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The antibacterial effects of an aqueous extract of yerba-mate (*Ilex paraguariensis*) leaves and twigs on pathogenic bacteria

Efeito antibacteriano de extrato aquoso de folhas e palitos de erva-mate em bactérias patogênicas

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RESUMO

A pesquisa sobre a atividade antibacteriana da erva-mate (EM) e sobre seus resíduos industriais carece de exploração mais aprofundada. O objetivo do trabalho foi avaliar a atividade antibacteriana contra bactérias patogênicas e o conteúdo polifenólico de folhas e galhos de YM extraídos por diferentes tempos de extração. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes* e *Escherichia coli* foram testados contra extrato aquoso de folhas e galhos de EM e o modo de ação foi avaliado por microscopia. Os resultados mostraram que o extrato aquoso dos galhos inibiu ambas as espécies de *Staphylococcus* quando a extração ocorreu por 18h. O extrato das folhas apresentou inibição contra *S. aureus*. *L. monocytogenes* e *E. coli* não foram inibidas. A extração realizada por 10 minutos não produziu atividade antibacteriana. Não foi observada diferença significativa na concentração de polifenóis totais ou taninos condensados nos extratos extraídos por 18 horas. A microscopia mostrou alterações na morfologia da célula bacteriana de *S. aureus*. EM e seu subproduto são uma fonte promissora de compostos funcionais.
Palavras-chave: *Ilex paraguariensis*, antimicrobiano, resíduos, antioxidante.

ABSTRACT

Research on the antibacterial activity of yerba-mate (YM) and its industrial residues has not been explored sufficiently. This study aimed to evaluate the antibacterial activity against pathogenic bacteria and the polyphenolic content of YM leaves and twigs extracted over different periods. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, and *Escherichia coli* were tested against an aqueous extract from YM leaves and twigs, and the mode of action was evaluated by microscopy. Results showed that the aqueous twigs extract inhibited both Staphylococcus species when the extraction was done for 18 hours. The leaf extract showed inhibition against *S. aureus*. *However*, *L. monocytogenes* and *E. coli* were not inhibited. Extraction performed for 10 minutes did not yield any antibacterial activity. No significant difference was observed in the total polyphenols or condensed tannins concentration in the extracts produced after 18 hours of extraction. Microscopy revealed changes in the morphology of the *S. aureus* bacterial cell. YM and its byproduct represent a promising source of functional compounds.

Keywords: *Ilex paraguariensis*, antimicrobial, byproducts, antioxidant.

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Introduction

Yerba mate (*Ilex paraguariensis* A. St.-Hil.) (YM) is native to Brazil, where its leaves are employed for various purposes, primarily in preparing beverages such as *chimarrão* and *tereré*. It is a traditional and cultural product not only in the southernmost states of Brazil but also in Argentina, Uruguay, and Paraguay. Various studies have associated YM with a variety of health benefits, including antioxidant properties, vasodilatory function, protection of DNA against oxidative-induced damage, hypoglycemic effects, inhibition of glycation, and atherosclerosis, as recently reported in the critical review by Vasconcellos, Frazzon, and Noreña (2022). These attributes of YM are closely linked to its phenolic profile, although the presence of minerals, vitamins, amino acids, xanthines, saponins, and organic acids also plays a significant role in the health benefits of YM (ZIELIENSKI et al., 2021).

The Brazilian production of YM is approximately 935 thousand tons, involving around 700 processing industries. According to Penteado Junior and Goulart (2017), the increase in food processing has led to greater organic waste generation. The YM sector generates twig waste as a by-product of its processing, which is currently utilized as organic fertilizer or for combustion in the sector's steam-generating furnaces. However, this by-product has a low calorific value, and a large volume is generated (approximately 2% of the YM used in processing becomes residual sticks), presenting a challenge for the industries that currently process the plant (PENTEADO JR.; GOULART, 2017). Nevertheless, evidence has indicated that these materials have significant potential for use as sources of high-value-added agents, such as phenolic, antioxidant, and antimicrobial compounds (GNOATO *et al.*, 2005; BURRIS *et al.*, 2012).

The demand for natural food preservatives has been driven by consumer preference for products with fewer or no synthetic chemical compounds. Moreover, the emergence and proliferation of pathogenic microorganisms have proven to be a serious public health issue worldwide, prompting research efforts in various fields of life and health sciences to study and address this problem (KHORSHIDIAN *et al.*, 2018). In this context, the search for non-toxic substances that can serve as biopreservatives and do not affect the organoleptic characteristics of the final products remains a critical challenge and priority in the food sector (KHORSHIDIAN *et al.*, 2018).

Water is a safe, abundant, inexpensive solvent with no environmental impact, and it is a material familiar to stakeholders in the food chain. It would be easier to disseminate knowledge with small family agroindustries and agribusinesses (CAXAMBÚ *et al.*, 2016; TROJAIKE *et al.*, 2019; FLECK *et al.*, 2021, 2023). Water has been utilized to produce phenolic-rich aqueous extracts with antioxidant, antimicrobial, and herbicidal activities from various food by-product matrices, proving to be an important sustainable strategy for producing functional ingredients for both food and pharmaceutical applications (SANT'ANNA *et al.*, 2017; CAXAMBÚ *et al.*, 2016; TROJAIKE *et al.*, 2019; FLECK *et al.*, 2021, 2023). However, this approach has not been applied to YM processing by-products, representing a significant research gap. Therefore, this study aimed to evaluate the antimicrobial activity of aqueous extracts from YM twigs and leaves obtained at different extraction periods against *S. aureus*, a bacterium of significant public health interest.

Materials and methods

ISSN 2237-1966

Plant material

Yerba mate leaves and twigs were obtained in November 2021 in Ilópolis (RS, Brazil) (28°55'37 "S 52°07'26"W) and were purchased from a local company producing commercial YM. Leaves were dried at 60 ºC for 24 hours in a static oven, whereas twigs were not dried. The samples were crushed in an industrial blender (Metvisa, model LAR4) and passed through 5 mm sieves. Samples were stored at -18 ºC in sealed plastic bags in the dark until analysis.

Chemicals and microorganisms

The culture media plate count agar, potato dextrose agar, and yeast extract were purchased from Biolog (Belo Horizonte, Minas Gerais, Brazil). Disodium phosphate, glycine, hydrochloric acid, sodium chloride, potassium chloride, and 2M Folin-Ciocalteu solution were from Exôdo Científica (Sumaré, São Paulo, Brazil); gallic acid, sodium citrate, sodium acetate, and acetic acid were acquired from Dinâmica (Indaiatuba, São Paulo, Brazil); ethanol was purchased from Itajá (Goianésia, Goiás, Brazil). Rutin, caffeic acid, and epicatechin were purchased from Sigma Aldrich (St. Louis, Missouri, USA). *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Staphylococcus epidermidis* ATCC 35984, and *Escherichia coli* ATCC 25922 were the indicator strains and were kept frozen in brain heart infusion broth (Difco, Detroit, Michigan, USA) containing 20% glycerol at -21 °C.

Aqueous extracts

Hot water was used as a solvent as per the literature (CAXAMBÚ *et al.*, 2016; TROJAIKE *et al.*, 2019). The YM twigs and leaves, separately, were boiled in distilled water for 10 minutes at a ratio of 10:1 (volume/mass). After the extraction period, the solution was filtered using Whatman no. 1 filter paper into falcon tubes with lids. The entire system had been previously sterilized, and filtration was performed in a laminar flow hood to guarantee the absence of microorganisms in the extract (CAXAMBÚ *et al.*, 2016).

Antibacterial activity

The evaluation of antimicrobial activity was conducted using the agar diffusion method (FLECK et al., 2022, 2023), where suspensions of 10^8 colony-forming units per mL (CFU/mL) of *L. monocytogenes*, *S. aureus*, *E. coli*, *and S. epidermidis* were spread on plate count agar cultivation medium. The CFUs were determined by pelletizing the microorganisms from 100 µL of the microorganism culture in yeast extract broth, which was centrifuged for 10 minutes to obtain the pellet. Aliquots of 20 µL of the extracts were then applied to the agar and the plates were incubated at 37 °C for 24 hours, with the presence of halos indicating the occurrence of antimicrobial activity. 1 mL of sterile 0.85% saline solution was used for the negative control. The presence of halos is indicative of the occurrence of antimicrobial activity.

Mode of action

The anti-*Staphylococcal* mode of action was evaluated by scanning electron microscopy. The

ISSN 2237-1966

direct contact of the extract with the bacteria was evaluated as described by Lappe *et al*. (2009). Biomass pellets of 10^7 CFU/mL of the microorganism were diluted with 1 mL of the aqueous extract of YM leaves and twigs and incubated at 37 °C for approximately 90 minutes. The biomass was then washed with a 0.85% saline solution, followed by concentration through centrifugation, with subsequent washing in a 0.1 mol/L phosphate buffer solution, and fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in a 0.12 mol/L phosphate buffer for 10 days. Subsequently, they were post-fixed in 2% (w/v) osmium tetroxide in the same buffer solution for 45 minutes. The samples were dehydrated in a graded series of acetone (30–100%) and embedded in Araldite Durcupan for 72 hours at 60 °C. Thin sections were cut on a microtome (UPC-20, Leica), mounted on grids, covered with collodion film, and post-stained with 2% uranyl acetate and Reynold's lead citrate. The material was observed using a JEOL JEM 1200ExII electron microscope (JEOL, Tokyo, Japan) at 120 kV.

Phenolic compounds and antioxidant activity

The total phenolic content, monomeric anthocyanins, condensed tannins, phenolic acids, and compounds with antioxidant activity by the ABTS method of the samples were evaluated using spectrophotometric methods as described in the literature and recently published procedures (SANT'ANNA *et al.*, 2017; FLECK *et al.*, 2022).

The concentration of total polyphenols was determined by the Folin-Ciocalteau method described by Singleton and Rossi (1965), where the extracts reacted with distilled water, Folin-Ciocalteau reagent, and saturated sodium carbonate solution at room temperature. The absorbance reading was measured at 765 nm against a blank using a UV/VIS spectrophotometer. For quantification, a standard curve was used with a gallic acid solution, and the total polyphenol content was expressed in mg of gallic acid equivalent per mL.

For phenolic acids, the methodology described by Mazza *et al*. (1995) was used, where the extracts were diluted in acidified ethanol solutions and diluted hydrochloric acid solution, and the absorbance reading was carried out in quartz cuvettes at 320 nm. A standard curve was used with a caffeic acid solution, and the final concentration was expressed in mg of caffeic acid equivalent per mL.

For flavonols, the methodology was also described by Mazza *et al*. (1995); the extracts were diluted in acidified ethanol solutions and diluted hydrochloric acid solution, and the absorbance was measured in quartz cuvettes at 360 nm. A standard curve was used with an epicatechin solution, and the final concentration was expressed in mg of epicatechin equivalent per mL.

To determine condensed tannins, the method described by Price *et al*. (1978) involves the reaction of the extract with a vanillin solution and the subsequent measurement of absorbance of the reaction mixture after 15 minutes at 500 nm. For quantification, a standard curve was prepared using catechin, and the content of condensed tannins was expressed in milligrams of catechin equivalent per milliliter.

The antioxidant activity was measured by the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay, which involves the generation of the ABTS

ISSN 2237-1966

chromophore radical through the oxidation of ABTS with potassium persulfate (RE *et al.*, 1999). The ABTS radical cation is produced via the reaction between the ABTS stock solution (7 mmol/L) and potassium persulfate (140 mmol/L final concentration). This mixture was allowed to stand in the dark for 12 hours at room temperature prior to use. For the assay, the $ABTS^{+}$ solution was diluted with sodium phosphate buffer (5 mmol/L; pH 7) until it reached an absorbance of 0.7 (±0.02) at 734 nm. Samples of 10 µL of the extract were mixed with 1 mL of the diluted $ABTS^{+}$ solution, and the absorbance at 734 nm was monitored for 6 minutes. The results were finally expressed as a percentage of free radical inhibition relative to the control sample.

Data analysis

The extracts' antimicrobial and antioxidant activity data were statistically compared by analysis of variance, followed by Tukey's test. Significant differences between the extraction methods were considered at $p < 0.05$.

Results and discussion

The results of the antibacterial activity of aqueous extracts from YM leaves and twigs against the studied pathogenic bacteria are presented in Table 1. The aqueous extract from YM processing twigs exhibited inhibitory activity against both studied species of *Staphylococcus* but did not inhibit *L. monocytogenes* and *E. coli* when the extraction duration was 18 hours. The leaf extract could inhibit *S. aureus* when water served as the extracting solvent and inhibited *L. monocytogenes* with 18 hours of extraction. However, neither extract could inhibit the pathogenic bacteria when the extraction occurred over a short duration (10 minutes).

The inability of aqueous extracts to inhibit bacteria when the extraction is performed for 10 minutes is consistent with findings from other by-product materials such as spent coffee grounds (SANT'ANNA *et al.*, 2016), sugarcane bagasse (GIRARDI; PADILHA; SANT'ANNA, 2019), although they were effective when using jaboticaba peel (FLECK *et al.*, 2022; 2023), *pinhão* seed coat (TROJAIKE *et al.*, 2019), and pecan nutshell (CAXAMBÚ *et al.*, 2016), studied under the same conditions. The samples could not inhibit E. coli, a Gram-negative bacterium, likely due to the architecture of the outer membrane of Gram-negative microorganisms, preventing the penetration of many antimicrobials into the cytoplasmic membrane.

Table 1. Antibacterial activity of yerba mate material against pathogenic bacteria.

	YM leaves		YM twigs	
	Extraction for 10 min	Extraction for 18h	Extraction for 10 min	Extraction for 18h
S. aureus	-	+	-	+
S. epidermidis	-	-	-	++
L. monocytogenes	-		-	-
E. coli	-	-	-	-

- Without inhibition halo; + inhibition halo of 1 mm; ++ inhibition halo of 2 mm.

Between 7–10% of YM leaves dry weight consists of phenolic compounds, predominantly quinic

ISSN 2237-1966

acid, a cyclic polyol, caffeic acid, ferulic acid, and p-coumaric acids (VASCONCELLOS; FRAZZON; NOREÑA, 2022). In Gram-positive bacteria, tannins are believed to react with and inhibit the biosynthesis of cell wall components (JONES *et al.*, 1994). Tannins with potential antibacterial, antifungal, and virucidal actions have been isolated from plants and/or plant components that contain antimicrobial activities and which are commercially available to consumers (KHAMENEH *et al.*, 2019). Phenolic acids alter the polar, non-polar, and electron-accepting components of bacterial cells. After exposure to gallic acid and ferulic acid, the electron acceptor capacity increases for Gram-positive bacteria and decreases for Gram-negative bacteria. This suggests that acids are electrophilic products and seem to interact significantly with bacterial surface components (BORGES *et al.*, 2013). Fleck *et al.* (2022) observed that phenolic acids were one of the main components of the polyphenolic class responsible for the antibacterial activity of jaboticaba peel aqueous extract.

Table 2 shows the total phenolic content, condensed tannins, phenolic acids, and the antioxidant capacity of the extracts that exhibited antibacterial activity (extraction for 18 hours). Results indicate a significant concentration of condensed tannins and phenolic acids, which may explain the observed antibacterial capacity. Also, the antioxidant activity of the extracts should be further explored in subsequent studies since it can act synergistically on Gram-negative bacteria cell walls with other antimicrobial compounds by weakening the outer membrane through a chelating mechanism of divalent cations, thereby facilitating inhibition.(NOHYNEK *et al.*, 2006).

Table 2. Polyphenolics and antioxidant activity of yerba mate 18-h aqueous extracts.

Sample	TPC	CT	PA	ABTS
Leaves	1058.24±100.25^{a}	0.652±0.052^{a}	38.09±1.52^{a}	80.55±3.74^{a}
Twigs	1030.17±75.37^{a}	0.854±0.092^{a}	25.47±2.89^{b}	72.52±1.98^{b}

TPC: Total polyphenol content: expressed in mg GAE mL^{-1}; CT: Condensed Tannins: expressed in mg EE/mL; PA: Phenolic Acids: expressed in mg CAE/mL; ABTS: antioxidant activity by scavenging free radicals: expressed in %. ^{a,b} different letters between lines in the same column indicate statistical differences compared to control samples (no treatment) ($p < 0.05$).

Since YM leaves and twigs exhibited antibacterial activity against *S. aureus* cells, they were selected as the target bacteria to evaluate the mode of action. *S. aureus* is a significant pathogenic bacterium, identified as one of the microorganisms for which the development of new antibiotics is urgently needed due to resistance against existing antibiotics (WORLD HEALTH ORGANIZATION, 2017). Additionally, its ability to form biofilms provides high resistance, ensuring the microorganism's survival in various environments (SINGH *et al.*, 2010). The effect of both YM extracts on *S. aureus* is presented in Figure 1. Figure 1A displays intact bacterial cells, typical of a control test. When the cells were treated with YM leaf extract (Figure 1B), alterations in the bacterial cell wall were observed, consistent with the findings of Fleck *et al.* (2022, 2023), who reported that the aqueous extract of jaboticaba peel inhibits *S. aureus*. Their findings indicated that the action is significantly associated with the presence of phenolic acids but not with the concentration of condensed tannins, with the mode of action is clearly linked to alterations in the microorganism's cell wall. Figure 1C shows more profound changes in the bacterial cell wall due to the YM twig extract.

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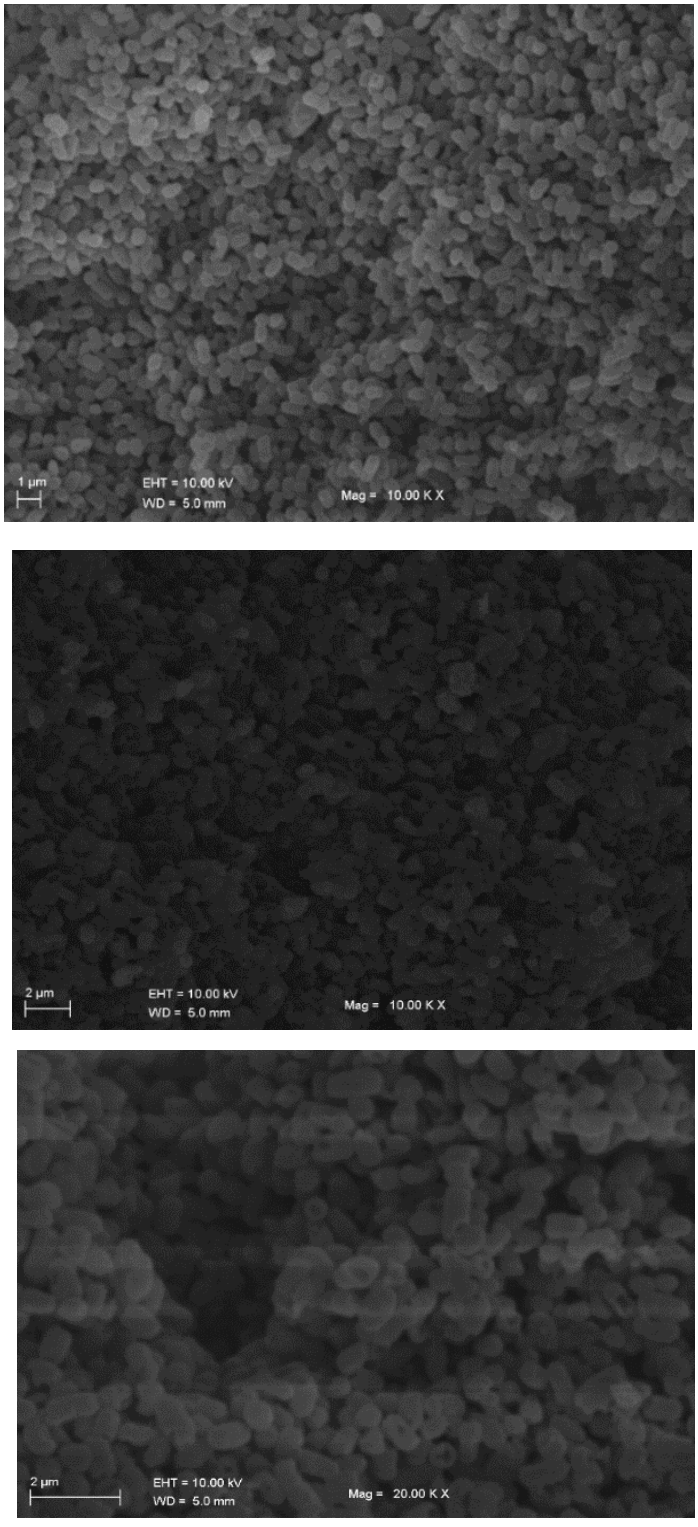

Figure 1. Scanning electron microscopy of *S. aureus* cells under the effects of the control treatment (A), aqueous extract of yerba mate leaves (B), and twigs from yerba mate processing (C) extracted for 18 hours. Source: the authors.

Although the action of phenolic compounds has not been completely clarified, these compounds stand out for involving antibacterial action at the cellular level of microorganisms (FLECK *et al.*, 2022). It is suggested that the referred activity is related to the permeability of the cell membrane through the presence of hydrogen bonds of phenolic compounds to enzymes or due to modifications

ISSN 2237-1966

of the cell wall in relation to interactions with the bacterial cell membrane (BOUARAB-CHIBANE *et al.*, 2019). Borges *et al.* (2013) posited that phenolic acids are capable of modifying the electron acceptors of microorganism cells, which for Gram-positive bacteria tends to increase after exposure to gallic acid and ferulic acid and decrease for Gram-negative bacteria, demonstrating the interaction of electrophilic acids on the bacterial surface. This is in line with the study by Nohynek *et al.* (2006), which proposes that phenolic compounds exhibit antimicrobial activity in Gram-negative bacteria due to the chelating power of divalent cations in their external membranes. Molva and Baysal (2014) used scanning electron microscopy and found damage to the structure and cellular components of bacteria when testing the antibacterial activity of grape seed extract on vegetative cells and endospores of *Alicyclobacillus acidoterrestris* DSM 3922, a Gram-positive bacterium, in apple juice, justifying the antibacterial activity against endospores as dependent on the sporulation medium.

As the mechanisms of antimicrobial action have not been fully clarified, it is believed that the antimicrobial activity is related to the cellular structure and the availability of the compounds in the extract. The absence or low inhibitory activity may be related to the low concentration of the compounds in the extracts obtained. Moreover, the extraction mode and particle characteristics are among the numerous factors that can influence the antioxidant capacity and phenolic content of extracts, which justifies the results obtained in the present work, taking into account the results from other works in the literature over the last years.

Conclusion

Aqueous extracts from yerba mate leaves and twigs are phenolic-rich infusions with interesting antibacterial activity against *S. aureus* and *S. epidermidis*. Short extraction durations (10 minutes) were not effective, and a longer extraction period (18 hours) should be employed to obtain antibacterial activity. Additionally, scanning electron microscopy results showed that they change the cell morphology of *S. aureus*, thereby being an interesting biopreservative to control Gram-positive pathogenic bacteria.

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