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Antioxidant and antimicrobial activities of a purified polysaccharide from yerba mate (*Ilex paraguariensis*)

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ABSTRACT

This study investigated the antioxidant, antimicrobial and cytotoxic properties of a purified yerba mate polysaccharide. The yerba mate polysaccharide showed a prominent antioxidant activity as evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH•)-radical scavenging activity (IC_{50} = 1.25 ± 0.10 mg/mL), 3-ethyl benzothiazoline-6-sulphonic acid (ABTS•+)-radical scavenging activity (IC_{50} = 0.41 ± 0.05 mg/mL), and hydroxyl scavenging activity (IC_{50} = 3.36 ± 0.31 mg/mL). The antioxidant activity evaluated as the ferric ion reduction power (FRAP) and oxygen radical absorbance radical assay (ORAC), expressed as trolox equivalents, were 20.84 ± 1.61 μM TE/-mg and 556.30 ± 12.83 μM TE/mg, respectively. The purified yerba mate polysaccharide presented high antimicrobial activity against several bacterial and fungal strains; however, no cytotoxicity against all four tumor human cell lines assessed.

1. Introduction

The yerba mate (YM) (*Ilex paraguariensis* A. St. Hil.) is a plant that grows naturally in Paraguay, Uruguay, Argentina and Brazil. The powder of YM leaves and thin stems is used for the preparation of several stimulant drinks. The three most important forms of consumption are chimarrão (hot water extract of green dried leaves; mate in Spanish speaking countries), tererê (cold water extract of green dried leaves) and mate tea (hot water extract of toasted leaves) [1,2].

The yerba mate is associated with numerous health benefits among which it is important to mention its antioxidant properties [3,4] vasodilators properties [5,6], hypoglycemic effects [7,8] and fat loss properties [9]. Different chemical components responsible for the yerba mate-based beverages' positive health outcomes have been identified, such as vitamins, minerals, polyphenols, xanthines, saponins, phenolic compounds, amino acids, enzymes, cellulose, lignin and organic acids [10].

The biological and functional properties of yerba mate are frequently associated with compounds from secondary metabolism. Yerba mate is known to be rich in phenolic acids such as caffeic and chlorogenic acids and their derivatives, in addition to flavan-3-ols such as (+)-catechin [11,12]. Other compounds frequently found in the extracts are: gallic, syringic, ferulic and *p*-coumaric acids, rutin, methylxanthines (caffeine and theobromine), saponins and tannins [1,8]. A few years ago, a polysaccharide from yerba mate leaves was purified and its chemical structure identified as a rhamnogalacturonan I (RG-I) 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 (Fig. 1) [13]. This RG-I presented an anti-inflammatory action demonstrated by its capability of decreasing tissue expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and a potential adjuvant action in sepsis treatment [13]. In addition to this, the yerba mate RG-I was able to inhibit the gastric lesions induced by ethanol in rats [14].

In the past years, the scientific community has been increasingly interested in the investigation of natural polysaccharides from different sources, including rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) type pectins, owing to their promising pharmacological and biological activities [15–20]. Based on these considerations, efforts to enlarge the spectrum of the biological actions attributable to the yerba mate RG-I are quite interesting and desirable. Thus, the aim of the present work was to evaluate the antioxidant, antimicrobial and cytotoxic potentials against tumoral cells and porcine liver cells of this polysaccharide.

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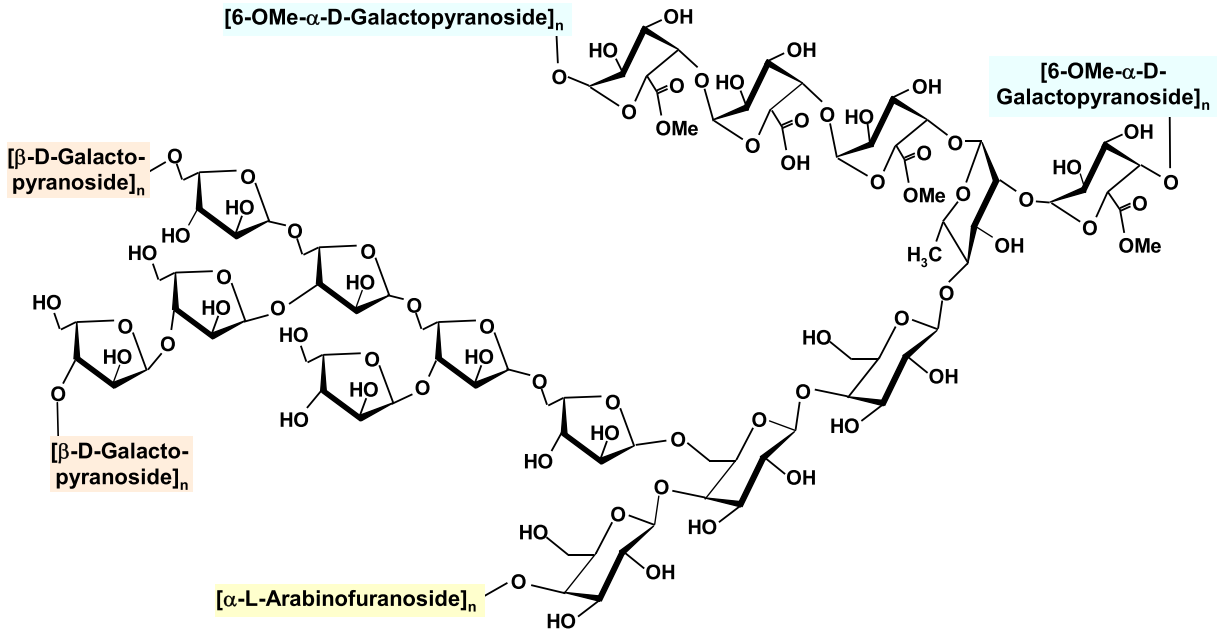

Fig. 1. Structure of the YM purified polysaccharide (rhamnogalacturonan I), according to Dartora et al. (2013) [13].

2. Material and methods

2.1. Plant material

I. paraguariensis A. St. Hil. plant (dry leaves and stems, stored at vacuum) was obtained from a reliable producer in Southern Brazil. The plant was collected during the January crop of 2016. The region of production presents subtropical climate, average altitude of 923.5 m and during 2016 the registered rainfall was of 800–1800 mm. The material was ground using an electric grinder and the standardization of granulometry was made by sieves of 20 mesh. The obtained powder was stored in a desiccator at room temperature (average 25 °C), and protected from light, until further analysis.

2.2. Standards and reagents

Ethanol, chloroform, butanol, anthrone, rhamnose, sulfuric acid, sodium carbonate, Bradford reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteau reagent, ascorbic acid, methanol, 2,4,6-tripyridyl-s-triazine (TPTZ), hydrochloric acid, ferric chloride, 3-ethyl benzothiazoline-6-sulphonic acid (ABTS), potassium persulfate, salicylic acid, iron sulphate, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), fluorescein, dialysis membrane (7000 Da) were purchased from Sigma-Aldrich Co (St Louis, MO). RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) were purchased from Gibco Invitrogen Life Technologies (California, USA). All other general laboratory reagents were purchased from Panreac Quimica S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). All other reagents used in the experiments were of analytical grade.

2.3. Polysaccharide extraction and purification

One hundred (100) g of dried powdered mate were extracted three times with 500 mL of ethanol (70%) for 3 h at room temperature and agitation of 120 rpm to remove low molecular weight compounds and for depigmentation. The sample was filtered and the procedure was repeated three times. Insoluble material was dried to remove ethanol completely. The polysaccharide was extracted and purified from the insoluble material as previously described [13] with some modifications. The residue of the extraction was dissolved in distilled water and subjected to a hot extraction at 80 °C, for 15 min. Thereafter the extract was autoclaved for 20 min. The soluble fraction was precipitated with 3 volumes of ethanol and kept overnight in the refrigerator. The material was centrifuged at 4000 rpm for 15 min, and the precipitate redissolved in distilled water. To remove the protein fraction, the material was treated using the Sevag method [21]. The material was precipitated with 3 volumes of ethanol for 24 h and centrifuged at 4000 rpm for 15 min. The precipitate was re-dissolved in a small volume of distilled water and dialyzed for 24 h against distilled water. To remove DNA contamination, a volume of 4 mL DNAse solution (2 U/mL in 100 mM Tris buffer, pH 7.5, containing 25 mM $MgCl_2$ and 5 mM $CaCl_2$) was added to the polysaccharide solution, and the mixture was incubated at 37 °C for 20 min. The DNA-free polysaccharide was precipitated with ethanol, centrifuged and dialyzed. Finally, the polysaccharide was lyophilized, weighed, and stored at −20 °C for further analysis.

2.4. Chemical analysis

The presence of phenolics and proteins in the yerba mate polysaccharide was evaluated by using the Folin-Ciocalteu method [22] and Bradford's method [23], respectively. For the first determination, a standard curve was constructed using gallic acid and the results were expressed as gallic acid equivalents/mg of material. For the evaluation of protein, bovine serum albumin was used as a standard and the results were expressed as albumin equivalents (AE)/mg of material.

2.5. Ultraviolet-visible (UV–Vis) and Fourier transform infrared (FTIR) spectroscopy

The purified yerba mate polysaccharide was re-dissolved in water to obtain a concentration of 0.25 mg/mL. The UV–Vis spectra of the solution between 190 and 800 nm were then recorded using a spectrophotometer (Beckman Coulter DU640 B, USA). Prior to FTIR analysis, the polysaccharide was dried and desiccated in a vacuum jar. Thereafter,

an amount of 2 mg of the dried sample was mixed with 200 mg KBr of spectroscopic grade and compressed into pellets at a pressure of about 1 MPa. Sample spectra were obtained in triplicates using an average of 128 scans in the range between 500 cm^{-1} and 4000 cm^{-1} with a spectral resolution of 2 cm^{-1}. Peak heights and areas of the FTIR spectra were determined by means of the Opus software version 6.5 normalized by maximum and minimum peaks.

2.6. Biological activities

2.6.1. Antioxidant activity evaluation

Five different methods were used to evaluate the antioxidant activity of the YM polysaccharide: reduction power of the ferric ion (FRAP), oxygen radical absorbance radical assay (ORAC), reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), reduction of the 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonate) cation (ABTS•+), and hydroxyl radical scavenging activity. Successive dilutions of the stock solution were made and used for assaying the antioxidant activity of the sample. FRAP and ORAC were evaluated as described previously [24]. Standard curves were constructed with trolox ($r^2 = 0.99$) and the results were expressed as mmol trolox equivalents (TE)/mg lyophilisate material. The DPPH and the ABTS assays were conducted as described previously [25]. The lyophilisate concentrations (mg/mL) providing 50% antioxidant activity were calculated from the graphs of antioxidant activity against the sample concentrations. Trolox was used as a positive control and water was used as negative control. The results were expressed as IC_{50} values (sample concentration providing 50% of antioxidant activity). The hydroxyl radical scavenging activity of the YM polysaccharide was measured having as principle the Fenton's reaction [21]. The results were also expressed as IC_{50} values. Ascorbic acid was used as positive control whereas the negative control was water.

2.6.2. Antimicrobial activity evaluation

For antibacterial activity assay, the following Gram-negative bacteria were chosen: *Escherichia coli* (ATCC 35210), *Salmonella enteritidis* (ATCC), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), along with the following Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973). For the antifungal tests the following microfungi were assessed: *Aspergillus fumigatus* (1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Candida crusei* (human isolate), *Penicillium funiculosum* (ATCC 36839) and *Penicillium verrucosum* var. *cyclopium* (food isolate). In order to investigate the antimicrobial potential of the YM polysaccharide against these foodborne pathogens and spoilage agents, a modified microdilution technique was applied [26]. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. Both minimum bactericidal (MBCs) and minimum fungicidal (MFCs) concentrations were determined by serial subcultivation of a 2 mL sample into microtiter plates containing 100 mL of broth per well and further incubation for 48 h at 37 °C or 72 h at 28 °C, as previously described [27]. The lowest concentrations with no visible growth were nominated as MBC/MFC, corresponding to 99.5% killing of the original inoculum. In the antibacterial bioassays, streptomycin (ICN-Galenika, Belgrade, Serbia) and ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls, while in antifungal tests the commercial fungicides bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Sabac, Serbia) were applied (at the concentration of 1 mg/mL in sterile physiological saline); 30% ethanol was employed as negative control.

2.6.3. Cytotoxicity in human tumor cell lines

Four human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF-7, NCI-H460 HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (1.0×10^4 cells/well) in 96-well plates. The sulforhodamine B assay was performed according to a procedure previously described by the authors [28]. Ellipticine was used as positive control.

2.6.4. Cytotoxicity in non-tumor liver cells primary culture

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to a procedure established by the authors [29]. It was designated as PLP2. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and commercial in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Ellipticine was used as positive control.

2.7. Statistical analysis

Three repetitions of the sample and triplicates for each concentration were carried out in all assays. The results were reported as mean ± standard error. The IC_{50} values and graphics were obtained from the logarithmic non-linear regression curve derived from the plotted data using the GraphPad Prism software (version 5.0).

3. Results and discussion

3.1. Yield and structural analysis of yerba mate polysaccharide

In order to obtain the pure polysaccharide previously illustrated by Fig. 1 [13], the sequential methods shown in Fig. 2 were used. An amount of 1.2 g of the dried pure polysaccharide was obtained starting from 100 g of yerba mate. Phenolics and proteins were not detected by chemical methods. In addition, the UV–Vis spectrum of the yerba mate polysaccharide showed no absorption peaks at 260 and 280 nm (Fig. 3). Significant contamination by proteins, peptides, DNA and phenolics is thus unlikely.

FTIR spectroscopy is usually used for identifying characteristic organic groups in the polysaccharide. As illustrated by Fig. 4, vibrations and glycosidic bonds typical of polysaccharide structures are apparent. The absorption peak at 767 cm^{-1} indicates the presence of pyranoses in α-configuration [30]. The absorption at 1072 cm^{-1} can be attributed to the stretching vibration of the C-O-C glycosidic bond vibrations and side group C—O—H link bonds [31]. The spectrum shows absorption bands at 1013, 1076 and 1100 cm^{-1}, which are characteristic of pectin polymers. The rhamnogalacturonan structure can be confirmed by the stretching vibration bands of the ester carbonyl at 1610 and 1737 cm^{-1} corresponding to asymmetric carbonyl stretching of carboxylate groups, overlapped by the water absorption band. The bands around 1442 cm^{-1} and 1374 cm^{-1} for C—H band stretching are due to asymmetric and symmetric bending vibrations, respectively [32]. The band at 1609 cm^{-1} can be attributed to the C═O stretching vibration of uronic acid [33]. Strong and wide absorption bands at about 2800–3500 cm^{-1} for C—H and O—H stretching vibrations and the strong absorption in the region of 1000–1125 cm^{-1} indicates the presence of the functional groups C—OH, C—O—C and C—C [34].

3.2. Antioxidant activities of the yerba mate polysaccharide

In vitro antioxidant models are based on the transfer of a single electron (DPPH•, ABTS•+, FRAP) or the transfer of hydrogen atoms (hydroxyl and ORAC) [35]. The results of three assays, DPPH, ABTS, and hydroxyl radical scavenging activities, are shown in Fig. 5. The IC_{50} values of the scavenging activities were 1.25 ± 0.10 mg/mL, 0.41 ±

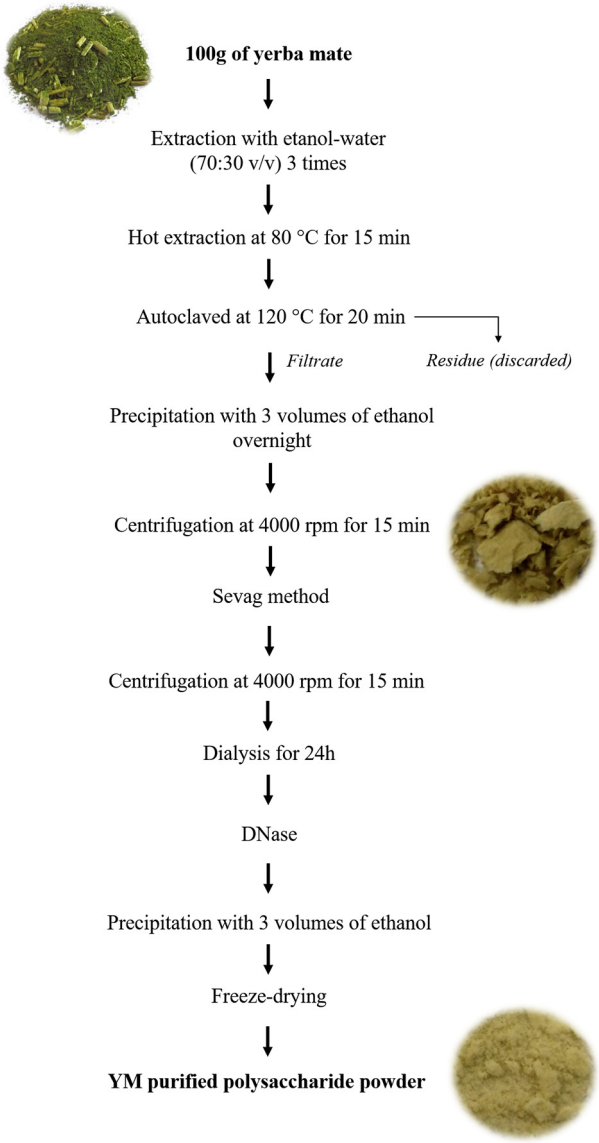

Fig. 2. Diagram for the obtainment of purified yerba mate polysaccharide.

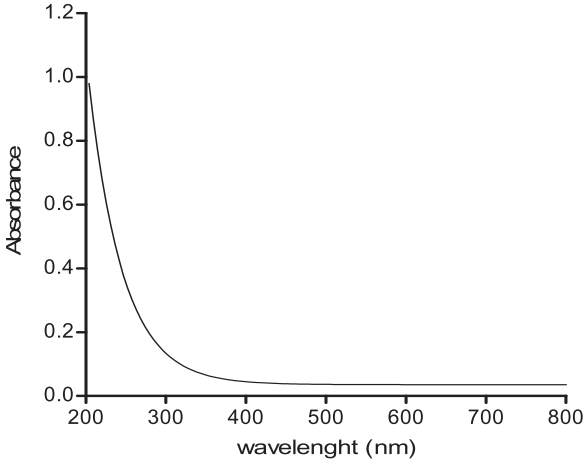

Fig. 3. UV–Vis spectrum of the purified yerba mate polysaccharide.

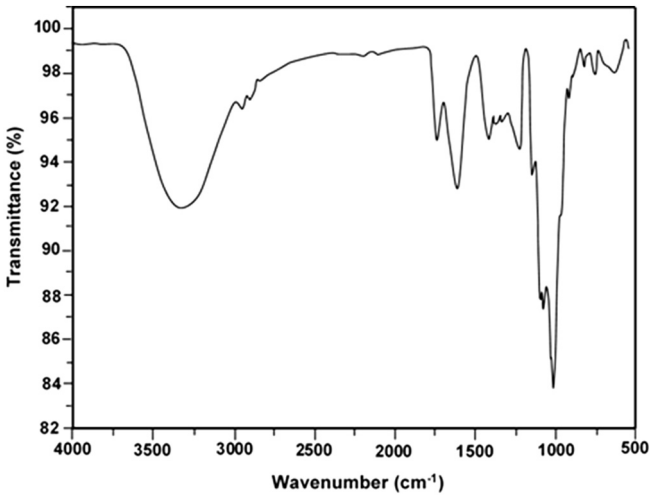

Fig. 4. FTIR spectrum of the purified yerba mate polysaccharide.

0.05 mg/mL and 3.36 ± 0.31 mg/mL, respectively. FRAP and ORAC, expressed as equivalents of Trolox, were 20.84 ± 1.61 μM TE/mg and 556.30 ± 12.83 μM TE/mg, respectively.

The mechanism by which polysaccharides act as antioxidants is still not a consensus among researchers. While some researchers strongly relate the structures of the polysaccharides to their antioxidant activity [36,37], others suggest that the antioxidant activities of complex carbohydrates should be attributed to their phenolic and protein components or contaminants rather than to the carbohydrate moieties, especially when the analyses were carried out using crude or semi-purified polysaccharides [16,38–40]. Recent analyses conducted with the highly homogeneous polysaccharide fractions from *Ilex latifolia* [36] *Mesona chinensis* [37], *Bryopsis plumose* [41], *Malva aegyptiaca* [42], *Plantago notata* [43], *Dendrobium officinale* [44], and *Schisandra sphenanthera* [45] have confirmed antioxidant activities of polysaccharides evaluated by different methods. The mechanisms involved, however, remain largely unknown. In general, functional groups such as —OH, —COOH and C═O, largely found in polysaccharides in addition to anionic and cationic functional groups, such as uronic acids, have been related to the antioxidant activities of polysaccharides [35]. Moderate molecular weight, water solubility, triple helix stereo-configuration and higher degrees of branching are also considered factors that might favour antioxidant activity [45].

3.3. Antimicrobial activities of the yerba mate polysaccharide

Antibacterial and antifungal activities of the YM polysaccharide are presented in Tables 1 and 2, respectively. The polysaccharide had prominent antimicrobial effects against Gram-negative bacteria (*Enterobacter cloacae*, *Salmonella enteritidis*, and *Salmonella typhimurium*), Gram-positive bacteria (*Bacillus cereus*, *Micrococcus flavus*, *Staphylococcus aureus*, and *Listeria monocytogenes*) and against *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus ochraceus*, *Aspergillus niger*, *Candida crusei*, *Penicillium funiculosum*, and *Penicillium verrucosum* var. *cyclopium*. According to MIC values, bioactive extracts from natural products can be classified into strong inhibitors (MIC below 0.5 mg/mL), moderate inhibitors (MIC between 0.6 and 1.5 mg/mL) and weak inhibitors (MIC above 1.6 mg/mL) [25]. According to these parameters, the herein assessed YM polysaccharide can be considered a strong inhibitor against *B. cereus*, *M. flavus*, *E cloacae*, *S. enteritidis* and *S. typhimurium*; however, a weak inhibitor of *E. coli*. Regarding the antifungal bioassays, the YM polysaccharide seems to be a strong inhibitor against all tested fungi, except for *A. niger*.

It was reported elsewhere that the YM polysaccharide prevents lethality caused by poly-microbial sepsis in mice, which was attributed to its action in reducing the neutrophil infiltration, on its turn an

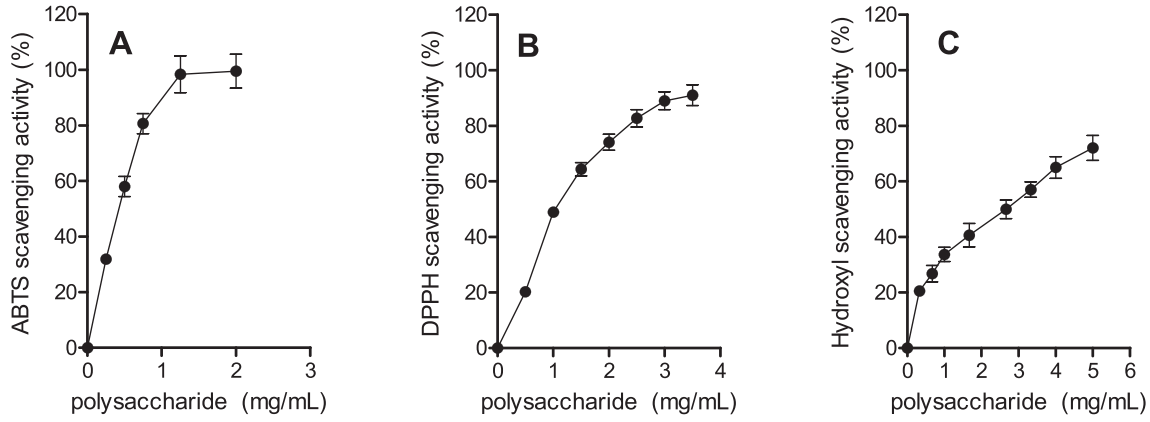

Fig. 5. Antioxidant activity of the purified yerba mate polysaccharide. A: (ABTS•+)-radical scavenging activity; B: (DPPH•)-radical scavenging activity; C: Hydroxyl scavenging activity.

important alteration associated to sepsis [13]. Although there is no doubt that diminution of neutrophil infiltration may be beneficial in sepsis, an equal or even higher weight must be attributed to the observed antimicrobial action of the polysaccharide. After all, an antimicrobial effect represents a direct action on the infectious agents, which, in principle, is more effective than indirect and marginal mechanisms such as neutrophil infiltration.

Antimicrobial activities have also been described for polysaccharides isolated from *Quercus brantii* leaves [46], *Olea europaea* leaves [47], *Capparis spinosa* leaves [48], and *Malva aegyptiaca* leaves [42]. The mechanisms involved in the antimicrobial activity of polysaccharides are worthy of further investigations. Differences in the cell membrane composition and structure can explain the slightly greater resistance among Gram-positive bacteria [48,49]. The YM polysaccharide showed antibacterial activity against all tested strains, except to *E. coli*. This result suggests that the inhibition of iron absorption by the bacteria is a possible mechanism of the antibacterial activity of polysaccharides. Iron is an important element for bacterial growth and the enterobactin secreted by *E. coli* has a high affinity for iron and can compete with the chelating activity of polysaccharides [50].

3.4. Antiproliferative and cytotoxic actions of the yerba mate polysaccharide

The YM polysaccharide did not show hepatotoxicity in PLP2 cells, up to the maximum concentration tested (GI_{50} > 400 mg/mL), what endorses its safe use in the case of potential applications as a food additive or nutraceutical. The same is valid for the four tumor human cell lines assessed namely MCF-7, NCI-H460, HeLa and HepG2 (GI_{50} > 400 mg/mL). However, there are reports on the direct action of polysaccharides on the tumor cells or by enhancing the immune function of the organism, exerting thus an indirect antitumor activity [51]. Different rhamnogalacturan I and II type polysaccharides (RG-I and RG-II) have been described as possessing potentially antitumoral activities. For example, a RG-II-type polysaccharide isolated from mature leaves of green tea, presented antitumor and anti-metastatic activities *via* activation of macrophages and natural killer cells [52]. RG-II isolated from the leaves of *Panax ginseng* inhibited tumor growth by activating dendritic cell-mediated $CD8^+$ T cells [53]. Furthermore, a RG-I domain-rich pectin from potato inhibited the proliferation of HT-29 cells and induced significant G2/M cell cycle arrest [54]. Our results suggest that the YM polysaccharide does not act directly on the tumor cell. However, it could be able to act indirectly if one takes into account what was found for the RG-II-type polysaccharide from green tea leaves. Additional approaches are necessary to clarify this question.

Table 1
Antibacterial activity of the yerba mate polysaccharide.

Compounds	YM polysaccharide		Streptomycin		Ampicillin	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram positive						
Bacillus cereus	0.30	0.40	0.10	0.20	0.25	0.40
Micrococcus flavus	0.30	0.40	0.20	0.30	0.25	0.40
Staphylococcus aureus	0.60	0.80	0.04	0.10	0.25	0.45
Listeria monocytogenes	0.60	0.80	0.20	0.30	0.40	0.50
Gram negative						
Escherichia coli	>1.60	>1.60	0.20	0.30	0.40	0.50
Enterobacter cloacae	0.30	0.40	0.20	0.30	0.25	0.50
Salmonella enteritidis	0.40	0.60	0.15	0.30	0.30	0.60
Salmonella typhimurium	0.40	0.60	0.25	0.50	0.40	0.75

MIC = minimum inhibitory concentration (mg/mL); MBC = minimum bactericidal concentration (mg/mL).

4. Conclusion

The results of the present work indicate that the YM polysaccharide possesses antioxidant activities corroborated by different *in vitro* methods. The YM polysaccharide also presents antibacterial and antifungal activities, which can help to explain mitigation of sepsis caused by this macromolecule according to a previous report. For these and other reasons, the YM polysaccharide can be considered to be potentially useful for the pharmaceutical and food industries.

Conflicts of interest

The authors declare no conflict of interests.

Table 2
Antifungal activity of the yerba mate polysaccharide.

Compound	YM polysaccharide		Ketoconazole		Bifonazole	
	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus fumigatus	0.40	0.60	0.25	0.50	0.15	0.20
Aspergillus versicolor	0.20	0.40	0.20	0.50	0.10	0.20
Aspergillus ochraceus	0.40	0.60	1.50	2.00	0.15	0.20
Aspergillus niger	0.60	0.80	0.20	0.50	0.15	0.20
Candida crusei	0.30	0.40	0.075	0.15	0.05	0.10
Penicillium funiculosum	0.40	0.60	0.20	0.50	0.20	0.25
Penicillium verrucosum var. *cyclopium*	0.30	0.60	0.20	0.30	0.10	0.20

MIC = minimum inhibitory concentration (mg/mL); MFC = minimum fungicidal concentration (mg/mL).

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