Antimicrobial Activity of Cashew Apple Flavor Compounds

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The antimicrobial activity of the 10 most abundant flavor compounds of the cashew *Anacardium occidentale* (Anacardiaceae) apple has been tested. Most of them exhibited some activity against one or more of 14 microorganisms. Noticeably, (*E*)-2-hexenal showed activity against all of the microorganisms tested, including Gram-negative bacteria. In addition, the antibacterial activity of indole against *Escherichia coli* was enhanced 4-fold by combining it with a sublethal amount of (*E*)-2-hexenal. Furthermore, the minimum inhibitory concentration of this combination was found to be bactericidal by the time-kill curve method.

INTRODUCTION

As our search for antimicrobial agents from tropical fruits and vegetables continues, we recently reported the isolation of antibacterial nonisoprenoid alkyl side chain phenolic compounds such as anacardic acids, cardols, methylcardols, and cardanols from the cashew *Anacardium occidentale* L. (Anacardiaceae) nut shell oil (Himejima and Kubo, 1991). Interestingly, among these phenolics isolated, the three anacardic acids, 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**), were also previously identified in the cashew *A. occidentale* apple (Kubo et al., 1986), which has been a dietary constituent consumed by many people. In recent years the cashew 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 relatively neglected until recently, although it is available in far greater tonnage. A number of processes have now been developed for converting the apple into various products such as juice, jam, syrup, chutney, and beverage (Winterhalter, 1991). In fact, cashew apple juice is now one of the most popular juices in Brazil.

The above-mentioned anacardic acids (**1**–**3**) were found to exhibit potent antibacterial activity against Gram-positive bacteria and weak antifungal activity against molds (Himejima and Kubo, 1991). Since the need for new antimicrobial agents persists, these anacardic acids may be considered for practical use. Besides the anacardic acids, the cashew apple may contain other antimicrobial principles since isolation of the anacardic acids was not guided by an antimicrobial assay. The active principles from a regularly imbibed beverage like cashew apple juice may be superior as antimicrobial agents as compared to many nonnatural products. This prompted its further investigation. The cashew apple is desirable because of its specific aroma impression, in addition to its high vitamin C content (Cecchi and Rodriguez-Amaya, 1981). Since the antimicrobial activity of its aroma components has not yet been investigated, we examined the antimicrobial activity of the 10 major aroma components of the cashew apple.

MATERIALS AND METHODS

Chemicals. Anacardic acid (**1**) was from our previous study (Kubo et al., 1986). The authentic car-3-ene (**4**), (*E*)-2-hexenal (**6**), furfural (**7**), hexanal (**8**), benzaldehyde (**9**), nonanal (**10**), 2-methylpentan-1-ol (**11**), and indole (**15**) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Limonene (**5**), α-terpinene (**12**), and β-caryophyllene (**13**) were purchased from Sigma Chemical Co. (St. Louis, MO). Vitamin C was obtained from ICN Biochemicals, Inc. (Costa Mesa, CA). For the antimicrobial assays, all chemicals were first dissolved in *N*,*N*-dimethylformamide (DMF) which was purchased from EM Science (Gibbstown, NJ).

Microorganisms and Media. All test microorganisms were purchased from American Type Culture Collection (Rockville, MD). They are *Bacillus subtilis* ATCC 9372, *Brevibacterium 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, *Proteus vulgaris* ATCC 13315, *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 (NYG broth), 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 shake-cultured at 30 °C. *B. ammoniagenes* and *E. aerogenes* were stationarily cultured at 30 °C. All other microorganisms were stationarily cultured at 37 °C.

MIC and MBC Determinations. The minimum inhibitory concentration (MIC) was measured by the 2-fold serial broth dilution method as previously described (Taniguchi and Satomura, 1972; Kubo et al., 1992a). 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 a 2-day-old culture of the test microorganisms (5-day-old culture of *P. chrysogenum* and *T. mentagrophytes*) was inoculated. After 2 days of cultivation (3 days for *P. ovale* and 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 was 800 μg/mL, unless otherwise specified, because of solubility limitations in the water-based media of some of the samples.

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The minimum bactericidal concentration (MBC) was the lowest concentration of the test compound that decreased the initial inoculum by >99.9%. After the MIC was determined, 10-fold dilutions from each test tube showing no turbidity were plated onto the chemical-free, appropriate agar medium. After 18–48 h of incubation, MBC break points were determined by using rejection values as previously described (Pearson et al., 1980).

Time–Kill Curve Studies. Bactericidal kinetic assay for *E. coli* was performed in NYG broth containing the appropriate concentrations of (*E*)-2-hexenal (6). The initial inoculum was approximately 1×10^7 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 NYG agar. The plates were incubated at 30 °C for 18–24 h before counting.

Combination Studies. The combination data were obtained by a broth checkerboard method (Norden et al., 1979). The 2-fold dilutions of (*E*)-2-hexenal (6) were tested in combination with concentrations of 2-fold dilutions of the other. The MICs and MBCs were determined by using the same method as described above. The tests were repeated at least twice.

To confirm the finding of the checkerboard experiments with the (*E*)-2-hexenal and indole (15) combination on *E. coli*, time–kill curves were established. A series of 2-fold dilutions of indole was tested in combination with 200 μg/mL (subinhibitory concentration) of (*E*)-2-hexenal. 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.

RESULTS AND DISCUSSION

The aroma components of the fresh cashew apple have been previously reported. Thus, we selected from the list reported (MacLeod and de Troconis, 1982; Maciel et al., 1986) for our study the 10 most abundant aroma compounds identified in the cashew apple, namely car-3-ene (4), limonene (5), (*E*)-2-hexenal (6), furfural (7), hexanal (8), benzaldehyde (9), nonanal (10), 2-methylpentan-1-ol (11), α-terpinene (12), and β-caryophyllene (13) (Figure 1) in decreasing concentration. Among them, hexanal, car-3-ene, limonene, (*E*)-2-hexenal, and benzaldehyde were described as being important for the odor quality (Winterhalter, 1991). Interestingly, among the 10 major aroma compounds, 5 were aldehyde compounds.

Throughout this experiment the broth dilution method was used despite its time-consuming disadvantage. This was because these aroma compounds are all volatile and, more importantly, water-insoluble (Himejima et al., 1992; Kubo et al., 1992c).

The above-mentioned 10 aroma components (4–13) were tested against 14 selected microorganisms. The activities of the individual components are listed in Table I. Among the Gram-positive bacteria tested, *P. acnes* was the most sensitive bacterium. Thus, seven of the compounds tested showed activity against this bacterium with MICs ranging from 6.25 to 800 μg/mL. Among them, β-caryophyllene was the most potent against this bacterium with an MIC of 6.25 μg/mL, while hexanal was the least with an MIC of 800 μg/mL. By contrast to *P. acnes*, *S. aureus* was the least sensitive Gram-positive bacterium. Thus, only (*E*)-2-hexenal and nonanal showed weak activity with MICs of both being 400 μg/mL. Furfural, 2-methylpentan-1-ol, and benzaldehyde did not show any activity against the bacteria tested up to 800 μg/mL but did show some activity against fungi.

Most noticeably, in this experiment, (*E*)-2-hexenal (6) exhibited antimicrobial activity against all of the microorganisms tested including four Gram-negative bacteria, *P. aeruginosa*, *E. aerogenes*, *E. coli*, and *P. vulgaris*, with MICs of either 200 or 400 μg/mL. This is interesting since few phytochemicals exhibit activity against Gram-negative bacteria, especially the *Pseudomonas* species. (*E*)-2-

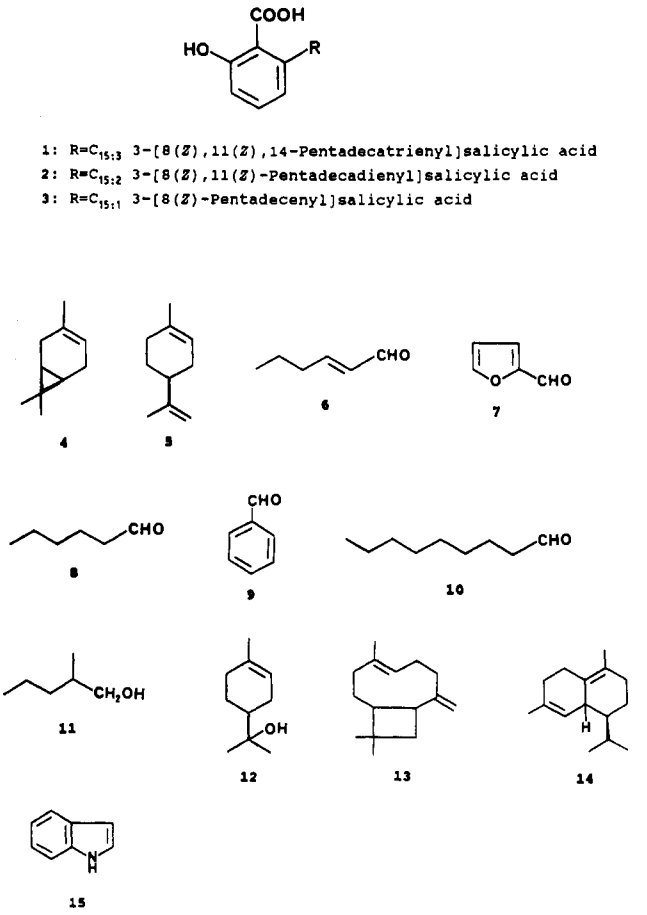

Figure 1. Structures of the anacardic acids (1–3), 10 major flavor compounds (4–13) from the cashew apple, δ-cadinene (14), and indole (15).

Table I. Antimicrobial Activity of the Volatile Flavor Components of Cashew Apple

	MIC against microorganisms[a] tested, μg/mL									
	4[b]	5[b]	6[b]	7[b]	8[b]	9[b]	10[b]	11[b]	12[b]	13[b]
Bs	>800	800	400	>800	>800	>800	400	>800	>800	50
Ba	>800	>800	400	>800	>800	>800	400	>800	>800	100
Sa	>800	>800	400	>800	>800	>800	400	>800	>800	>800
Sm	50	100	400	>800	>800	>800	>800	>800	100	>800
Pac	50	50	50	>800	800	>800	100	>800	50	6.25
Pae	>800	>800	400	>800	>800	>800	>800	>800	>800	>800
Ea	>800	>800	400	>800	>800	>800	>800	>800	>800	>800
Ec	>800	>800	400	>800	>800	>800	>800	>800	>800	>800
Pv	>800	400	200	>800	>800	>800	100	>800	400	>800
Sc	50	50	200	>800	>800	>800	100	>800	50	>800
Cu	100	200	100	>800	>800	>800	100	>800	100	>800
Po	>800	200	50	400	800	>800	100	800	800	>800
Pc	–[c]	>800	50	100	>800	200	400	>800	>800	>800
Tm	–	–	50	400	>800	800	100	>800	>800	>800

[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*; Pv, *P. vulgaris*; Sc, *S. cerevisiae*; Cu, *C. utilis*; Po, *P. ovale*; Pc, *P. chrysogenum*; Tm, *T. mentagrophytes*. [b] 4, Car-3-ene; 5, limonene; 6, (*E*)-2-hexenal; 7, furfural; 8, hexanal; 9, benzaldehyde; 10, nonanal; 11, 2-methylpentan-1-ol; 12, α-terpinene; 13, β-caryophyllene. [c] –, not tested.

Hexenal is one of the rare phytochemicals that exhibited antibacterial activity against *P. aeruginosa*. In addition, (*E*)-2-hexenal exhibited broad antimicrobial activity. The activity ranging from 50 to 400 μg/mL may not be potent enough to be used exclusively for the control of specific microorganisms but is broad enough to be utilized as preservatives, disinfectants, *etc.* Its strong odor may need to be masked with other fragrance or flavor compounds. However, (*E*)-2-hexenal, known as "leaf aldehyde", is

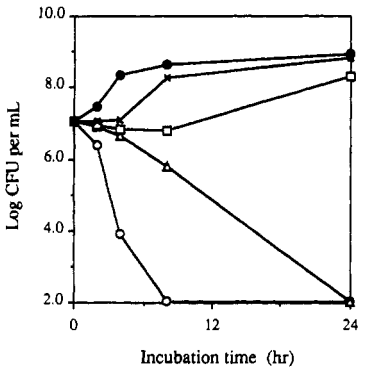

Figure 2. Bactericidal effect of (*E*)-2-hexenal (6) on growth of *E. coli* ATCC 9637. A 48-h culture was inoculated into the NYG broth containing 800 (○), 400 (△), 200 (□), and 100 μg/mL (×) (*E*)-2-hexenal and without (*E*)-2-hexenal (●) (control).

characterized by a pronounced green odor of leaves. Thus, it smells pleasantly green and apple-like (Bauer et al., 1990). This volatile compound is also found in many vegetables, fruits, and beverages (Renold et al., 1974; Bauer et al., 1990; Etiévant, 1991; Nykänen and Nykänen, 1991; Flament, 1991). Interestingly, both hexanal (8) and nonanal (10) isolated from the same source did not show any significant activity compared to (*E*)-2-hexenal. It seems that the enal group is essential to have biological activity.

The MIC, which is determined by measuring the turbidity after 48 h of incubation, does not fully characterize the antimicrobial activity of a sample. Therefore, we have investigated the antibacterial activity of (*E*)-2-hexenal against *E. coli* in more detail using the time–kill curve method. Thus, cultures of *E. coli*, with a cell density of 1×10^7 CFU/mL, were exposed to different concentrations of (*E*)-2-hexenal. The number of viable cells was determined following different periods of incubation. As illustrated in Figure 2, the MIC of (*E*)-2-hexenal is confirmed to be the MBC. Thus, its MIC of 400 μg/mL against *E. coli* was found to be bactericidal. Moreover, this compound at 800 μg/mL (2 × MIC), rapidly killed *E. coli* cells within 8 h of incubation.

The yield of the aroma components obtained by steam distillation from 1 kg of the Venezuelan fresh cashew apple was reported at about 3.6 μg (MacLeod and de Troconis, 1982). If this is the case, the concentration does not seem to be strong enough to control any microorganisms, particularly fungi, even if the aroma component of the cashew apple was assumed to consist of only (*E*)-2-hexenal, the most potent antimicrobial substance among the 10 aroma compounds tested. However, the cashew apple contains many other chemicals such as the aforementioned antibacterial anacardic acids (1–3). The combination of these substances may enhance the total antimicrobial activity (Himejima and Kubo, 1992; Kubo et al., 1992b, 1993; Kubo and Taniguchi, 1988).

The application of phytochemicals as antimicrobial agents has produced a keen interest. Needless to say, they are all biodegradable and, more importantly, renewable. However, their biological activity is usually not potent enough to be considered for practical applications. The moderate activity of (*E*)-2-hexenal is an example of this lack of potency. For these concerns, an attempt to enhance the activity of (*E*)-2-hexenal by combining it with other substances was made. The combination of more than two compounds may, in addition to enhancing and broadening the total activity, be superior to the use of a single antimicrobial compound. More importantly, it may also hinder the development of resistant mechanisms in microorganisms (Neu, 1992).

We first combined (*E*)-2-hexenal with vitamin C, known as an antioxidant, to retard oxidative destruction of this molecule, which possesses an easily oxidizable enal group (Kubo and Himejima, 1992). The rationale of this combination was based on the fact that oxidation is, in general, one of the most important detoxification (or metabolic) pathways in living organisms. More importantly, this combination was also suggested by the fact that the cashew apple contains a large amount of vitamin C. Thus, its ascorbic acid (vitamin C) content is reported to be greater than 6 times that for oranges (Cecchi and Rodriguez-Amaya, 1981). In addition, vitamin C (antioxidant) was found to enhance the antifungal activity of polygodial 16-fold against a dermatomycotic fungus *Pityrosporum ovale* (Kubo and Himejima, 1992). Therefore, this combination was tested against *P. ovale* in addition to *S. mutans* and *S. aureus*. The combination effects were studied by the broth checkerboard method (Norden, 1979). The combination effect against *P. ovale* was only additive, and there was no effect against the two bacteria.

(*E*)-2-Hexenal was also combined with anacardic acid (1) which was isolated from the same cashew apple and found to exhibit potent antibacterial activity against Gram-positive bacteria. Therefore, we tested this combination against two Gram-positive bacteria, *S. mutans* and *S. aureus*, keeping oral care products in mind since both anacardic acid and (*E*)-2-hexenal were isolated from the same edible cashew apple. However, no synergism was observed with this combination. The combination was only additive as far as the MIC was concerned. The MBC of anacardic acid (1) against *S. mutans* was reduced 4-fold by combining with half-MIC of (*E*)-2-hexenal. Thus, the MBC was lowered from 6.25 to 1.56 μg/mL (data not shown).

In addition, the combination study with (*E*)-2-hexenal was accomplished against Gram-negative bacteria, since (*E*)-2-hexenal is one of the rare phytochemicals which exhibited activity against them, especially *P. aeruginosa*. In our previous study of antimicrobial activity of green tea flavor compounds, indole was found to enhance the antibacterial activity of δ-cadinene (14) 128-fold against *S. mutans*. Thus, the MIC was lowered from 800 to 6.25 μg/mL by combining it with a sublethal amount of indole (15) (Kubo et al., 1992c). More importantly, indole was also found to exhibit activity against Gram-negative bacteria, including *P. aeruginosa*, with an MIC of 800 μg/mL. Therefore, the combination of (*E*)-2-hexenal with indole was tested against two Gram-negative bacteria, *P. aeruginosa* and *E. coli*. Indole did not increase the activity of (*E*)-2-hexenal against *P. aeruginosa* or *E. coli*. The combination effect was only additive. However, (*E*)-2-hexenal enhanced the activity of indole against *E. coli* 4-fold. Its MIC was lowered from 800 to 200 μg/mL by combining it with 200 μg/mL (=half-MIC) of (*E*)-2-hexenal. In addition, the MIC value of this combination was confirmed as the MBC by the time–kill curve method as shown in Figure 3.

Although the cashew apple contains various antimicrobial principles such as anacardic acids and volatile compounds described in this paper, it is easily spoiled in nature, especially by fungal infection. This limits its use only to local utilization and prevents the exportation of the fresh fruit into other countries. The reason for this is that the activity of the antimicrobial principles is not potent enough for defense from fungal attack since they were found in the fresh apple in only minute concentra-

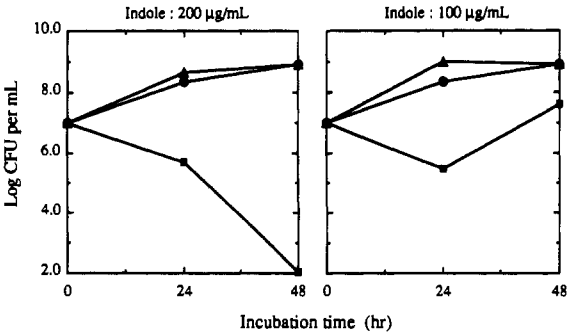

Figure 3. Combination effects of indole with half-MIC of (*E*)-2-hexenal against *E. coli* ATCC 9637. A 48-h culture was inoculated into the NYG broth containing 200 μg/mL (*E*)-2-hexenal (●) and 200 (left) or 100 (right) μg/mL indole (▲) alone and in combination (■).

tions. The concentrations of antifungal compounds such as (*E*)-2-hexenal, furfural, benzaldehyde, and 2-methylpentan-1-ol in the apple are not enough to protect the fruit from fungal attack. However, having found the antimicrobial activities of these compounds, they may be applied for use in products such as cosmetics and disinfectants. In addition to their antimicrobial activities, another benefit of these volatile compounds includes the addition of fragrance to the products.

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