4) contained five signals corresponding to that of a (1→4)-linked-β-xylan.[14,15] The signal at δ 101.9 corresponded to the anomeric region in a β-configuration, as confirmed by the ^1H NMR spectrum (H-1 signal at δ 4.3), while the signals at δ 75.8, 74.2, and 72.8 corresponded to C-4, C-3, and C-2, respectively, and δ 63.4 arose from C-5.[14,15] Methylation data confirmed the (1→4)-linkages as the main mass fragments, using $NaBD_4$ as the reducing agent in GC-MS analysis, which occurred at *m/z* 87, 102, 118, 129, and 189, characterizing the only derivative as 2,3-Me_2-xylitol acetate. Traces of 2,3,4-Me_3-Xyl from the nonreducing end-units were also found. Although *O*-acetyl groups, which are widely found in higher plants,[16] could be present, they were not identified since alkali was used to extract the polymer.

Xylans are the most representative hemicelluloses from higher plants[17] and have an enormous structural variability between species, although the backbones are mainly constituted of β-(1→4)-linked xylopyranosyl units, as observed in *P. niruri*. Linear xylans, however, are considered to be rare polymers, although linear xylans were also characterized from intact lichens[14] and lichen photobionts[15] when the same purification procedures were carried out. Heteroxylans containing acidic monosaccharides are common polymers found in the secondary plant cell wall, but special attention is given to this class of polysaccharides since they play an important role in cereals due to their

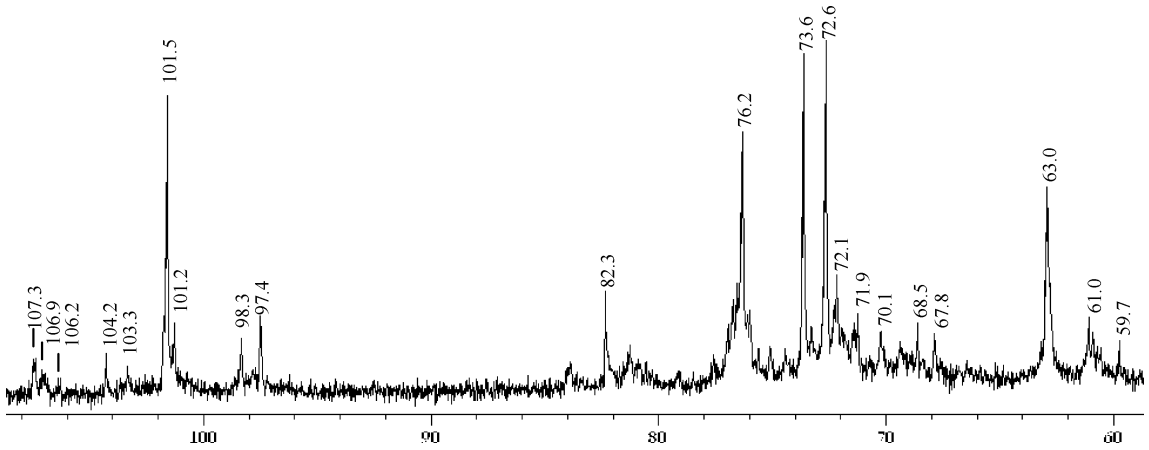

Figure 3. ^{13}C NMR spectrum of the purified fraction AX. Solvent is D_2O at 50 °C; numerical values are in δ (ppm).

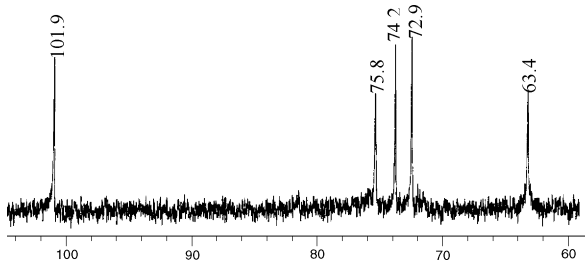

Figure 4. ^{13}C NMR spectrum of the purified β-xylan (HX). Solvent is Me_2SO-d_6 at 50 °C; numerical values are in δ (ppm).

properties in the bread-making processes[18] and also to their additional biological properties.[10]

Experimental Section

General Experimental Procedures. All fractions were evaporated at <40 °C under reduced pressure. The centrifugation conditions were 9000 rpm for 15 min, at 25 °C. Uronic acids were estimated using the improved *m*-hydroxybiphenyl method.[19] The alditol acetate derivatives were analyzed by GC-MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap model 810-R12 mass spectrometer, using a DB-225 capillary column (30 m × 0.25 mm i.d.) programmed from 50 to 220 °C at 40 °C/min. The partially *O*-methylated alditol acetates also analyzed by GC-MS were carried out from 50 to 215 °C at 40 °C/min, and a DB-225 column was used. ^{13}C NMR experiments were obtained using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Samples were dissolved in D_2O or Me_2SO-d_6 and examined at 50 °C. Chemical shifts (δ in ppm) are expressed relative to the resonance of Me_4Si (TMS; $\delta = 0$). Carboxy reduction of the heteroxylan (AX) was carried out using two successive cycles with carbodiimide−$NaBH_4$, according to Taylor and Conrad.[20] The homogeneity and molar mass of AX were determined by high-performance size-exclusion chromatography (HPSEC-MALLS), using a Waters 510 HPLC pump at 0.6 mL/min, with four gel permeation columns in series with exclusion sizes of 1 million to 5000 Da, using a refractive index (RI) detector, and the eluent was 0.1 mol/L aqueous $NaNO_2$ with 200 ppm aqueous NaN_3. AX was dissolved in Milli-Q water, previously filtered in membrane (0.22 μm), and injected (250 μL loop) at a 2 mg/mL concentration. The specific refractive index increment (dn/dc) was also determined ($dn/dc = 0.142$).

Plant Material. Dried powder obtained from the whole plant (*P. niruri* L.) (300 g) was donated by the Pharmaceutical Industry "As Ervas Curam Ltda." The identity of plant species was confirmed by Ms. N. K. Takemori and Prof. Dr. C. Bona (Botany Department, Federal University of Paraná, Curitiba, Brazil), and a sample is deposited in the Herbarium of UFPR (UPCB), as voucher no. 42822.

Defatting Procedure. Lipids were extracted successively with refluxing EtOH (1.5 h, twice), $CHCl_3$−MeOH (2:1 v/v, 1.5 h, twice), and acetone (1 h). The dried defatted residue was then submitted to successive aqueous and alkaline extractions.

Polysaccharide Extraction. The above residue was successively extracted under reflux with water (6 h, three times), 2% aqueous KOH containing traces of $NaBH_4$ (1.5 h, twice), 5% aqueous KOH (1.5 h, twice), and 15% aqueous KOH (1 h, twice). The alkaline extracts were neutralized with HOAc and dialyzed against tap water. All extracts were concentrated under reduced pressure and freeze-dried. The alkaline extracts were submitted to polysaccharide purification procedures.

Polysaccharide Purification. (a) Polysaccharide obtained from 2% aqueous KOH extraction: This fraction was submitted to a freeze−thawing procedure,[21] and the supernatant then submitted to a precipitation process, using Fehling's solution.[22] The precipitated fraction was purified by membrane dialysis with a 16 kDa cutoff followed by a ultrafiltration of the retained material, using membranes of 0.22 μm and 30 000 Da porosities, sequentially. (b) Polysaccharide obtained from 15% aqueous KOH extraction: This material was submitted to several procedures of freezing and thawing, until no more precipitate was formed; the obtained precipitate was then purified by Fehling precipitation.

Monosaccharide Composition. The polysaccharides were hydrolyzed with 1 M TFA at 100 °C for 8 h, followed by evaporation to dryness and successive reduction with $NaBH_4$ or $NaBD_4$ and acetylation with Ac_2O−pyridine (1:1, v/v; 2 mL) at room temperature for 12 h.[23,24] The resulting alditol acetates obtained were analyzed by GC-MS as indicated above and identified by their typical retention times and electron impact profiles.

Methylation Analysis. Per-*O*-methylation of the isolated polysaccharides was carried out using powdered NaOH in Me_2SO−MeI.[25] The products were converted into partially *O*-methylated aldose acetates by treatment with refluxing 2% MeOH−HCl for 2 h, followed by neutralization with Ag_2CO_3. Total acid hydrolysis was carried out with 0.5 M H_2SO_4 for 12 h at 100 °C, which was neutralized with $BaCO_3$ and filtered, and the filtrate evaporated to dryness. The products were converted into partially *O*-methylated alditol acetates as described above and analyzed by GC-MS (as described above).

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