A Polysaccharide from a Tea (Infusion) of *Maytenus ilicifolia* Leaves with Anti-ulcer Protective Effects

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Maytenus ilicifolia is a medicinal plant used as a tea (infusion) for treatment of stomach ulcers. This tea furnished a polysaccharide after several purification steps, consisting of a freezing−thawing process, Fehling precipitation, ultrafiltration, and dialysis. It consisted of arabinose, galactose, galacturonic acid, 4-*O*-methylglucuronic acid, rhamnose, and glucose in a 42:41:6:5:4:2 molar ratio. Methylation analysis, controlled Smith degradation, and NMR spectroscopy indicated that it was a type II arabinogalactan containing a (1→3)-linked *β*-D-Gal*p* main chain, substituted at O-6 by (1→6)-linked *β*-D-Gal*p* chains, which were mainly substituted at O-3 by (1→5)- and (1→3)-linked α-L-Ara*f* chains, and nonreducing end-units of α-L-Ara*f* and 4-*O*-Me-Glc*p*A. This polysaccharide significantly inhibited ethanol-induced gastric lesions in rats with an ED_{50} of 9.3 mg/kg, suggesting that the arabinogalactan liberated from the infusion has a protective anti-ulcer effect.

Maytenus ilicifolia Mart. ex Reissek (Celastraceae) is a medicinal plant locally known as "espinheira-santa" and is found in Paraguay, Uruguay, Argentina, and Southern Brazil.[1] Its leaves are widely used as a tea (infusion) for effective treatment of stomach ulcers and gastritis,[2] and recently, it has been determined that the aqueous extract of *M. ilicifolia* leaves induces vasorelaxation.[3] Several secondary metabolites have been identified or chemically characterized, namely, triterpenes,[4,5] sesquiterpenes,[6] catechin and epicatechin,[7] glucosides,[1] and flavonoid glycosides.[8,9] However, when such infusions are ingested, not only secondary metabolites but also primary metabolites are ingested, including polysaccharides.

Many herbs used in popular medicine have been reported to contain polysaccharides possessing a great variety of suggested biological activities, including antiviral, antitumor, immunostimulating, anti-inflammatory, anticomplementary, anticoagulant, hypoglycemic, and anti-ulcer.[10−13] Arabinogalactans (AG) are polysaccharides often reported to be immunologically active,[14,15] and an anti-ulcer activity has also been suggested.[12] They are essential structural polymers in the cell wall of plants and a main component of many gums and exudates,[16,17] sometimes affording highly viscous solutions, with wide industrial application. Their chemical structures are very complex,[18] and members of this class of polysaccharides may exist as a pectin component[19] or linked to proteins.[16,20] The AG found in pectin fractions are mainly type I arabinogalactans (AGI), which consist of a (1→4)-linked *β*-Gal*p* main chain and can be linked to type I rhamnogalacturonan (RGI). The type II arabinogalactans (AGII) consist of a (1→3)-linked *β*-Gal*p* main chain, substituted at O-6 by (1→6)-linked *β*-Gal*p* side chains.[19]

We have previously reported the isolation and characterization of an AGI, which was extracted with hot aqueous 2% KOH,[21] and we now report the isolation and structural analysis of a polysaccharide liberated by popular aqueous extraction (infusion) of *M. ilicifolia* leaves and determine its protective anti-ulcer effect.

Results and Discussion

Leaves of *M. ilicifolia* were extracted by infusion with boiling H_2O, and the extract was treated with excess EtOH, to obtain a crude precipitate of polysaccharides (1.3% yield). It was deproteinated and submitted to a freezing−thawing process and Fehling precipitation.[22] The latter furnished a supernatant component, which was heterogeneous when analyzed by high-performance size-exclusion chromatography (HPSEC) and contained 10% glucose (GC-MS). After α-amylase treatment, the content of glucose was reduced to 2%. This fraction was then submitted to ultrafiltration with 300 and 30 kDa cutoff membranes and dialysis with a 16 kDa cutoff membrane, successively, to give four fractions. HPSEC analysis showed that the retained material on dialysis contained a homogeneous polysaccharide (AG) with an average molar mass (M_w) of 11 400 g/mol ($dn/dc = 0.254$). It contained arabinose, galactose, uronic acid, rhamnose, and glucose in a 42:41:11:4:2 molar ratio. The absolute configuration for the neutral monosaccharides was established by GC-MS analysis as their (−)-2-octyl glycoside acetates[23] and determined as L-Ara, D-Gal, L-Rha, and D-Glc, according to retention times and electron impact spectra from their sugar standards.

The ^{13}C NMR spectrum of the AG (Figure 1A) showed a highly complex polysaccharide, with many signals in the C-1 region (δ 99.3−109.1). Although it contained 11% uronic acids, no signal was observed for the carboxyl groups, due to spectral conditions. Signals at δ 106.5, 106.9, 107.5, and 109.1 corresponded to C-1 of α-L-Ara*f* units,[17,24,25] and those at δ 103.2 to C-1 of *β*-D-Gal*p* units.[17,24−26] The signals at δ 16.7 and 99.7 were attributed to CH_3-6 and C-1 of α-L-Rha*p* units, respectively.[26,27] The α- and *β*-configurations of the monosaccharides were confirmed by the determination of $J_{C-1,H-1}$ couplings (Figure 2). All Ara*f* units have α-configurations (J = 180, 175, and 176 Hz), the Gal*p* units *β*-configurations (J = 162 Hz), and the Rha*p* units α-configurations (J = 174 Hz).[28,29]

Methylation analysis (Table 1) showed that AG is a highly branched polysaccharide, containing nonreducing end-units of Ara*f* (2,3,5-Me_3-Ara) (21.5%) and Gal*p* (2,3,4,6-Me_4-Gal) (2%). The arabinosyl units were substituted at O-5 (Ara*f*) and/or O-4 (Ara*p*), and O-3, according to 2,3-Me_2-Ara (14.5%) and 2,5-Me_2-Ara (6%) derivatives, respectively. However, according to the predominant ^{13}C NMR signals of AG at δ 106.5 to 109.1 (Figure 1A), the great majority of arabinosyl units are in the α-Ara*f* form. The galactopyranosyl units are 3-*O*-, 3,6-di-*O*-, and 6-*O*-substituted according to 2,4,6-Me_3-Gal (7%), 2,4-Me_2-Gal (33%), and 2,3,4-Me_3-Gal (3%) methylated derivatives, respectively. 4-*O*-Substituted glucopyranosyl

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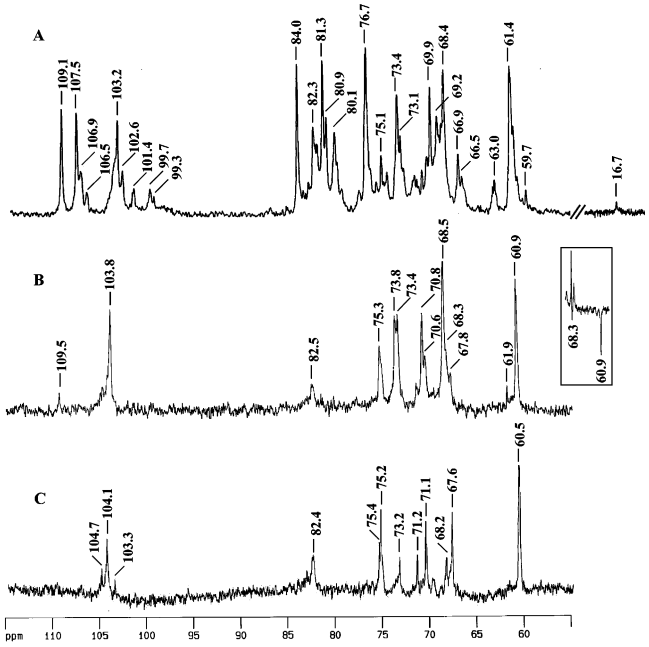

Figure 1. ^{13}C NMR spectra of AG (A), AG-OR (B), and AG-OR2 (C): solvent D_2O (A) and DMSO-d_6 (B and C), at 50 °C, numerical values are in δ, ppm. DEPT of AG-OR (inset in B).

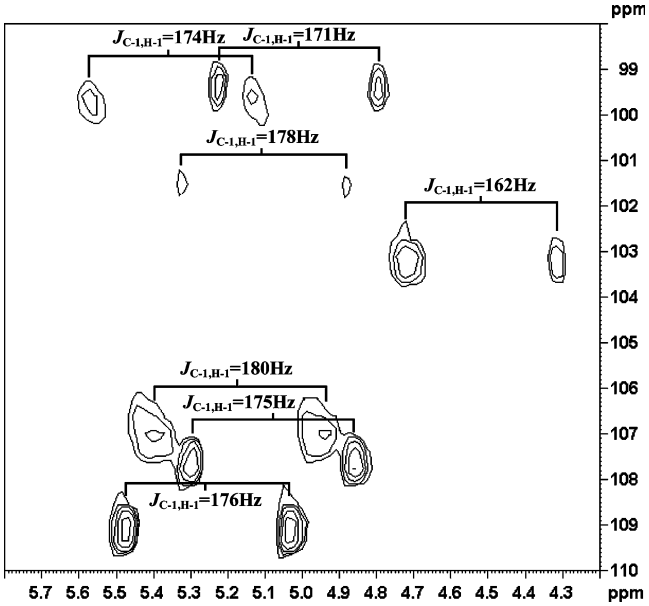

Figure 2. 2D ^{1}H/^{13}C-coupled NMR of the anomeric region of AG: solvent D_2O, at 50 °C, numerical values are in δ, ppm.

units were also present in a small proportion, as shown by the 2,3,6-Me_3-Glc (2%) derivative.

The structure of the uronic acid found in AG was determined after reduction of its carboxyl group. This process transformed the acid monosaccharide to its corresponding neutral monosaccharide. The neutral product (AG-CR) contained arabinose, galactose, 4-*O*-methylglucose, rhamnose, and glucose in a 42:47:5:4:2 molar ratio. The increase of Gal units from 41 to 47% and the appearance of 4-*O*-Me-Glc after reduction indicated that galacturonic acid and 4-*O*-methylglucuronic acid residues were present in AG. Therefore, the signal of ^{13}C NMR (Figure 1A) at δ 99.3 with J = 171 Hz (Figure 2) was attributed to C-1 of the α-Gal*p*A units,[27] and that at δ 59.7 to −CHO*C*H$_3$-4 of the 4-*O*-Me-Glc*p*A units.[30]

Methylation analysis of AG-CR (Table 1) showed that the content of 4-*O*-Me-Glc*p*A residues present in the native AG are nonreducing end-units according to the presence of 2,3,4,6-Me_4-Glc (5%). Also,

Table 1. Profile of Partially *O*-Methylated Alditol Acetates Obtained by Methylation Analysis

O-Me-alditol acetate	linkages	t_R[a]	mol % AG[b]	AG-CR	AG-OR	AG-OR2
2,3,5-Me_3-Ara	terminal	0.834	21.5	20	3	
3,4-Me_2-Rha	2-	0.967		1		
2,5-Me_2-Ara	3-	0.973	6	5.5		
2,3,4,6-Me_4-Glc	terminal	1.000		5		
2,3-Me_2-Ara	5-	1.025	14.5	12		
2,3,4,6-Me_4-Gal	terminal	1.039	2	4	19	20
3-Me-Rha	2,4-	1.172		3		
2,4,6-Me_3-Gal	3-	1.242	7	6.5	24	63
2,3,6-Me_3-Gal	4-	1.268		5		
2,3,6-Me_3-Glc	4-	1.291	2	2		
2,3,4-Me_3-Gal	6-	1.411	3	4	29	3
2,4-Me_2-Gal	3,6-	1.824	33	32	25	14

[a] t_R = relative retention time compared with that of 2,3,4,6-tetra-*O*-methylglucitol. [b] The content of uronic acid of AG was 11%, and that of AG-CR, AG-OR, and AG-OR2 was 0%, according to the method of Filisetti-Cozzi and Carpita.[33]

the galacturonic acid residues present in AG are 4-*O*-substituted, as was shown by the appearance of the 2,3,6-Me_3-Gal (5%) methylated derivative in AG-CR. The presence of 3,4-Me_2-Rha (1%) and 3-Me-Rha (3%) indicates that 2-*O*- and 2,4-di-*O*-substituted Rha*p* residues are also present. 4-*O*-Substituted galacturonic acid and 2-*O*- and 2,4-di-*O*-substituted rhamnose are normal components of type I rhamnogalacturonans.[19] These polysaccharides consist of repeating (1→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p* groups, often having C-4 of the rhamnosyl units substituted by an arabinan, galactan, or arabinogalactan sequence.[19] Thus, it is proposed that galacturonic acid and rhamnose result from an RGI.

Controlled Smith degradation[31] of AG was carried out to characterize its main chain. From 500 mg of AG, 130 mg of an oxidation-resistant polysaccharide (AG-OR) was obtained. It contained galactose and arabinose in a 97:3 molar ratio. The uronic acids and the great majority of arabinosyl units were eliminated on periodate oxidation, indicating that these residues were components of side chains. The ^{13}C NMR spectrum of AG-OR (Figure 1B) contained signals at δ 103.8 and 109.5 of the C-1 of β-D-Gal*p* and α-L-Ara*f* units, respectively. Those at δ 68.3 (DEPT, inset Figure 1B) and 82.5 belong to C-6- and C-3-linked β-D-Gal*p* units, respectively. Methylation analysis (Table 1) showed that the arabinosyl units present in AG-OR are all nonreducing end-units, by the presence of 2,3,5-Me_3-Ara (3%) only. Nonreducing end-units of Gal*p* are also present, according to the appearance of the 2,3,4,6-Me_4-Gal (19%) derivative. The other derivatives found in AG-OR were 2,4,6-Me_3-Gal (24%), 2,3,4-Me_3-Gal (29%), and 2,4-Me_2-Gal (25%), indicating that the AG molecular core is constituted by 3-*O*-, 6-*O*-, and 3,6-di-*O*-substituted galactopyranosyl units.

AG-OR (80 mg) was submitted to a further degradation, giving rise to 25 mg of an oxidation-resistant polysaccharide (AG-OR2). It contained 100% galactosyl units, which were nonreducing end-units (20%), 6-*O*- (3%) and 3,6-di-*O*-substituted (14%), and mainly 3-*O*-substituted (63%), according to the appearance of 2,3,4,6-Me_4-Gal, 2,3,4-Me_3-Gal, 2,4-Me_2-Gal, and 2,4,6-Me_3-Gal derivatives, respectively. In agreement, the ^{13}C NMR analysis of AG-OR2 (Figure 1C) showed an increase of a signal at δ 82.4 of C-3 3-*O*-substituted β-D-Gal*p* units when compared with AG-OR (Figure 1B).

According to our results, AG is a type II arabinogalactan containing a (1→3)-linked β-D-Gal*p* main chain, substituted at O-6 by (1→6)-linked β-D-Gal*p* side chains. The side chains are substituted at O-3 by (1→5)- and (1→3)-linked α-L-Ara*f* chains and nonreducing end-units of α-L-Ara*f* and 4-*O*-Me-Glc*p*A. This arabinogalactan is probably linked to a type I rhamnogalacturonan through C-4 of some of the rhamnosyl units. The structures suggested for AG, RGI, AG-OR, and AG-OR2 are showed in Figure 3.

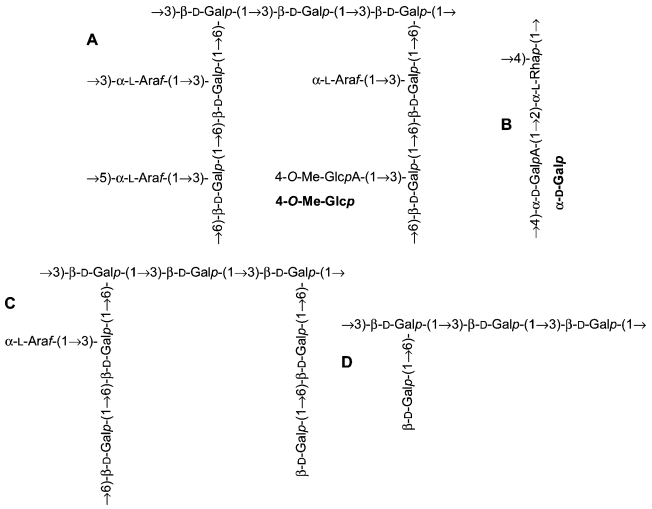

Figure 3. Schemes of the structures suggested for AG (A), RGI (B), AG-OR (C), and AG-OR2 (D). The neutral monosaccharide obtained after reduction of AG is shown in bold.

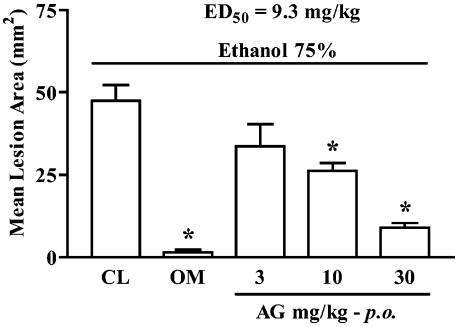

Figure 4. Protective effect of AG (3, 10, and 30 mg/kg, po) against ethanol-induced gastric lesions (CL: control, H_2O 0.1 mL/100 g, po; OM: omeprazole, 40 mg/kg, po). The results are expressed as mean ± SEM ($n = 6$). Statistical comparison was performed using analysis of variance (ANOVA) followed by Tukey's test. *$p < 0.05$ when compared to control group.

M. ilicifolia is widely used for stomach ulcers and gastritis treatment. However, up to the present, the compound responsible for this property is not known. The present investigation has led to the structural characterization of a polysaccharide belonging to a class of polymers whose anti-ulcer activity was suggested.[12] To determine if the biological properties of the *M. ilicifolia* infusion could be due to the type II arabinogalactan, its anti-ulcer activity was evaluated. Oral treatment with AG (10 and 30 mg/kg) reduced the gastric lesions induced by EtOH by 45 and 81%, with $ED_{50} = 9.3$ mg/kg (control value = 121 ± 11, mean ± SEM). Omeprazole, a positive control of the test, reduced the gastric lesions by EtOH by 97% (Figure 4). The pretreatment with AG practically abolished the ethanol-induced gastric damage, suggesting a potential ability of the arabinogalactan as a direct cytoprotective agent. Possible mechanisms for anti-ulcer effects suggested are the ability to bind to the surface mucosa and function as a protective coating, antisecretory activity, mucosal protection by increased mucus synthesis, and radical scavenging.[12,13,32]

The present investigation has led to the structural characterization of a polysaccharide from *M. ilicifolia* leaves, which has a protective anti-ulcer effect. Moreover, this compound is liberated by a mild procedure used in the folk medicine (infusion), and thus, when people drink it, they are ingesting, among other compounds, this polysaccharide.

Experimental Section

General Experimental Procedures. All extracts (see below) 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 according to the colorimetric method of Filisetti-Cozzi and Carpita, using *m*-hydroxybiphenyl.[33] The sample (100 mg) was carboxy-reduced with $NaBH_4$ via the carbodiimide ester.[34] Homogeneities and average molar mass (M_w) were determined by high-performance size-exclusion chromatography (HPSEC) coupled to a multiangle laser light scattering (MALLS) and refractive index detectors.[35] 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 aqueous $NaNO_2$ with 200 ppm aqueous NaN_3 at 0.6 mL/min. Samples, previously filtered through a membrane (0.22 μm; Millipore), were injected (250 μL loop) at a 1 mg/mL concentration. The specific refractive index increment (d*n*/d*c*) was also determined. Results were processed with software provided by the manufacturer (Wyatt Technologies). The alditol acetates and acetylated octyl glycosides were analyzed by GC-MS, using a Varian Saturn 2000R-3800 gas chromatograph coupled to a Varian ion-trap 2000R mass spectrometer with a DB-225 and a DB-23 capillary column (30 m × 0.25 mm), respectively, and helium as carrier gas. The alditol acetates and acetylated octyl glycosides analyses were carried out from 50 to 220 °C at 40 and 10 °C/min, respectively, and maintaining the temperature at 220 °C. The GC-MS analyses of the partially *O*-methylated alditol acetate derivatives (DB-225) were carried out from 50 to 215 °C at 40 °C/min, maintaining at 215 °C. ^{13}C NMR and 2D $^1H/^{13}C$-coupled NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer with a 5 mm inverse probe, at 50 °C in D_2O or DMSO-d_6. Chemical shifts of the samples are expressed in ppm (δ) relative to acetone-d_6 or DMSO-d_6, at δ 30.2 and 39.5, respectively. 2D $^1H/^{13}C$-coupled NMR was obtained after repeated D_2O dissolution, followed by evaporation.

Plant Material. Leaves of *M. ilicifolia* Mart. ex Reissek (Celastraceae) (2 kg), collected in the region of Curitiba (Southern Brazil) in July 2003, were donated by the Central de Produção e Comercialização de Plantas Medicinais, Aromáticas e Condimentares do Paraná Ltda. The plant was identified by Prof. Olavo Guimarães (Botany Department, Federal University of Paraná, Curitiba, Brazil) and is deposited in the Herbarium of UFPR, as voucher no. 30842.

Extraction and Purification of the Type II Arabinogalactan (AG). A 1 L amount of boiling H_2O was poured over dried, ground leaves (100 g), the container was then closed, and the extraction was allowed to proceed until room temperature was reached (~6 h). A total of 2 kg of dried leaves was used. All extracts were evaporated to a small volume, and EtOH was added (× 3 volumes). The resulting precipitates were dissolved in H_2O and treated with 5% aqueous TCA for protein removal. The TCA was removed by neutralization with NaOH and the solution dialyzed. The deproteinated product was submitted to freeze−thawing until no more precipitate appeared. The water-soluble fraction was treated with Fehling solution,[22] 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 H^+ form cation-exchange resin. The Fehling supernatant (10 mg/mL) was treated with α-amylase from *Bacillus licheniformis* (Sigma). The reaction was carried out using 100 units/mL of the enzyme in 20 mM phosphate buffer pH 6.9, containing 6.7 mM NaCl, at 20 °C for 30 min. The enzyme was removed by TCA precipitation. The resulting fraction was then successively submitted to ultrafiltration with 300 and 30 kDa cutoff membranes and dialysis with a 16 kDa cutoff membrane.

Monosaccharide Analysis. Monosaccharide components and their ratios were determined after hydrolysis of the polysaccharide (2 mg) with 2 M TFA (1.5 mL) at 100 °C for 8 h. The solution was evaporated to dryness and the residue dissolved in H_2O (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 co-evaporation with MeOH. Acetylation was carried out with Ac_2O−pyridine (1:1, v/v; 2 mL) at room temperature for 12 h. The solution was added to excess ice−water, which was extracted with $CHCl_3$. This was evaporated to dryness at room temperature to give alditol acetates,[36,37] which were analyzed by GC-MS and identified by their typical retention times and electron impact spectra.

Preparation and Analysis of Derived Acetylated Octyl Glycosides. Monosaccharide standards and the mixture obtained from the hydrolyzed polysaccharide (2 M TFA, at 100 °C for 8 h) were derivatized using 300 μL of (−)-2-octanol containing 10 μL of 1 M TFA[23] and maintained at 100 °C for 14 h. The resulting octyl glycosides were then acetylated as described above and analyzed by GC-MS.

Methylation Analysis. The polysaccharides (5 mg) were methylated according to the method of Ciucanu and Kerek, using powdered NaOH in DMSO−MeI.[38] The per-*O*-methylated derivatives were 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.[39] 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 resulting partially *O*-methylated alditol acetates were identified by their typical retention times and electron impact spectra.[40]

Controlled Smith Degradation. The AG (300 mg) was oxidized in 0.05 M $NaIO_4$ (30 mL) at 25 °C in the dark for 72 h. To stop the reaction, 1,2-ethanediol was added, the solution was dialyzed, and the resulting polyaldehydes were reduced with $NaBH_4$. After neutralization with HOAc followed by dialysis, the material was partially hydrolyzed with TFA, pH 2.0, for 30 min at 100 °C.[31] The neutralized material was dialyzed (2 kDa cutoff membranes) and the retained solution freeze-dried.

Animals. Female Wistar rats (180−200 g) from UFPR colony were maintained under standard laboratory conditions (12 h light/dark cycle, temperature 22 ± 2 °C). Standard pellet food (Nuvital, Curitiba/PR, Brazil) and H_2O 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.

Induction of Acute Gastric Lesions in Rats. Fasted rats ($n = 6$) were orally treated with vehicle (H_2O, 0.1 mL/100 g body weight), omeprazole (40 mg/kg), or the AG (3, 10, 30 mg/kg) 1 h before administration of 75% EtOH (0.5 mL/200 g, po). Animals were killed by cervical dislocation 1 h after treatment.[41] The severity of the gastric lesion was quantified using the following formula: injured area (mm^2) = length of lesion (mm) × width of lesion (mm), as previously described.[42]

Statistical Analysis. Data were expressed as mean ± 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 $p < 0.05$. The effective dose 50 (ED_{50}) was calculated by fitting the data to the equation $V_i/V_o = 1/(1 + [I]/IC_{50})$ using the KhaleidaGraph 3.0 for Windows program (Synergy Software, PA). V_i = total activity, V_o = remaining activity.

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