

## Research Note

# Aqueous Extracts of Yerba Mate (*Ilex paraguariensis*) as a Natural Antimicrobial against *Escherichia coli* O157:H7 in a Microbiological Medium and pH 6.0 Apple Juice

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## ABSTRACT

*Ilex paraguariensis* is popularly used in the preparation of a tea infusion (yerba mate), most commonly produced and consumed in the South American countries of Uruguay, Paraguay, Argentina, and Brazil. In this study, aqueous extracts of commercial tea, derived from the holly plant species *I. paraguariensis* were evaluated for their ability to inhibit or inactivate *Escherichia coli* O157:H7 in a microbiological medium and modified apple juice. Dialyzed, lyophilized aqueous extracts were screened for antimicrobial activity against *E. coli* O157:H7 strains ATCC 43894 and ‘Cider’ in tryptic soy broth (TSB) and apple juice (adjusted to pH 6.0 to allow for growth of the bacterium). A mixture of the two strains was used as the inoculum when apple juice was used as the medium. MBCs were determined to be ca. 5 and 10 mg/ml for ATCC 43894 and ‘Cider’, respectively, in TSB. Higher concentrations of the extract were required to inactivate *E. coli* O157:H7 in pH-adjusted apple juice. An approximate 4.5-log reduction was observed for *E. coli* O157:H7 treated with 40 mg/ml extract. It was concluded that aqueous extracts from commercial yerba mate have potential to be used as antimicrobials in foods and beverages against pathogenic *E. coli* O157:H7.

Increasing incidence of foodborne illnesses along with consumer demand for more natural foods have prompted the need for novel antimicrobials. Foodborne illnesses are a continuous threat to public health. Scallan et al. (40) estimated that the 31 major foodborne pathogens account for nearly 9.4 million people becoming sick, for more than 55,961 hospitalizations, and for 1,351 deaths from foodborne illness each year in the United States, with an estimated economic cost of \$152 billion annually (41).

*Escherichia coli* O157:H7 is a toxin-producing enteropathogen responsible for a hemorrhagic form of colitis, bloody diarrhea, and hemolytic uremic syndrome. Apple juice, which is a high-acid food (pH < 4.6), was believed at one time to be inhibitory to the survival and growth of *E. coli* O157:H7 (30). However, multiple foodborne illness outbreaks have been associated with the consumption of high-acid, unpasteurized fruit juices (10, 46). Regulations were developed in 2001 by the U.S. Food and Drug Administration (21 CFR 120) that require treatments to achieve 5-log reductions of *E. coli* O157:H7 in fruit juices. Traditionally, weak organic acids have been added as preserving agents in juices; however, consumers are less accepting of perceived “chemical additives” as preservatives and desire more natural products. This shift in

consumer preferences has pressed the food industry to examine more natural sources of antimicrobials, such as plant extracts.

Teas, mainly from the species *Camellia sinensis*, contain components that provide antimicrobial agent activity against a variety of gram-negative and gram-positive foodborne pathogens (1, 4, 12, 23, 24, 26, 36, 38, 43, 44, 47, 48). However, little research has been done on the use of yerba mate tea as a food antimicrobial. Yerba mate is a popular tea infusion from the perennial plant *Ilex paraguariensis* and is traditionally found, commercially produced, and primarily consumed in the South American countries Paraguay, Uruguay, Brazil and Argentina. With a demand for more healthy and natural foods, this tea is quickly increasing in popularity in the United States (20). While a variety of medicinal and pharmacological properties for yerba mate has been characterized (2, 3, 5–7, 9, 13, 15–18, 32), limited research has been conducted on its effectiveness as an antimicrobial (8, 22, 26, 37, 43) and even less for its ability to preserve foods (35) and beverages such as apple juice, as an additive.

Typically, plant extracts that have been used as antimicrobial food preservatives have a negative sensory effect on the flavors and odors of the food. The flavor of yerba mate has been described as bitter, acidic, astringent, hay-like, green, humid, toasted, and paper-like (11). While these terms seem negative, a study incorporating yerba mate

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as an antioxidant into precooked chicken meatballs resulted in no effect on the taste or smell of the food product (35), indicating its potential sensory acceptance in foods. However, to date, no sensory-acceptance testing has been performed for use of yerba mate at its active antimicrobial level in juices.

The goal of this study was to determine the effectiveness of yerba mate aqueous extracts against *E. coli* O157:H7, one of the most common pathogens associated with outbreaks in apple juice, in optimal growth conditions.

## MATERIALS AND METHODS

**Culture preparation.** *E. coli* O157:H7 ATCC 43894 and strain 'Cider' were stock cultures obtained from the Department of Food Science and Technology at the University of Tennessee–Knoxville. All stock cultures were grown in tryptic soy broth (TSB; BD, Franklin Lakes, NJ) and stored at  $-20^{\circ}\text{C}$  in TSB and glycerol. Working cultures were obtained by inoculating 50 ml of TSB with 100- $\mu\text{l}$  stock cultures and incubating for 24 h at  $35$  to  $37^{\circ}\text{C}$ . After incubation, the cultures were diluted in 0.1% peptone to ca. 5.0 to 6.0 log CFU/ml and tested for antimicrobial activity.

**Aqueous extraction.** Dried leaves of a single commercial brand of yerba mate (100% leaves; Taragui, Argentina) were purchased from a local international supermarket. Dried plant tissue was finely ground ( $<1$  mm) with a coffee grinder (Braun, Kronberg, Germany) for 15 to 20 s. Extracts, obtained by adding sterile water at a ratio of 3.6 ml to 1 g of ground tissue, were allowed to stand for 2 h at  $4^{\circ}\text{C}$ , with occasional mixing to maximize extraction, and were subsequently centrifuged at  $5,000 \times g$  for 30 min to remove larger particles. Aqueous extracts were then dialyzed against deionized water for 36 h by using a 3500 MWCO SnakeSkin pleated dialysis tubing (Pierce Biotechnology, Rockford, IL) to remove low-molecular-weight compounds. Resulting extracts were centrifuged at  $5,000 \times g$  for 30 min to remove sediment, and the supernatant was frozen at  $-80^{\circ}\text{C}$ . Frozen extracts were then lyophilized by using the VirTis AdVantage Plus BenchTop freeze dryer (SP Industries, Gardiner, NY). This process was repeated until a sufficient quantity of lyophilized extract had been collected to allow for the antimicrobial assays. Lyophilized extracts were stored at room temperature in a sealed container.

**Phenolic determination.** Lyophilized extract (25 mg) was resuspended in 5 ml of 95% ethanol to a final concentration of 5 mg/ml, filtered through Whatman no. 4 paper, and analyzed for total phenolics. Total phenolics were quantified spectrophotometrically at 725 nm by using Folin-Ciocalteu reagent (31), with caffeic acid as the standard. The result is the mean value of nine replications.

**Time-kill assays.** Dialyzed, lyophilized aqueous extracts (0 to 1,000 mg) were diluted in 10 ml of sterile water and filter sterilized with 0.22- $\mu\text{m}$  Express PES Membrane (Millipore, Billerica, MA). Diluted extracts (10 ml) were mixed with bacteria harvested at the late-logarithmic phase and diluted to ca. 5.0 to 6.0 log CFU/ml. Bacteria and extracts were incubated in TSB and apple juice (pH adjusted to 6.0 by using 1 N NaOH to allow the *E. coli* strains utilized to grow; Laura Lynn Organic Apple Juice, Ingles Markets, Black Mountain, NC) at  $35$  to  $37^{\circ}\text{C}$ , and at regular intervals (0, 3, 6, 12, and 24 h) a bacterial suspension sample was collected, serially diluted in 0.1% peptone, plated in duplicate with tryptic soy agar (BD), incubated for 24 h at  $35$  to  $37^{\circ}\text{C}$ , and then

CFU were enumerated. All experiments were duplicated, and average values (expressed in log CFU per milliliter) were reported. The MIC and MBC were determined for each strain in TSB and for the combined strains in apple juice. MIC was determined as the lowest concentration tested that inhibited bacterial growth above the original inoculum level of ca. 5.0 to 6.0 log CFU/ml after 24 h. The MBC was determined as the lowest concentration tested where bacterial death (inactivation) was observed after 24 h.

**Statistical analysis.** Data were analyzed as a completely randomized design with four replicates. Analysis of variance was done by using the general linear model procedure of SAS (version 9.2, SAS Institute, Cary, NC). Least significant differences (LSD) were used to compare treatment mean values when significant differences ( $P < 0.05$ ) were found. Error bars represent 95% confidence intervals for the mean by using LSD.

## RESULTS AND DISCUSSION

We obtained ca. of 12 g of lyophilized extract from the extraction and processing of 280 g of commercial yerba mate tea. Quantitative analysis of the phenolics in the rehydrated aqueous extracts (5 mg of lyophilized extract per ml) showed that the extracts contained ca. 79  $\mu\text{g}$  of caffeic acid equivalent per ml. Thus, every milligram of lyophilized extract or gram of tea represented ca. 16 or ca. 687  $\mu\text{g}$  of caffeic acid equivalent per ml, respectively. This is similar to results found by Filip and others (14), with caffeic acid content constituting 0.023% of dried weight. For every milligram of dried weight, there was 23  $\mu\text{g}$  of caffeic acid. However, Mazzafera (29) determined caffeic acid content as 340 mg for every serving of tea (50 to 60 g) brewed in the traditional manner. This caffeic acid content was nearly 20 times higher than what we found, most likely because of preparation, brand, and cultivar. In comparison, ground coffee has been shown to have a total phenolic content of 52.5 to 57 mg of gallic acid equivalents per g (27).

The dialyzed, lyophilized aqueous extracts of yerba mate were evaluated for their ability to inhibit growth of and/or inactivate *E. coli* O157:H7 in a microbiological medium and apple juice (pH adjusted to 6.0). Lyophilized extracts were diluted in 10 ml of sterile water and tested against *E. coli* O157:H7 at 0 to 20 mg/ml for a microbiological medium and 0 to 40 mg/ml for modified apple juice. Processed extracts derived from commercially available yerba mate were effective at inhibiting and inactivating both strains of *E. coli* O157:H7 in a microbiological medium and modified apple juice (Figs. 1 and 2).

MICs were determined to be 10 mg/ml against both *E. coli* O157:H7 strain ATCC 43894 and 'Cider' in a microbiological medium (Fig. 1A and 1B). MBCs were determined to be 5 mg/ml for *E. coli* O157:H7 strain ATCC 43894 and 10 mg/ml for *E. coli* O157:H7 strain 'Cider' in a microbiological medium (Fig. 1A and 1B). An approximate 4.5-log reduction was observed for *E. coli* O157:H7 treated with 40 mg/ml extract in modified apple juice, which is near to the pathogen reduction requirement of 21 CFR 120.

An aqueous extraction protocol for yerba mate was used to obtain extracts with antimicrobial activity against *E. coli* O157:H7. Crude extracts and many individual

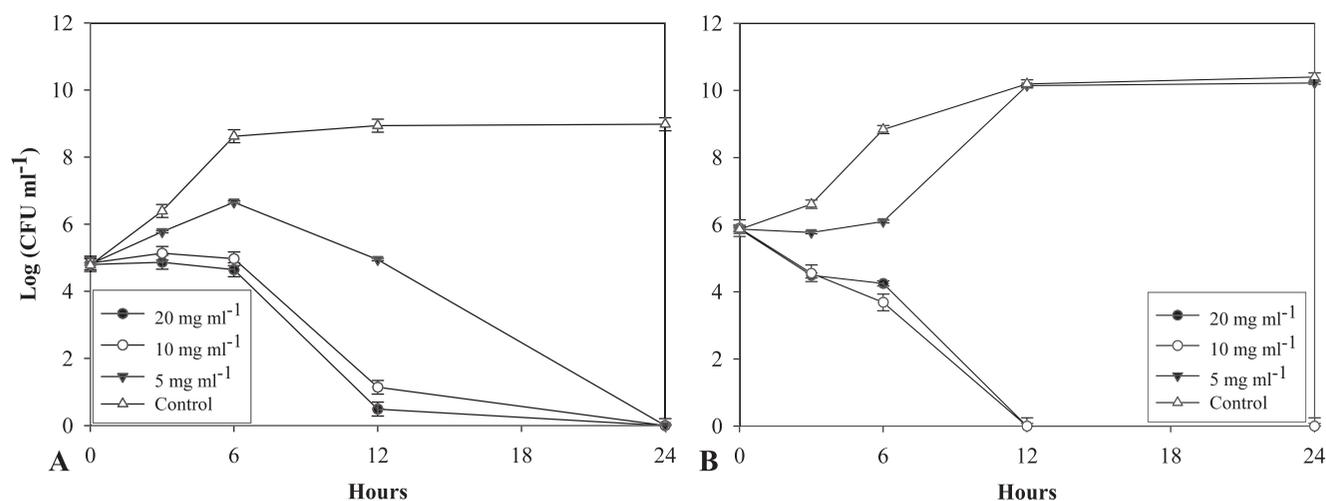


FIGURE 1. Antimicrobial activity of yerba mate extracts at 0, 5, 10, and 20 mg/ml against *Escherichia coli* O157:H7 (A) strain ATCC 43894 and (B) strain 'Cider' in tryptic soy broth. Error bars represent 95% confidence intervals for the mean by using least significant differences ( $P < 0.05$ ).

compounds in the more commonly consumed green and black teas have been extensively studied for broad antimicrobial activity (25, 43, 44, 47, 48). However, limited research has been conducted on extracts and compounds possessing antimicrobial activity derived from yerba mate (8, 22, 26, 39, 42, 45). While many of the compounds found in yerba mate extracts are known (14, 19, 22, 26), the identification of those compound(s) contributing to antimicrobial activity and whether they have combined additive or synergistic effects are not entirely known.

Caffeine, ursolic acid, and chlorogenic acid are three common compounds that have been identified from yerba mate. However, none has demonstrated antimicrobial activity against *E. coli* at 400  $\mu\text{g/ml}$  (26), a concentration much lower than tested here. Other compounds isolated from yerba mate include the polyphenols, caffeic acid, caffeoyl derivative, caffeoylshikimic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin, and theobro-

mine (19, 28), which may provide antimicrobial activity against *E. coli* O157:H7. In pure form, caffeic and chlorogenic acids have demonstrated activity against gram-negative bacteria (21, 34). Several flavonols extracted from other plant species but that can also be found in yerba mate have been examined for their antimicrobial activity—kaempferol, quercetin, and rutin (33, 37). However, in previous studies neither kaempferol nor quercetin was found to inhibit *E. coli* (33, 34). *n*-Hexane distillate extracts of yerba mate have been shown to be effective antimicrobial agents (26). Ten main compounds were identified in the *n*-hexane distillate extracts of yerba mate and included linalool,  $\alpha$ -ionone,  $\beta$ -ionone,  $\alpha$ -terpineol, octanoic acid, geraniol, 1-octanol, nerolidol, geranylacetone, and eugenol (26). These distillate compounds tested were only weakly active against *E. coli* (26). Compounds present in our aqueous extracts demonstrating antimicrobial activity might differ from those reported by Kubo et al. (26), since we utilized a water-based extraction. Further, we observed inhibition and inactivation of *E. coli* O157:H7, whereas Kubo et al. (26) and Sari et al. (39) only demonstrated weak to no activity against nonpathogenic and pathogenic *E. coli*, respectively. One possibility of these differences could be that one or more of the identified compounds are acting interactively and contributing to the antimicrobial activity against *E. coli* O157:H7.

While in this study we found that aqueous extracts from yerba mate provide antimicrobial activity against *E. coli* O157:H7 at a relatively low level (5 to 10 mg/ml), Montanha (as cited in (42)) determined that neither a crude ethanolic extract (100 mg/ml) nor saponin fraction (100 mg/ml) from yerba mate demonstrated antimicrobial activity against *E. coli*. Hongpattarakere (22) determined the concentration of aqueous methanolic crude extract required to inhibit *E. coli* O157:H7 was 150 mg/ml, and no observable activity was detected at 30 to 60 mg/ml. Here, we demonstrated activity at much lower concentrations (5 to 10 mg/ml) for the complete inactivation of two strains of *E. coli* O157:H7.

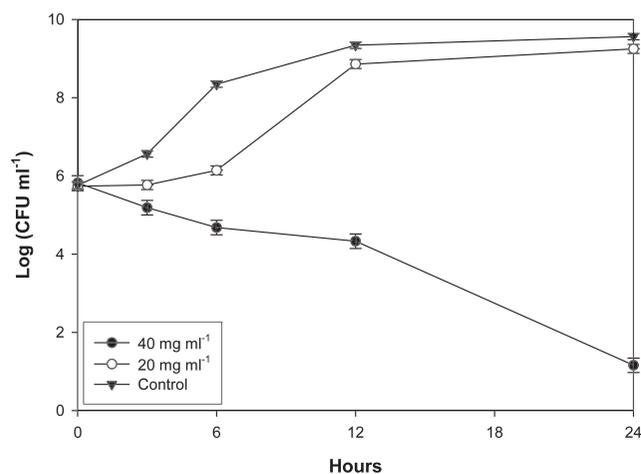


FIGURE 2. Antimicrobial activity of yerba mate extracts at 0, 20, and 40 mg/ml against a mixture of *Escherichia coli* O157:H7 strains ATCC 43894 and 'Cider' in modified apple juice. Error bars represent 95% confidence intervals for the mean by using least significant differences ( $P < 0.05$ ).

Typically, higher concentrations of antimicrobials are required in food systems to achieve the same levels of bacterial inactivation because of interaction of food components with antimicrobial compounds. In the present study, ca. four- to eightfold more lyophilized extract was required in modified apple juice than in a microbiological medium. However, even in a food system, less extract was required than that used by Hongpattarakere (22) to inactivate and achieve an almost 5-log reduction in *E. coli* O157:H7.

One chief goal of the food and beverage industry is to protect consumers from the harmful effects of foodborne pathogens. Dialyzed, lyophilized aqueous extracts of yerba mate demonstrated bactericidal activity against *E. coli* O157:H7 in a microbiological medium as well as in a beverage system. Results demonstrated that relatively low concentrations of yerba mate aqueous extracts provide antimicrobial activity against *E. coli* O157:H7 in modified apple juice. Consumer demand for natural products has prompted researchers to find natural sources of antimicrobials for use in foods as alternatives or in addition to currently used regulated antimicrobials. Here it was demonstrated that the antimicrobial activity of a biobased antimicrobial, yerba mate extract, could be used in beverages for protection against *E. coli* O157:H7.

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