Naturally Occurring Anti-*Salmonella* Agents

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Polygodial and (2*E*)-hexenal were found to possess antibacterial activity against *Salmonella choleraesuis* with the minimum bactericidal concentrations (MBC) of 50 μg/mL (0.17 mM) and 100 μg/mL (0.98 mM), respectively. The time kill curve study showed that these two α,β-unsaturated aldehydes were bactericidal against this food-borne bacterium at any stage of growth. However, they showed different effects on the growth of *S. choleraesuis*. The combination of polygodial and anethole exhibited strong synergism on their bacteriostatic action but only marginal synergism on their bactericidal action.

Keywords: *Salmonella choleraesuis; bactericidal activity; polygodial; (2E)-hexenal; anethole; surfactant*

INTRODUCTION

Salmonellosis is the most frequently occurring bacterial food-borne illness. It results following the ingestion of viable cells of a member of the genus *Salmonella*. Over 2500 serovars of *Salmonella* exist, all of which are pathogenic for humans. The salmonellae are Gram-negative non-spore-forming rods that ferment glucose, usually with gas, but usually do not ferment lactose or sucrose. In general, Gram-negative bacteria easily develop resistance to commonly used antibiotics because of their adaptability (*1*, *2*). This resistance involves the enzymic inactivation of antibiotics in resistant bacteria (*3*), and such resistant genes are often transferred to other bacteria by a variety of gene transfer mechanisms. Hence, there is a great need for effective antibacterial agents with new modes of action. Phytochemicals have the potential of filling this need because their structures are different from those of the more-studied microbial sources; therefore, their modes of action may very likely differ. The selected phytochemicals from our previous studies were tested for their anti-*Salmonella* activity. To search for anti-*Salmonella* agents, *Salmonella choleraesuis* was selected because this bacterium most frequently causes septicemia, although septicemia can be caused by any *Salmonella* (*4*).

The study to search for anti-*Salmonella* agents was initiated by the request to solve the problem of contamination in the fruit of *Piper nigrum* (Piperaceae), commonly known as pepper, by *Salmonella* in the Amazon basin. On the basis of the preliminary survey, this contamination has likely been caused by increased large-scale poultry farms around the area, because important sources of *Salmonella* contamination for foods are poultry and rodents. For example, chickens may be infected with any number of types of *Salmonella*, which are then found in their fecal matter. Rodents, rats, mice, and bats exposed to the chicken feces may then contaminate unprotected pepper with their feces and thus spread *Salmonella* bacteria (*4*).

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In our preliminary screening, several phytochemicals previously characterized as antibacterial agents against Gram-negative bacteria were found to exhibit antibacterial activity against *S. choleraesuis*. Therefore, further evaluation of these compounds may provide new insights into their antibacterial action on a molecular basis.

MATERIALS AND METHODS

Chemicals. Polygodial and anethole (*5*), farnesol (*6*), and (2*E*)-hexenal (*7*) were available from our previous works. Chloramphenicol, gentamycin, and sorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO). All other compounds tested in the antimicrobial assay were obtained from Aldrich Chemical Co. (Milwaukee, WI). For the experiment, all the compounds were first dissolved in *N*,*N*-dimethylformamide (DMF) which was purchased from EM Science (Gibbstown, NJ). The concentration of DMF in each medium was always 1%. The highest concentration tested was 1600 μg/mL.

Test Strains. The test strains, *Salmonella choleraesuis* ATCC 35640, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, and *Proteus vulgaris* ATCC 13315, used for this study were purchased from American Type Culture Collection (Rockville, MD).

Medium. NYG broth (0.8% nutrient broth, 0.5% yeast extract, 0.1% glucose) was used for the antibacterial assay. Nutrient broth was obtained from BBL Microbiology System (Cockeysville, MD). Yeast extract was purchased from Difco Lab (Detroit, MI).

Antibacterial Assay. Broth macrodilution methods were used as previously described (*5*−*7*) with slight modifications. Briefly, serial 2-fold dilutions of the test compounds were prepared in DMF, and 30 μL of each dilution was added to 3 mL of NYG broth. These were inoculated with 30 μL of an overnight culture of *S. choleraesuis*. After incubation of the cultures at 37 °C for 48 h, the minimum inhibitory concentration (MIC) was determined as the lowest concentration of the test compound that demonstrated no visible growth. The minimum bactericidal concentration (MBC) was determined as follows. After the determination of the MIC, 100-fold dilutions with drug-free NYG broth from each tube showing no turbidity were incubated at 37 °C for 48 h. The MBC was the lowest concentration of the test compound that showed no visible growth in the drug-free cultivation. The antibacterial assay of (2*E*)-hexenal against *Salmonella typhimurium*, *Kleb-*

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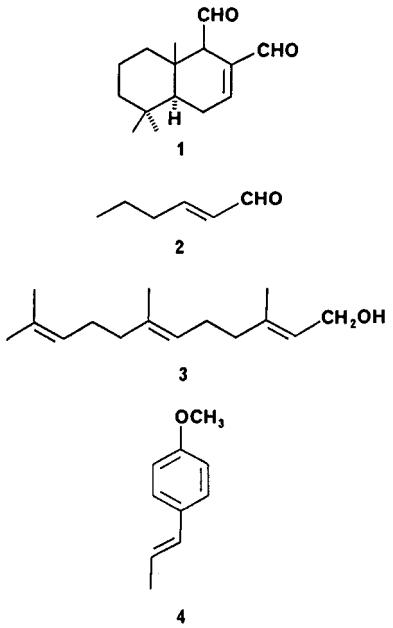

Figure 1. Chemical structures of polygodial (**1**), (2*E*)-hexenal (**2**), farnesol (**3**), and anethole (**4**).

Table 1. Antibacterial Activity (μg/mL) of the Selected Compounds against *Salmonella choleraesuis*

compounds tested	MIC[a]	MBC[a]
(2*E*)-hexenal	100	100
hexanal	400	800
hexanol	>1600	>1600
hexanoic acid	400	400
sorbic acid	400	400
benzoic acid	800	800
anisic acid	800	800
polygodial	50	50
geraniol	400	800
farnesol	12.5	400
anethole	200	200
eugenol	400	400
indole	800	800
gentamycin	12.5	12.5

[a] −, Not tested.

siella pneumonia, and *Helicobacter pylori* was also carried out by Panlabs (Taipei, Taiwan) and the MICs obtained were 400, 800, and 800 μg/mL, respectively.

Combination studies were performed by a broth checkerboard method (*8*). A series of 2-fold dilutions of one compound were tested in combination with 2-fold dilutions of the other compounds. The assays were performed in triplicate.

Time Kill Study. The cultivation of bacteria with each compound was done in the same manner as that described above for MIC. Samples were withdrawn at selected time points, and serial dilutions were performed in sterile saline before the samples were plated onto NYG agar plates. After the plates were incubated at 37 °C for 16 h, colony-forming units (CFU) were determined.

RESULTS

In a preliminary screening, the 10 phytochemicals selected from our previous studies and their structurally related compounds were tested for their antibacterial activity against *S. choleraesuis*. Among them, polygodial (**1**) (Figure 1), a bicyclic sesquiterpene dialdehyde, was found to possess the most potent activity against this food-borne bacterium, followed by (2*E*)-hexenal (**2**), an aliphatic α,β-unsaturated aldehyde (Table 1). These two aldehydes were further studied in detail. Polygodial

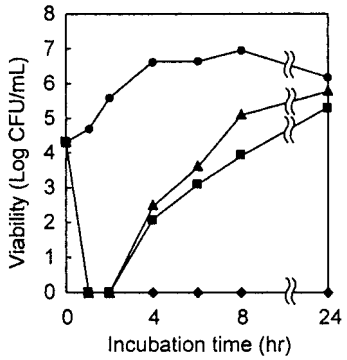

Figure 2. Bactericidal effect of polygodial against *S. choleraesuis*. Exponentially growing cells of *S. choleraesuis* were inoculated at 37 °C in NYG broth with 0 (●), 12.5 (▲), 25 (■), or 50 (◆) μg/mL of polygodial. Viability was established by the number of colonies formed on NYG plate after incubation at 30 °C for 24 h.

exhibited the activity with both MIC and MBC of 50 μg/mL (0.17 mM), suggesting that no residual bacteriostatic activity is involved. This sesquiterpene dialdehyde was first isolated as a pungent principle from sprouts of *Polygonum hydropiper* (Polygonaceae) (*9*), known as "tade" and used as a food spice in Japan. Subsequently it was isolated from two East African *Warburgia* trees, *W. ugandensis* and *W. stuhlmannii* (*10*), and these two plants are also locally used as food spice (*11*). The potent fungicidal activity of polygodial, especially against yeasts such as *Candida albicans* and *Saccharomyces cerevisiae*, was subsequently reported, although it possessed little or no activity against several bacteria (*12, 13*). In the current experiment, polygodial did not exhibit any antibacterial activity against the four common Gram-negative bacteria tested, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Proteus vulgaris* up to 800 μg/mL. Hence, the result obtained against *S. choleraesuis* was unexpected. It should be added that farnesol (**3**), a common sesquiterpene alcohol, was noted to exhibit the most potent activity against *S. choleraesuis* with a MIC of 12.5 μg/mL. However, lethality was needed at 400 μg/mL, suggesting that residual bacteriostatic activity is involved.

The bactericidal activity of polygodial against *S. choleraesuis* was confirmed by the time kill curve experiment as shown in Figure 2. Cultures of *S. choleraesuis*, with a cell density of 4.4×10^4 CFU/mL, were exposed to three different concentrations of polygodial. The number of viable cells was determined following different periods of incubation with polygodial. It shows that MIC significantly reduced the growth rate, but that the final cells recover count was not different from that of the control. It should be noted that lethality occurred quickly, within the first 1 h after adding polygodial. This rapid lethality very likely indicates that the antibacterial activity of polygodial against *S. choleraesuis* is associated with the disruption of the membrane, similar to its effect found against *S. cerevisiae* (*14*).

The potency of the antibacterial activity against *S. choleraesuis* was followed by (2*E*)-hexenal with both MIC and MBC of 100 μg/mL (0.98 mM), indicating that no residual bacteriostatic activity was involved. In contrast to polygodial, (2*E*)-hexenal exhibits broad antimicrobial activity (*15*). For example, its antibacterial activity against *E. coli*, *P. aeruginosa*, *E. aerogenes*, and *P. vulgaris* (*7*), as well as *H. pylori* (*16*), was previously reported. Because of this broad antimicrobial activity

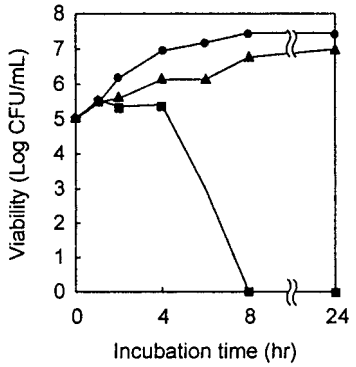

Figure 3. Bactericidal effect of (2*E*)-hexenal against *S. choleraesuis*. Exponentially growing cells of *S. choleraesuis* were inoculated at 37 °C in NYG broth with 0 (●), 50 (▲), or 100 (■) μg/mL of (2*E*)-hexenal. Viability was established by the number of colonies formed on NYG plate after incubation at 30 °C for 24 h.

and availability, this aliphatic α,β-unsaturated aldehyde, known as "leaf aldehyde" (*17*) and widely distributed in many edible plants (*18*), was studied in more detail. In our continuing search for antimicrobial agents from edible plants, (2*E*)-hexenal was previously characterized as an antimicrobial agent from the volatile fraction of the cashew apple (*19*) and olive oil (*15*). In contrast to (2*E*)-hexenal, hexanol did not show any activity up to 1600 μg/mL, but hexanal and hexanoic acid still exhibited some activity, though to a lesser extent than (2*E*)-hexenal. Thus, the conjugated double bond is not essential to elicit the activity although it increases it.

The MIC and MBC values against *S. choleraesuis* were noted to be variable to some degree. The maximum extent and rate of antimicrobial activity are known to vary with the experimental conditions such as the seed culture mediums, the physiological age of the culture, and the type of culture medium. The variation observed may be caused in part by volatilization of the test compounds from the test medium during the incubation time. This postulate can be supported by the observation that concentrations of the nonvolatile compounds tested, such as polygodial, were relatively constant compared to that of volatile (2*E*)-hexenal. Nonetheless, the activity against *S. choleraesuis* seems to be more affected in general by experimental conditions than other microorganisms.

The bactericidal effect of (2*E*)-hexenal was also confirmed by the time kill curve experiment as shown in Figure 3. Cultures of *S. choleraesuis*, with a cell density of 1×10^5 CFU/mL, were exposed to two different concentrations of (2*E*)-hexenal. The number of viable cells was determined following different periods of incubation with (2*E*)-hexenal. The result verifies that MIC and MBC are the same. It shows that $^1/_2$MIC slowed the growth rate, but that the final cell count was not significantly different from that of the control. Lethality occurred slower than with polygodial. The result obtained indicates that the mode of antibacterial actions of polygodial and (2*E*)-hexenal against *S. choleraesuis* differ to some extent.

The effects of (2*E*)-hexenal against *S. choleraesuis* were further tested during holding viable cell number in the presence of chloramphenicol which is known to restrict cell division by inhibiting protein synthesis. Figure 4 shows that the effect of this antibiotic against *S. choleraesuis* cells is bacteriostatic for the first 3 h of

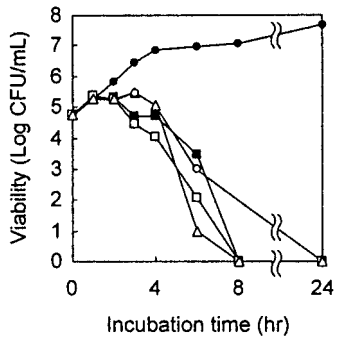

Figure 4. Effect of (2*E*)-hexenal in the presence of chloramphenicol against *S. choleraesuis*. Exponentially growing cells were inoculated into NYG broth and then cultured at 37 °C. Chloramphenicol at 0 μg/mL (●) or 6.25 μg/mL (○) was added to the culture after 1 h cultivation. (2E)-Hexenal (100 μg/mL) was added at 1 (■), 2 (□), and 3 (△) h.

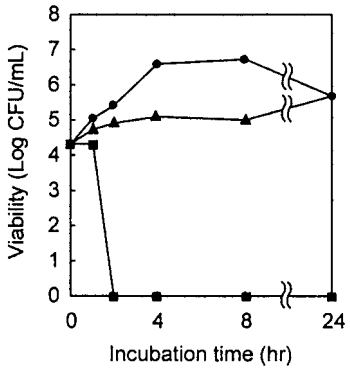

Figure 5. Bactericidal effect of anethole against *S. choleraesuis*. Exponentially growing cells of *S. choleraesuis* were inoculated at 37 °C in NYG broth with 0 (●), 100 (▲), or 200 (■) μg/mL of anethole. Viability was established by the number of colonies formed on NYG plate after incubation at 30 °C for 24 h.

incubation after addition of the drug. Chloramphenicol is known to be bacteriostatic for a wide range of Gram-positive and Gram-negative bacteria, but this antibiotic expressed bactericidal effect against *S. choleraesuis* after 8 h of incubation. In the presence of chloramphenicol, (2*E*)-hexenal decreased viable cell numbers a little more quickly than in its absence. The inhibition of cell division by chloramphenicol did not influence the bactericidal effects of (2*E*)-hexenal, and this observation excludes several modes of action. It is, thus, not likely that the reduced bacterial viability is due to interaction with the synthesis of macromolecules such as DNA, RNA, and proteins.

In previous reports, the antifungal activity of polygodial against *Saccharomyces cerevisiae* was described to be significantly enhanced when used in combination with anethole (**4**) (*5*). Hence, polygodial was combined with anethole to see whether the same combination effect could also be observed against *S. choleraesuis*. Anethole itself exhibits antibacterial activity against this food-borne bacterium with both MIC and MBC of 200 μg/mL (1.35 mM). Similar to polygodial and (2*E*)-hexenal, no differences in MIC and MBC were noted, suggesting that no residual bacteriostatic activity was involved. The bactericidal effect of anethole was confirmed by the time kill curve method as shown in Figure 5. The lethality occurred more slowly than that of polygodial, occurring 4 h after adding anethole. It shows that $^1/_2$MIC reduced the growth rate, but that the final cell count was not significantly different from that of

the control. *S. choleraesuis* is one of the few Gram-negative bacteria susceptible to anethole, which thus resembles polygodial.

The combination of polygodial and anethole synergistically retarded the growth rate of *S. choleraesuis* to a large extent, but this combination showed only marginal synergism on their bactericidal action. Thus, *S. choleraesuis* cells appeared to adapt to this combination stress, eventually recovering and growing normally. These results may indicate possibly different antimicrobial mechanisms of the combination of yeasts and bacteria, or more specifically, between *S. cerevisiae* and *S. choleraesuis*. Anethole was also combined with (2*E*)-hexenal to see if the combination had any enhancing activity. This combination also exhibited strong synergism on their bacteriostatic action, but only marginal synergism on their bactericidal action. The reason for the residual bacteriostatic activity against *S. choleraesuis* remains unknown.

DISCUSSION

The present study shows that polygodial was bactericidal against *S. choleraesuis* and killed the cells quickly. Polygodial possesses potent antifungal activity, so its mode of action has been extensively studied using *S. cerevisiae* as a model. In essence, the fungicidal activity of polygodial is likely exerted by its multiple functions but primarily comes from its ability to act as a nonionic surface-active agent (surfactant), thereby disrupting lipid−protein interface (*14*). This surfactant concept can also be applicable in part against *S. choleraesuis* because the lethality against this food-borne bacterium occurred remarkably quickly in the time kill experiment, within the first 1 h after adding polygodial. This rapid lethality observed supports its ability to function as a nonionic surfactant. If this is the case, polygodial very likely targets the extracytoplasmic region as a nonionic surfactant and thus does not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms. A further study to investigate this idea was not performed because polygodial falls short of the broad spectrum of activity as far as Gram-negative bacteria are concerned. *S. choleraesuis* was the only Gram-negative bacterium susceptible to polygodial, indicating that *S. choleraesuis* differs from other Gram-negative bacteria in some aspects. This difference may be caused by their different permeability of the outer membrane layer, as this layer plays a major role in the general resistance of Gram-negative bacteria. Gram-negative bacteria are surrounded by an outer lipopolysaccharidic membrane, and this functions as an effective but less specific barrier (*20*). In general, antibacterial activity against Gram-negative bacteria decreases by increasing the lipophilicity of molecules. However, the antibacterial agents against *S. choleraesuis* characterized so far are not the case, but they are rather similar to those against Gram-positive bacteria and fungi. If the selective elimination of *Salmonella* bacteria is desirable, polygodial may be considered to be a superior anti-*Salmonella* agent.

In contrast to polygodial, (2*E*)-hexenal is noted to possess a broad antimicrobial spectrum (*7, 17*). Although the precise mode of antimicrobial action of this alkenal is not yet clear, it should be a nonspecific mechanism due to its broad spectrum. (2*E*)-Hexenal unlikely acts as a surfactant but likely permeates by passive diffusion across the plasma membrane. Once inside cells, its α,β-unsaturated aldehyde moiety readily reacts with biologically important nucleophilic groups. For example, this aldehyde moiety is known to react with sulfhydryl groups mainly by 1,4-additions under physiological conditions (*19*). Sulfhydryl groups in proteins and lower-molecula-weight compounds such as glutathione are known to play an important role in the living cell. However, the precise target molecule remains unclear.

In our continuing search for antimicrobial agents from plants, a number of active principles have been characterized. However, only a few of them showed activity against Gram-negative bacteria, especially the *Pseudomonas* species. Among the compounds we characterized as antibacterial agents, (2*E*)-hexenal is one of the two phytochemicals characterized as antibacterial agents against *Pseudomonas aeruginosa*. We first characterized (2*E*)-hexenal as the principal antimicrobial agent from the cashew apple and subsequently olive oil. This common α,β-unsaturated aldehyde is known as "leaf aldehyde" (*18*) and is widely distributed. It may be a key defense chemical (post-inhibitin) against microbial attacks.

Currently, no appropriate anti-*Salmonella* agent for pepper is available. Hence, the phytochemicals characterized as anti-*Salmonella* agents can be applicable to disinfection and prevention of the contamination. For example, high concentrations are needed to cause the loss of viability, but (2*E*)-hexenal may be considered to be a genuine anti-*Salmonella* agent because of its easy availability and wide distribution in many edible plants such as apples, pears, grapes, strawberries, kiwi, tomatoes, olives, etc. (*19*). In the case of disinfecting the *Salmonella* contaminated pepper, this volatile α,β-unsaturated aldehyde seems to fit nicely for the purpose. This application is currently under examination and the results will be reported elsewhere. Also, anti-*Salmonella* phytochemicals can be mixed into artificial fodder to eliminate *Salmonella* at its sources.

It is conceivable that the anti-*Salmonella* activity of common phytochemicals such as (2*E*)-hexenal may play a role from an ecological point of view. It appears that plants contain a variety of antibacterial agents against *Salmonella* serovars, so that *Salmonella* can very likely be controlled in nature when chickens are continuously fed plant-based foods. This postulate can be supported by the fact that (2*E*)-hexenal is formed mainly after damage to the leaf (*19*). In the Amazon basin, *Salmonella* contamination of pepper has been increasingly noted with an increase in large-scale poultry farms. This may be caused by the shift of chicken feed from plant-based natural foods to artificial fodders.

Safety is a primary consideration for anti-*Salmonella* agents, especially those in food products, which may be utilized in unregulated quantities on a regular basis. The anti-*Salmonella* agents isolated from plants being used as food spices and/or characterized as flavor substances in many edible plants should be superior to nonnatural agents with respect to safety.

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