Chemical and Biological Properties of an Arabinogalactan from *Phyllanthus niruri*

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Phyllanthus niruri is a well-known herb widely used medicinally in Asia, Africa, and South America. Aqueous extraction of the intact plant provided an acidic arabinogalactan, which was characterized chemically, and its effects on peritoneal macrophage activation were determined. Methylation analyses and ^{13}C NMR spectroscopy showed it to have a complex structure with a (1→4)-linked β-Gal*p* main chain, substituted by rhamnose, galacturonic acid, arabinose, xylose, galactose, and glucose-containing side chains, with nonreducing end-units of arabinofuranose, xylopyranose, galactopyranose, and glucopyranose. In immunological studies, the arabinogalactan stimulated superoxide anion production, when tested using peritoneal macrophages of mice, but did not interfere with the nitric oxide pathway. Thus, traditional aqueous extraction methods, such as decoction and infusion, provide a major polysaccharide, which stimulates an intense biological response in macrophages: this could represent an interesting approach in phytotherapeutic treatments.

Plants of the genus *Phyllanthus* are part of the Euphorbiaceae family, comprising more than 600 species, which are widely distributed throughout South America, Asia, and Africa. *Phyllanthus niruri* L., known locally as *quebra pedra* (stone breaker), is one of the most common species found in Brazil.[1] The earliest investigation on *P. niruri* was carried out by Parelle and co-workers in the 1960s, when they isolated an alkaloid named norsecurinine.[2] Several studies have appeared on other *Phyllanthus* spp., which provided information on identification and characterization of their secondary metabolites, which included alkaloids, flavonoids, lignans, polyphenols, triterpenoids, and vitamin C,[3] besides glycosides of quercetin, kaempferol,[4] and the phyllantostatins.[5]

A variety of extracts from *Phyllanthus* spp. and their metabolites have been examined to determine their biological activities. These included inhibition of hepatitis B virus,[6] hypoglycemic, hypotensive, and diuretic effects,[7] and antinociceptive,[8] antitumor,[5] antioxidative,[9] and anti-inflammatory[10] properties. These extracts appear to stimulate the immune system by increasing the natural killer (NK) and antibody-dependent cellular cytotoxicity (ADCC)[11] and nitric oxide (NO) production with mouse peritoneal macrophages.[12]

Recent studies on the biological activities of medicinal plants gave rise to the concept that crude extracts may enhance certain biological activities when compared to individual secondary compounds.[8]

Polysaccharides[13] have biological properties including antiviral,[14] leischmanicidal,[15] and respiratory burst alterations of macrophages and NO, gamma interferon (IFN-γ),[16] and tumoral necrosis factor alpha (TNF α)[17] induction. Certain polysaccharides can thus act as "biological response modifiers" (BRM) in terms of immunomodulatory and antitumor effects.[18−20] A promising class of these polymers is arabinogalactans (AGs), which are highly branched polymers that have been shown to be involved as immunomodulatory agents in *Viscum album*,[21] *Plantago major*,[22] *Salvia officinalis*,[23] and *Anadenanthera colubrina*.[17]

Extracts of *P. niruri* showed interesting immunological properties,[12] but data on its polysaccharide components are limited.[24] We now report an arabinogalactan from the aqueous extracts and demonstrate in vitro macrophage activation by $O_2^{\bullet-}$ production.

Results and Discussion

The dried intact plant was subjected to three distinct aqueous extraction procedures, two of which represent popular ways of extraction, such as a tea by infusion or decoction, and a third that is an exhaustive aqueous extraction. The purpose was to compare the yields and carbohydrate content of each procedure. The tea as an infusion (INF) gave 10.1%, while the decoction (DEC) gave 7.2% yields. The exhaustive extraction procedure afforded about 11.0% yield considering the dried weight of the coarsely milled plant.

After precipitation of polysaccharide with excess EtOH, the yields obtained for precipitates of infusion (INF-P) and decoction (DEC-P) were 3.1% and 0.9%, respectively. These yields of precipitate were lower than that obtained on exhaustive aqueous extraction (EAE-P), which was 4.2%. A higher yield from the most vigorous extraction was expected.

After the extraction procedures, all three fractions were subjected to identical purification steps, which consisted of freeze−thawing followed by dialysis of the water-soluble fractions with a 16 kDa cutoff membrane and ultrafiltration of the retained material, using successive membranes with pores of 0.22 μm, 30 kDa, and 10 kDa. The fractions containing polysaccharides were filtered through the membranes and named AG-DEC (decoction), AG-INF (infusion), and AG-EAE (exhaustive aqueous extraction). These fractions were examined by HPSEC, and AG-INF (Figure 1B) and AG-EAE (Figure 1C) were homogeneous and had the same molar mass of 3.5×10^4 g/mol and d*n*/d*c* of 0.305. AG-DEC (Figure 1A) was heterogeneous, but its main peak was similar to those of AG-INF and AG-EAE, suggesting

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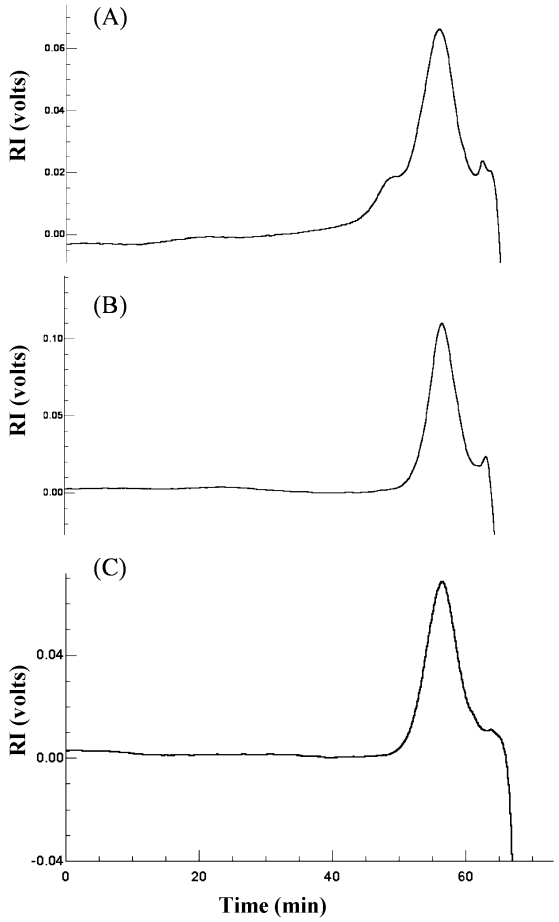

Figure 1. HPSEC elution profile of AG-DEC (A), AG-INF (B), and AG-EAE (C), using a refractive index detector.

Table 1. Monosaccharide Composition of AG-DEC, AG-INF, and AG-EAE[a]

	monosaccharide composition (mol %)						
fraction	Rha	Ara	Xyl	Man	Gal	Glc	UA[b]
AG-DEC	5	25	5	6	32	16	11
AG-INF	7	24	4	9	27	20	9
AG-EAE	8	35	9	4	21	8	15

[a] Arabinogalactan from decoction (AG-DEC), infusion (AG-INF), and exhaustive aqueous extraction (AG-EAE). [b] Uronic acid (UA).

that AG-DEC has basically the same acidic arabinogalactan with different molar masses.

The monosaccharide components of AG-INF, AG-DEC, and AG-EAE were mainly arabinose, galactose, and glucose, with smaller proportions of rhamnose, xylose, and mannose. The percentage compositions were similar (Table 1). There was also a resemblance between the ^{13}C NMR spectra of fractions AG-INF, AG-DEC, and AG-EAE (Figure 2A, 2B, 2C, respectively). However, some differences can be observed in Figure 2C, perhaps due to better resolved signals.

In view of the higher yield of AG-EAE, further chemical analyses and biological experiments were carried out only with this fraction. Methylation analyses, involving conversion of per-*O*-methylated polysaccharides to partially *O*-methylated alditol acetates were carried out for both native (AG-EAE) and carboxy-reduced (CRAG-EAE) polysaccharides. However, the latter was the most reliable, since the partially *O*-methylated galacturonic acid structures of AG-EAE cannot be detected by GC-MS.

After hydrolysis, CRAG-EAE resulted in Rha, Ara, Xyl, Man, Gal, and Glc in a molar ratio of 14:18:7:5:48:8 (GC-MS), which had a greater galactose content, arising from reduction of galacturonic acid in AG-EAE, whose ratio was 8:35:9:4:21:8 (Table 1).

Table 2 shows the methylation data for CRAG-EAE. It contained nonreducing ends of Ara*f* (16%), Xyl*p* (3%), Gal*p* (6%), and Glc*p* (3%). Some side chains were composed of 2-*O*- (8%), 3-*O*- (1%), and 2,4-di-*O*- (3%) substituted Rha*p*, 5-*O*- (3%) and 2-*O*-substituted Ara*f* (1%), and 4-*O*-substituted Xyl*p* (4%), besides smaller amounts of 3-*O*- (2%) and 4-*O*-(3%) Glc*p*-substituted units. Small amounts of 6-*O*-substituted Gal*p* units (1%) were also found, and some of these were substituted at O-3 (3%). The main chain consisted of (1→4)-linked Gal*p* units (36%), with small amounts of substituents at O-3 (2%), O-2 (2%), and O-6 (2%) and disubstituents at O-3,6 (1%). The rhamnose and galacturonic acid components were not present as nonreducing ends, and the increase in the percentage of the 2,3,6-Me_3-Gal derivative after carboxy-reduction could be due to the presence of a pectic chain formed by Gal*p*A (1→4)-linked to Rha*p* at O-2 (8%), as previously found.[25,26]

The ^{13}C NMR spectra from AG-INF, AG-DEC, and AG-EAE (Figures 2A, 2B, 2C, respectively) contain many C-1 signals, which also reflect the complexity of the polysaccharides. For example, AG-EAE gives rise to 11 such signals, each representing one or more structures. Of these, low-field signals from δ 107.1 to 109.5 arise from α-Ara*f* units,[25,27] while that at δ 102.0 is from β-Gal*p*A units.[28] Signals at δ 103.7 and 102.8 are from β-Gal*p* units,[25−28] while the one at δ 98.0 is from α-Gal*p*A.[25] Other assignable signals are at δ 17.3 and 17.0 (CH_3) from nonsubstituted and substituted α-Rha*p*[27,28] and at δ ~175.6 (COOH) from galacturonic acid units.[25,27]

The AGs found in *P. niruri* extracts are relatively small molecules, but with a high structural complexity. They can be classified as pectic arabinogalactans type I. Proteins or peptides were not found in any of the three purified samples.

Figure 3 shows the effects of AG-EAE on macrophage viability. No significant loss was observed following 2 or 24 h of exposure to the polysaccharide. However, a decrease was found after 48 h, when concentrations of 50, 100, and 250 μg/mL were tested. The effect was thus time and dose dependent, and a maximum inhibition of macrophage viability (35%) occurred at 100 μg/mL after 48 h of exposure.

Figure 4A shows the AG-EAE macrophage activation via $O_2^{\bullet-}$ production after 2 h of exposure of cells to the arabinogalactan. The stimulus was 1.5-fold at 50 μg/mL, reaching 3-fold for the highest tested concentration (250 μg/mL).

To evaluate the role of AG-EAE in macrophage functions as tested by NO production, concentrations of 5, 10, and 50 μg/mL were used. In contrast to data observed for other polysaccharides, such as galactomannans[15] and mannans,[29] AG-EAE neither stimulated this pathway (Figure 4B) nor interfered in the NO pathway, when tested alone or in combination with IFN-γ, the latter being a recognized agent that stimulates the NO pathway in macrophages.[30] A control in the presence of polymyxin B, an antibiotic known by its LPS-neutralizing effect,[30] was also carried out and indicated that AG-EAE was LPS-free.

Previous reports showed immunomodulatory responses using *Phyllanthus* extracts, as with aqueous and acetone extracts of *P. niruri*, when the plant stimulated macrophage activation via NO production.[12] We have not detected

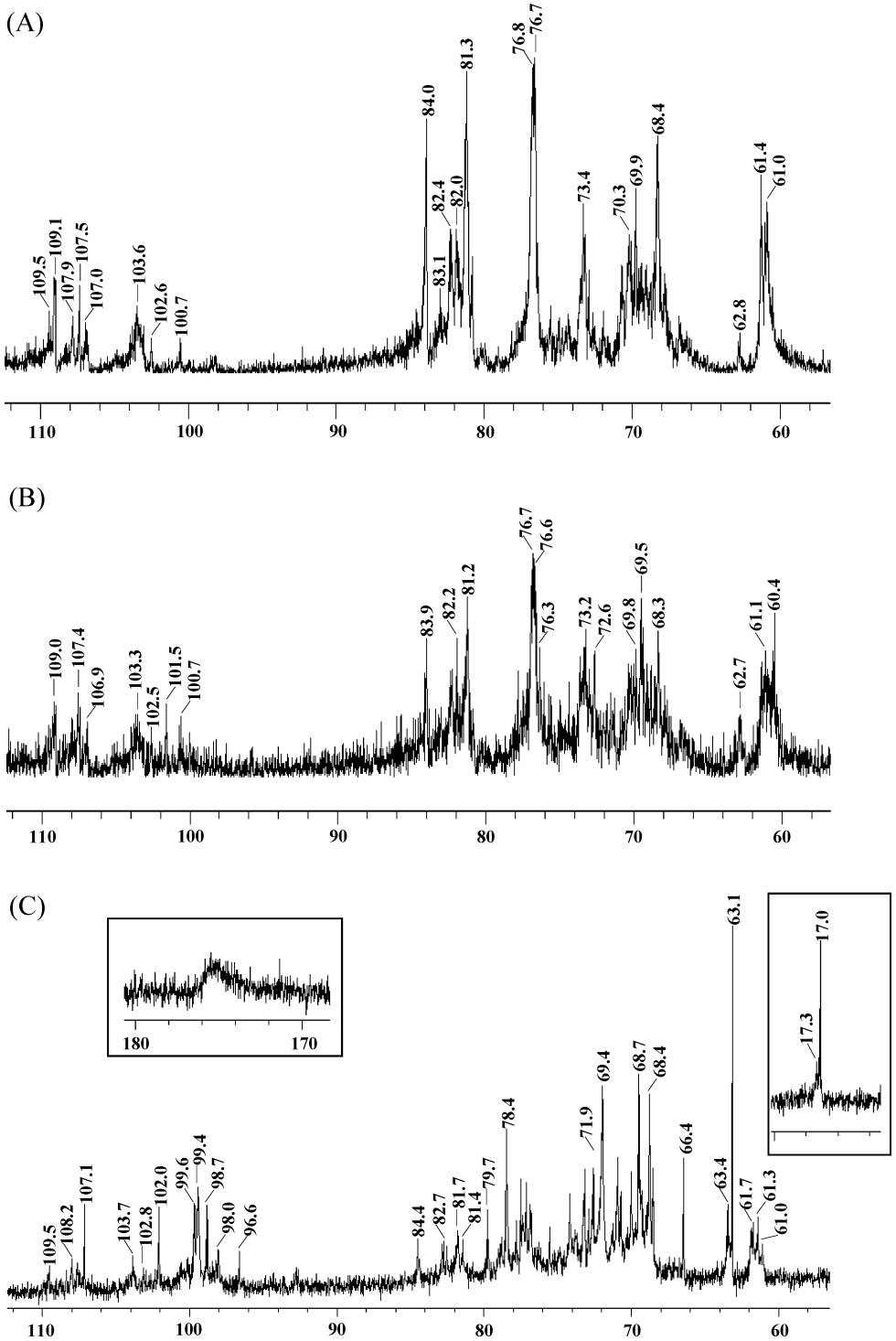

Figure 2. ^{13}C NMR spectra of AG-INF (A), AG-DEC (B), and AG-EAE (C). Solvent D_2O at 50 °C, numerical values in δ (ppm).

any increase in NO production in our experiments, and therefore no NO effect could be attributed to our AG. However, it is the first time that an $O_2^{\bullet-}$ production arising from *P. niruri* extracts was described, and this is of great interest, as it can be attributed to the major polysaccharide component present in its tea preparations.

Considering that the acidic arabinogalactan occurs in traditional tea extracts, either by infusion or decoction, AG is significant, as it was able to generate a high in vitro macrophage response. Although the in vivo accessibility of these molecules to macrophages is not known, these data are relevant from the biological point of view. Further studies aiming at comprehension of how traditional plant remedies are effective for many human disorders are currently in progress in our laboratory.

Experimental Section

General Experimental Procedures. All aqueous solutions were evaporated at <40 °C under reduced pressure. Centrifugation was carried out at 12500*g* for 15 min, at 25 °C. Uronic acids were estimated using an improved *m*-hydroxybiphenyl method.[31] Alditol acetate mixtures (see below) were analyzed by GC-MS in the electron impact mode, 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.), injector temperature

Table 2. Partially *O*-Methylalditol Acetates Formed on Methylation Analysis of Carboxy-Reduced Arabinogalactan (CRAG-EAE) from *P. niruri*[a]

alditol acetate	t_R[b]	mol %
2,3,5-Me_3-Ara	0.84	16
2,3,4-Me_3-Xyl	0.89	3
3,5-Me_2-Ara	0.95	1
3,4-Me_2-Rha	0.96	8
2,4-Me_2-Rha	0.98	1
2,3,4,6-Me_4-Glc	1.00	3
2,3-Me_2-Ara	1.02	3
2,3,4,6-Me_4-Gal	1.04	6
2,3-Me_2-Xyl	1.05	4
3-Me-Rha	1.16	3
2,4,6-Me_3-Glc	1.23	2
2,3,6-Me_3-Gal	1.25	36
2,3,6-Me_3-Glc	1.27	3
2,3,4-Me_3-Gal	1.38	1
2,6-Me_2-Gal	1.44	2
3,6-Me_2-Gal	1.53	2
2,3-Me_2-Gal	1.70	2
2,4-Me_2-Gal	1.76	3
2-Me-Gal	1.97	1

[a] Obtained using a DB-225 column at 215 °C. [b] t_R = relative retention time compared with that of the acetate of 2,3,4,6-tetra-*O*-methylglucitol.

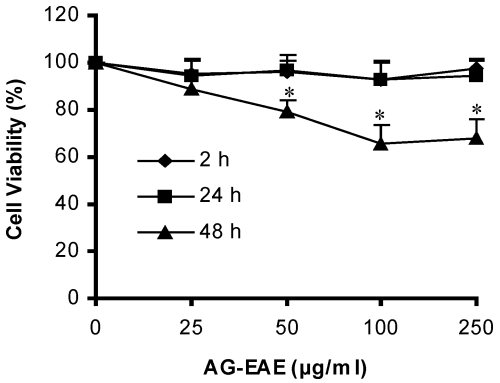

Figure 3. Effects of AG-EAE on macrophage viability. Adherent macrophages were incubated in different concentrations of AG-EAE. The medium was then removed and MTT was added, followed by incubation for 3 h. Excess MTT was removed and formazan crystals were dissolved by addition of DMSO. The absorbance was measured at 550 nm. Values are means ± SD of three experiments, each one in triplicate. *Significantly different from control, $p < 0.05$. Control (100%) corresponds to the medium in the absence of AG-EAE complexes. (◆) 2 h, (■) 24 h, and (▲) 48 h exposure to AG-EAE.

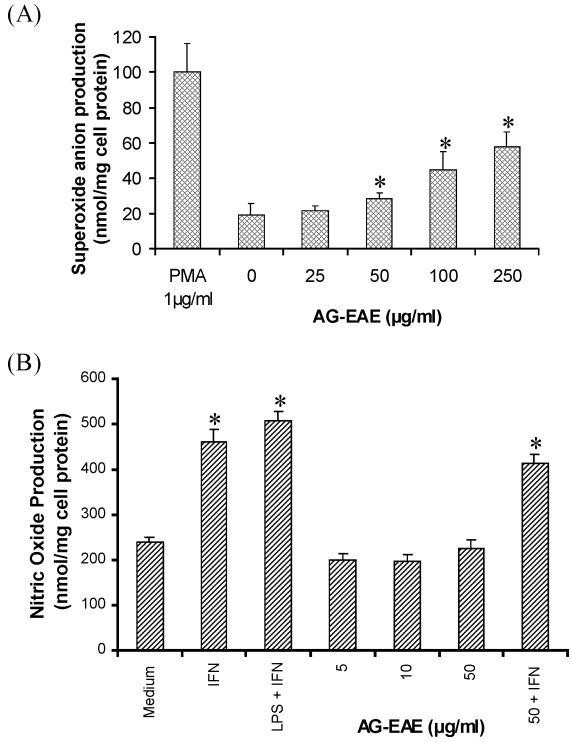

Figure 4. Effects of AG-EAE on superoxide anion and nitric oxide production by macrophages. (A) Adherent macrophages were incubated with HBSS containing ferricytochrome *c* (80 µM) and different concentrations of AG-EAE. PMA (1 µg/mL) as positive control was used. After 2 h the supernatant was removed and the absorbance measured at 550 nm. Results are expressed as nmol of superoxide anion/mg of cell protein. Values are means ± SD of three experiments, each one in triplicate. *Significantly different from experiments performed in the absence of AG-EAE (negative control). (B) Effect of AG-EAE on nitric oxide production by macrophages. Adherent macrophages were incubated for 48 h in the absence (negative control) or presence of AG-EAE. LPS (50 µg/mL) plus IFN-γ (26 U/mL) was used as a positive control for NO production. NO accumulation was measured in the supernatant using the Griess reaction and calculated as nmol nitrite/mg cell protein. AG-EAE was incubated in the absence or presence of IFN-γ (26 U/mL). Values are means ± SD of four experiments, each one in triplicate. *Significantly different from control (medium); $p < 0.05$.

250 °C, programmed from 50 to 220 °C at 40 °C/min, then hold. Helium was the carrier gas. ^{13}C NMR experiments were performed using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Samples were dissolved in D_2O and examined at 50 °C. Chemical shifts are expressed in ppm (δ), based on the resonance of acetone at δ 30.2. The homogeneity and molar mass of fractions 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 Ultrahydrogel columns in series (each column 7.8 × 300 mm) with exclusion sizes of 1×10^6, 4×10^5, 8×10^4, and 5×10^3 Da, using a refractive index (RI) detector, the eluent being 0.1 M aqueous $NaNO_2$ with 200 ppm aqueous NaN_3. Samples were dissolved in Milli-Q water, filtered through a cellulose acetate Millipore membrane (0.22 µm), and injected (250 µL loop) at a 1 mg/mL concentration. The specific refractive index increment (d*n*/d*c*) was also determined for AG-EAE and AG-INF. Carboxy-reduction of AG-EAE was carried out using two successive cycles with carbodiimide-$NaBH_4$, according to Taylor and Conrad.[32]

Plant Material. *P. niruri* L. (1.4 kg) was donated by the Pharmaceutical Industry "As Ervas Curam Ltda.". The material was obtained from the entire plant, previously oven-dried and coarsely milled. Its identity was confirmed by Ms. Nathieli Keila Takemori and Prof. Dr. Cleusa Bona, Botany Department, Federal University of Paraná, Curitiba, Brazil (UFPR), and a sample of the plant is deposited in the Herbarium of UFPR (UPCB) as voucher no. 42822.

Polysaccharide Extraction. Three distinct aqueous extractions were carried out, namely, (a) infusion: 3 L of boiling H_2O was poured over the dried material (200 g), the recipient was then closed, and the extraction proceeded until 25 °C was reached (~6 h), (b) decoction: the dried material (200 g) was submitted to reflux in distilled H_2O (3 L) for 15 min, and (c) exhaustive aqueous extraction: the dried material (1 kg) was first submitted to a defatting procedure with refluxing $CHCl_3$–MeOH (2:1 v/v, 2 h, × 3), and the residual material was refluxed with 8 L of distilled H_2O (×10), sequentially, for 6 h each. The extracts were combined and concentrated under reduced pressure to a small volume and freeze-dried. After 60 h, no more carbohydrate was detected by the phenol–sulfuric acid method.[33]

Polysaccharide Fractionation and Purification. Each of the above fractions, dissolved in a small volume of H_2O, was added to ethanol (×3), and the resulting precipitate was then dissolved in water, which was freeze–thawed until no more precipitate appeared. The H_2O-soluble fractions were then dialyzed using a 16 kDa cutoff membrane, followed by sequential ultrafiltrations of the retained material, using membranes of 0.22 µm porosity, 30 kDa and a 10 kDa cutoff. The fractions

that passed through all the three membrane ultrafiltrations were further analyzed. They were from the infusion (AG-INF), decoction (AG-DEC), and exhaustive aqueous extraction (AG-EAE).

Monosaccharide Composition of Polysaccharides. 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.[34,35] The resulting alditol acetates were then examined by GC-MS (see above) and identified by their typical retention times and electron impact profiles.[36,37]

Methylation Analyses of Polysaccharides. Per-*O*-methylation of the three polysaccharide fractions was carried out using powdered NaOH in DMSO−MeI.[38] The products were converted into partially *O*-methylated aldoses by 3% MeOH−HCl at 80 °C for 2 h and neutralized (Ag_2CO_3), followed by hydrolysis with 0.5 M H_2SO_4 for 16 h at 100 °C and neutralization ($BaCO_3$). The resulting mixtures of partially *O*-methylated aldoses were reduced with $NaBD_4$ and then treated with Ac_2O−pyridine to give partially *O*-methylated alditol acetates, which were analyzed by GC-MS as described above for alditol acetates. The temperature program ranged from 50 to 215 °C at 40 °C/min, then hold.[36,37]

Experimental Animals. Swiss mice received a standard laboratory diet (Purina). All recommendations of the Brazilian national law (no. 6638, 05/11/1979) for scientific management of animals were respected.

Macrophage Isolation. Peritoneal macrophages of mice were collected by infusing their peritoneal cavity with ice-cold PBS. The cells were plated in a culture medium (minimum essential medium (MEM), 5% fetal bovine serum, and antibiotics) or Hank's balanced saline solution (HBSS) to give 5×10^5 cells/well in 96-well dishes. After incubation for 1 h at 37 °C under 5% CO_2 in a humidified incubator, nonadherent cells were removed by washing with phosphate-buffered saline (PBS) at 37 °C.[39]

Cell Viability. Adherent macrophages were incubated for 2, 24, and 48 h in various concentrations of AG-EAE (25−250 μg/mL). An evaluation of toxicity was performed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), according to the description of Reilly and co-workers.[40]

Superoxide Anion Production. Adherent macrophages were incubated in a standard reaction mixture consisting of HBSS containing ferricytochrome *c* (80 μM) in the presence or absence of phorbol 12-myristate 13-acetate (PMA) (1 μg/mL). To the standard reaction mixture was added AG-EAE (25−250 μg/mL). Controls in the absence of the polysaccharide and in the presence of adequate amounts of DMSO (solvent of PMA) were obtained. The amount of superoxide anion released was calculated by dividing the difference in absorbance of the samples, with or without superoxide dismutase, by the extinction molar coefficient $\epsilon_{550\ nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ for reduced cytochrome *c*.[39] Results are expressed as nmol/mg cell protein.

Nitric Oxide Production. For measurement of nitric oxide production, adherent macrophages (5×10^5/well) were incubated with AG-EAE (5−50 μg/mL) in the presence or absence of either IFN-γ (26 U/mL) or LPS (50 ng/mL) plus IFN-γ (26 U/mL) as control. After 48 h, NO production was determined by measuring nitrite/nitrate in the culture medium, using the Griess reaction.[41,42] For control of an eventual LPS contamination of AG-EAE, a sample was previously treated with polymyxin B (50 μg/mL) for 1 h before examination.[30]

Protein Determination. After removal of the reaction mixture by centrifugation, the cells were washed with PBS at 37 °C and then processed according to Sasada et al.[39] The protein content was determined according to Bradford,[43] using bovine serum albumin as standard.

Statistical Analysis. Statistical analysis of data was carried out using an analysis of variance (ANOVA) test and the test of Tukey for mean comparison.

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