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INFORME BREVE

Antimicrobial activity of yerba mate (*Ilex paraguariensis* St. Hil.) against food pathogens

José G. Prado Martin*, Ernani Porto, Severino M. de Alencar, Eduardo M. da Glória, Cristina B. Corrêa, and Ingridy S. Ribeiro Cabral

Departamento de Agroindústria, Alimentos e Nutrição, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo, Piracicaba, São Paulo, Brasil

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Abstract
Yerba mate (*Ilex paraguariensis* St. Hil.) has been studied for its important biological activities mainly attributed to phenolic compounds. This study evaluated the antimicrobial activity of methanolic and ethanolic extracts of yerba mate against food pathogens, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* through minimum inhibitory (MIC) and bactericidal (MBC) concentrations, in addition to the determination of chemical composition by gas chromatography with mass spectrometry (GC-MS) and phenolic content. The most effective extract had its activity evaluated under different pH conditions by growth curve analysis. All microorganisms except *E. coli* were inhibited. The ethanolic extract showed the lowest MIC/MBC (0.78/0.78 mg/ml), the highest phenolic content (193.9 g.GAE/kg) and the presence of chlorogenic acid derivatives, especially 3-O-caffeoylquinic and caffeic acid. This extract was able to inhibit microbial growth at pH 7 and 8.

PALABRAS CLAVE
Yerba mate;
Actividad antimicrobiana;
Staphylococcus aureus;
Listeria monocytogenes;
Salmonella Enteritidis

Actividad antimicrobiana de la yerba mate (*Ilex paraguariensis* St. Hil.) contra patógenos alimentarios

Resumen
La actividad biológica de la yerba mate (*Ilex paraguariensis* St. Hil.) ya ha sido descrita. Dicha actividad generalmente se ha asociado a la presencia de compuestos fenólicos. Este estudio evaluó la actividad antimicrobiana de los extractos etanólicos y metanólicos de la yerba mate contra patógenos alimentarios como *Staphylococcus aureus*, *Listeria*

*Corresponding author.
E-mail address: gui.pmartin@gmail.com (J.G. Prado Martin).

monocytogenes, Salmonella Enteritidis y *Escherichia coli* mediante la determinación de la concentración inhibitoria (CIM) y bactericida mínima (CBM). También se efectuó el análisis de la composición química por cromatografía gaseosa-espectrometría de masas (CG-EM) y se determinó el contenido de compuestos fenólicos. El extracto con mayor capacidad inhibitoria se evaluó en diferentes condiciones de pH, por análisis de curvas de crecimiento. Todos los microorganismos fueron inhibidos, excepto *E. coli*. El extracto etanólico mostró la menor CIM/CBM (0,78/0,78 mg/ml), el más alto contenido de fenólicos totales (193,9 g.EAG/kg) y la presencia de derivados clorogénicos, principalmente ácido 3-O-cafeoilquínico y cafeico. Este extracto fue capaz de inhibir el crecimiento microbiano a pH 7 y 8.

In recent decades, research has shown the potential exploitation of plant products as a bioactive compound source for industrial interest. Leaves, stems and flowers may present biological activities[1]. In addition, the study of products used in infusions such as teas and beverages has gained increasing prominence[14]. Among these products, yerba mate stands out, being a product widely used by South American populations both as a source of caffeine in place of or in addition to tea and coffee; also as a therapeutic agent due to its known pharmacological properties such as antioxidant, anti-inflammatory, antitumor, and weight reducing activities[2].

In the last years, different investigations have revealed the antimicrobial potential of yerba mate, whose spectrum of activity includes gram-positive and gram-negative bacteria and fungi[4,5]. These bioactivities are strictly related to the presence of different classes of compounds, mainly phenolics, whose main representatives in yerba mate are gallic, syringic, caffeic, ferulic and ρ-coumaric acids[11]. However, there are few studies about the influence of pH conditions on the activity of these compounds in crude extracts; these data are extremely important, since this variable is vital for antimicrobial effectiveness.

This study evaluated the antimicrobial activity against food pathogens of the methanolic and ethanolic extracts of yerba mate (*Ilex paraguariensis* St. Hil.) used to prepare the typical hot mate-based beverage *chimarrão* and its relation to the content of phenolic compounds. The composition of the extract with the highest antimicrobial activity and phenolic contents was determined by gas chromatography with mass spectrometry (GC-MS) and its antimicrobial activity was evaluated under different pH conditions.

Yerba mate was acquired from the local trade in Campinas, São Paulo, Brazil. The extracts were obtained by percolation. The sample (1:8 w/v) was extracted in hydroethanolic (40:60) and hydromethanolic (30:70) solutions (Synth®, Diadema, Brazil) and maintained under refrigeration for 96 h, with filtering using qualitative filter paper 12.5 μm (Qualy®) every 24 h. The extract was evaporated in a rotary evaporator at 45 °C (Tecnal®). Then, the final extract was freeze-dried (Liotop®L101) and kept under refrigeration. For the tests, the extracts were dissolved in tryptic soy broth (TSB) (Difco®, Franklin Lakes, USA).

Antimicrobial activity from the strain collection of the Laboratório de Higiene e Laticínios (ESALQ/USP), was evaluated against *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 07644, *Salmonella* Enteritidis ATCC 13076 and *Escherichia coli* ATCC 25922. All antimicrobial tests were performed in triplicate. Antimicrobial screening was performed by agar diffusion[6]. Two hundred microliters of standardized inoculum ($1x10^8$ CFU/ml) of each organism were transferred to 200 ml of TSB plus 0.7% bacteriological agar (final population of $1.5\ x10^5$ CFU/ml). Seventy milliliters of this preparation were transferred to Petri dishes, in which 8 mm diameter wells were produced by vacuum pump and 40 μl of extracts were distributed (100 mg/ml). Negative (40 μl of TSB) and positive control (40 μl of chlorhexidine 0.12% v/v) were tested.

For MIC determination, the macrobroth dilution method was performed in 96-well microplate[3]. The extract concentrations were obtained by 2-fold serial dilution in the microplate, ranging from 25 mg/ml to 0.78 mg/ml after the addition of inoculated TSB ($1\text{-}2\ x\ 10^5$ CFU/ml). The final volume for each well was 200 μl. Positive (200 μl of TSB added of 0.12% chlorhexidine v/v) and negative control (200 μl of sterile TSB) were tested. Two hundred microliters of sterile TSB were used for broth sterility control. After incubation (35 °C/24 h), all wells received 30 μl of resazurin (0.01% w/v) (Sigma-Aldrich®, St. Louis, USA) in order to detect bacterial growth in the wells. Any evidence of color change was considered to be bacterial growth. For MBC determination, 10 μl of broth were removed from the wells considered inhibitory and sown in tryptic soy agar (TSA) (35 °C/24 h). MBC was considered as the lowest concentration at which no growth of colonies on the culture medium surface was observed.

The effectiveness of the yerba mate extract showing the best antimicrobial activity was evaluated at pH 6, 7 and 8 using 96-well microplates[9]. All wells received 100 μl of sterile TSB. One hundred μl of extracts were added into the first row of each column and then 2-fold serial dilution was performed (final concentrations ranging from 25 mg/ml to 0.78 mg/ml), after adding 100 μl of inoculated broth ($1\text{-}2\ x\ 10^5$ CFU/ml). Control groups: 200 μl of inoculated TSB (negative), 200 μl of inoculated TSB added of chlorhexidine 0.12% v/v (positive) and 200 μl of sterile TSB plus extract (white). The microplates

Table 1 Antibacterial activity of methanolic and ethanolic extracts of yerba mate

	S. aureus		*L. monocytogenes*		*S.* Enteritidis		*E. coli*	
	M	E	M	E	M	E	M	E
Inhibition zones (mm)	21.67±0.57	23.67±0.57	10.00±0.00	10.33±0.58	10.00±0.00	13.67±0.57	-	-
MIC	1.56	0.78	3.13	3.13	6.25	3.13	-	-
MBC	3.13	0.78	3.13	3.13	6.25	6.25	-	-

MIC: minimum inhibitory concentration (mg/ml); MBC: minimum bactericidal concentration (mg/ml); M: methanolic extract; E: ethanolic extract; Averages of triplicates ± standard deviation; -: No inhibition

were incubated in a multilabel plate reader (Victor™X3, PerkinElmer®) (35 °C/18 h) with absorbance readings performed at 1 hour intervals (600 nm).

The total phenolic content was determined with the Folin-Ciocalteau reagent[13]. Five hundred microliters of extracts (10 mg/ml) were mixed with 2.5 ml of the Folin-Ciocalteau reagent (1:10) and 2.0 ml of sodium carbonate solution (Na_2CO_3) (4% w/v) (Dinâmica®, Diadema, Brazil). After 2 hours of incubation, the absorbance reading was performed at 740 nm in visible light spectrophotometer (Femto® Plus). The total phenolic content was expressed as gallic acid equivalent (GAE) in g per kg of sample from the gallic acid standard curve. For the gallic acid, the curve was established by plotting concentration (ranging from 2.5 μg/ml to 50 μg/ml) *versus* absorbance (nm) ($y = 42.71x + 0.3187$; $R^2 = 0.9997$, where y is the absorbance and x is the concentration).

The most active extract was submitted to GC-MS analysis (Shimadzu® GC-2010 coupled to Shimadzu® QP 2010 Plus). The sample purification was performed by the solid phase extraction technique (SPE). Then, the extracts were derivatized adding 100 μl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) reagent (Sigma-Aldrich®, St. Louis, USA). The derivatization product was diluted in hexane and the supernatant was transferred to a vial. The chromatographic analysis was carried out as follows: separation in capillary column RTX5MS (30 m × 0.25 mm × 0.25 μm), injection temperature of 280 °C and initial volume of 0.5 μl in "splitless" mode, and detection at 280 °C operating in "scanning" mode (m/z 40-800). The chromatographic conditions were: initial temperature of 80 °C (1 min), heating to 250 °C (20 °C/min) (1 min), 300 °C (6 °C/min) (5 min), 310 °C (15 °C/min) (10 min) and 320 °C (20 °C/min) (10 min). Integration was performed using the LabSolutions-CGMS software and the identification of the compounds of interest was performed by comparison of data obtained from GC-MS and the Wiley 8 Library (supplied with the equipment).

The statistical analysis was performed using the Statistical Analysis System software (SAS 2002). The Tukey's test (0.5% probability) was used to compare means.

The methanolic and ethanolic extracts of yerba mate inhibited all evaluated microorganisms, except *E. coli* (Table 1). The ethanolic extract produced the largest inhibition zones and the lowest MIC on *S. aureus* and *S.* Enteritidis; for *L. monocytogenes*, the values were similar. Furthermore, it showed the lowest MBC on *S. aureus*, which demonstrates its high *in vitro* bactericidal activity.

The study by De Biasi *et al.*[5] found inhibitory activity in the ethanolic extract of yerba mate against several microorganisms and only *E. coli* was not inhibited, agreeing with our results. However, Vaquero *et al.*[14] reported the antimicrobial potential of yerba mate infusion against *E. coli*, which was the most inhibited microorganism among all evaluated ones. Cogo *et al.*[4] reported antimicrobial activity of ethanolic extracts of yerba mate leaves against *Helicobacter pylori* in MIC as low as those found in this study. These findings reinforce the importance of yerba mate as a research source for new natural antimicrobials.

Table 2 Chemical composition of yerba mate ethanolic extract determined by GC-MS

Compounds[a]	RT	Percentage of relative area[b]	Ion (m/z, abundance between parenthesis)
Caffeic acid	11.00	2.99	219 (100), 396 (95), 73 (72), 381 (24), 191 (12), 45 (13); 401 (M+)
5-O-caffeoylquinic acid	19.99	28.45	345 (100), 73 (94), 255 (58), 307 (41), 147 (21), 397 (15); 786 (M+)
4-O-caffeoylquinic acid	20.64	20.83	307 (100), 73 (72), 255 (46), 219 (15), 489 (15), 147 (12); 786 (M+)
3-O-caffeoylquinic acid	20.93	34.02	307 (100), 73 (76), 345 (68), 255 (30), 447 (21), 147 (17); 771 (M+)

RT: retention time (min).
[a]All compounds with similarity percentage > 80%.
[b]Peak area in relation to total percentage of peak areas.

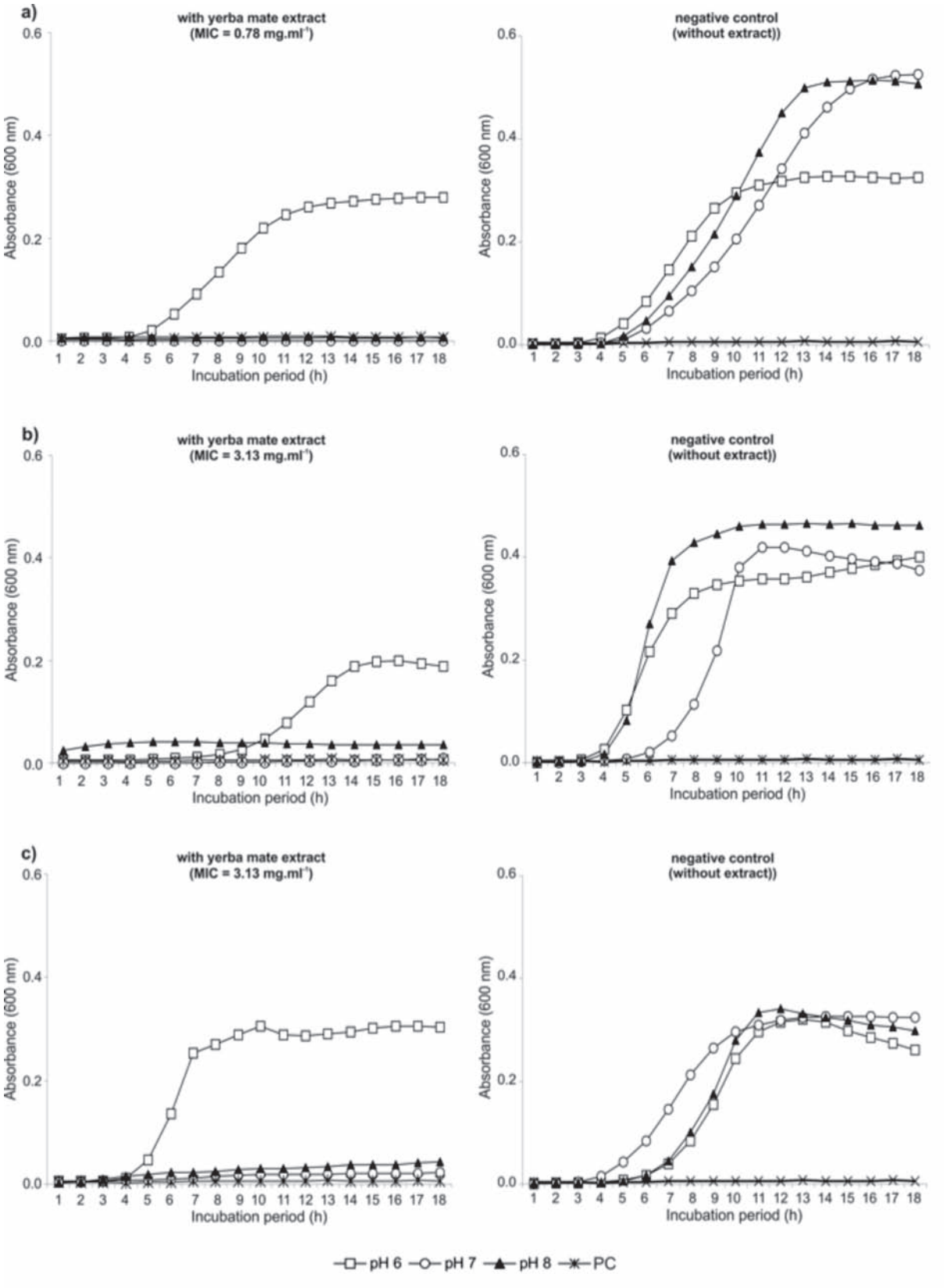

Figure 1 Growth curves of a) *S. aureus*, b) *L. monocytogenes* and c) *S.* Enteritidis under action of yerba mate ethanol extract different pH values. PC = positive control (chlorhexidine 0.12%).

The ethanolic extract showed the highest total phenolic content. Significant differences were observed between the ethanolic and methanolic extracts; ethanol 60% (v/v) (193.9 g GAE/kg) was the solvent which best extracted these compounds in comparison to methanol 70% (v/v) (173.0 g GAE/kg). Relating this data to the antimicrobial activity, it could be inferred that the phenolic contents of the extracts are directly related to their antimicrobial potential.

For presenting the best antimicrobial activity, the ethanolic extract of yerba mate was selected for GC-MS analysis. Its components in greatest abundance were 3-O-caffeoylquinic acid (34.02%), 5-O-caffeoylquinic acid (28.45%), 4-O-caffeoylquinic acid (20.83%) and caffeic acid (2.99%) (Table 2). Caffeoylquinic acids were the major phenolic compounds and their presence has been reported in previous studies[11]. These compounds have several important biological activities such as antioxidant and antiviral activities[10]. Detected in a lower amount, caffeic acid has been reported in plant extracts as having antimicrobial activity against gram-positive and gram-negative bacteria and fungi[7].

The effect of pH on the antimicrobial activity was assessed by growth curve analysis for *S. aureus, L. monocytogenes* and *S.* Enteritidis, in MIC values at neutral pH (Table 1). The extract inhibited the growth of *S. aureus* at pH 7 and 8, which was not observed at pH 6, being unable to inhibit the growth of this microorganism (Figure 1). This behavior was also observed for *L. monocytogenes*, and the extract was ineffective only at pH 6. The growth curve of *S.* Enteritidis demonstrated a slight tendency to growth at pH 8; the extract was not able to inhibit bacterial growth at pH 6, as in other cases.

Gutierrez et al.[9] showed a decrease of antilisterial activity of natural products at pH 6 in comparison to neutral pH, which is in agreement with our results; however, a considerable increase in the lag phase was observed at pH 5. Wen et al.[15] reported an increase in antilisterial activity of phenolic acids with decreasing pH; in the case of chlorogenic acid, better antibacterial activity was observed at a higher pH. According to the authors, this discrepancy may be related to changes in the ionization state and proportion of dissociated molecules at different pH values, resulting in decreased activity of chlorogenic acid at a lower pH.

Since the values of dissociation constants (*pK*) of phenolic acids are generally higher than 8, the concentration of phenoxide ions is low in solutions with pH lower than 7[8]. The chlorogenic acid has a *pK* value of ≈ 8.5[12]. If the antimicrobial activity is related to the concentration of phenoxide ions, the higher the pH of the medium, the more pronounced it would be. The increased antimicrobial activity probably results from the ability of negatively charged phenoxide ions to change the electrochemical balance of the bacterial microenvironment, facilitating cell death[8].

According to our results, it could be concluded that yerba mate presents antimicrobial activity against food pathogens. The ethanolic extract was more effective than the methanolic extract in inhibiting *S. aureus, L. monocytogenes* and *S.* Enteritidis. The antimicrobial activity observed seems to be related to the presence of compounds derived from chlorogenic acid of known biological activities. The ethanolic extract was active at neutral and at pH 8. Yerba mate is thus a potential source for the extraction of antimicrobial compounds for use by the food industry as a natural preservative in foods and beverages.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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