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Rhamnogalacturonan from *Ilex paraguariensis*: A potential adjuvant in sepsis treatment

Nessana Dartora[a], Lauro M. de Souza[a], Simone M.M. Paiva[a], Camila T. Scoparo[a], Marcello Iacomini[a], Philip A.J. Gorin[a], Yanna D. Rattmann[a,b,*], Guilherme L. Sassaki[a,*]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-980, Curitiba, PR, Brazil
[b] Departamento de Saúde Comunitária, Universidade Federal do Paraná, Rua Padre Camargo, 280, CEP 80060-240, Alto da Glória, Curitiba, PR, Brazil

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ABSTRACT

The present study evaluated the anti-inflammatory activity of a polysaccharide from maté, using a clinically relevant model of sepsis induced by cecal ligation and puncture (CLP). A polysaccharide from maté (SPI) was obtained from aqueous extraction followed by fractionation, being identified as a rhamnogalacturonan with a main chain of →4)-6-*O*Me-α-D-Gal*p*A-(1→ groups, interrupted by α-L-Rha*p* units, substituted by a type I arabinogalactan. SPI was tested against induced-polymicrobial sepsis, at doses of 3, 7 and 10 mg/kg. Via oral administration, SPI prevented the late mortality of infected mice by a rate of 60% at 10 mg/kg, in comparison with untreated mice Dexamethasone, used as positive control, was slightly less effective, with an overall survival rate of 16.7% of mice at the end of the observation period. SPI also affected neutrophil influx, avoiding its accumulation in lungs, and significantly decreased tissue expression of iNOS and COX-2. In this context, maté is a potential nutraceutical, and its polysaccharide a promising adjuvant for sepsis treatment, being consumed as tea-like beverages with no related adverse effects.

1. Introduction

Ilex paraguariensis (known as maté, erva-mate or yerba-mate) is a native plant from Argentina, Paraguay, Uruguay and southern Brazil, where it has considerable socioeconomic relevance. Leaves and branches from *I. paraguariensis* are used to prepare commercial tea-like beverages or then sold after being processed and packed. The processing usually consists of blanching (*sapeco*) and milling, and these leaves are prepared as infusions that are appreciated as a hot beverage called *chimarrão* (Filip, Lopez, Giberti, Coussio, & Ferraro, 2001).

The consumption of maté has increased due to its health benefits, attributed to the presence of secondary metabolites, such as methylxanthines, polyphenols and saponins, readily extracted from leaf tissue during infusion preparations. In fact, an increasing number of biological applications have been claimed for maté infusion-components, such as hepatoprotective, choleretic, diuretic, hypocholesterolemic, antirheumatic, anti-thrombotic, anti-inflammatory, anti-obesity and anti-ageing (Anderson & Fogh, 2001; Dartora et al., 2011; Filip et al., 2001; Filip, Lotito, Ferraro, & Fraga, 2000; Gorzalczany et al., 2001; Gugliucci & Menini, 2002; Mucillo-Baisch, Johnston & Paganini-Stein, 1998; Pittler & Ernst, 2004; Souza et al., 2011).

Currently, the secondary metabolites from *I. paraguariensis* are the main focus of investigations, lacking information about its primary metabolites. However, with the infusions not only secondary but also primary metabolites, including polysaccharides, are ingested. It is known that many herbs used in folk medicine contain polysaccharides with a recognized variety of biological activities, including antiviral, antitumor, immunostimulation, anti-inflammatory, anticomplementary, anticoagulant, hypoglycemic, and anti-ulcer effects (Capek et al., 2003; Cipriani et al., 2006; Nergard et al., 2005; Srivastava & Kulshveshtha, 1989; Yamada, 1994).

Nevertheless, there are very few reports dealing with the ability of polysaccharides in reducing mortality caused by sepsis. This is a considerable health hazard and a main cause of morbidity and mortality in many intensive care units. It represents a state of overproduction of proinflammatory mediators which frequently occurs

Abbreviations: AcOH, acetic acid; Ac_2O, acetic anhydride; CLP, cecal ligation and puncture; COX-2, cyclooxygenase-2; EtOH, ethanol; HPSEC, high-performance size-exclusion chromatography; IPI, insoluble fraction (after freeze-thawing); MPO, myeloperoxidase; PI, crude polysaccharide fraction; SPI, soluble fraction (after freeze-thawing); TMSP-d_4, 2,2,3,3-tetradeuterium-3-trimethylilsilylpropionate.

* Corresponding authors at: Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-980, Curitiba, PR, Brazil. Tel.: +55 41 3361 1577; fax: +55 41 3266 2042.
E-mail addresses: yannadr@yahoo.com.br (Y.D. Rattmann), sassaki@ufpr.br (G.L. Sassaki).

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after various noxious injuries, especially bacterial infection, as a consequence of abdominal surgery, appendicitis, perforated ulcers, or an ischemic bowel, and so on (Angus et al., 2001).

Considering the above, we now report the isolation and structural analysis of a polysaccharide via hot aqueous extraction of *I. paraguariensis* (*chimarrão*) and its pharmacological action against murine sepsis, by evaluating the effects on lethality, neutrophil migration and levels of expression of proinflammatory enzymes.

2. Materials and methods

2.1. Plant material

Leaves from *I. paraguariensis* were collected randomly from a homogeneous group of cultivars, with geographical coordinates 27°37′15″ south, 52°22′47″ west at a 765 m altitude (Barão de Cotegipe, State of Rio Grande do Sul). Harvesting was carried out during the winter, July 2009.

In order to simulate the commercial maté, the leaves were dried in an oven with air circulation at 30 °C for 24 h. Thereafter, they were exposed to flames (*sapeco*) at 180 °C for 5 min, yielding a product with residual moisture of ∼15%, then dried at 65 °C for 90 min (moisture ∼5%) and ground, providing *chimarrão*.

2.2. Polysaccharide extraction and fractionation

The ground leaves (100 g) were submitted to aqueous extraction (100 °C, 500 mL, ×3). The extracts were combined and evaporated to a small volume. High-molecular weight components (mainly polysaccharides) were precipitated by addition of cold EtOH (3 vol.), and separated by centrifugation (8.000 rpm at 4 °C, 20 min). The sediment was dissolved in H_2O, dialyzed against tap water for 48 h to remove the remaining low-molecular weight compounds, giving rise to a crude polysaccharide fraction (PI). This was frozen and then allowed to thaw at room temperature (Gorin & Iacomini, 1984), resulting in soluble (SPI) and insoluble fractions (IPI) which were separated by centrifugation as described above. The insoluble fraction was not analyzed in this study due to its lower yield and difficult solubilization.

2.3. Monosaccharide analysis

SPI (2 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, the solution then evaporated, and the residue dissolved in water (1 mL). The resulting monosaccharide mixture was examined by thin layer chromatography (TLC) silica-gel 60 (Merck), developed with ethyl acetate:acetic acid:*n*-propanol:water (4:2:2:1, v/v), then stained with orcinol-sulfuric acid (Sassaki, Souza, Cipriani, & Iacomini, 2008; Skipski, 1975). The monosaccharides were then reduced with 2 mg $NaBH_4$ yielding alditols which were acetylated in Ac_2O-pyridine (1:1, v/v, 0.5 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a; Wolfrom & Thompson, 1963b). The resulting alditol acetates were extracted with $CHCl_3$, and analyzed by gas chromatography–mass spectrometry (GC–MS – Varian, Saturn 2000R, Ion-Trap detector), using a DB-225-MS column (30 m × 0.25 mm × 0.25 μm) programmed from 50 to 220 °C at 40 °C/min, with He as carrier gas. Components were identified by their typical retention times and electron ionization (EI – 70 eV) spectra. The uronic acid content of SPI was determined using the colorimetric *m*-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991).

Carboxy-reduction of SPI (10 mg) was carried out by the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, giving a product (SPI-CR), having its uronic acid carboxyl groups reduced to primary alcohols.

2.4. Methylation analysis

SPI and SPI-CR (10 mg) were per-*O*-methylated in alkaline DMSO solution by addition of iodomethane as described by Ciucanu and Kerek (1984). The alkylated polysaccharides were hydrolyzed in 72% (v/v) aq. H_2SO_4 (0.5 mL, v/v, 1 h, 0 °C), followed by dilution to 8% (v/v). The solution was kept at 100 °C for 17 h, then neutralized with $BaCO_3$, filtered and evaporated to dryness (Saeman, Moore, Mitchell, & Millet, 1954). The hydrolyzate was reduced with NaB^2H_4 and then acetylated, giving rise to partially *O*-methylated alditol acetates (PMAAs) and analyzed by GC–MS, as the above, but with final temperature of 215 °C. Their identification was based on their retention times and EI-MS spectra (70 eV) by comparison with standards and an EI-MS library (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.5. NMR analysis

1D and 2D NMR experiments (^1H, ^{13}C, HSQC and HMBC) were carried out using a Bruker Avance *III* 400 MHz spectrometer. The samples (40–50 mg) were dissolved in D_2O and the ^1H and ^{13}C chemical shifts were expressed in ppm (δ) relative to TMSP-d_4 (2,2,3,3-tetradeuterium-3-trimethylilsilylpropionate; $\delta = 0$ for ^{13}C and ^1H) at 70 °C.

2.6. Animals

Male albino Swiss mice (3 months old, weighing 30 g) were used in biological tests. They were maintained under standard conditions, with a constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C). Standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available ad libitum. All experimental procedures were previously approved by the Institutional Ethics Committee of the University (authorization number 430).

2.7. Sepsis induction by cecal ligation and puncture (CLP)

Mice were randomly grouped into five clusters of 10 mice: sham-operation, CLP plus vehicle (water p.o.), CLP plus SPI 3 mg/kg, p.o.; CLP plus SPI 7 mg/kg, p.o.; CLP plus SPI 10 mg/kg, p.o. The mice average weight was 30 g, thus, 25 μL of each SPI solution was found appropriate to be administered by oral way for avoiding regurgitation. Ketamine (80 mg/kg) and xylazine (20 mg/kg) were injected intraperitoneally to anesthetize the mice before surgical procedures. Polymicrobial sepsis was induced by CLP as previously described (Rittirsch, Huber-Lang, Flierl, & Ward, 2009). A midline incision of ∼1.5 cm was carried out on the abdomen. The cecum was carefully exposed and 50% of the distal moiety was ligated. The cecum was then punctured thrice with a sterile 16-gauge needle and squeezed to extrude the fecal material from the wounds. The cecum was replaced and the abdomen was stitched surgically. Sham-control animals were treated identically, but no cecal ligation or puncture was carried out. Each mouse received subcutaneous sterile saline injection (1 mL) for fluid resuscitation after surgery. The mice were then kept on a heating pad (35 °C) until they recovered from the anaesthesia. Food and water, ad libitum, were provided throughout the experiment. The survival rate was monitored for 7 days, each 12 h. During this period, water (vehicle) and drugs were orally administered daily. Dexamethasone was commercially purchased and subcutaneously administered at doses of 0.5 mg/kg (Longhi-Balbinot et al., 2012; Rocha Lapa, Silva, Almeida Cabrini, & Santos, 2012; Silva et al., 2012).

In another experimental set (1 h before surgery), mice were orally treated with vehicle, SPI (3, 7 or 10 mg/kg, p.o.) or dexamethasone (0.5 mg/kg s.c.). After 6 h post-operation, mice were sacrificed. Their lung and ileum tissues were collected and frozen for further

use to determine the myeloperoxidase (MPO) activity and tissue expression of iNOS and cyclooxygenase-2 (COX-2), respectively.

2.8. Lung MPO activity

The MPO activity was measure in order to determine the neutrophil influx, according to established protocols (Bradley, Priebat, Christensen, & Rothstein, 1982). Briefly, the lung tissue was homogenized in 0.5 mL of 50 mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 14,000 rpm for 15 min at 4 °C. Supernatants were then assayed at a 1:20 dilution in a reaction buffer (9.6 mM 3,3,5,5-tetramethylbenzine, 150 nM H_2O_2 in 50 mM potassium phosphate buffer), and read at 620 nm. Results are expressed as change in optical density per milligram of protein (measured by Bradford assay).

2.9. Western blot analysis

The samples of ileum were washed twice with PBS and then homogenized and lyzed in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na_3VO_4, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen)). Total proteins (20 μg) were separated on 8% SDS-PAGE (Sigma) at 80 V for 2 h. Separated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Bio-Rad) at 100 V for 120 min. Membranes were blocked with blocking buffer containing 3% low fat milk powder, Tris-buffered saline solution (Bio-Rad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 h. Membranes were then incubated with primary antibodies of both iNOS and COX-2 (Santa Cruz Biotechnology, dilution of 1:1000) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-mouse IgG – Santa Cruz Biotechnology, dilution of 1:5000) at room temperature, for 60 min. The detection of COX-1 (constitutive isoform) proteins was used for normalization and quantification of iNOS and COX-2 respectively. Prestained markers (Invitrogen) were used for molecular mass determinations. Immunoreactive bands were detected by chemiluminescence enhancement (Bio-Rad).

2.10. Statistical analysis

Data were expressed as means ± SEM of five or ten mice examined in each group. Statistical error was determined by one-way ANOVA; the post hoc test was Bonferroni's. Calculations performed with Graphpad Prism 5.0. p Values < 0.05 were considered significant.

3. Results

3.1. Isolation and chemical analysis of the polysaccharide

In order to obtain polysaccharides similar to those of commercial products, the leaves from *I. paraguariensis* were submitted to a process of blanching and milling, then extracted with water at 100 °C. The polysaccharides were precipitated with excess ethanol, obtained as sediments on centrifugation, which were dialyzed against tap water and freeze-dried to give a crude polysaccharide fraction (PI – 3.3 g).

Fractionation of PI was carried out by a freeze-thawing procedure, resulting in cold water-soluble (SPI, 3.2 g) and a discarded insoluble fraction. The monosaccharide composition of SPI was arabinose, uronic acid, galactose, rhamnose, and glucose in a 4:3:2:0.5:0.4 molar ratio. TLC analysis of the SPI hydrolyzate indicated that galacturonic acid was also present.

3.1.1. NMR data

The $^1H/^{13}C$ HSQC spectrum of SPI (Fig. 1) showed the presence of units of α-D-galactopyranosyluronic acid with signals at δ 100.2/4.96 attributed to C-1/H-1. The presence of pectins was confirmed by detection of the sequence of (1 → 4)-linked α-galacturonic acid residues, which gives a fingerprint cross peak at δ 78.6/4.44 (C-4/H-4), indicating this type of linkage. The latter was confirmed by the presence of other signals of Gal*p*A units at δ 68.1/3.74 (C-2/H-2), 68.4/3.98 (C-3/H-3), 70.6/5.07 (C-5/H-5), and 170.6 (C-6), consistent with (1 → 4)-linked α-D-Gal*p*A units (Cipriani, Mellinger, Gorin, & Iacomini, 2004; Ovodova et al., 2009; Popov et al., 2011).

Methyl esters of galacturonic acid are commonly found in type II ramnogalacturonanas, and the HSQC signal at δ 52.8/3.74 suggested the presence of $-CO_2\underline{CH_3}$ units. The ester signal was confirmed by HMBC experiment, which gave a cross peak at δ 171.0/3.74, indicating the methyl protons have long range correlation with the carboxyl group ($-\underline{C}O_2-C\underline{H_3}$). However, signals at δ 71.7/4.67 (C-5/H-5) also suggest also the presence of non-esterified Gal*p*A units (Popov et al., 2011; Renard, Lahaye, Mutter, Voragen, & Thibault, 1998) and the overall NMR data indicate that most of the Gal*p*A units of SPI are esterified (Fig. 1).

Type I and II rhamnogalacturonans are commonly found as constituents of pectins, the major components of primary cell walls of dicotyledonos. These polysaccharides are formed by long sequences of (1 → 4)-linked α-D-polygalacturonic acid, interrupted by units of α-L-Rha*p* (Carpita & Gibeatu, 1993). The $^1H/^{13}C$ HSQC signals at δ 99.3/5.14 (C-1/H-1), 16.8/1.25 (C-6/H-6) and 76.6/3.94 (C-2/H-2) were consistent with (1 → 2)-linked α-L-Rha*p* units of a rhamnogalacturonan (Renard et al., 1998).

Acetyl groups can also be present in rhamnogalacturonans, frequently as substituents at *O*-2 or *O*-3 of Gal*p*A units (Lerouge, O'Neill, Darvill, & Albersheim, 1993; Popov et al., 2011). When this substitution occurs, the chemical shifts of C-2/H-2 and C-3/H-3 from 6-*O*Me-Gal*p*A-units would appear at a downfield region, superimposed on the signal of C-5/H-5 (δ 70.6/5.07) (Popov et al., 2011). However, a signal at δ 20.4/2.14 (Fig. 1), typical from acetyl groups, was present in our $^1H/^{13}C$ HSQC spectrum, so that they are attached to SPI-polysaccharide, but its actual position could not be determined.

The main chain of rhamnogalacturonans is often substituted by side chains of arabinans, galactans, or arabinogalactans. Type I and type II arabinogalactans are classified according to their main chain, the former consisting of a main chain of β-D-Gal*p* (1 → 4)-linked, while the latter has a (1 → 3)-linked β-D-Gal*p* main chain, substituted at *O*-6 by β-D-Gal*p* side chains (Carpita & Gibeatu, 1993). The $^1H/^{13}C$ HSQC spectrum of SPI (Fig. 1) contained signals at δ 103.3/4.51 (C-1/H-1) and δ 77.6/4.19 (C-4/H-4) consistent with (1 → 4)-linked β-D-Gal*p* units. The edited-HSQC experiment gave a negative signal δ 67.3/3.97, consistent with a substituted HO-6 of β-D-Gal*p*. In this case, the C-4/H-4 signals of these units are shifted to δ 74.8/3.65 (Carpita & Gibeatu, 1993).

Arabinose is frequently found as a component linked to C-6 β-D-Gal*p*, appearing as terminal units or forming 3- or 5-*O*-linked chains (Carpita & Gibeatu, 1993). The HSQC spectrum of SPI had typical signals of (1 → 5)-linked α-L-Ara*f* units, at δ 109.1/5.25 (C-1/H-1) and δ 64.5/3.71 (C-5/H-5) (Delgobo, Gorin, Jones, & Iacomini, 1998).

3.1.2. Methylation analysis

Methylation analysis (Table 1) showed that SPI is a highly branched polysaccharide, containing nonreducing end-units of Ara*f* (17.5%), and Gal*p* (3.1%). The arabinofuranosyl units are substituted at *O*-5 (12.2%), *O*-3 (11.0%) and *O*-3,5-disubstituted (5.2%). Gal*p* units are, mainly, 4-*O*- (10.7%) and 4,6-di-*O*-substituted (3.7%). The presence of 6-*O*- and 3,6-di-*O*-substituted galactopyranosyl

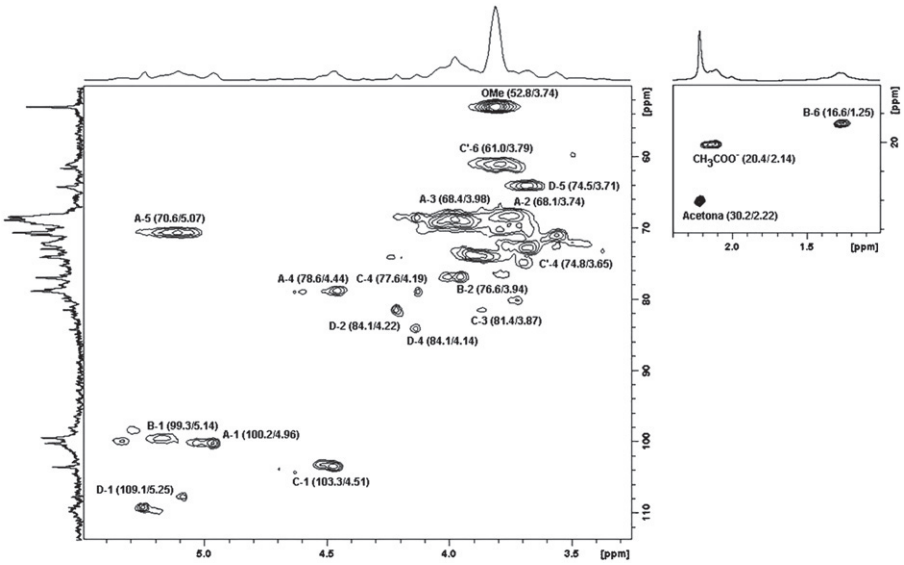

Fig. 1. $^{1}H/^{13}C$ HSQC NMR spectrum of SPI, solvent D_2O at 70 °C; numerical values are in δ ppm. (A) (6-*O*Me-α-D-Gal*p*A), (B) (α-L-Rha*p*), (C) (β-D-Gal*p*) and (D) (α-L-Ara*f*). The letters are followed by the carbon number of the monosaccharide unit.

units was observed in low amounts, as well as 2,4-*O*-substituted rhamnopyranosyl units (3.3%).

The structure of the uronic acid found in SPI, as well as its linkage types, was determined by carboxy reduction, followed by methylation analysis. The neutral product (SPI-CR) showed an increase in 2,3,6-Me_3-galactitol acetate, confirming that (1 → 4)-linked galacturonic acid residues were present.

The NMR data and methylation analysis of SPI suggest a polysaccharide composed by an arabinogalactan type I, linked to a type II rhamnogalacturonan (Fig. 2).

3.2. Biological experiments

3.2.1. Protective effect of SPI on CLP-induced sepsis in mice

In order to investigate the effects of SPI isolated from *I. paraguariensis*, an induction of polymicrobial sepsis was carried out in mice, by cecal ligation and puncture (CLP). This model mimics sepsis in humans, caused by pathogens derived from the intestinal tract, and is considered to closely simulate a clinical situation (Otero-Anton et al., 2001). SPI was tested at doses of 3, 7 and 10 mg/kg, and their effect on the survival rate of infected mice was determined.

Mice treated with vehicle started to die between 12 h and 24 h after CLP, with a death rate reaching 75% and 95% at 24 h and 84 h post-CLP, respectively. The overall mortality at the end of the observation period was 100%, and the area under the curve was 2.700 (arbitrary units). The lethality was markedly delayed in mice treated, orally, with SPI and their areas under the curve were increased to 5.640, 8.400 and 10.680 after administrating 3, 7 and 10 mg/kg, respectively. At the end of the period, the overall survival in these SPI groups was 20%, 40% and 60%. No death was observed in the sham-operated mice and its corresponding area under the lethality curve was 16.800 (arbitrary units). Dexamethasone, used as positive control, showed a significant improvement in survival (area under curve 10.100), with an overall survival rate of 16.7% at the end of the observation period (Fig. 3A).

Table 1
Profile of partially *O*-methylated alditol acetates and monosaccharide linkages of SPI.

*O*Me-Alditol acetate	Structure	t_R[a]	mol%[b]
2,3,5-Me_3-Ara	Ara*f*-(1→	0.805	17.5
2,5-Me_2-Ara	→3)-Ara*f*-(1→	0.965	11.0
2,3,4,6-Me_4-Glc	Glc*p*-(1→	1.000	1.0
2,3-Me_2-Ara	→5)-Ara*f*-(1→	1.030	12.2
2,3,4,6-Me_4-Gal	Gal*p*-(1→	1.051	3.1
3-Me-Rha	→2,4)-Rha*p*-(1→	1.207	3.3
2-Me-Ara	→3,5)-Ara*f*-(1→	1.289	5.2
2,3,6-Me_3-Gal	→4)-Glc*p*-(1→	1.362	10.7
2,3,4-Me_3-Gal	→6)-Gal*p*-(1→	1.487	1.1
2,3-Me_2-Gal	→4,6)-Gal*p*-(1→	1.623	3.7
2,4-Me_2-Gal	→6)-Gal*p*-(1→	1.824	1.2

[a] t_R = relative retention time to 2,3,4,6-tetra-O-metilglucitol acetate.
[b] The galacturonic acid content of SPI was 30.0%, according to the spectrophotometric method of Filisetti-Cozzi and Carpita (1991).

3.2.2. Inhibition of MPO activity

Since MPO is a lysosomal enzyme of polymorphonuclear leukocytes that acts as a catalyst in the production of hypochlorous acid (powerful oxidant), the effects of SPI on MPO activity were also investigated. CLP surgery markedly increased the MPO levels in the lung tissues in comparison with the Sham group (55.8%) (Fig. 3B). This rise in tissue MPO was significantly prevented by SPI at 3, 7 and 10 mg/kg, with an inhibition of 7.4%, 20.7% and 38.4%, respectively, vs. vehicle group (Fig. 3B). In order to evaluate the percentual of inhibition, the results were also normalized relative to Sham group, using a ratio factor based on basal response (Sham): CLP response with a numerical value of 0.558. This normalization gave the follow inhibitions: 13.3%, 37.1% and 68.8%, respectively. Dexamethasone, the anti-inflammatory control, strongly inhibited the MPO activity in lungs at 33.7% (60.4 normalized), being statistically close to SPI at a dose of 10 mg/kg. SPI could prevent the increase of MPO activity, indirectly indicating a reduction in neutrophil recruitment to lung, and consequently avoiding tissue damage by oxidative processes (Fig. 3B).

3.2.3. Inhibition of INOS and COX-2 expression

To better investigate the expression of proinflammatory enzymes in the ileum, the levels of iNOS and COX-2 were determined by immunoblotting after different treatments (Fig. 4A). CLP increased the levels of iNOS in comparison with the Sham group in 55.7%, and SPI at 10 mg/kg decreased the levels of iNOS by 29.5% (or 53.0% normalized by Sham group) (Fig. 4B). The COX-2 expression in the ileum was reduced by 16.8% and 32.3% (or 30.0% and

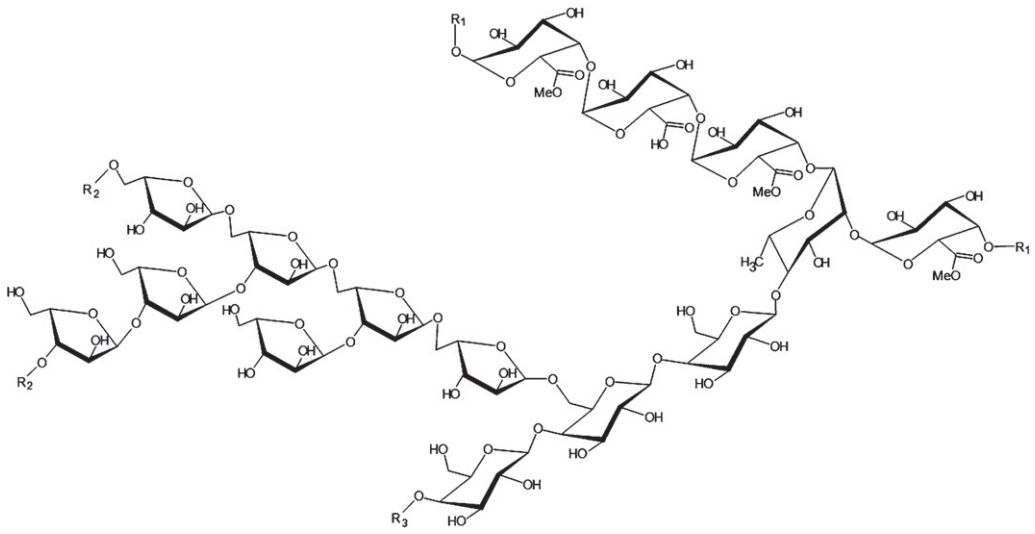

Fig. 2. Suggested structure for SPI isolated from *I. paraguariensis*, where R_1 are 6-*O*Me-α-D-Gal*p*A units, R_2 are β-D-Gal*p* units and R_3 are α-L-Ara*f* units.

57.8% – normalized by 0.559 ratio factor) after treating with SPI at 7 and 10 mg/kg respectively (Fig. 4B). Dexamethasone significantly affected both iNOS and COX-2 expression, reducing by 43.2% and 38.7% (77.6% and 69.2 normalized by Sham group), respectively (Fig. 4B and C). In contrast, the level of COX-1 (constitutive isoform) remained the same under these conditions (Fig. 4A).

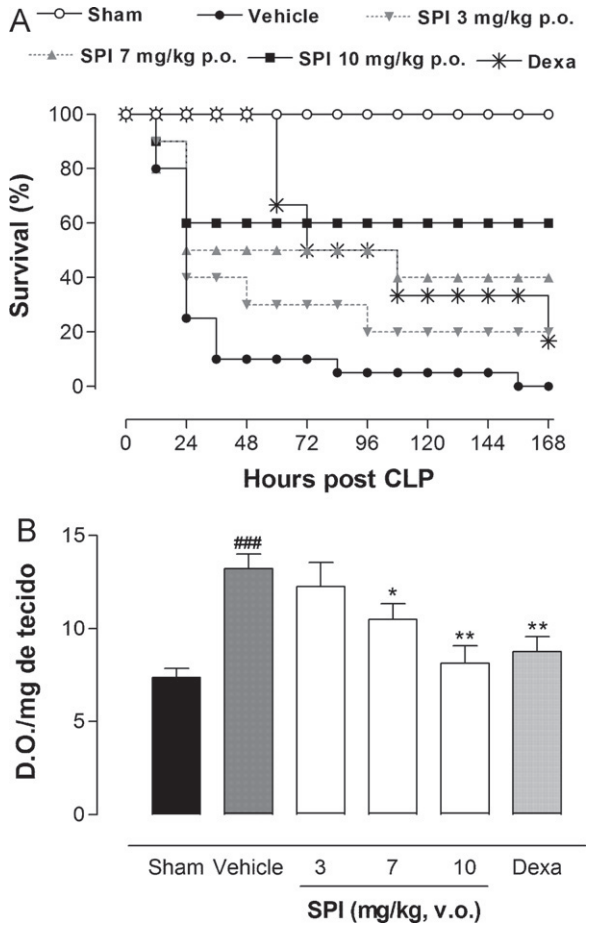

Fig. 3. Effect of polysaccharide SPI on survival rate (A) and on MPO activity (B) in sepsis. SPI protects against sepsis-induced lethality and inhibits MPO activity. Mice (10 animals/group) were orally administered various doses of SPI (3, 7 or 10 mg/kg), vehicle (water) and dexamethasone (0.5 mg/kg s.c.). Values represent means ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$, indicated value versus CLP plus vehicle group; $^{###}p < 0.001$, CLP plus vehicle versus sham. ANOVA followed by Bonferroni's test.

4. Discussion

I. paraguariensis is a popular plant cultivated in southern Brazil and neighboring countries, and its leaves are used in the preparation of several types of beverages. On drinking such beverages, not only secondary metabolites, but also a great variety of primary metabolites, including polysaccharides, are ingested. We have now determined the chemical structure of a soluble polysaccharide from hot aqueous extraction of the commercial product (*chimarrão*) prepared from *I. paraguariensis*.

On the basis of chemical data, the polysaccharide SPI consists of a rhamnogalacturonan formed by a long sequence of →4)-6-*O*Me-α-D-Gal*p*A-(1→ units, interspersed by some α-L-Rha*p* residues, substituted by side chains of type I arabinogalactans.

Many polysaccharides isolated from plants have shown immune responses *in vivo* and *in vitro*. There are also many reports of polysaccharide-induced nonspecific resistance against diverse microbial pathogens (Caillot et al., 2009; Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012). Additionally, polysaccharides isolated from *Agaricus bisporus*, *Lactarius rufus* (Ruthes et al., 2012), *Panax ginseng* (Lim et al., 2002) and *Bacteroides fragilis* (Tzianabos, Kasper, Cisneiros, Smith, & Onderdonk, 1995), had its anti-septicaemic effect determined. However, these compounds were tested subcutaneously.

Here, we clearly demonstrated that SPI polysaccharide prevents lethality caused by polymicrobial sepsis in mice, which was more effective than dexamethasone, a steroidal anti-inflammatory, used as positive control in this work. Furthermore, SPI was administered by oral way, which represents a therapeutic advantage. This beneficial effect seems to be, at least in part, due to a reduction in neutrophil migration, as observed using the MPO assay, where the enzyme inhibition was up to 68%. Neutrophil infiltration is an important pathophysiological alteration associated with sepsis. These cells directly damage tissue by releasing proinflammatory mediators, such as cytokines, reactive oxygen species and lysosomal enzymes, such as MPO, which amplify the systemic inflammatory response and cause multiple organ failure (Landry & Oliver, 2001).

Furthermore, SPI decreased the levels of the both pro-inflammatory enzymes, iNOS and COX-2, whose role in the

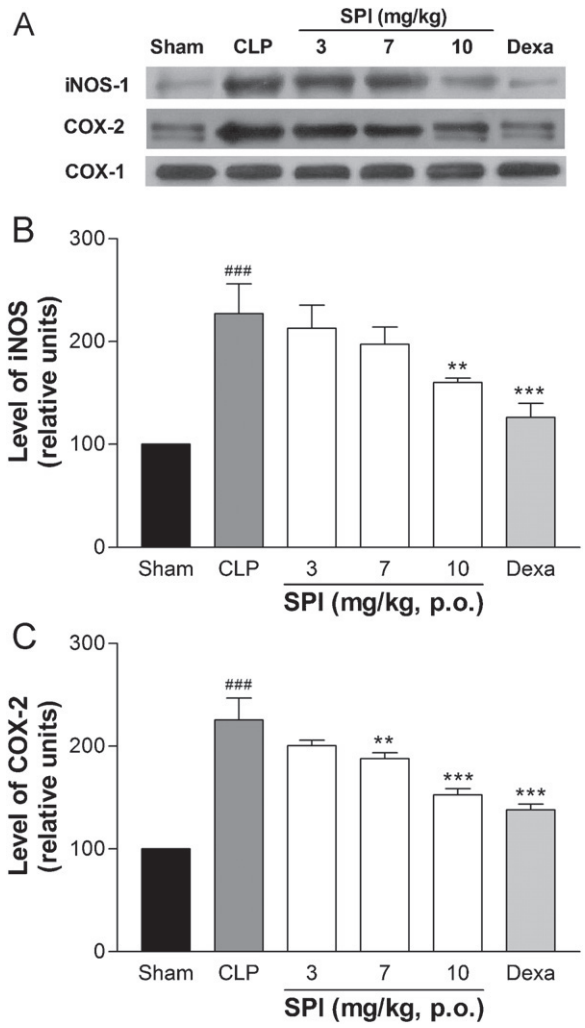

Fig. 4. Effect of polysaccharide SPI on iNOS (B) and COX-2 (C) expression in the ileum tissue of infected mice. Mice were treated with SPI 3, 7 or 10 mg/kg, p.o., or dexamethasone. The levels of iNOS and COX-2 were determined by Western blot analysis. (A) Representative immunoblots. Results are shown as the means ± SEM of 3–4 different experiments. $^{###}p < 0.001$, CLP plus vehicle versus sham. $^{**}p < 0.01$ and $^{***}p < 0.001$, SPI versus vehicle.

pathophysiology of inflammatory states such as sepsis is being increasingly recognized.

iNOS is induced in response to inflammatory stimuli such as bacterial lipopolysaccharide (LPS) and proinflammatory cytokines (for example, IL-1, TNF-α). When expressed, iNOS produces high amounts of NO over long periods, which causes cellular damage (Tinker & Wallace, 2006).

Cyclooxygenase-2 (COX-2) is another important product of inflammation from cells of the innate immune system, catalyzing the formation of inflammatory prostanoids such as prostaglandins and tromboxane, which can mediate a significant inflammatory response (Hayashi, Sumi, Ueno, Murase, & Takada, 2011). Systemic COX-2 is increasingly recognized as an important mediator in sepsis-induced inflammation. It was previously shown that COX-2-knockouted mice are naturally protected from inflammation by sepsis-induced and consequently from death (Ejima et al., 2003).

Puanfpraphant and Mejia (2009) investigated the anti-inflammatory properties of different constituents of maté and evaluated potential interactions between them. They found that the chlorogenic acids tested alone had no effects on the decrease in the proinflammatory markers. Otherwise, the saponins present in the maté inhibited the iNOS/NO system, preferably in the system COX-2/PGE-2 and quercetin, COX-2/PGE-2, preferably at iNOS/NO. They also noted that the mixture of saponins plus quercetin maté was able to inhibit the expression of proinflammatory cytokine IL-1β, nuclear translocation of subunit 65 of NF-κB, as well as the reduction of NO and PGE-2, suggesting synergy. Consequently our results suggest that in addition to the compounds of secondary metabolism, a polysaccharide from *I. paraguariensis* also acts on these enzymes, reducing inflammatory lesion and suggesting that, at least in part, the anti-inflammatory activity of maté can be attributable to its polysaccharides.

The activation of nuclear factor-kappaB (NF-κB) signaling pathway has been reported in multiple inflammatory diseases, because its stimulation results in increased gene expression and biosynthesis of proinflammatory mediators (Brown & Jones, 2004). The role of NF-κB pathway in sepsis outcome is well defined (Brown & Jones, 2004; Cohen, 2002). Since SPI decreased tissue expression of pro-inflammatory proteins (iNOS and COX-2), we propose that SPI can downregulating the activation of NF-κB signaling pathway. It has been confirmed for various sources of natural products (Tian, Fan, Zhang, Jiang, & Zhang, 2012; Zhang et al., 2011), but demands a further investigation using SPI.

In previous studies, pectins from sweet pepper (Popov et al., 2011) and celery (Ovodova et al., 2009) were evaluated in sepsis model, acting against release of proinflammatory cytokines and decreasing the mortality of LPS-induced septic mice. In such studies, the authors observed that some characteristics, such as molecular weight, amounts of acidic monosaccharides, among other structural details, were keys for activity. This was confirmed following partial acid hydrolysis, when the isolated galacturonan backbone was more efficient than the entire polysaccharide. Thus, considering that the SPI backbone is composed of an RGI, these can be the main factor for its anti-sepsis activity.

Furthermore, pectin with different degrees of methyl-esterification was able to inhibit iNOS and COX-2 expressions, but with different potencies, in which the greater the degree of esterification, the greater the effect on these enzymes proinflammatory (Chen et al., 2006). Thus, the antiseptic effect of SP can also be related to the presence of methyl ester groups in its structure.

It is noteworthy that isolated SPI is more effective in combating sepsis than the aqueous extract of *I. paraguariensis*, or with other compounds isolated from same plant such as the saponins and quercetin (Puanfpraphant & Mejia, 2009). Thus, the present results enhance the nutraceutical role of *I. paraguariensis* and their primary chemical components. Furthermore, it could also suggest a new option as an antisepsis adjuvant and drug development from edible plant products.

Conflict of interest

The authors have declared no conflict of interest.

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