Antimicrobial Activity of Green Tea Flavor Components and Their Combination Effects

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The antimicrobial activity of the 10 most abundant volatile components of green tea flavor (1-10) was examined. The activity of each volatile was moderate but broad in spectrum. Most of the volatiles tested inhibited the growth of one of the most important cariogenic bacteria, *Streptococcus mutans*. Among them, nerolidol (4) was the most potent; linalool (1) was the least effective. In addition, indole (7) significantly enhanced the activity of δ-cadinene (2) and caryophyllene (10) against *S. mutans*. These two sesquiterpene hydrocarbons also showed potent activity against a dermatomycotic bacterium, *Propionibacterium acnes*. Lastly, but most importantly, indole inhibited the growth of all of the Gram-negative bacteria tested, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Escherichia coli*.

INTRODUCTION

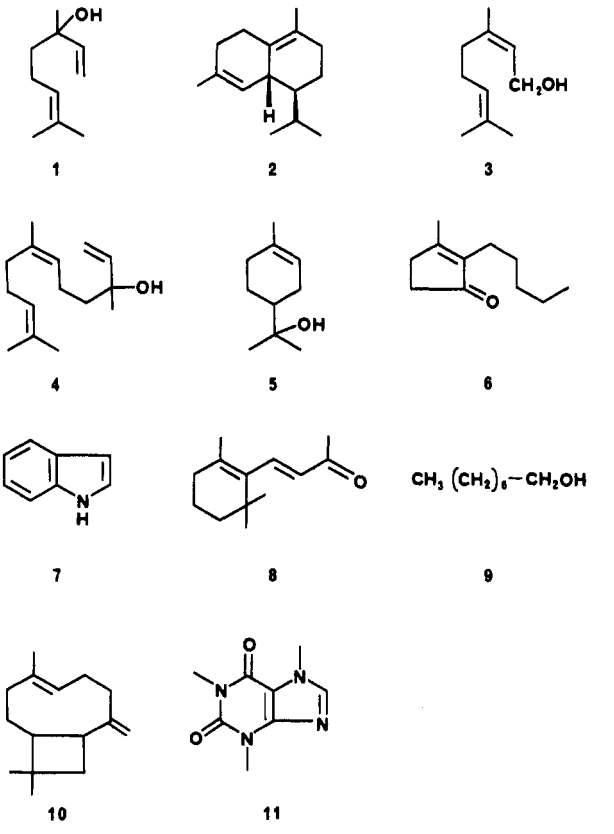
Tea is one of the most widely consumed beverages in the world. Its popularity is attributed to its pleasant flavor, combined with its stimulating effects. There are many types of tea, including green tea, black tea, and oolong tea, and each has several subclassifications (Eden, 1976). Recently, green tea flavor has been used in ice cream, candy, soft drinks, etc. All of them are prepared from what is basically the same plant, *Camellia sinensis* L. (Theaceae) and its varieties, by different manufacturing processes.

It has been said that those who continuously drink a large amount of green tea have less tooth decay. After a year of continuous surveillance at elementary schools, this was proven (Onishi et al., 1981a). Onishi et al. also reported that the green tea extract contained many active substances for caries prevention (Onishi et al., 1981b). The active principles have not yet been thoroughly defined, although several polar polyphenolic compounds in the green tea have already been reported as moderate antibacterial principles (Sakanaka et al., 1989) against *Streptococcus mutans*. This is one of the bacteria responsible for causing dental caries (Hamada and Slade, 1980). The MICs of these polyphenols, reported against *S. mutans*, were at most 250 μg/mL (Sakanaka et al., 1989). Interestingly, the antimicrobial activity of nonpolar substances in green tea, particularly volatile flavor compounds, has not yet been investigated. Therefore, we examined the antimicrobial activity of 10 major flavor constituents of green tea against *S. mutans*.

The need for new antimicrobial agents in cosmetics is pressing. The lack of effective preservatives to control microorganisms which putrefy cosmetic products is a major problem to be solved. Moreover, the control of specific microorganisms that cause skin, hair, and oral problems is becoming even more important. In contrast to medicines which are used to heal ill people, antimicrobial agents in cosmetics are repeatedly applied to healthy skin, hair, and teeth, often for long periods. Therefore, the safety of these products is the first consideration. It is possible that edible plants, beverages, and food spices may be a superior source of new antimicrobial agents. With this in mind, the same green tea flavor compounds were also tested against 12 additional selected microorganisms (Himejima and Kubo, 1991).

MATERIALS AND METHODS

Chemicals. The authentic indole (7) was purchased from Sigma Chemical Co. (St. Louis, MO). Geraniol (3) was obtained



from Johnson Matthey (Ward Hill, MA). Linalool (1), nerolidol (4), α-terpineol (5), δ-cadinene (2), β-ionone (8), 1-octanol (9), caryophyllene (10), and *cis*-jasmone (6) were gifts from Takasago International Co. (Tokyo). They were all used for the assay without purification, except for δ-cadinene, which was further purified by a combination of various chromatographic methods. Caffeine was previously isolated from the leaves of *Ilex paraguayensis* (Aquifoliaceae) which is known as "mate tea". *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ).

Microorganisms and Media. All microorganisms used for the antimicrobial assay were purchased from American Type Culture Collection (Rockville, MD, USA). They are *Bacillus*

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Table I. Antimicrobial Activity of the Components in the Distillate

	MIC against microorganism[a] tested, μg/mL										
	1	2	3	4	5	6	7	8	9	10	11
Bs	800	50	400	25	800	800	400	100	400	50	>400
Ba	800	50	400	25	>800	>800	800	100	400	100	>400
Sa	>800	>800	800	50	>800	>800	400	200	800	>800	>400
Sm	1600	800	400	25	400	800	800	100	400	>1600	>400
Pac	200	3.13	400	25	100	400	200	25	200	6.25	>400
Pae	>800	>800	>800	>800	>800	>800	800	>800	>800	>800	>400
Ea	>800	>800	>800	>800	>800	>800	800	>800	>800	>800	>400
Ec	>800	>800	800	>800	800	>800	800	>800	400	>800	>400
Sc	800	>800	400	>800	800	800	>800	>800	400	>800	>400
Cu	400	>800	400	>800	800	800	>800	400	200	>800	>400
Po	400	>800	200	800	400	200	200	>800	100	>800	>400
Pc	800	>800	200	800	400	200	50	400	200	>800	>400
Tm	200	>800	200	12.5	200	200	100	50	200	>800	>400

[a] Bs, *B. subtilis*; Ba, *B. ammoniagenes*; Sa, *S. aureus*; Sm, *S. mutans*; Pac, *P. acnes*; Pae, *P. aeruginosa*; Ea, *E. aerogenes*; Ec, *E. coli*; Sc, *S. cerevisiae*; Cu, *C. utilis*; Po, *P. ovale*; Pc, *P. chrysogenum*; Tm, *T. mentagrophytes*.

subtilis ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Staphylococcus aureus* ATCC 12598, *Streptococcus mutans* ATCC 25175, *Propionibacterium acnes* ATCC 11827, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Saccharomyces cerevisiae* ATCC 7754, *Candida utilis* ATCC 9226, *Pityrosporum ovale* ATCC 14521, *Trichophyton mentagrophytes* ATCC 18748, and *Penicillium chrysogenum* ATCC 10106.

The culture media for the bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco), and 0.1% glucose, except in the case of *S. mutans*. For the culture of *S. mutans*, 3.7% brain heart infusion broth (Difco) was utilized. The culture for the fungi consisted of 2.5% malt extract broth (BBL), except in the cases 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 used.

Cultivation of Microorganisms. The freeze-dried microorganisms purchased from ATCC were reactivated as follows: *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 stationarily at 30 °C. All other microorganisms were cultured stationarily at 37 °C.

Antimicrobial Assay. The bioassay was performed by a broth dilution method. Thus, the test compound was first dissolved in DMF, and 30 μL of the sample solution was added to 3 mL of the appropriate medium. Then, 30 μL of 2-day-old culture of the test microorganisms (5-day old of *T. mentagrophytes* and *P. chrysogenum*) was inoculated. The highest concentration used for the assay was 800 μg/mL unless otherwise specified because of solubility limitation of the samples in DMF and/or the water-based media. For the antimicrobial assay, all microorganisms were cultured stationarily, except *T. mentagrophytes* and *P. chrysogenum*, which were cultured with shaking. After 2 days (5 days for *T. mentagrophytes* and *P. chrysogenum*), the growth of the microorganisms, except *P. ovale*, *T. mentagrophytes*, and *P. chrysogenum*, was examined as a function of turbidity (OD at 660 nm). That of the three fungi was justified with the naked eye. The minimal inhibitory concentration (MIC) against microorganisms was measured by 2-fold serial broth dilution. Incidentally, it should be noted that the concentration of DMF in each medium was always 1%, which did not affect the growth of any of the microorganisms employed. The lowest concentration of the test compound in which no growth occurred was defined as the MIC.

The combination data against *S. mutans* and *P. acnes* were obtained according to the 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. Each bacterium was tested at least twice with the checkerboard method.

RESULTS AND DISCUSSION

The complex green tea flavor contains over 100 volatile compounds (Flament, 1991; Yamaguchi and Shibamoto, 1981). Hence, the 10 most abundant volatile flavor constituents identified in green tea, namely linalool, δ-cadinene, geraniol, nerolidol, α-terpineol, *cis*-jasmone, indole, β-ionone, 1-octanol, and caryophyllene, in decreasing concentration, were selected for our assay from the list reported previously (Nose et al., 1971). Most of the teas seem to consist of almost the same components, although the compositions differed as a result of the manufacturing process (Kiribuchi and Yamanishi, 1963; Owuor et al., 1986; Hazarika et al., 1984). Furthermore, these volatile compounds are identified in many edible plants, food spices, and beverages and are frequently used for fragrance and flavor (Bauer et al., 1991). For example, the most abundant volatile in green tea flavor, linalool (**1**), was also found in food spices such as coriander, lavender, sage, and thyme (Maarse, 1991), often, even, as the main component. There is no doubt that these volatile compounds have long been consumed by many people. Despite their occurrence in relatively large amounts, several green tea flavor components such as cubenol and α-muurolene were unable to be assayed because of limited availability.

The 13 microorganisms (Table I) were selected for the assay. Before antimicrobial activity of an individual compound is discussed, it should be emphasized that the broth dilution method was used throughout this experiment, since these nonpolar flavor compounds tested were not soluble in water. In fact, they did not show any activity by the paper disk method since these water-insoluble compounds might not diffuse into the media and/or because these volatiles were partially or even entirely evaporated from the paper disk when the solvent was removed.

The antimicrobial activity of the 10 selected flavor compounds (**1**–**10**) is listed in Table I. The compounds tested, with the exception of caryophyllene (**10**), exhibited some activity against *S. mutans*. Among them, nerolidol (**4**) was the most potent, with an MIC of 25 μg/mL. Linalool (**1**) was the least effective, with an MIC of 1600 μg/mL. Thus, the activity of each compound against *S. mutans* was moderate or weak. One of the most characteristic tea constituents, caffeine (**11**), was also assayed. It did not show any activity against *S. mutans*, although the highest concentration tested was 400 μg/mL because of its limited solubility in DMF. However, the total activity of a cup of green tea was reported to be enough to control this cariogenic bacterium (Onishi et al., 1981a). As far as the volatile components alone are concerned, it does not seem to be potent enough.

The yield of the volatile flavor compounds obtained by steam distillation from 16 kg of green tea was reported at

Table II. MICs of Green Tea Flavor Compounds in Combination with Half-MIC of Indole against *S. mutans* and *P. acnes*

compd tested	MIC against microorganism tested, μg/mL	
	S. mutans	*P. acnes*
linalool	1600 → 800	
δ-cadinene	800 → 6.25	3.13 → 1.56
geraniol	400 → 200	
nerolidol	50 → 12.5	
caryophyllene	>1600 → 6.25	6.25 → 3.13

about 5.6 g (Yamanishi et al., 1970). If this is the case, theoretically a cup of green tea prepared with 2 g (the usual amount for a commercial tea bag) of the tea leaves in 100 mL of hot water contains a total of 7 μg/mL of volatiles. This concentration does not seem to be strong enough to control *S. mutans*, even if the volatile component of tea was assumed to consist of only nerolidol (**4**), the most potent antibacterial substance against *S. mutans*, among the 10 flavor compounds tested. However, the MIC is the number obtained on the basis of test tube assay. Moreover, it should be noted that tea contains many other chemicals such as the aforementioned antibacterial polyphenolic compounds. The combination of these substances may enhance the total antibacterial activity against *S. mutans*. In addition, it differs from the in vivo assay, especially from continuous drinking of a large amount of green tea.

Because of these concerns, we attempted to enhance the antibacterial activity against *S. mutans* through combination of two or more green tea flavor compounds. The rational basis for an approach such as this is still in an embryonic stage. In our preliminary combination study, indole (**7**) was found to enhance the antibacterial activity of several other compounds against *S. mutans*. Therefore, the detailed study with the four most abundant green tea flavor compounds (**1**–**4**), in combination with indole, was carried out. Table II shows the MICs of these compounds in combination with 400 μg/mL of indole (equivalent to half-MIC for *S. mutans*). In this combination, the activity of three terpene alcohols, linalool (**1**), geraniol (**3**), and nerolidol (**4**), against *S. mutans* was not significantly increased. Their MICs were enhanced by only 2–4-fold. In contrast, the activity of δ-cadinene (**2**), the most abundant sesquiterpene hydrocarbon in green tea flavor, was dramatically enhanced, and its MIC was lowered from 800 to 6.25 μg/mL. On the basis of this finding, the other sesquiterpene hydrocarbon, caryophyllene (**10**), was also tested in combination with indole. It should be noted that caryophyllene did not exhibit any activity against *S. mutans* up to 1600 μg/mL. As expected, indole also significantly increased the activity of **10**. In this case, the MIC of **10** was lowered to 6.25 μg/mL. The enhancing activity of indole seems to depend on the chemicals that it combines with. Although we have previously described potentiation of antifungal activity of several antibiotics, especially against *S. cerevisiae* and *C. utilis* (Kubo and Taniguchi, 1988), this is the first report of potentiation of antibacterial activity against *S. mutans* by the combination of two substances. Interestingly, a large quantity of indole is contained in jasmin. Jasmin is sometimes added to tea for flavor. The basics of this combination effect are currently under investigation.

Besides the activity against *S. mutans*, the same volatile compounds (**1**–**10**) also showed activity against all of the other microorganisms tested. The antimicrobial activity of each compound exhibited a moderate but broad spectrum. Among the microorganisms tested, *P. acnes* was the most sensitive, with MICs between 3.13 and 400 μg/mL. *P. acnes*, one of the bacteria responsible for acne, produces a lipase that hydrolyzes sebum triglycerides to free fatty acids, causing inflammation and comedones (Matsuoka, 1983). The two most active compounds, δ-cadinene (**2**) and caryophyllene (**10**), may be particularly useful as protection from *P. acnes* infection. The activity of these sesquiterpene hydrocarbons against other microorganisms was mainly limited to Gram-positive bacteria, with MICs between 50 and 100 μg/mL. In addition, since the antimicrobial activity of δ-cadinene and caryophyllene was significantly synergized against *S. mutans* by indole, these two sesquiterpene hydrocarbons were also tested in combination with indole to examine if it also had the same enhancing activity against *P. acnes*. The result, unexpectedly, was that indole did not exhibit any meaningful enhancing activity against this bacterium. This is shown in Table II. The enhancing activity of indole seems to depend on not only the chemicals being combined but also the test microorganisms.

In contrast to *P. acnes*, *S. aureus* was the least sensitive Gram-positive bacterium. Only nerolidol (**4**), β-ionone (**8**), indole (**7**), geraniol (**3**), and 1-octanol (**9**) showed any activity, with MICs of 50, 200, 400, 800, and 800 μg/mL, respectively.

Most noticeably, in this experiment, indole (**7**) exhibited antibacterial activity against all of the Gram-negative bacteria tested, *P. aeruginosa*, *E. aerogenes*, and *E. coli*. The MICs were 800, 800, and 400 μg/mL, respectively. The antibacterial activity of indole against several *Pseudomonas* species was previously reported; however, it was isolated from microbial fermentation (Oimomi et al., 1974; Matsuda et al., 1990). Generally, few phytochemicals exhibit activity against Gram-negative bacteria, especially *Pseudomonas* species. In addition, geraniol (**3**), α-terpineol (**5**), and 1-octanol (**9**) also showed weak activity against *E. coli*, with the MICs of 800, 800, and 400 μg/mL, respectively.

In addition to antibacterial activity, most of the green tea flavor compounds tested exhibited antifungal activity against *P. ovale*, *S. cerevisiae*, *C. utilis*, *T. mentagrophytes*, and *P. chrysogenum*. Most significantly, the aforementioned antibacterial nerolidol (**4**) inhibited the growth of *T. mentagrophytes* at 12.5 μg/mL. Moreover, all other volatiles except δ-cadinene and caryophyllene also inhibited the growth of *T. mentagrophytes*, with MICs between 50 and 200 μg/mL. This fungus occurs primarily on human hair and causes human dermatomycosis. Similarly, the growth of another dermatomycotic fungus, *P. ovale*, was also inhibited by the same flavor components.

In conclusion, the green tea flavor compounds described herein may be considered potential antimicrobial agents for cosmetic and food products. For example, antibiotics such as penicillin, erythromycin, and tetracycline effective prevented dental caries in vitro and in vivo (McClure and Hewitt, 1946; Stephan et al., 1952; Fitzgerald, 1972), but they resulted in derangement of oral and intestinal bacterial floras. These are obviously undesirable and unacceptable side effects (Fitzgerald, 1972). The compounds identified in a common beverage such as tea should not cause these undesirable side effects. Since green tea has been continuously consumed by many people for centuries, either the extract or purified flavor compounds of green tea may be considered safe for practical use in such things as oral care products.

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