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Antibacterial Activity of *Gundelia tournefortii* Compounds against *Salmonella choleraesuis*

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ABSTRACT: Aliphatic (2E)-alkenals and alkanals characterized from the fresh leaves of the coriander *Gundelia tournefortii* were found to possess bactericidal activity against *Salmonella choleraesuis* ssp. choleraesuis (2E)-Dodecenal (C12) was the most effective against this food-borne bacterium with the minimum bactericidal concentration (MBC) of 6.25 µg/mL (34 µM), followed by (2E)-undecenal (C11) with an MBC of 12.5 µg/mL (74 µM). The time-kill curve study showed that these α,ß-unsaturated aldehydes are bactericidal against *S. choleraesuis* at any growth stage and that their bactericidal action comes in part from the ability to act as nonionic surfactants.

Keywords: Anti-Salmonella activity; *Gundelia tournefortii*; *Salmonella choleraesuis*; surfactant activity

INTRODUCTION

Gundelia is a genus of plants in the sunflower family. The best known species is *Gundelia tournefortii*. It is found in the semi-aried areas of Lebanon, Syria, Palestine, Israel, Jordan, Iraq, Iran, Azerbaijan, Armenia, and Anatolia. *Gundelia tournefortii* is a perennial growing to 0.4 m (1ft 4in). The flowers are hermaphrodite (have both male and female organs) and are pollinated by Insects (Aburjai *et al.* 2001; Abutbul *et al.* 2005). The leaves, stems, roots, and undeveloped flower buds of *G. tournefortii*, colloquially known as tumble thistle, are edible when they first sprout in early spring(Samani, Rafieian-Kopaei, and Azimi 2013). The plant becomes progressively drier over the summer, it leaves yellowing and growing spikes. Before dying, it detaches from the root to be pushed around by the wind and disperse its seeds for the following year's harvest. Sold in markets in Jerusalem, Syria, Iraq and Lebanon, it's also gathered in the wild in Turkey (Al-Salt 2012). Among Palestinian citizens of Israel in the north of the country, a popular dish using the plant consists of the dethorned heads covered in olive oil and fried, and then simmered with lemon juice. The underground part of this plant is used as fresh food in different part of Iran (Samani *et al.* 2013).

The antibacterial activity of Gundelia juice has recently been reported. Although precise active principles have not been examined, volatile compounds such as (2E)-hexenal and (3E)-hexenal were suggested as possible active compounds. Much information is available on the identification of volatile compounds of the fruit essential oils (2-4), but limited information exists on those of the fresh leaves in the literature. Recently, 13 volatile compounds, including 11 straight chain aldehydes, have been described as volatile compounds from the fresh leaves of *Gundelia* genius (Kubo *et al.* 2004).

The salmonellosis is one of the most frequently occurring bacterial food-borne illnesses. It results following the ingestion of viable cells of a member of the genus Salmonella. There are over 2500 serovars of Salmonella, all of which are pathogenic for humans. Currently, no appropriate anti-Salmonella agent for food is available; hence, safe and effective anti-Salmonella agents are urgently needed. Phytochemicals characterized from edible plants have the potential of filling this need because their structures are different from those of the well-studied microbial sources; therefore, their modes of action may very likely differ. Hence, the volatile compounds characterized from the fresh leaves of *Gundelia tournefortii* were tested to search for anti-Salmonella agents. *Salmonella choleraesuis* ssp. Choleraesuis was selected as an example because this bacterium most frequently causes septicemia, even though septicemia can be caused by any *Salmonella* (Davies and Breslin 2003).

MATERIALS AND METHODS

Chemicals

Both (2E)-alkenals and alkanals, linalool, and geraniolof Aldrich Chemical Co. Chloramphenicol and gentamicin sulfate were supplied by Sigma Chemical Co. were used. N,N-Dimethyl formamide (DMF) was obtained from EM Science. The test strains *S. choleraesuis* ssp. Choleraesuis ATCC35640, *E. coli* ATCC 9673, Enterobacteraerogenes ATCC 13048, *Pseudomonas aeruginosa* ATCC 10145, and *Proteus vulgaris* ATCC 13315 were prepared based on the American Type Culture Collection. NYG broth (0.8% nutrient broth, 0.5% yeast extract, and 0.1% glucose) was used for the antibacterial assay.

The nutrient broth was used based on BBL Microbiology System. The bacterial cells including *S. choleraesuis, E. coli, E. aerogenes, P. aeruginosa, and P. vulgaris* were precultured in 3 mL of NYG broth without shaking at 37°C for 16 h. The preculture was used for the following antibacterial assay and time-kill study.

Antibacterial Assay

The test compounds were first dissolved in DMF, and the highest concentration tested was 1600 µg/mL. It should be noted, however, higher concentrations reported might not be accurate because of their solubility limitation in the water-based medium. The final concentration of DMF in each medium was 1%, which did not affect the growth of the test strain. Broth macrodilution methods were used as previously described (Kubo, Lunde, and Kubo 1996) 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.

Thirty microliters of the exponentially growing bacterial cells of *S. choleraesuis, E. coli, E. aerogenes, P. aeruginosa, and P. vulgaris* (final 1.0 ×105 CFU/mL) were inoculated into the broth. After the cultures were incubated at 37°C for 24 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. 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 (Feron *et al.* 1991).

The antibacterial assay of (2E)-hexenal against *Salmonella typhimurium* and *Klebsiella pneumonia* was also carried out by Panlabs (Taipei, Taiwan), and the MICs obtained are 400 and 800 µg/mL, respectively.

Time-Kill Study

The cultivation with each compound was done the same as the above MIC assay. Samples were drawn 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 counted.

Preparation of Bacterial Cell Membrane

Exponentially growing *G. tournefortii* above-ground organs were harvested and then washed twice with distilled water. After centrifugation, the organs was suspended in 50 mMTris-HCl buffer (pH 7.4) containing 0.5 M sucrose and 20 mM $MgCl_2$, and then, it was disrupted by sonication using a Branson Sonifier 450 (Danbury, CT) for 2 min at 4°C. After the cell suspension was centrifuged at 15000g for 20 min, the supernatant was centrifuged at 105000g for 90 min. The resultant precipitate was washed by centrifugation at 105000g for 60 min with 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 M sucrose and 10 mM $MgCl_2$. The precipitate was resuspended in the same buffer (Haraguchi *et al.* 1992).

Enzyme Assay

The NADH oxidase activity was assayed by measuring the decrease in the absorbance at 340 nm. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.5), 200 µM NADH, and a membrane fraction (equivalent to 2 mg of protein) with or without (2E)-hexenal and (2E)-undecenal (Dancey, Levine, and Shapiro 1976).

RESULTS AND DISCUSSION

Among the 13 major volatile compounds characterized from the fresh leaves of the G. tournefortii, 11 of them were acyclic aldehyde compounds and typical products of oxidative cleavage of unsaturated fatty acids, including decanal, (2E)-decenal (C10), (2E)-dodecenal (C12), nonane, linalool, tetradecanal, (2E)-undecenal (C11), dodecanal, (2E)-tridecenal (C13), octanal, undecanal, nonanal, and (2E)-hexenal (C6) in decreasing order (7). The antibacterial activity of the individual compounds, except nonane and tetradecanal, was tested against *S. choleraesuis, E. coli, E. aerogenes, P. aeruginosa,* and *P. vulgaris* using a broth dilution method. The results obtained using *S. choleraesuis* are listed in Table 1.

Table 1: Antibacterial Activity (µg/mL) of *G. tournefortii* Compounds Characterized from the Coriander Fresh Leaves against *S. choleraesuis* ssp. choleraesuis ATCC 35640.

Compounds tested	MIC	MBC	Compounds tested	MIC	MBC
decanal	100	100	(2E)-tridecenal	25	200
(2E)-decenal	50	50	octanal	200	400
(2E)-dodecenal	6.25	6.25	undecanal	100	100
nonane	a		nonanal	100	200
linalool	400	800	(2E)-hexenal	100	100
tetradecanal			geraniol	200	400
(2E)-undecenal	12.5	12.5	gentamicin	12.5	12.5
dodecanal	100	100			

All of the aldehydes tested were effective against this food-borne bacterium, and the activity is correlated with the hydrophobic alkyl (tail) chain length from the hydrophilic aldehyde group (head). Thus, *S. choleraesuis* showed different susceptibilities to aldehydes possessing different chain lengths. It appears that the activity increased with increasing carbon chain length up to (2E)-dodecenal (C12). (2E)-Dodecenal is the most effective bactericide against *S. choleraesuis* with an MBC of 6.25 íg/mL (34 íM), followed by (2E)-undecenal (C11) with an MBC of 12.5 µg/mL (74 íM). The antibacterial assay of (2E)-hexenal against *S. typhimurium* and *K. pneumonia* was also carried out by Panlabs (Taipei, Taiwan), and the MICs obtained are 400 and 800 µg/mL, respectively. The result is broadly similar to those of the corresponding alcohols against many microorganisms (Kubo, Muroi, and Kubo 1995), indicating the similarity of their mode of action at least in part. The range of antibacterial activity of the (2E)-alkenals tested against *S. choleraesuis* is between 6.25 and 400íg/mL, and MICs and MBCs are markedly the same, indicating that their activity is bactericidal. Both MIC and MBC of the most potent (2E)-dodecenal are slightly more potent than those of gentamycin. On the other hand, (2E)-hexenal was the only active compound against the other Gram-negative bacteria tested, *E. coli, E. aerogenes, P. aeruginosa, and P. vulgaris*, and the range of MBC was 400-800 µg/mL. The other (2E)-alkenals tested did not show any antibacterial activity against these Gram-negative bacteria up to 800 µg/mL. The bactericidal effect of (2E)-dodecenal against *S. choleraesuis* was confirmed by the time-kill curve method as shown in Fig. 1. This method measures viable counts over time of microbial colonies plated on agar medium. The cultures of this food-borne bacterium, with a cell density of 1×105 CFU/mL, were exposed to three different concentrations of (2E)-dodecenal, the 1/4MIC, 1/2MIC, and MIC. The number of viable cells was determined following different periods of incubation with (2E)-dodecenal. The result verifies that MIC and MBC are the same. Notably, lethality occurs quickly, within the first 1 h after the addition of this α,ß-unsaturated aldehyde. This rapid lethality very likely indicates that the antibacterial activity of (2E)-dodecenal against *S. choleraesuisis* associated with the disruption of the membrane. In addition, the bactericidal effect of dodecanal (C12) against *S. choleraesuis* was also confirmed by the time-kill curve experiment (data not illustrated), and the result obtained is similar to those of (2E)-dodecenal.

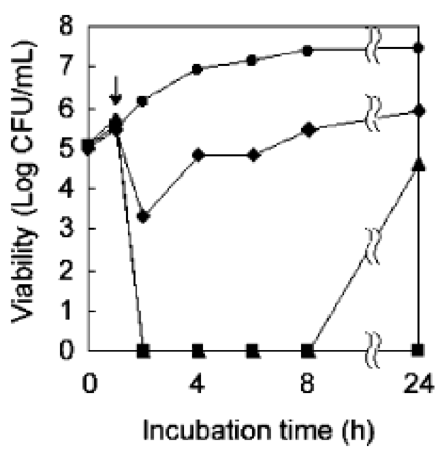

Fig. 1. Effect of (2*E*)-dodecenal on the growth of *S. choleraesuis*. Exponentially growing cells were inoculated into NYG broth and then cultured without shaking at 37 °C. The arrow indicates the time when the drug was added. (2*E*)-Dodecenal: 0 (●), 1.56 (◊), 3.13 (▲), and 6.25 (■) *µ*g/mL. The representative data are presented among triplicates on separate occasions.

The bactericidal activity of (2E)-hexenal (C6) against *S. choleraesuis* was also confirmed by the time-kill curve experiment (Kubo and Fujita 2001). The cultures of *S. choleraesuis*, with a cell density of 1 × 105 CFU/mL, were exposed to two different concentrations of (2E)-hexenal. The number of viable cells was determined following different periods of incubation with (2E)-hexenal. The result verifies that MIC and MBC are the same. Notably, lethality occurred 7 h after the addition of this α,ß-unsaturated aldehyde. It seems that the mode of antibacterial activity of (2E)-hexenal and (2E)-dodecenal against *S. choleraesuis* differs to some extent. (2E)-Hexenal is known to exhibit broad antimicrobial activity (Nakamura and Hatanaka 2002). For example, its antibacterial activity against *E. coli, P. aeruginosa, E. aerogenes, and P. vulgaris* as well as Helicobacter pylori (16) was previously reported.

In this study, (2E)-hexenal was found to be effective against *P. aeruginosa* with an MBC of 800 µg/mL. This troublesome bacterium is the most resistant organism to phytochemicals, followed by *E. coli* and *E. aerogenes* (Kubo *et al.* 1996).

The activity of (2E)-alkenals against *P. aeruginosa* decreased with an increasing carbon chain length (data not shown). (2E)-Decenal did not exhibit any antibacterial activity against *P. aeruginosa* up to 1600 µg/mL. The different susceptibilities between *S. choleraesuis* and *P. aeruginosa* may be caused by their different permeabilities of the outer membrane layer since this plays a major role in the general resistance of Gram-negative bacteria especially to lipophilic antibiotics. The outer membrane is known to surround most Gram-negative bacteria and this function as an effective but less specific barrier (Nikaido 1994). It is logical to assume that most of the lipophilic (2E)-alkenal molecules being dissolved in the medium are incorporated into the outer membrane and hence hardly reach the plasma membrane of *P. aeruginosa*. Notably, (2E)-hexenal was the only active compound against all of the Gram-negative bacteria tested. In our continuing search for antimicrobial agents from plants, a number of active principles have been characterized. However, only a few of them are known to show activity against Gram-negative bacteria, especially the Pseudomonas species. (2E)-Hexenal is one of the rare phytochemicals found as antibacterial agents against P. aeruginosa. The bactericidal effect of (2E)-dodecenal against *S. choleraesuis* occurred faster than that of (2E)-hexenal (Kubo and Fujita 2001). The phenomenon observed very likely indicates that the primary action of (2E)-dodecenal is on the cell membrane.

We first characterized (2E)-hexenal as the principal antimicrobial agent from the cashew apple and subsequently olive oil (Kubo, Lunde, and Kubo 1995). Soon after, we became aware that this common ?,ß-unsaturated aldehyde known as "leaf aldehyde" (Hatanaka 1993) is widely distributed in many plants. (2E)-Hexenal may be a key defense chemical (post inhibition) in plants against microbial attacks. 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 phytochemicals characterized as anti-Salmonella agents isolated from plants being used as food spices and/or characterized as flavor substances in many edible plants should be superior as compared to a nonnatural one (Witz 1989).

The antibacterial activity of (2E)-alkenals is nonspecific, and the potency of the activity against *S. choleraesuis* was distinctly increased with each additional CH_2 group up to (2E)-dodecenal. In the time-kill experiment of (2E)-dodecenal against *S. choleraesuis*, (i) lethality occurred notably quickly, within the first 1 h after the addition of (2E)-dodecenal, (ii) the bactericidal activity was found at any growth stage, and (iii) (2E)-dodecenal rapidly killed *S. choleraesuis* cells in which cell division was inhibited by chloramphenicol. Taking together this study and our previous report (Kubo and Fujita 2001), the antibacterial activity of (2E)-dodecenal against *S. choleraesuisis* mediated primarily due to its nonionic surfactant property, although it cannot be inferred that membrane damage is the only cause of the lethal effect.

The greater bactericidal activity of (2E)-dodecenal than that of (2E)-hexenal against *S. choleraesuisis* due primarily to a balance between the hydrophilicity of the unsaturated aldehyde subunit and the hydrophobicity of the alkyl portion of the molecule similar to their action against *Saccharomyces* cere Visiae (Guijarro and Lagunas 1984). The possibility of the anti-*Salmonella* mechanism of the amphipathic (2E)-dodecenal is due largely to their nonionic surfactant property. On the other hand, short chain (2E)-hexenal showed somewhat different effects on the growth of *S. choleraesuis*.

The amount of (2E)-alkenals entering into the cytosolor lipid bilayer is dependent on the length of the alkyl chain. The short chain (2E)-alkenals enter the cell by passive diffusion across the plasma membrane and/or through porin channels (22).The more lipophilic long chain (2E)-alkenals molecules being dissolved in the medium are incorporated into the lipid bilayers, similar to those found for alkanols (13, 23). In contrast to alkanols, R,â-unsaturated aldehydes are chemically highlyreactive substances and they readily react with biologically important nucleophilic groups, such as sulfhydryl, amino, orhydroxyl. The main reaction appears to be 1,4-additionunder physiological conditions, although the formation of Schiff bases is also possible. Once inside the cells, (2E)-alkenals may react with various intercellular components. For instance, bacteria are known to protect themselves against reactive oxygen species in various ways, and some of the most ubiquitous systems include glutathione (Brul and Coote 1999). It appears that (2E)-dodecenal mainly acts as a surfactant and then inhibits various cellular functions in an ordered sequential mechanism, while (2E)-hexenal behaves reversely. In our previous experiment, (2E)-undecenal rapidly adsorbed onto the surface of *S. cere* Visiaecells but (2E)-hexenal slightly adsorbed. It appears that S. cere Visiae showed different affinities to (2E)-alkenal having different alkyl chain lengths, and this may support the fore mentioned postulate (Kubo *et al*. 2003).

The same series of (2E)-alkenals has been reported to inhibit the succinate-supported respiration of intact mitochondria isolated from rat liver, and the potency increased with increasing chain length up to (2E)-undecenal, similar to those found for alkanols(Hammond and Kubo 2000). On the other hand, (2E)-alkenals have not been reported to inhibit the bacterial membrane respiratory system. In this study, we also confirmed that neither (2E)-hexenal nor (2E)-undecenal inhibited NADH oxidase prepared from the membrane fraction of *P. aeruginosa* IFO 3080 cells up to 100µM.

In the flesh leaves of *Gundelia tournefortii*, decanal, (2E)-decenal, and 2E)-dodecenal were reported to be the most abundant volatile compounds, accounting for more than 85% of total amounts of the identified compounds. In a previous paper, (2E)-decenal was also reported to be the most abundant flavor compound of whole cilantro (Shrivashankara *et al.* 2003). The alkanals described, decanal, dodecanal, octanal, undecanal, and nonanal, were granted a "generally recognized as safe" status. In addition, (2E)-hexenal is the predominant volatile component, which has been found in vegetative portions of virtually all plant species (Lanciotti *et al.* 2003), and was previously reported to be negative for the mutagenicity test. Moreover, (2E)-alkenals are known to possess a broad antimicrobial spectrum including *E. coli, B. subtilis, and methicillin resistant S. aureus* (Andersen *et al.* 1994). It needs to be clarified if the aldehydes in the leaves of *Gundelia tournefortii* contribute to the antibacterial activity observed with salsa sauce. In addition, many other compounds in salsa sauce may also have antimicrobial activity. For example, citral, geraniol, and carvacrol were previously reported to show antibacterial activity against *S. typhimurium* (Kim *et al.* 1995). The bactericidal effect of geraniol against *S. choleraesuis* was confirmed by the time-kill curve experiment as shown in Fig. 2. Similarly, the bactericidal activity of linalool against this food-borne bacterium was confirmed by the time-kill curve method (data not illustrated), and the result obtained is similar to those found for geraniol. Antimicrobial compounds in salsa sauce may synergize or antagonize one another, and this possibility should not be overlooked (Kubo and Himejima 1991).

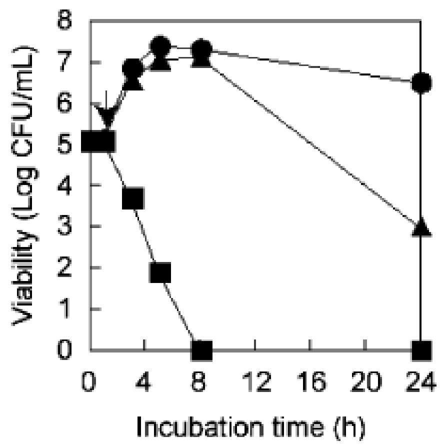

Fig. 2. Effect of geraniol on the growth of *S. choleraesuis*. Exponentially growing cells were inoculated into NYG broth and then cultured without shaking at 37°C. The arrow indicates the time when the drug was added. Geraniol: 0 (●), 200 (▲), and 400 (■) μg/mL. The representative data are presented among triplicates on separate occasions.

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