Bactericidal Activity of Anacardic Acids against *Streptococcus mutans* and Their Potentiation

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Anacardic acids isolated from the cashew *Anacardium occidentale* L. (Anacardiaceae) apple and a series of their synthetic analogs, 6-alkylsalicylic acids, were found to be bactericidal against *Streptococcus mutans* by the time–kill curve method. The maximum antibacterial activity of 6-alkylsalicylic acids against this cariogenic bacterium showed when the carbon length comprised 12 carbon atoms in the side chain. To enhance the bactericidal activity of anacardic acids, various combinations were tested. Synergistic bactericidal activity was found in the combination of anacardic acid [6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid] and anethole or linalool.

INTRODUCTION

Dental caries is one of the most ubiquitous infectious diseases in developed countries. Interactive elements, including nutritional status and sugar intake and the presence of cariogenic microflora, are contributors to this disease. Many recent studies have concluded that *Streptococcus mutans* is a causative organism of dental caries (Hamada and Slade, 1980; de Jong et al., 1984). This bacterium adheres firmly to smooth tooth surfaces and facilitates the accumulation of other oral microorganisms. Predominant in plaque, *S. mutans* and accumulated microorganisms create organic acids, such as lactic acid, that gradually destroy enamel, leaving an opening susceptible to bacterial degradation, thus forming a cavity (Hamada and Slade, 1980; Loesche, 1986). Theoretically, dental caries can be prevented by eliminating *S. mutans*.

In our continuing search for antimicrobial agents from edible plants, food spices, and beverages (Kubo et al., 1991, 1992), we reported antibacterial activity of 16 phenolic compounds, which have a C_{15} non-isoprenoid alkyl side chain with zero to three double bonds, isolated from the cashew *Anacardium occidentale* L. (Anacardiaceae) nut shell oil. Most of them exhibited potent antibacterial activity against primarily Gram-positive bacteria, among which *S. mutans* was one of the most susceptible bacteria (Himejima and Kubo, 1991).

In recent years, the cashew *A. occidentale* apple has increased in value, especially in the countries where it is grown, such as Brazil. The nut is obviously an important product of the cashew tree, but this tree also yields the pear-shaped apple to which the nut is attached. The cashew apple was neglected until recently, although it is available in far greater tonnage. A number of processes have now been developed for converting the cashew apple into various products such as juice, jam, syrup, chutney, and beverage (Winterhalter, 1991). The cashew apple juice is, for example, one of the most popular juices in Brazil today. Interestingly, among the phenolic compounds isolated from the cashew nut shell oil, 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid (1), 6-[8(*Z*),11(*Z*)-pentadecadienyl]salicylic acid (2), and 6-[8(*Z*)-pentadecenyl]salicylic acid (3), otherwise known as anacardic acids, were also identified in the cashew apple (Kubo et al., 1986), which has been continuously consumed by many people. The active principles from a regularly imbibed beverage such as the cashew apple juice may be superior as anticavity agents compared to many nonnatural products, since the use of these nonnatural products accompanies a potential risk of undesirable side effects (Fitzgerald, 1972). This is in light of the application of anticavity agents from edible plants to oral care products.

In a previous paper, we reported the antibacterial activity against *S. mutans* of these anacardic acids (1–3) with the minimum inhibitory concentrations (MICs) which ranged from 1.56 to 6.25 μg/mL (Himejima and Kubo, 1991). However, the MIC, which is determined by measuring the turbidity after 48 h of incubation, does not fully characterize their antibacterial activity. To be ideal anticaries agents, these anacardic acids should possess bactericidal activity against this cariogenic bacterium (Fitzgerald, 1972). Therefore, we have investigated their antibacterial activity against this cariogenic bacterium in more detail.

MATERIALS AND METHODS

Chemicals. The anacardic acids 1–4 (Figure 1) were previously isolated from the cashew *A. occidentale* nut shell oil (Kubo et al., 1986). Their repurification was achieved by recycle HPLC (Kubo and Nakatsu, 1991) using an ODS C_{18} column. Since the minor anacardic acid 4 was separated only in minute amounts, it was also derived by hydrogenation over Pd–C from the mixture of anacardic acids (1–4). The serial analogs of anacardic acid with the side chain of various lengths (7–11) were previously synthesized (Kubo et al., 1993). Salicylic acid (5) and 3-methylsalicylic acid (6) were purchased from Sigma Chemical Co. (St. Louis, MO). Anethole (12) and linalool (13) used for the combination studies were also from Sigma, and indole (14) was from Aldrich Chemical Co. (Milwaukee, WI). For antimicrobial assay experiments, all chemicals were first dissolved in *N,N*-dimethylformamide (DMF) that was purchased from EM Science (Gibbstown, NJ).

Microorganisms 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, Detroit) and incubated stationary for 2 days at 37 °C before the assay. Although only one strain of *S. mutans* was tested, compounds active against this strain are expected to retain a similar order of activity against a variety of strains of this species. On the other hand, the possibility of resistance among strains is always present. With these qualifications in mind, the experiments were carried out.

MIC and MBC Determinations. To evaluate MICs, a broth dilution method was performed (Kubo et al., 1992; Kubo, 1994). 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. This test broth was then inoculated with 30 μL of a 2-day-old

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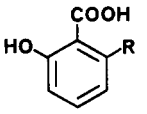

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1: $C_{15:3}$, 6-[8(Z),11(Z),14-*n*-Pentadecatrienyl]salicylic acid

2: $C_{15:2}$, 6-[8(Z),11(Z)-*n*-Pentadecadienyl]salicylic acid

3: $C_{15:1}$, 6-[8(Z)-*n*-Pentadecenyl]salicylic acid

4: $C_{15:0}$, 6-*n*-Pentadecylsalicylic acid

5: H, Salicylic acid

6: CH_3, 6-Methylsalicylic acid

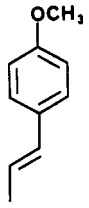
7: $C_{5:0}$, 6-*n*-Pentylsalicylic acid

8: $C_{8:0}$, 6-*n*-Octylsalicylic acid

9: $C_{10:0}$, 6-*n*-Decylsalicylic acid

10: $C_{12:0}$, 6-*n*-Dodecylsalicylic acid

11: $C_{20:0}$, 6-*n*-Eicosylsalicylic acid



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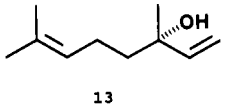

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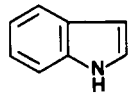

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Figure 1. Structures of natural (1–4) and synthetic (5–11) anacardic acids, anethole (12), linalool (13), and indole (14).

Table I. MICs and MBCs of Anacardic Acid (1–4) for *S. mutans* ATCC 25175

anacardic acid	MIC, μg/mL	MBC, μg/mL
1 ($C_{15:3}$)	1.56	6.25
2 ($C_{15:2}$)	3.13	3.13
3 ($C_{15:1}$)	6.25	6.25
4 ($C_{15:0}$)	>800	*a*

[a] Not tested.

culture of *S. mutans*. The highest concentration used for the assay was 800 μg/mL, unless otherwise specified, because of solubility limitation in DMF or the water-based media of some of the samples. The lowest concentration of the test compound resulting in complete inhibition of visible growth after 2 days of incubation at 37 °C represented the MIC.

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

To assess the precision of MIC and MBC procedures, all assays were carried out at least twice.

Growth Studies. Bactericidal kinetic assays were performed in BHI broth containing the appropriate concentrations of test compounds. The initial inoculum was 1–8 × 10^5 colony forming units (CFU)/mL. Samples were removed at 0, 2, 4, 8, and 24 h of incubation. The number of CFU per milliliter was determined by serial 10-fold dilutions and plating onto BHI agar. The plates were incubated at 37 °C for 2 days before counting.

Combination Studies. Combination studies were performed by time–kill studies (Norden et al., 1979; Krogstad and Moellering, 1986; Eliopoulos, 1989). A series of 2-fold dilutions of anacardic acid (1) or its analog (10) was tested in combination with 2-fold dilutions of anethole, linalool, or indole. In every case, the highest concentration of each compound added to the bacterial culture was equal to the previously determined MIC. At 0, 24, and 48 h of incubation, aliquots were removed from each tube, and the number of CFU per milliliter was determined as described above.

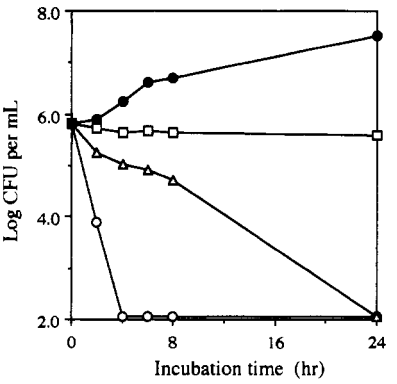

Figure 2. Bactericidal effects of $C_{15:3}$ anacardic acid (1) on *S. mutans* ATCC 25175. The number of viable cells was determined in BHI broth in the presence of 25 (○), 6.25 (△), and 1.56 μg/mL (□) of anacardic acid and was also determined in the control culture (●).

Results of the time–kill studies were interpreted as synergistic when an increase in killing of ≥2 $\log_{10}$ CFU/mL at 48 h was measured with the combination in comparison with the most active compound alone. An increased killing of 1–2 $\log_{10}$ CFU/mL was interpreted as additive. If either compound alone had an excellent bactericidal activity, the effect of the combination could not be interpreted (Norden et al., 1979; Krogstad and Moellering, 1986; Eliopoulos, 1989).

RESULTS AND DISCUSSION

All of the anacardic acids (1–3) isolated from the cashew apple possess a C_{15} alkyl side chain with one to three double bonds. 6-Pentadecylsalicylic acid (4), also known as $C_{15:0}$ anacardic acid, was isolated from the cashew nut shell oil but not identified in the cashew apple. This anacardic acid was also tested as the simplest key molecule for comparison.

The MICs and MBCs of anacardic acids 1–3 isolated from the cashew apple for *S. mutans* are listed in Table I. These anacardic acids (1–3) isolated from the cashew apple showed bactericidal activity, and MBC to MIC ratios were no greater than 4. Thus, the MBC (6.25 μg/mL) of $C_{15:3}$ anacardic acid (1) was 4-fold higher than its MIC (1.56 μg/mL). The MBC was the same as the corresponding MIC in $C_{15:2}$ anacardic acid (2) and $C_{15:1}$ anacardic acid (3).

The bactericidal activity of $C_{15:3}$ anacardic acid (1) was established by the time–kill curve method, as illustrated in Figure 2. Thus, cultures of *S. mutans*, with a cell density of 8 × 10^5 CFU/mL, were exposed to different concentrations of $C_{15:3}$ anacardic acid. The number of viable cells was determined at following different periods of incubation with 1. This anacardic acid showed MBC against *S. mutans* at 6.25 μg/mL. Moreover, this compound at 25 μg/mL (4 × MBC) rapidly killed this bacterium within 4 h of incubation.

Gellerman et al. (1969) previously reported that a decrease in the number of double bonds in the side chain decreases the antibacterial activity of anacardic acids. In addition, a similar result was observed with their molluscicidal activity (Kubo et al., 1986). Our result also indicated that the antibacterial activity of anacardic acids 1–3 against *S. mutans* slightly decreased with decreasing number of double bonds. By contrast, the MICs against this cariogenic bacterium differed largely between $C_{15:0}$ anacardic acid (4) and $C_{15:1}$ anacardic acid (3). More precisely, 4 did not exhibit any activity against this cariogenic bacterium up to 800 μg/mL, while the MIC of 3 was as low as 6.25 μg/mL (Table I).

Table II. Antibacterial Activity of Salicylic Acid (5) and 6-Alkylsalicylic Acids (6–11) against *S. mutans* ATCC 25175

compound	MIC, μg/mL	compound	MIC, μg/mL
salicylic acid (5)	>800	6-*n*-decylsalicylic acid (9)	3.13
6-methylsalicylic acid (6)	>800	6-*n*-dodecylsalicylic acid (10)	1.56
6-*n*-pentylsalicylic acid (7)	200	6-*n*-pentadecylsalicylic acid (4)	>800
6-*n*-octylsalicylic acid (8)	50	6-*n*-eicosylsalicylic acid (11)	>800

Table III. Number of CFU of *S. mutans* after a 48-h Exposure to Anacardic Acid (1) and Anethole (12) or Linalool (13)[a]

		$\log_{10}$ CFU/mL at following anacardic acid (1) concn			
compound	concn, μg/mL	0 μg/mL	0.39 μg/mL	0.78 μg/mL	1.56 μg/mL
none		7.9	7.7	7.0	4.3
anethole	50	7.8	*b*	6.7	2.6 (a)[c]
	100	8.1	*b*	2.8 (s)	<2.0 (s)
	200 (MIC)	4.6	<2.0 (s)	<2.0 (s)	<2.0 (s)
linalool	400	7.5	*b*	5.9 (a)	<2.0 (s)
	800	7.0	3.9 (s)	<2.0 (s)	<2.0 (s)
	1600 (MIC)	<2.0	<2.0	<2.0	<2.0

[a] Initial inoculum was 5.5 ($\log_{10}$ CFU/mL). [b] Not tested. [c] (a) and (s): the combination was interpreted as additive (a) or synergistic (s). See Materials and Methods.

Table IV. Number of CFU of *S. mutans* after a 48-h Exposure to 6-*n*-Dodecylsalicylic Acid (10) and Anethole (12)[a]

	$\log_{10}$ CFU/mL at following 6-*n*-dodecylsalicylic acid (10) concn			
anethole concn, μg/mL	0 μg/mL	0.39 μg/mL	0.78 μg/mL	1.56 μg/mL
0	7.9	6.8	7.4	3.3
50	7.8	*b*	4.0 (s)[c]	<2.0
100	8.1	5.4 (a)	<2.0 (s)	<2.0
200 (MIC)	4.6	<2.0 (s)	<2.0 (s)	<2.0

[a] Initial inoculum was 5.0 ($\log_{10}$ CFU/mL). [b] Not tested. [c] (a) and (s): the combination was interpreted as additive (a) or synergistic (s).

To investigate the relationships between the antibacterial activity and the alkyl side-chain lengths of anacardic acids, this simplest $C_{15:0}$ anacardic acid (4) was selected as a standard, despite of the lack of potency in the activity against *S. mutans*. This anacardic acid is the most stable, and more importantly, the synthesis of its various derivatives can be easily accomplished according to the method previously reported (Yamagiwa et al., 1987).

The MICs of salicylic acid (5) and a series of anacardic acid analogs with various side-chain lengths (6–11) for *S. mutans* are shown in Table II. The maximum activity against this bacterium occurred at the C_{12} side-chain length with a MIC of 1.56 μg/mL. This potency is comparable with that of 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid (1), the most active anacardic acid isolated from the cashew apple. This result indicates that the activity is affected not only by the degree of unsaturation in the side chain but also by the lengths of the side chain. Interestingly, the activity dropped off rapidly against this cariogenic bacterium above C_{12}. Thus, as mentioned above, anacardic acid 4 possessing a C_{15} alkyl side chain no longer exhibited any activity up to 800 μg/mL. It is evident from these results that the mechanism of bactericidal action of anacardic acids against *S. mutans* is due to a balance between hydrophilic and hydrophobic parts of the molecule, similar to their cytotoxic activity (Itokawa et al., 1989).

Our data indicated that the antibacterial activity of anacardic acids 1–3 alone against *S. mutans* may be potent enough to be considered for practical use. However, combining more than two compounds is generally thought to produce a more favorable outcome to obtain the enhancement of each antimicrobial activity, as well as to retard the development of resistance mechanisms in microorganisms. Because of these concerns, we attempted to enhance the antibacterial activity, especially the bactericidal activity of $C_{15:3}$ anacardic acid (1) against *S. mutans*, through combination with other natural substances. We also tested 6-*n*-dodecylsalicylic acid (10), the most effective compound among the synthetic analogs of anacardic acid, for this combination study.

We first combined $C_{15:0}$ anacardic acid (1) with indole (14), a green tea flavor compound. In our previous study, indole was found to enhance the antibacterial activity of δ-cadinene and β-caryophyllene, sesquiterpene hydrocarbons identified in the same green tea flavor, against *S. mutans* (Kubo et al., 1992). However, no synergism was observed with this combination. The combination was at most additive (an increased killing of 1.5 $\log_{10}$ CFU/mL) when 800 μg/mL of indole and 1.56 μg/mL of 1 were added together to *S. mutans* culture.

$C_{15:3}$ anacardic acid (1) was also combined with anethole (12) and linalool (13), two antibacterial flavor compounds. We previously reported that anethole isolated from the seed of *Pimpinella anisum* (Umbelliferae) and linalool identified in green tea flavor exhibited moderate antibacterial activity against *S. mutans* (Kubo and Himejima,

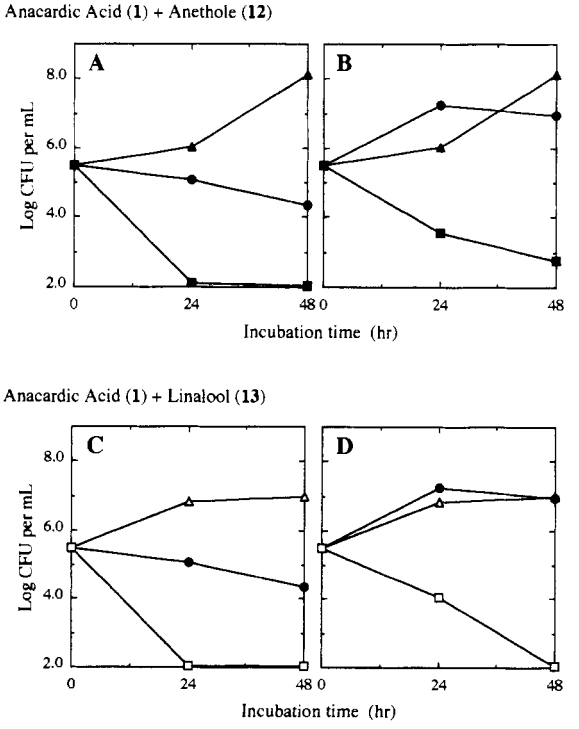

Figure 3. Time–kill curves of *S. mutans* showing synergism between $C_{15:3}$ anacardic acid (1) and anethole (12) and between 1 and linalool (13). Compounds were added to *S. mutans* cultures at the following concentrations: (A, B) 1.56 (A) or 0.78 μg/mL (B) of 1 (●) and 100 μg/mL of anethole (▲) alone and in combination (■); (C, D) 1.56 (C) or 0.78 μg/mL (D) of 1 (●) and 800 μg/mL of linalool (△) alone and in combination (□). The number of viable cells was determined after 24 and 48 h of incubation.

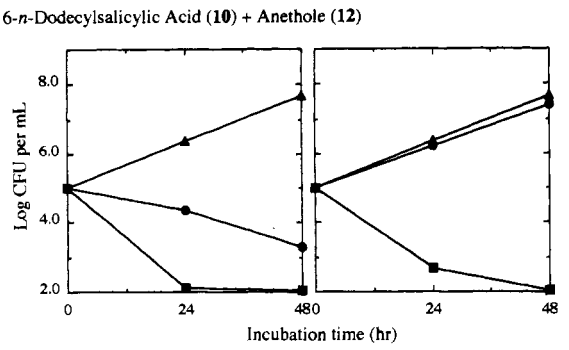

Figure 4. Time–kill curves of *S. mutans* showing synergism between 6-*n*-dodecylsalicylic acid (**10**) and anethole (**12**). The number of viable cells was counted in BHI broth containing 1.56 (left) or 0.78 μg/mL (right) of **10** (●) and 100 μg/mL of anethole (▲) alone and in combination (■).

1991; Kubo et al., 1992). In addition, both compounds have been used as flavor ingredients in many foods and beverages (Furia and Bellanca, 1975). As shown in Table III, the combinations acted synergistically against *S. mutans* when they were carried out at the following concentrations: 100 μg/mL of anethole and 0.78 μg/mL (or higher) of **1**, 200 μg/mL of anethole and 0.39 μg/mL (or higher) of **1**, 400 μg/mL of linalool and 1.56 μg/mL of **1**, 800 μg/mL of linalool and 0.39 μg/mL (or higher) of **1**. The time–kill curves proved this synergism more clearly (Figure 3). For example, the combination of **1** at 0.78 μg/mL with 100 μg/mL of anethole exhibited synergy, with an increased killing of 4.2 $\log_{10}$ CFU/mL after 48 h of incubation. The combination with **1** (0.78 μg/mL) and linalool (800 μg/mL) also exhibited synergy and was found to be bactericidal by the time–kill curve for *S. mutans*.

Similarly, the combination of 0.78 μg/mL of 6-*n*-dodecylsalicylic acid (**10**) with 100 μg/mL of anethole (**12**) was also synergistic (Table IV) and proved to be bactericidal against *S. mutans* by the time–kill curve method (Figure 4).

In addition to their potent antibacterial activity against *S. mutans*, another benefit of these anacardic acids is their antiplaque activity. However, because of their potent antibacterial activity against this cariogenic bacterium, we were unable to study further details of this antiplaque activity.

Overall, the anacardic acids (**1–3**) and some of their synthetic analogs seem to satisfy most of the requirements as proposed by Fitzgerald (1972) to make them the ideal anticaries agents.

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