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Acidic heteroxylans from medicinal plants and their anti-ulcer activity

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ABSTRACT

Xylans are the main hemicelluloses found in higher plants, and are often present in phytotherapic medicines. An acidic heteroxylan was obtained from *Maytenus ilicifolia* leaves by hot 10% aqueous KOH extraction. This was subjected to freeze-thawing process, giving insoluble and soluble fractions and the latter treated with Fehling solution. Its insoluble fraction (MI-HX) was further examined. The acidic heteroxylan gave xylose, galactose, glucose, and 4-*O*-methylglucuronic acid in a 76:6:9:9 molar ratio and methylation analyses and ^{13}C NMR spectroscopy showed its main chain consists of 4-*O*-linked β-D-Xyl*p* units. This polysaccharide and another acidic heteroxylan from *Phyllanthus niruri* had anti-ulcer activity and were able to reduce gastric lesions induced by ethanol by 65% and 78%, with ED_{50} = 40.0 and 20.4 mg/kg, each respectively. These results suggest that this class of polysaccharide has a protective anti-ulcer effect, and that there is a relation between its chemical structure and biological function.

1. Introduction

The use of phytotherapeutic drugs in secondary or primary medical treatment is increasing worldwide. The efficacy of different classes of secondary metabolites, such as alkaloids, flavonoids, and polyphenols from higher plants has been widely demonstrated (Halberstein, 2005).

Studies on the efficacy of primary metabolites, especially carbohydrates, have also been carried out, involving their chemical structure related to their biological and/or industrial importance (Bohn & BeMiller, 1995).

Some researchers have attributed enhanced, synergic biological activity to crude or partially fractionated extracts, compared with their purified components. This is consistent with the interaction of different chemicals in a pill to interfere with the final result of a given treatment (Halberstein, 2005). Crude plant extracts or dried powdered plants used in phytomedical treatments are composed of more than the active metabolite primarily responsible for the biological response. Thus, based on the presence of xylans in some therapeutic pills, the following investigation has been carried out.

Xylan-based polysaccharides are the main hemicelluloses found in higher plant cell-walls, and depending on the plant organ, may be present in up to 30% of its dry weight, significant in terms of pill production. They are present in all higher plants and have a great structural variability, although their main chain is commonly composed of β-(1 → 4)-Xyl*p* residues (Joseleau, Comtat, & Ruel, 1992).

Studies have already showed biological properties of xylans, as immunological (Ebringerová, Hromadková, Alfodi, & Hribalová, 1998; Ebringerová, Hromadková, Malovíková, & Hribalová, 2002), anti-complementary (Samuelsen et al., 1999), and antitussive (Kardosová, Malovíková, Pätoprstý, Nosál'ová, & Matáková, 2002).

Two native Brazilian plant species were chosen for the present study, which are widely used in medicinal pills as well as in tea preparations. *Maytenus ilicifolia* is traditionally used and scientifically proven to be efficient in treating and preventing stomach ulcers (Cipriani et al., 2006; Souza-Formigoni et al., 1991). The chemical structure of its acidic heteroxylan is now determined. Conversely, the chemical structure of the acidic heteroxylan from *Phyllanthus niruri* is already known (Mellinger, Carbonero, Cipriani, Gorin, & Iacomini, 2005), although any biological activity for it has not been demonstrated. We now investigate the anti-ulcer activity of both acidic heteroxylans.

2. Experimental

2.1. Plant material

Leaves of *M. ilicifolia* Mart. ex Reissek (Celastraceae) were collected in Curitiba (Southern Brazil) in July 2003, and identified by Dr. Olavo Guimarães, Department of Botany, Federal University of Paraná. A voucher specimen (No. 30842) is deposited at the Herbarium of Federal University of Paraná.

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Phyllanthus niruri was donated by the Pharmaceutical Industry "As Ervas Curam Ltda". Mellinger et al. (2005) described the extraction and structural analysis of its heteroxylan.

2.2. General analytical methods

All extracts were evaporated at <40 °C under reduced pressure. The centrifugation conditions were 10,000 rpm for 15 min, at 25 °C. Uronic acid contents were determined using the improved *m*-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991). Carboxy-reduction of the polysaccharide (10 mg) was carried out by the carbodiimide method (Taylor & Conrad, 1972), using $NaBD_4$ as the reducing agent, in order to detect and characterize the uronic acid residues in the monosaccharide and methylation analyses.

2.3. Extraction and isolation of acidic heteroxylan from M. ilicifolia

Ground leaves of *M. ilicifolia* (350 g) were defatted with $CHCl_3$–MeOH (2:1, v/v; 1 L) at 60 °C for 2 h (3×). The residue was extracted with water (1 L, 100 °C, 3×) followed by 2% aq. KOH (1 L, 100 °C, 3X), in the presence of a trace of $NaBH_4$, to remove pectic polysaccharides (Cipriani, Mellinger, Gorin, & Iacomini, 2004) and then 10% aq. KOH (1 L) at 100 °C for 3 h (3×). The aqueous and 2% aq. KOH extracts were not examined. After neutralization with HOAc, the 10% aq. KOH extract was dialyzed and freeze-dried. It was solubilized in water and then frozen and thawed at 4 °C to form a precipitate, the process being repeated until a precipitate no longer appeared. The water-soluble fraction was treated with Fehling solution (Jones & Stoodley, 1965), and the resulting insoluble Cu^{2+} complex was isolated by centrifugation. Both the supernatant and the insoluble complex were neutralized with HOAc, dialyzed, and deionized with cation-exchange resin (H^+ form).

2.4. HPSEC examination of polysaccharide

Homogeneity and average molar mass (M_w) of the polysaccharide was determined by high-performance size-exclusion chromatography (HPSEC) coupled to a refractive index detector (Reed, 1995). Four gel permeation ultrahydrogel columns in series, with exclusion sizes of 7×10^6, 4×10^5, 8×10^4, and 5×10^3 Da, were used. The eluent was 0.1 mol/L aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL/min. The sample, previously filtered through a membrane (0.22 μm), was injected (250 μL loop) at a concentration of 1 mg/mL. Specific refractive index increment (dn/dc) was also determined and the results were processed with a software provided by the manufacturer (Wyatt Technologies).

2.5. Monosaccharide analysis

Monosaccharides were liberated from the polysaccharide (2 mg) with 2 M TFA (1 mL) at 100 °C for 8 h. The solution was evaporated to dryness and the residue dissolved in water (1 mL) to which $NaBH_4$ (2 mg) was added. After 18 h, HOAc was added, the solution evaporated to dryness and the resulting boric acid removed as trimethyl borate by repeated evaporations of added MeOH (Wolfrom & Thompson, 1963a). Acetylation was carried out with Ac_2O-pyridine (1:1, v/v; 2 mL) at room temperature for 12 h, the solution added to excess ice-water and extracted with $CHCl_3$. This was evaporated to dryness at room temperature to give alditol acetates (Wolfrom & Thompson, 1963b). These were analyzed by GC–MS (Varian Saturn 2000R—3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225 column (30 m × 0.25 mm) programmed from 50 to 220 °C at 40 °C/min, and helium as carrier gas. Components were identified by their typical retention times and electron impact spectra (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

2.6. Methylation analysis

According to Ciucanu and Kerek (1984), the polysaccharide (5 mg) was dissolved in DMSO (1 mL), followed by addition of powdered NaOH (300 mg), and MeI (1 mL). The mixture was vigorously shaken for 30 min and then left for 18 h at rest. After neutralization with HOAc, the per-*O*-methylated derivative was extracted with $CHCl_3$. It was hydrolyzed with 50% v/v H_2SO_4 (0.5 mL) at 0 °C for 1 h, followed by dilution to 8% p/v and treatment at 100 °C for 17 h (Saeman, Moore, Mitchell, & Millet, 1954). The resulting mixture of *O*-methyl aldoses was neutralized with $BaCO_3$, filtered, reduced with $NaBD_4$ and acetylated as described above to give a mixture of partially *O*-methylated alditol acetates, which were analyzed by GC–MS. The conditions were those described for alditol acetates, except that GC–MS analysis was carried out from 50 to 215 °C at 40 °C/min. The resulting partially *O*-methylated alditol acetates were identified by their typical retention times and electron impact spectra (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.7. NMR spectroscopy

1D and 2D NMR spectra were obtained using a 400-MHz Bruker model DRX Avance spectrometer with a 5-mm inverse probe, at 70 °C in D_2O. Chemical shifts (δ) are expressed in ppm relative to acetone, at δ30.2.

2.8. Animals

Female Wistar rats (180–200 g) from the Federal University of Paraná colony were maintained under standard laboratory conditions (12 h light/dark cycle, temperature 22 ± 2 °C). Standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available *ad libitum*. The animals were deprived of food 15–18 h prior to the experiment. The experimental protocol using animals was performed according to the "Principles of Laboratory Animal Care" (NIH Publication 85-23, revised 1985) adopted by UFPR.

2.9. Induction of acute gastric lesions in rats

Fasted rats ($n = 6$) were orally fed with vehicle (water, 0.1 mL/100 g body weight), omeprazole (40 mg/kg), MI-HX or PN-HX (10, 30, 100 mg/kg), 1 h before administration of 80% EtOH (0.5 mL/200 g, p.o.). Animals were killed by cervical dislocation 1 h after treatment (Robert, Nezamis, Lancaster, & Hauchar, 1979). The severity of the gastric lesion was quantified using the formula: injured area (mm^2) = length of lesion (mm) × width of lesion (mm), as previously described (Zayachkivska et al., 2005).

2.10. Statistical analysis of gastric lesion rate

Data were expressed as means ± SEM. Statistical significance of the results was determined using a one-way analysis of variance followed by the Bonferroni test. Data were considered different at a significance level of $p < .05$. The effective dose 50 (ED_{50}) was calculated by fitting the data into the equation: $V_i/V_o = 1/(1 + [I]/IC_{50})$ using the KhaleidaGraph 3.0 for a Windows program (Synergy Software, PA, USA). V_i, total activity; V_o, remaining activity.

3. Results and discussion

Defatted leaves of *M. ilicifolia* were successively extracted under reflux, with water, 2% and 10% aq. KOH. The aqueous and the 2% aq. KOH extracts, rich in pectins, were not analyzed in this study. The 10% alkaline extract was neutralized (HOAc) and dialyzed against tap water to give rise to the polysaccharidic fraction (3.7% yield),

which was submitted to freeze-thawing. The supernatant was then treated with Fehling solution giving a precipitate and a supernatant component. HPSEC analysis showed that the former was homogeneous, with M_w = 59,600 g/mol and dn/dc = 0.165 (Fig. 1). The purified polysaccharide (MI-HX, 1.2% yield) had 9% of uronic acids according to *m*-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991).

In order to characterize the uronic acid units of MI-HX, it was carboxy-reduced using carbodiimide/$NaBD_4$ prior to monosaccharide analysis, which gave rise to xylose, galactose, glucose, and 4-*O*-methylglucose. Derived alditol acetates gave corresponding GC-MS signals in a 76:6:9:9 molar ratio (Table 1), with the 4-*O*-methylglucitol acetate providing ions with *m*/*z* 89, 131, 191 corresponding to C-6 di-deuteration. Galactitol and glucitol acetate ions with *m*/*z* representative of galacturonic and glucuronic acids were not observed.

Methylation analysis (Table 2) was carried out on carboxy-reduced MI-HX. The principal Xyl*p* units were found to be substituted at O-4 and O-2,4, according to the formation of alditol acetates of 2,3-Me_2-Xyl (65%) and 3-Me-Xyl (9%) derivatives, respectively. The 4-OMe-Glc*p*A units present in MI-HX were non-reducing end- (8%) and 2-*O*-substituted (2%), according to the appearance of alditol acetates of 2,3,4,6-Me_4-Glc-1d-6d_2 (*m*/*z* 89, 102, 118, 131, 147, 163, 207) and 3,4,6-Me_3-Glc-1d-6d_2 (*m*/*z* 88, 89, 130, 131, 147, 163, 190), respectively. The Glc*p* units were substituted at *O*-4,6 as demonstrated by the presence of alditol acetates 2,3-Me_2-Glc (7%). Nonreducing end-units of Xyl*p* (2,3,4-Me_3-Xyl; 4%) and Gal*p* (2,3,4,6-Me_4-Gal; 5%) were also present.

The ^{13}C NMR spectrum of MI-HX (Fig. 2A) contained six signals in the anomeric region at δ 97.9–104.2. Those at δ 102.2, 101.6, and 101.1 were from C-1 of nonreducing end, 4-*O*- and 2,4-di-*O*-substituted β-D-Xyl*p* units, respectively (Ebringerová et al., 1998; Kardosová et al., 2002; Maurer-Menestrina, Sassaki, Simas, Gorin, & Iacomini, 2003; Mellinger et al., 2005). The signal at δ 97.9 corresponding to C-1 of nonreducing end-units of 4-OMe-α-D-Glc*p*A (Ebringerová et al., 1998; Kardosová et al., 2002; Maurer-Menestrina et al., 2003), whereas that at δ 104.2 was from C-1 of nonreducing end-units of β-D-Gal*p* (Gorin & Mazurek, 1975; Mellinger et al., 2005). The C-1 signal from glucopyranosyl units was assigned using 2D NMR techniques (HMQC, COSY, and TOCSY). A ^{13}C/^{1}H-1 signal at δ 99.0/4.92 was consistent with an α-glycosidic configuration. Complete connectivity occurred from *trans*-vicinal protons,

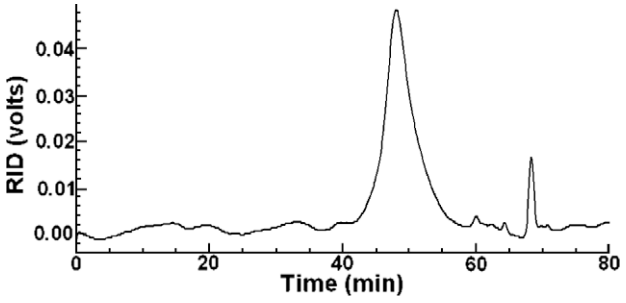

Fig. 1. Elution profile obtained on HPSEC analysis of MI-HX.

Table 1
Monosaccharide composition of the heteroxylans MI-HX and PN-HX

	Monosaccharide composition (mol %)						
	Rha	Ara	Xyl	Gal	Glc	GlcA	4-OMe-GlcA
MI-HX	–	–	76	6	9	–	9
PN-HX	5	6	59	7	1	6	16

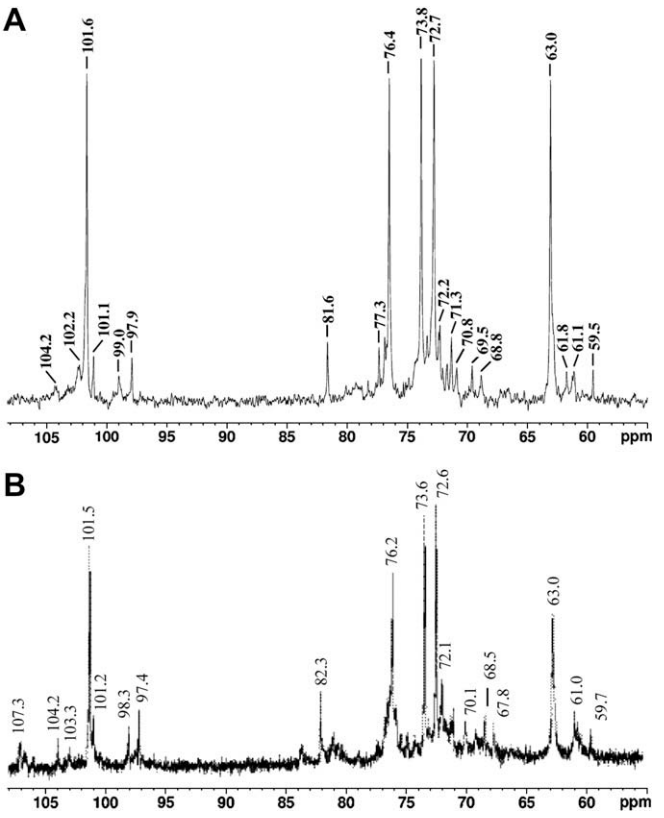

Fig. 2. ^{13}C NMR spectra of MI-HX (A) and PN-HX (B). Solvent D_2O at 70 °C; numerical values are in δ ppm.

from δ 4.92 (H-1) to 3.53 (H-2), and then to δ 3.70 (H-3), 3.59 (H-4), 3.43 (H-5), and 3.32 (H-6), confirming the α-glucopyranosyl structure. It followed that the signal at δ 99.0 arose from C-1 of 4,6-di-*O*-substituted α-D-Glc*p* units present in MI-HX.

The ^{13}C signals at δ 76.4, 73.8, 72.7, and 63.0 were from C-4, C-3, C-2, and C-5 of the (1 → 4)-linked β-D-Xyl*p* units (Kardosová et al., 2002; Maurer-Menestrina et al., 2003; Mellinger et al., 2005; Carbonero, Sassaki, Gorin, & Iacomini, 2002). Those at δ 81.6 and 59.5 corresponded to –*C*HOCH$_3$-4 and –CHO*C*H$_3$-4 from 4-OMe-α-D-Glc*p*A units, respectively (Maurer-Menestrina et al., 2003; Mellinger et al., 2005). These results indicated that MI-HX is an acidic heteroxylan containing a (1 → 4)-linked β-D-Xyl*p* main chain, commonly occurring in higher plants (Joseleau et al., 1992).

In order to determine whether the acidic heteroxylans from *M. ilicifolia* and *P. niruri* have anti-ulcer activity, oral treatment with 10, 30, and 100 mg/kg of MI-HX and PN-HX (*P. niruri* heteroxylan) were tested into an *in vivo* model. Reduced gastric lesions induced by EtOH were observed by 65% and 78%, with ED_{50} = 40.0 and 20.4 mg/kg, each respectively. Examination of omeprazole (40 mg/kg), a positive control for the test, showed reduction of the gastric lesions induced by EtOH by ~60% in both experiments (Fig. 3).

These results suggest a potential activity of these polysaccharides to act as direct cytoprotective agents. Possible mechanisms suggested for anti-ulcer effects of polysaccharides are the ability to: (1) bind to the mucosa surface and function as a protective coating; (2) diminish secretory activity; and (3) protect the mucosa by increasing mucus synthesis and/or scavenging radicals (Nergard et al., 2005; Yamada, 1994; Matsumoto, Moriguchi, & Yamada, 1993).

MI-HX and PN-HX had different anti-ulcer activity potentials, suggesting that this activity is influenced by differences in the

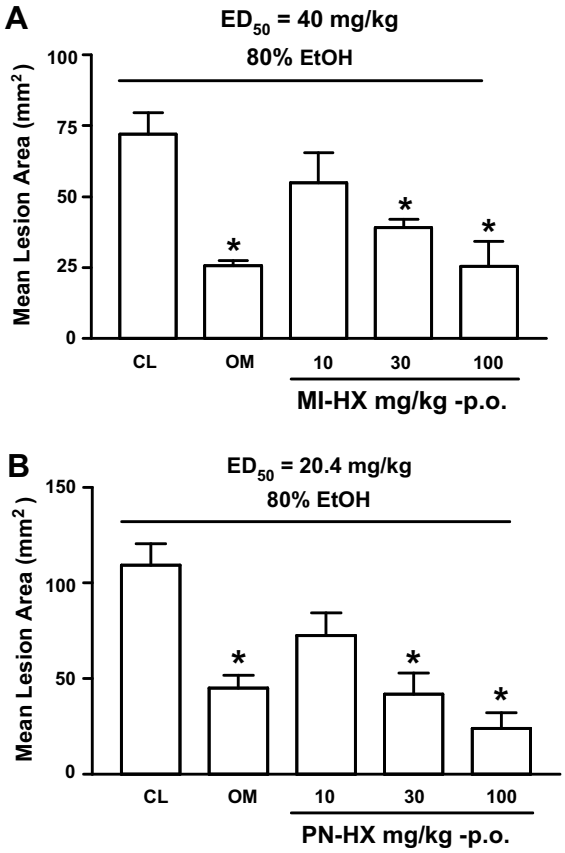

Fig. 3. Protective effect of (A) MI-HX and (B) PN-HX tested with doses of 10, 30, and 100 mg/kg, p.o., against ethanol-induced gastric lesions (CL: control, water 0.1 mL/100 g, p.o. and OM: omeprazole 40 mg/kg, p.o.). The results are expressed as means ± SEM ($n = 6$). Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni test. $^{*}p < .05$ when compared to control group.

polysaccharides structure. Comparing the structures (Fig. 2, and Tables 1 and 2), it can be observed that, even having the same main-chain of β-(1 → 4)-Xyl residues, PN-HX has a higher uronic acid content, and contains rhamnose, arabinose, and glucuronic acid, different from MI-HX. In addition, PH-HX has a higher molar mass (M_w of 160,000 g/mol). Arabinogalactans (Cipriani et al., 2006; Nergard et al., 2005) and pectic polysaccharides having higher uronic acid contents (Yamada, 1994) are known to have anti-ulcer activity. Thus, the presence of arabinose residues and a higher content of uronic acids can be related with the higher anti-ulcer activity of PN-HX.

Table 2
Partially *O*-methylalditol acetates formed on methylation analyses of carboxy-reduced heteroxylans

O-Me-alditol acetate	Linkages	MI-HX (mol %)	PN-HX (mol %)
2,3,4-Me_3-Xyl	Xyl*p*-(1 →	4	2
2,3-Me_2-Xyl	→ 4)-Xyl*p*-(1 →	65	46
3-Me-Xyl	→ 2,4)-Xyl*p*-(1 →	9	7[a]
Xyl	→ 2,3,4)-Xyl*p*-(1 →	–	4
2,3,4,6-Me_4-Glc[b]	4-OMe-Glc*p*A-(1 →	8	7
3,4,6-Me_3-Glc[b]	→ 2)-4-OMe-Glc*p*A-(1 →	2	17
2,3,5-Me_3-Ara	Ara*f*-(1 →	–	2
2,3,4,6-Me_4-Gal	Gal*p*-(1 →	5	6
2,3-Me_2-Ara	→ 5)-Ara*f*-(1 →	–	2
2,3-Me_2-Glc	→ 4,6)-Glc*p*-(1→	7	–
2-Me-Ara	→ 3,5)-Ara*f*-(1 →	–	1
3,4-Me_2-Rha	→ 2)-Rha*p*-(1 →	–	3
3-Me-Rha	→ 2,4)-Rha*p*-(1 →	–	3

[a] Due to peak overlapping, it was a mixture of both 2-Me-Xyl and 3-Me-Xyl in PN-HX only.
[b] Present with C-6 di-deuteration.

In conclusion, our results are important in different parameters: MI-HX is a biologically active acidic heteroxylan from *M. ilicifolia*, a traditional plant, which is already used to treat and prevent gastric ulcers. This polymer may be part of medicinal pills and its ingestion may increase such property. The positive result obtained with PN-HX does not focus on proposing a medicinal treatment based in *P. niruri* plant extracts, but has a wider meaning, especially in studying other natural sources rich in highly acidic xylans; mainly those which are discarded as industrial trash, and could eventually be useful into promoting an efficient pharmacotherapy using natural products. Finally, xylans from higher sources plants may be introduced as a significant part of medicinal pills, and may have the additional property of protecting the stomach from injuries promoted from other chemicals.

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