Antibacterial Activity against *Streptococcus mutans* of Mate Tea Flavor Components

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The 10 major volatile constituents were identified by GC-MS analysis as common flavor principles in mate tea obtained from various locations. Their antimicrobial activity against 13 microorganisms was tested. All of the volatiles tested exhibited moderate to weak activity with broad spectra. Some of them are bactericidal against one of the most important cariogenic bacteria, *Streptococcus mutans*. The antibacterial activity of the distillate against *S. mutans* was significantly enhanced by indole.

INTRODUCTION

Mate tea, often known as "yerba mate", is one of the most commonly consumed beverages in several South American countries, including Uruguay, Paraguay, Argentina, and Brazil. It is manufactured from the caffeine-rich leaves of *Ilex paraguayensis* St. Hil. (Aquifoliaceae) by a method similar to that used to produce green tea. Yerba mate has a very characteristic odor and is used in stimulants, bitter tonics, and diaphoretics. In addition, *I. paraguayensis* has been used as a folk medicine (Schultes and Raffauf, 1990). Besides caffeine, triterpenes and chlorogenic acid derivatives are also present in the leaves of *I. paraguayensis* (Gosmann et al., 1989; Clifford and Ramirez-Martinez, 1990).

Interestingly, mate tea is not drunk from a common tea cup but rather from a unique vessel which can be made from a variety of materials such as "calabash" and "bull horn". It is sipped through a metallic straw called a "bonbilla". Traditionally, mate tea is prepared in the following manner. Tiny yerba leaves are first placed into the vessel. Then, slowly, hot or cold water is added and imbibed, repeatedly, until the tea is no longer flavorful. This vessel is sometimes passed around and shared with other people as a custom to make close friendships. In Paraguay, mate tea is often made with ice water because of the hot climate. The tea made this way is called "terere" to which other herbs are occasionally added. In spite of its popularity, the composition of its flavor constituents was not reported until recently (Kawakami and Kobayashi, 1991).

It has been said that those who continuously drink a large amount of mate tea have less tooth decay. This old tradition attracted us to search for an anticavity principle which, for example, might control cariogenic bacteria such as *Streptococcus mutans*. Additional consideration for this experiment is the continuing need for new antimicrobial agents in cosmetics and other perishable products. The control of specific microorganisms which cause skin, hair, and teeth problems is becoming more important. In contrast to medicine, which generally uses antimicrobial agents for the treatment of ill people, antimicrobial agents for cosmetics are repeatedly applied to healthy skin, hair, and teeth. Hence, safety is the first consideration. Edible plants, beverages made from some of these plants, and food spices seem to be a good potential source of antimicrobial agents. In our continuing search for new antimicrobial agents from these sources (Himejima and Kubo, 1991; Kubo et al., 1991), the *n*-hexane extract of the yerba mate has been found to exhibit a rather broad spectrum of antimicrobial activity. Thus, active principles from a regularly imbibed beverage like yerba mate may be superior as microbial control agents as compared to many nonnatural products.

MATERIALS AND METHODS

Plant Materials. Nine samples of mate tea (yerba mate) were purchased in several locations in South America: Porto Alegre (one) and Sâo Paulo (three), Brazil; Asuncion (two), Paraguay; Montevideo (one), Uruguay; and Buenos Aires (two), Argentina.

Extraction and Identification. The yerba mate (Gaucho, produced by Moinhos Unidos, Brazil) (500 g) purchased in Sâo Paulo was extracted repeatedly with *n*-hexane (three times) at ambient temperature. The *n*-hexane extract (3.15 g) was divided into a distillate (0.36 g) and residue (2.15 g) by steam distillation. The distillate possessed the characteristic mate tea odor, but the residue did not exhibit any appreciable aroma. The distillate was analyzed by gas chromatography (GC). The GC trace indicated the presence of over 100 compounds in the distillate. Among the compounds identified by GC-MS, the 10 major compounds, linalool, α-ionone, β-ionone, α-terpineol, octanoic acid, geraniol, 1-octanol, nerolidol, geranylacetone, and eugenol, were selected for antimicrobial bioassay. Most of these volatiles were also found in the other eight yerba mate samples obtained from different locations.

Chemicals. Authentic α-ionone, β-ionone, linalool, nerolidol, 1-octanol, and α-terpineol were gifts from Takasago International Corp. (Tokyo, Japan). Geraniol was purchased from Johnson Matthey (Ward Hill, MA), and octanoic acid, geranylacetone, eugenol, and safrole were obtained from Sigma Chemical Co. (St. Louis, MO). For the antimicrobial assay experiments, all chemicals were first dissolved in *N*,*N*-dimethylformamide (DMF), which was purchased from EM Science (Gibbstown, NJ).

GC Analysis. Analytical gas chromatography (GC) of the distillate was performed on a Hewlett-Packard 5890 gas chromatograph fitted with a flame ionization detector (FID) and fitted with a glass capillary column (0.25 mm i.d. × 30 m) coated with Carbowax bonded 20 m. The carrier gas was helium at 1.5 mL/min, and the oven was programmed to increase temperature from 50 to 230 °C at 4 °C/min.

GC-MS Analysis. GC-MS analysis was carried out on a Hitachi M-808 double-focusing instrument equipped with a Hewlett-Packard 5890 gas chromatograph. The GC conditions were identical to those of the above analytical GC runs. Mass spectral data were acquired and processed by a built-in computer system (M-0101) developed by Takasago International. The components of the distillate were identified by comparing their GC retention times and MS fragmentations with those of the authentic samples.

Microorganisms and Media. All test microorganisms were purchased from American Type Culture Collection (Rockville, MD). They are *Bacillus subtilis* ATCC 9372, *Brevibacterium*

0021-8561/93/1441-0107$04.00/0

ammoniagenes ATCC 6872, *Staphylococcus aureus* ATCC 12598, *Streptococcus mutans* ATCC 25175, *Propionibacterium acnes* ATCC 11827, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 9637, *Saccharomyces cerevisiae* ATCC 7754, *Candida utilis* ATCC 9226, *Pityrosporum ovale* ATCC 14521, *Penicillium chrysogenum* ATCC 10106, and *Trichophyton mentagrophytes* ATCC 18748.

The culture media for the bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco), and 0.1% glucose, with the exception of *S. mutans*. For the culture of *S. mutans*, 3.7% brain heart infusion broth (Difco) was utilized. The culture media for the fungi consisted of 2.5% malt extract broth (BBL), with the exception of *P. ovale* and *T. mentagrophytes*. For the culture of *P. ovale*, 1% bactopeptone (Difco), 0.5% yeast extract, 1% glucose, and 0.1% corn oil were used, and for *T. mentagrophytes*, 1% bactopeptone and 4% glucose were utilized.

The freeze-dried microorganisms were prepared for testing by growing for 2 days (5 days for *P. chrysogenum* and *T. mentagrophytes*) in the following manner. *B. subtilis*, *S. cerevisiae*, *C. utilis*, *P. ovale*, *T. mentagrophytes*, and *P. chrysogenum* were cultured with shaking at 30 °C. *B. ammoniagenes* and *E. aerogenes* were cultured stationary at 30 °C. All other microorganisms were cultured stationary at 37 °C.

Antimicrobial Assay. The minimum inhibitory concentration (MIC) was measured by the 2-fold serial broth dilution method as previously described (Kubo et al., 1992). Briefly, the test compound was dissolved in DMF, and 30 μL of the sample solution was added to 3 mL of the appropriate medium, to which 30 μL of 2-day-old culture of test microorganisms (5-day-old of *P. chrysogenum* and *T. mentagrophytes*) was inoculated. After 2 days of cultivation (5 days for *P. chrysogenum* and *T. mentagrophytes*), the growth of the microorganisms, except *P. ovale*, *P. chrysogenum*, and *T. mentagrophytes*, was examined by turbidity (OD at 660 nm). That of the three fungi was examined with the naked eye. The lowest concentration of the test compound in which no growth occurred was defined as the MIC. The highest concentration used for the assay of the pure compounds was 800 mg/mL, unless otherwise specified, because of solubility limitations in the water-based media of some of the samples.

The combination data against *S. mutans* were obtained by a broth checkerboard method (Norden et al., 1979). The 2-fold dilutions of indole were tested in combination with concentrations of 2-fold dilutions of the other. The test was repeated three times.

Growth Studies. The combination was further studied against *S. mutans* with half-MIC of indole and varying 2-fold dilutions of the other. The culture test tubes were prepared as described above and incubated at 37 °C for 2 days. Growth was monitored every 6 h by measuring the absorbance (OD at 660 nm) increase. A viability study was conducted to examine combination effects in more detail. Thirty microliters of the 2-day-old culture was inoculated in 3 mL of brain heart infusion (BHI) broth containing the appropriate amount of the sample. This represented 4–8 × 10^5 colony forming units (CFU)/mL. Samples were taken at selected times, and serial dilutions were made in sterile saline before plating onto the surfaces of BHI agar. The plates were incubated at 37 °C for 2 days before counting.

RESULTS AND DISCUSSION

In our preliminary screening against four representative microorganisms (Taniguchi et al., 1978), the *n*-hexane extract of the yerba mate exhibited a rather broad spectrum of antimicrobial activity at the concentration of 1600 μg/mL. The extract was divided into distillate and residue fractions by steam distillation, and subsequently the antimicrobial activity of each fraction was tested at the same concentration. As a result, the distillate was found to have retained the original antimicrobial activity. Further analysis of the bioactive distillate, which possessed the characteristic odor of the mate tea, was performed by gas chromatography (GC); however, the GC indicated the presence of over 100 components. The composition of these compounds was varied and depended on the manufacturing process and tea leaves. However, all of the mate tea obtained from the different locations basically consisted of the same compounds. The 10 main compounds identified in the distillate by GC–MS were linalool (**1**), α-ionone (**2**), β-ionone (**3**), α-terpineol (**4**), octanoic acid (**5**), geraniol (**6**), 1-octanol (**7**), nerolidol (**8**), geranylacetone (**9**), and eugenol (**10**) (see Figure 1). The ratios of these compounds in other yerba mates obtained from different locations varied but basically were very similar. In fact, most of these 10 volatiles were identified in all 9 mate tea samples obtained from the different locations. This result is in general agreement with those reported recently (Kawakami and Kobayashi, 1991). In addition, the same compounds have been reported to be flavor components in *Camellia sinensis* teas such as green tea, black tea, and oolong tea, although their ratios differ. For example, linalool, β-ionone, α-terpineol, geraniol, 1-octanol, and nerolidol were also reported to be the major flavor constituents of green tea (Nose et al., 1971). Noticeably, a rather large amount (5.5% of the distillate) of safrole (**11**) (see Figure 1) was detected in one of the mate tea samples purchased in São Paulo but not in the others.

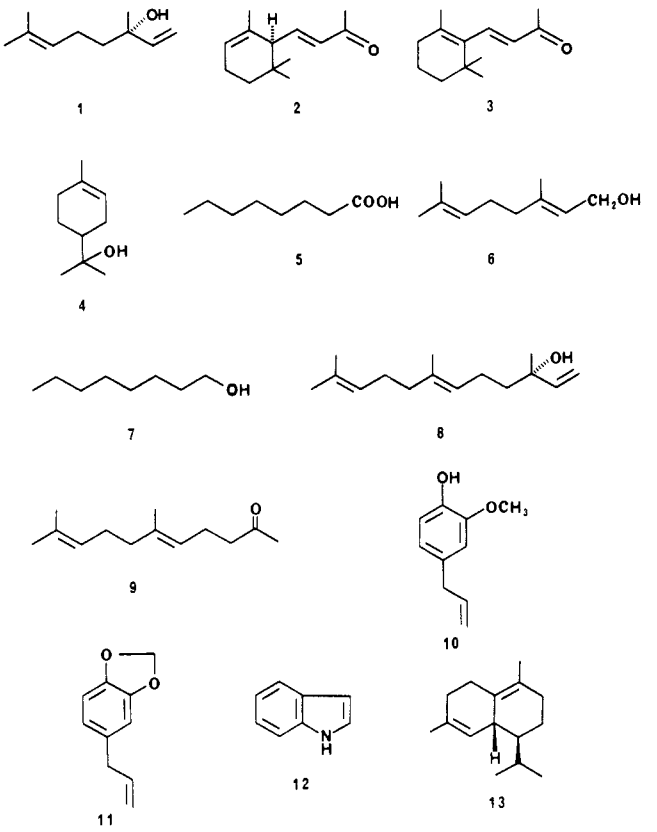

Figure 1. Structures of volatile compounds from mate tea flavor.

The antimicrobial activity of these individual components was tested against 13 selected microorganisms (Kubo et al., 1991). Despite having a relatively large yield, several substances could not be assayed because of their limited availability. It should be noted that throughout this experiment the broth dilution method was used since most of the lipophilic flavor compounds tested are not soluble in water. In fact, these compounds did not show any activity when tested using the paper disk method. The reason for the negative result using this assay is that these water-insoluble substances do not diffuse into the media. Another drawback to use of the paper disk method was the fact that these volatiles were partially, or even entirely, evaporated from the paper disk when solvent was removed.

Table I. Antimicrobial Activity of the 10 Main Components in the Distillate

	MIC against microorganisms[a] tested, μg/mL									
	1	2	3	4	5	6	7	8	9	10
Bs	800	100	100	800	200	400	400	25	100	400
Ba	800	100	100	>800	400	400	400	25	100	800
Pac	200	25	25	100	200	400	200	25	50	50
Sa	>800	>800	200	>800	400	800	400	50	>800	800
Sm	1600	100	100	400	1600	400	800	25	50	400
Pae	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800
Ea	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800
Ec	>800	>800	>800	800	>800	800	400	>800	>800	400
Sc	800	>800	>800	800	200	400	400	>800	>800	800
Cu	400	>800	400	800	200	400	200	>800	>800	800
Po	400	100	>800	400	200	200	100	800	400	200
Pc	800	400	400	400	100	200	200	800	100	200
Tm	200	–[b]	50	200	50	200	200	12.5	50	200

[a] Bs, *B. subtilis*; Ba, *B. ammoniagenes*; Pac, *P. acnes*; Sa, *S. aureus*; Sm, *S. mutans*; Pae, *P. aeruginosa*; Ea, *E. aerogenes*; Ec, *E. coli*; Sc, *S. cerevisiae*; Cu, *C. utilis*; Po, *P. ovale*; Pc, *P. chrysogenum*; Tm, *T. mentagrophytes*. [b] –, not tested.

As shown in Table I, the growths of five Gram-positive bacteria, *B. subtilis*, *B. ammoniagenes*, *P. acnes*, *S. aureus*, and *S. mutans*, and five fungi, *S. cerevisiae*, *C. utilis*, *P. ovale*, *P. chrysogenum*, and *T. mentagrophytes*, tested were all inhibited by at least one of these compounds. In the case of activity against Gram-negative bacteria, none of the compounds tested exhibited activity against *P. aeruginosa* and *E. aerogenes*, although eugenol (**10**) and 1-octanol (**7**) showed weak activity against *E. coli*, with MICs of 400 μg/mL. Few phytochemicals so far investigated exhibit activity against Gram-negative bacteria, especially against *Pseudomonas* species. In addition to the compounds listed above, the three most characteristic mate tea constituents, caffeine, ursolic acid, and chlorogenic acid, were also assayed. None of them showed any activity; the highest concentration tested was 400 μg/mL because of the limited solubility in DMF or the water-based media.

The work was centered on the investigation of the antibacterial activity of the flavor components against *S. mutans*, which is one of the bacteria responsible for causing dental caries (Hamada and Slade, 1980). All of the compounds tested exhibited some activity against this cariogenic bacterium. However, the range of activity of each compound was moderate to weak. Thus, among these 10 active compounds, nerolidol (**8**) was the most potent, with an MIC of 25 μg/mL, while linalool (**1**) and octanoic acid (**5**) were the least effective, each having an MIC of 1600 μg/mL. Noticeably, the MIC of geraniol (**6**) against *S. mutans* remained the same, even after 5 days, indicating that this monoterpene alcohol is a bactericide. This was confirmed by addition of 1% of 2-day-old medium containing 400 μg/mL geraniol into the geraniol-free medium. After 2 days of incubation, no recovery of *S. mutans* was observed. Thus, the MIC was proved to be the minimum bactericidal concentration (MBC) as well. Similarly, the concentration of each MIC of linalool and 1-octanol was found to be the MBC.

Some of these flavor components against *S. mutans* may be considered for practical use since they were identified in a daily beverage mate tea, which has long and commonly been consumed by many people. However, it is advisable to use them in combination with other compounds to make more unlikely the development of resistance mechanisms of *S. mutans*. In addition to enhancing and broadening the total activity, the combination of more than two compounds may, in general, be superior than the use of a single antimicrobial compound (Kubo and Taniguchi, 1988; Kubo and Himejima, 1991) to avoid the development of resistance mechanisms in microorganisms.

Interestingly, indole (**12**), the most abundant nitrogen-containing substance in green tea flavor (Nose et al., 1971), has been found to significantly enhance the activity against *S. mutans* of δ-cadinene (**13**), the second most abundant compound in green tea flavor (Nose et al., 1971); the MIC was reduced from 800 to 6.25 μg/mL when δ-cadinene was combined with a sublethal amount of indole (Kubo et al., 1992). However, this synergistic effect of indole against *S. mutans* was not observed in combination with nerolidol (**8**), the most potent substance against this cariogenic bacterium identified in mate tea flavor. In this combination, the effect of indole was found to be additive. Thus, the MIC was reduced only from 25 to 12.5 μg/mL. Also, the synergistic effect of indole against *S. mutans* was not the case in combination with linalool (**1**), the most abundant component in mate tea flavor. Again, the combination was only additive; the MIC (=MBC) was lowered from 1600 to 800 μg/mL.

Since the MIC and MBC alone do not well characterize the combination effect, the combination of linalool and indole was studied in more detail, as an example. First, the growth curves of *S. mutans* in the presence of linalool alone and in combination with indole were studied by measuring turbidity. As illustrated in Figure 2, linalool alone supressed the growth of this cariogenic bacterium at 1600 μg/mL, while 800 μg/mL showed little restriction of the growth. However, 800 μg/mL linalool in combination with 400 μg/mL indole (equivalent to half-MIC) suppressed the growth over a 48-h period (Figure 3). Moreover, 400 μg/mL linalool combined with 400 μg/mL indole still increased the culture lag time to 24 h, while the lag time for the culture containing linalool alone at 400 μg/mL was approximately 6 h. Second, to confirm these findings, time–kill curves were established. The killing effects of linalool and indole, both alone and in combination, are illustrated in Figures 2 and 3. *S. mutans* tolerated up to 400 μg/mL indole with little restriction of growth, while the cell numbers declined slowly in the presence of 800 μg/mL indole. Again, when linalool alone was tested against *S. mutans*, 1600 μg/mL proved bactericidal, 800 μg/mL suppressed the growth over 12 h of incubation and, 400 and 200 μg/mL reduced the growth very little, respectively. However, 800 μg/mL linalool in combination with 400 μg/mL indole showed bactericidal activity, and 400 μg/mL suppressed the growth, cell numbers increasing slowly over 32 h of incubation.

Although indole was detected in minute quantities in mate tea flavor (Kawakami and Kobayashi, 1991), it may play an important role in enhancing the total activity of the mate tea's other flavor components. On the basis of

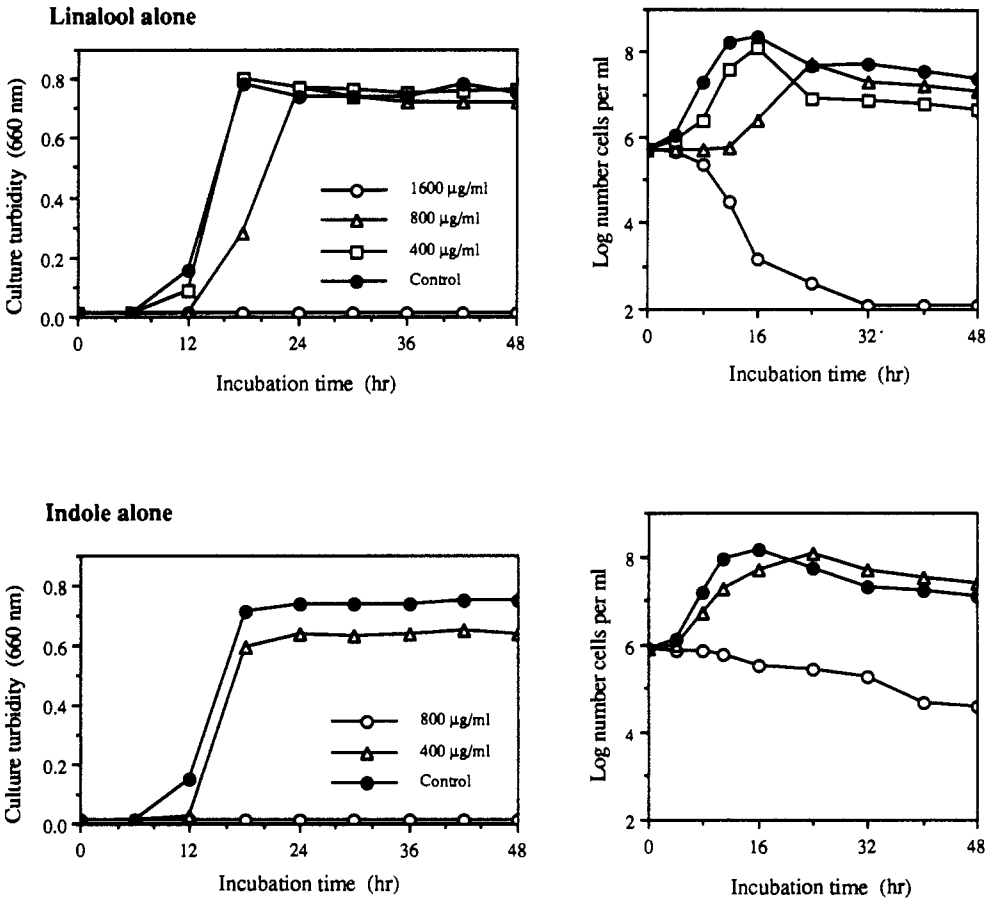

Figure 2. Effects of linalool and indole alone on growth of *S. mutans* ATCC 25175. A 48-h culture was diluted 1:100 into the fresh medium containing various concentrations of each linalool and indole.

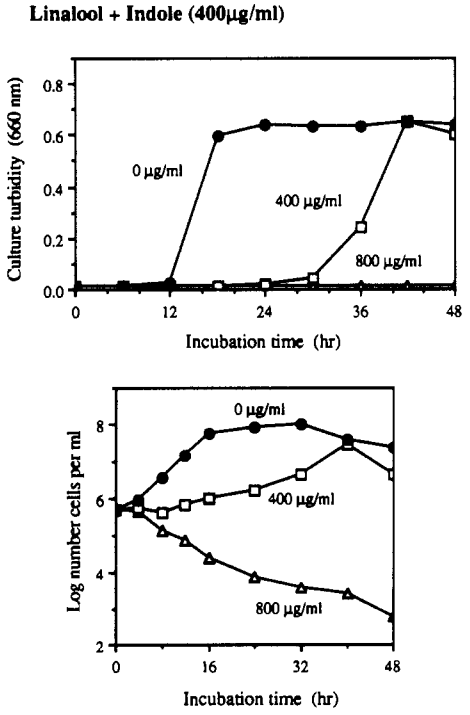

Figure 3. Combination effects of linalool with half-MIC of indole against *S. mutans* ATCC 25175. A 48-h culture was diluted 1:100 into the fresh medium containing 400 μg/mL indole in combination with 0 (●), 400 (□), and 800 (△) μg/mL linalool.

this consideration, 0.04% (400 μg/mL, equivalent of half-MIC) of indole was added into the distillate to examine if it had enhancing activity against *S. mutans*. As expected, the activity of the distillate was increased 16-fold; the MIC was lowered from 800 to 50 μg/mL. Jasmine, which contains a large amount of indole, is sometimes added for flavor to *C. sinensis* teas such as black tea and oolong tea. Similarly, jasmine may be added to mate tea to enhance its anticavity activity, in addition to enriching its flavor.

Dental caries are an infectious disease which is most frequently caused by any one of several plaque-forming organisms such as *Streptococci* and *Actinomyces*. *S. mutans* has been reported to be a particularly important cariogenic bacterium in humans. For the prevention of dental caries, many efforts are being undertaken to eliminate these cariogenic bacteria from oral flora. Although several antibiotics were found to effectively prevent dental caries in vitro and in vivo (Stephan et al., 1952; Fitzgerald, 1972), their use resulted in the derangement of oral and intestinal bacterial floras—obviously undesirable and unacceptable side effects (Fitzgerald, 1972).

Since the mate tea has been consumed daily by people, it would appear that its potential for human oral toxicity either may not be serious or has been overlooked. As a matter of fact, α-ionone, β-ionone, α-terpineol, nerolidol, eugenol, and linalool identified in the mate tea have been used as flavor ingredients in foods such as candy, ice cream, and chewing gum (Furia and Bellanca, 1975). Thus, these flavor components can be considered for use in oral care products, even though their antimicrobial activity is only moderate. It should be noted that safrole showed activity similar to that of the other compounds tested. However, it is advisable to omit safrole from the list of potential oral care compounds, since it is a suspected carcinogen.

ACKNOWLEDGMENT

We thank Dr. H. Tsuruta, Takasago International Corp., for GC–MS measurements and for the gifts of α-ionone, β-ionone, linalool, nerolidol, and α-terpineol, Dr. P. Moyna and Mr. T. Matsuo for assistance in obtaining mate tea from various locations, and Mr. M. Lewin for critical reading of the manuscript. H.M. thanks Takasago Institute for Interdisciplinary Science for financial support.

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Received for review February 4, 1992. Revised manuscript received June 15, 1992. Accepted November 11, 1992.