Combination Effects of Antibacterial Compounds in Green Tea Flavor against *Streptococcus mutans*

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Combinations of green tea flavor compounds with indole were tested for antibacterial activity against *Streptococcus mutans*, a bacterium responsible for causing dental caries. Synergism was found in the combination of sesquiterpene hydrocarbons (δ-cadinene and β-caryophyllene) with indole; their bactericidal activities increased from 128-fold to 256-fold. The combination effect was confirmed by time-kill curve assay which showed that 6.25 μg/mL δ-cadinene combined with 400 μg/mL (subinhibitory concentration) indole was bactericidal against *S. mutans*. Furthermore, the combination of 25 μg/mL δ-cadinene and 400 μg/mL indole reduced the number of viable cells at any stage of growth.

INTRODUCTION

Dental caries are among the most ubiquitous infectious diseases in developed countries. They stem from a variety of interactive elements, including nutritional status, diet (sugar intake), and the presence of cariogenic microflora. Many recent studies have concluded that *Streptococcus mutans* is the primary bacterium causing dental caries in both humans and experimental animals (Hamada and Slade, 1980; de Jong et al., 1984). This bacterium adheres firmly to smooth tooth surfaces and produces sticky water-insoluble glucans from dietary sucrose that faciliitate the accumulation of the other oral microorganisms. *S. mutans* and these other microorganisms in plaque create organic acids, such as lactic acid, that gradually destroy enamel, forming a cavity (Hamada and Slade, 1980; Loesche, 1986).

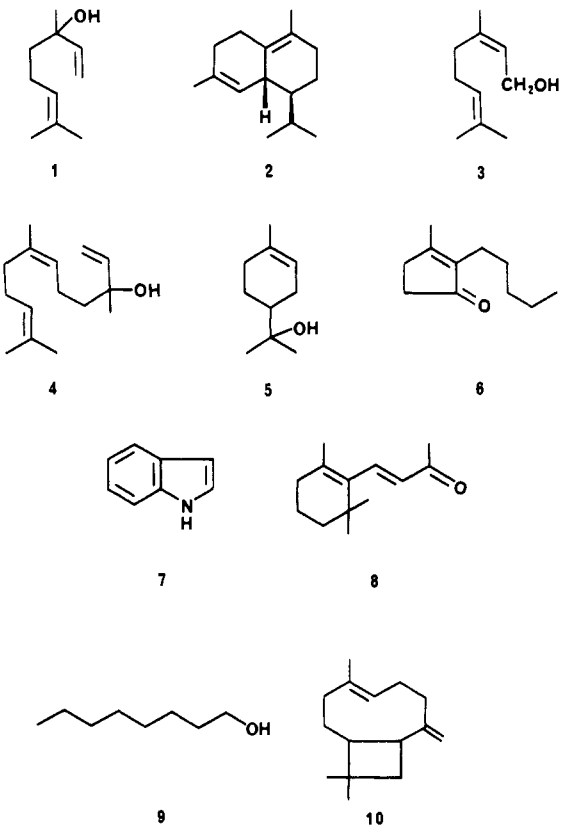
Theoretically, dental caries can be prevented by eliminating *S. mutans*. In our continuing search for naturally occurring antibacterial agents against this cariogenic bacterium, we have recently reported that green tea flavor compounds have antibacterial activity against *S. mutans* (Kubo et al., 1992). This was the first report of volatile substances in green tea showing antibacterial activities, although several polar polyphenolic compounds have already been reported as antibacterial and antiplaque compounds against *S. mutans* (Sakanaka et al., 1989, 1990; Hattori et al., 1990). These studies were based largely on the old tradition that those who continuously drink a large amount of green tea have less tooth decay, and a recent report has proved this (Onishi et al., 1981).

With the exception of β-caryophyllene (10), all nine other flavor compounds tested exhibited activity against *S. mutans*. The range of the activity of each compound was moderate to weak. Among these active compounds, nerolidol (4) was the most potent, with a minimum inhibitory concentration (MIC) of 25 μg/mL, while linalool (1) was the least effective, with an MIC of 1600 μg/mL. Noticeably, indole (7) was found to enhance the activity of two sesquiterpene hydrocarbons against *S. mutans*. Thus, the MIC of δ-cadinene (2) was reduced from 800 to 6.25 μg/mL when it was tested in combination with a subinhibitory concentration (half-MIC) of indole. More dramatically, the MIC of β-caryophyllene was lowered to 6.25 μg/mL by combining with indole, although it did not exhibit any activity against *S. mutans* up to 1600 μg/mL when it was assayed alone (Kubo et al., 1992).

The MIC, which is determined by measuring the growth of *S. mutans* in a test system after 48 h of incubation, does not well characterize the antibacterial activity of a sample. Therefore, we have investigated the activity of some of the key compounds in green tea flavor against this cariogenic bacterium in more detail and using other methods.

MATERIALS AND METHODS

Chemicals. The authentic linalool (1), δ-cadinene (2), nerolidol (4), α-terpineol (5), *cis*-jasmone (6), β-ionone (8), 1-octanol (9), and β-caryophyllene (10) were gifts from Takasago International Corp. (Tokyo, Japan). Indole (7), geraniol (3), and (-)-epicatechin were purchased from Sigma Chemical Co. (St. Louis, MO) (7, 3) and Johnson Matthey (Ward Hill, MA). (-)-Epigallocatechin was purchased from Funakoshi Co. Ltd. (Tokyo, Japan). All chemicals were used for the assay without purification, except for δ-cadinene which was purified by a combination



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of various chromatographic methods. For antimicrobial assay experiments, all chemicals were first dissolved in *N,N*-dimethylformamide (DMF) that was purchased from EM Science (Gibbstown, NJ).

Microorganism and Media. *S. mutans* ATCC 25175 was obtained from American Type Culture Collection (Rockville, MD). The freeze-dried culture of *S. mutans* was inoculated into 3.7% brain heart infusion broth (BHI; DIFCO Laboratories, Detroit, MI) and incubated stationary for 2 days at 37 °C before antimicrobial assay.

MIC and MBC Determinations. The MIC was determined by a broth dilution method as previously described (Kubo et al., 1992). Briefly, serial 2-fold dilutions of test compounds were made in DMF, and 30 μL of each dilution was added to 3 mL of BHI broth that was then inoculated with 30 μL of a 2-day-old culture of *S. mutans*. Because of the solubility limitation of the samples in DMF and/or the water-based media, the highest concentration used for the assay was 1600 μg/mL unless otherwise specified. The cultures were incubated stationary at 37 °C for 2 days. The MIC was defined as the lowest concentration of the test compound that demonstrated no visible growth.

The minimum bactericidal concentration (MBC) was the lowest concentration of antibacterial compound that decreased the initial inoculum concentration by >99.9%. After the MIC was determined, 10-fold dilutions from each tube showing no turbidity were plated onto the chemical-free BHI agar medium. After 2 days of incubation, MBC break points were determined by using rejection values as described by Pearson et al. (1980).

The MIC and MBC of each compound were determined at least twice.

Combination Studies. Combination studies were performed by a broth checkerboard method (Norden et al., 1979). A series of 2-fold dilutions of indole was tested in combination with 2-fold dilutions of the other. MICs and MBCs were determined by using the same method as described above. All combination studies were carried out at least twice.

Growth Studies. Combination was also studied with half-MIC of indole (7) and 2-fold dilutions of linalool or δ-cadinene. The culture tubes were prepared as described above and incubated at 37 °C for 2 days. Growth was monitored repeatedly by measuring the absorbance (OD) increase at 660 nm on a Milton Roy Spectronic 20D spectrophotometer (Rochester, NY). Viability studies were designed to examine combination effects in more detail. Thirty microliters of the 2-day-old culture was inoculated into 3 mL of BHI broth containing appropriate concentrations of the test compounds. The initial population was 1–8 × 10^5 colony-forming units (CFU)/mL. Samples were taken at selected times, and serial dilutions were made in sterile saline before plating onto BHI agar plate. The plates were incubated at 37 °C for 2 days before counting.

The effect of the combination between indole and δ-cadinene was further studied on *S. mutans* in different growth phases. The bacterial cultures starting at about 1 × 10^5 CFU/mL were prepared as described above, and both indole and δ-cadinene were added after 0, 4, 10, or 24 h of incubation at appropriate concentrations. Twenty-four and 48 h after the compounds were added, viable counts were determined as described above.

RESULTS AND DISCUSSION

MICs, the index for bacteriostatic activity, of the green tea flavor compounds (1–9) ranged from 25 to 1600 μg/mL. As the ideal anticaries agent, compounds should possess bactericidal activity against this cariogenic bacterium (Fitzgerald, 1972). We examined the minimum bactericidal concentrations (MBC) of the same flavor compounds for *S. mutans* (Table I). Noticeably, these compounds had bactericidal activity and MBC to MIC ratios were no greater than two in all compounds except nerolidol (4). The MBC was the same as the corresponding MIC in linalool (1), δ-cadinene (2), geraniol (3), and 1-octanol (9). The MBC was 2-fold higher than the corresponding MIC in α-terpineol (5), *cis*-jasmone (6), indole (7), and β-ionone (8). The MBC of nerolidol, the most potent compound, was 8-fold higher than the measured MIC (MIC = 25 μg/mL, MBC = 200 μg/mL).

Table I. MICs and MBCs of Green Tea Flavor Compounds against *S. mutans* ATCC 25175

compd tested	MIC, μg/mL	MBC, μg/mL
linalool	1600	1600
δ-cadinene	800	800
geraniol	400	400
nerolidol	25	200
α-terpineol	800	1600
cis-jasmone	800	1600
indole	800	1600
β-ionone	100	200
1-octanol	400	400
β-caryophyllene	>1600	*a*

[a] Not tested.

Table II. MICs and MBCs of Green Tea Flavor Compounds in Combination with 400 μg/mL (Half-MIC) Indole (7) for *S. mutans* ATCC 25175

compd tested	MIC, μg/mL	MBC, μg/mL
linalool	1600 → 800	1600 → 800
δ-cadinene	800 → 6.25	800 → 6.25
geraniol	400 → 200	400 → 400
nerolidol	25 → 12.5	200 → 25
β-caryophyllene	>1600 → 6.25	>1600 → 6.25

In the previous paper, we reported that indole (7) enhances the microbial activity of the other green tea flavor components (Kubo et al., 1992). Usually, the rationale for using more than two antimicrobial agents is to target a broad spectrum of microorganisms and to prevent resistance mechanisms developing in microorganisms. However, the results of our previous combination study showed that there was an obvious synergistic action between indole and sesquiterpene hydrocarbons in green tea flavor, such as δ-cadinene (2) and β-caryophyllene (10), on growth inhibition of *S. mutans*.

In this study, the MIC of the δ-cadinene and β-caryophyllene in combinatioin with indole was found to be the same as the MBC (Table II). The bactericidal activity of δ-cadinene and β-caryophyllene were increased 128-fold and more than 256-fold, respectively, when they were combined with 400 μg/mL of indole (equivalent to half-MIC). The combination of indole and three terpene alcohols, linalool (1), geraniol (3), and nerolidol (4) also showed bactericidal effects, although these combination effects were not as remarkable as those of the sesquiterpene hydrocarbons.

For a more critical assessment of the combination effect on *S. mutans*, growth studies were carried out by both measuring culture turbidity and enumerating viable cells. Linalool (1) and δ-cadinene (2) were chosen for the studies as the representatives of terpene alcohols and sesquiterpene hydrocarbons, respectively.

The growth curves of *S. mutans* in the presence of indole (7), linalool (1), and δ-cadinene (2) alone are illustrated in Figure 1, and those in combinations are in Figure 2. This bacterium tolerated up to 400 μg/mL indole with little restriction of growth, but the cell numbers declined slowly at 800 μg/mL. Linalool proved to be bactericidal at 1600 μg/mL and suppressed the growth over 12 h at 800 μg/mL. However, 800 μg/mL linalool plus 400 μg/mL indole showed bactericidal activity. The growth rate of *S. mutans* was suppressed in the presence of 400 μg/mL linalool combined with 400 μg/mL indole, but cell numbers did increase slowly over the 32-h period of the test.

S. mutans showed a different response in the presence of δ-cadinene (2) (Figure 3). The MIC of δ-cadinene alone

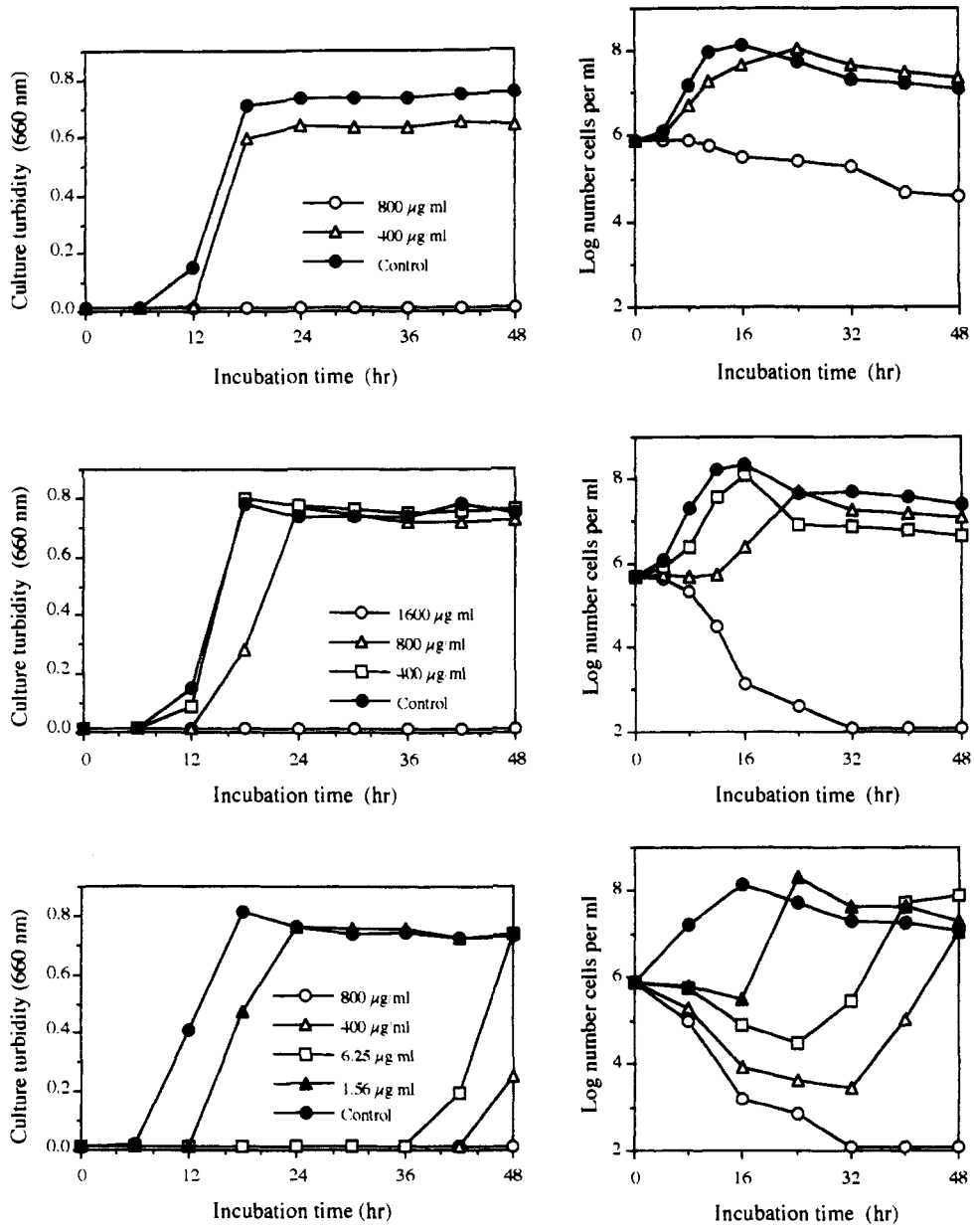

Figure 1. Growth curves of *S. mutans* in the presence of (upper panels) indole (7), (middle panels) linalool (1), and (lower panels) δ-cadinene (2) alone.

was as low as 800 μg/mL. Again, the inhibitory effect of a compound is not well characterized by MIC, as aforementioned. When culture growth for the first 48 h, in 6-h intervals, was measured, it was found that concentrations of δ-cadinene lower than 800 μg/mL did not completely inhibit growth of *S. mutans* but did increase the lag time in the growth curve (Figure 1). The time–kill curves proved this more clearly. A lethal effect was seen when 800 μg/mL δ-cadinene was employed. This compound at 400 μg/mL resulted in an apparent decrease in the viable cell count of *S. mutans* over 32 h of incubation, followed by an increase in cell numbers to the same final cell count as the control after 48 h. The same type of growth pattern was obtained at 6.25 μg/mL δ-cadinene. Thus, cell numbers of *S. mutans* declined slowly over 24 h, and then rapid growth occurred.

It has been previously reported that some Gram-negative bacteria showed this unusual growth pattern under certain conditions (Robach et al., 1977; Shih and Harris, 1977), e.g., *Salmonella typhimurium*, one of the food poisoning bacteria, in the presence of butylated hydroxyanisole (BHA) (Chang and Branen, 1975; Pierson et al., 1980), and *Clostridium perfringens* when cells are heated at 50 °C (Collee et al., 1961). In the latter microorganism, the phenomenon was known to be an injury–recovery process (Shoemaker and Pierson, 1976). The growth response of *S. mutans* to δ-cadinene is similar to an injury–recovery phenomenon. However, the same growth pattern was not observed in combination with indole (Figure 2). Even 6.25 μg/mL δ-cadinene combined with 400 μg/mL indole proved to be bactericidal against *S. mutans*. It is quite likely that 400 μg/mL indole caused lethal damage to the injured cells of *S. mutans*.

The effect of the combination of indole and δ-cadinene was further studied on *S. mutans* in the different growth phases. Although, at 6.25 μg/mL, δ-cadinene combined with 400 μg/mL indole was not bactericidal on exponentially growing *S. mutans*, 25 μg/mL δ-cadinene combined with indole showed bactericidal activity at any stage of growth, producing a $>2 \times \log_{10}$ reduction in CFU per milliliter at 24 h of incubation after the compounds were added. It should be noted that this combination killed the *S. mutans* cells even at the exponential growth stage in which metabolic and enzymatic activities are high. The

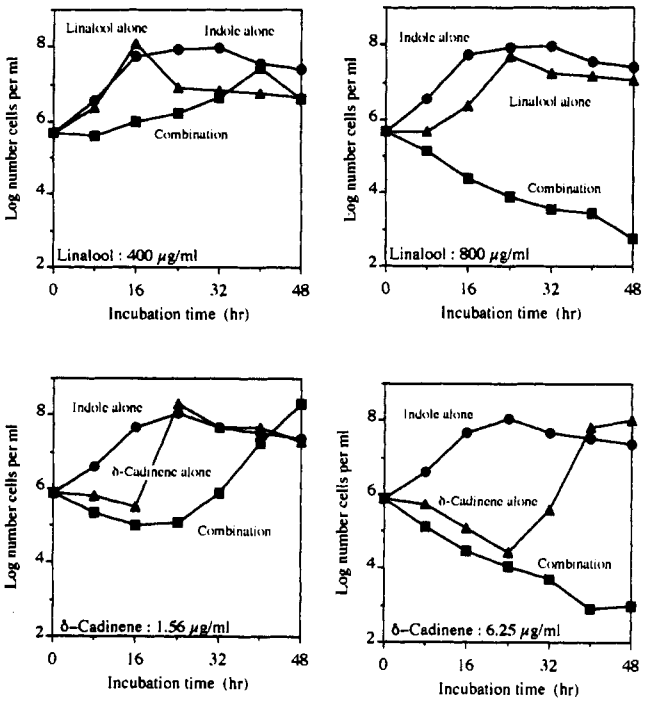

Figure 2. Bactericidal activity of (upper panels) linalool (1) and (lower panels) δ-cadinene (2) in combination with 400 μg/mL (half-MIC) indole (7) against *S. mutans*.

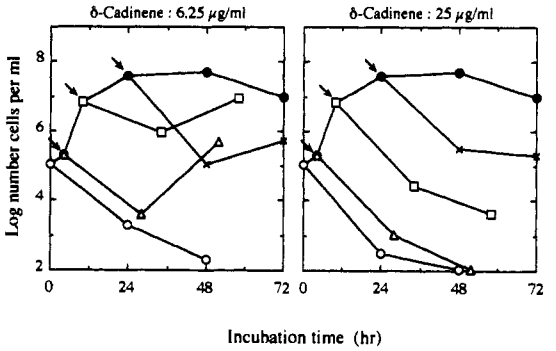

Figure 3. Effect of δ-cadinene in combination with 400 μg/mL (half-MIC) indole on *S. mutans* in the different growth phases. Compounds were added 0 (○), 4 (△), 8 (□), and 24 h (×) after the beginning of incubation [control (●)].

mechanisms of action of these compounds, used alone and in combination, are currently under investigation.

The combination study of the flavor compounds with polyphenolic compounds identified in green tea such as (−)-epicatechin and (−)-epigallocatechin was not conducted, because these polyphenols did not show any antibacterial activity against *S. mutans* up to 500 μg/mL by the broth dilution method. However, tea polyphenols have been reported to have antiplaque activity, inhibiting glucosyltransferase of *S. mutans* (Sakanaka et al., 1990; Hattori et al., 1990). It could be concluded that green tea extract is effective in the prevention of dental caries because of the antibacterial activity of flavor compounds together with the antiplaque activity of polyphenols.

As mentioned in our previous paper (Kubo et al., 1992), various compounds have been studied for possible use for caries control over decades (Stephan et al., 1952; Fitzgerald, 1972; Tanzer et al., 1977). The application of antibacterial agents is especially attractive, because it focuses on the eradication of the causal microorganisms, in contrast to fluoridation or dietary control. However, the use of antibiotics such as penicillin, erythromycin, and tetracycline are accompanied by a potential risk of undesirable and unacceptable side effects (Fitzgerald, 1972). Since green tea has been widely consumed by people as a daily beverage, extracts or purified flavor compounds of green tea may be safe, or risk-free, for use in oral care products. Overall, this study indicates that the bactericidal activities of green tea flavor compounds and the combination effects support their practical use for caries control.

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