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Green-Leaf-Derived C6-Aroma Compounds with Potent Antibacterial Action That Act on Both Gram-Negative and Gram-Positive Bacteria

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All eight C6-aliphatic alcohol and aldehyde compounds in naturally occurring green leaves showed bacteriostatic effects against *Staphylococcus aureus* IFO 12732, methicillin-resistant *S. aureus*, *Escherichia coli* IFO 3301, *E. coli* O157:H7, and *Salmonella enteritidis*, with bacteriostatic activities of less than 12.5 μg mL^{-1}. In this study, the susceptibility of Gram-positive bacteria tested was observed to be greater than that of Gram-negative bacteria. The bactericidal action of the aldehyde compounds was found to be much stronger than that of the alcohol compounds under both liquid and gaseous conditions. The most effective compound was (3*E*)-hexenal at concentrations of 0.1 and 1 μg mL^{-1}, which killed 2.1 $\times$ 10^5 cfu mL^{-1} of *S. aureus* IFO 12732 and 1.4 $\times$ 10^5 cfu mL^{-1} of *E. coli* IFO 3301, respectively, by direct contact with the compound. Lethality of (3*E*)-hexenal against *S. aureus* IFO 12732 and *E. coli* IFO 3301 was also observed as a result of gaseous contact at concentrations of 3 and 30 μg mL^{-1}, respectively. The bactericidal effects of 30 μg mL^{-1} (3*E*)-hexenal were thoroughly maintained throughout periods of 2 days and 1 day against *S. aureus* IFO 12732 and *E. coli* IFO 3301, respectively, by a complex formation with α-cyclodextrin.

KEYWORDS: Green-leaf-derived C6-aroma compounds; bacteriostatic effects; bactericidal effects; Gram-negative and Gram-positive bacteria; α-cyclodextrin

INTRODUCTION

The fresh scent emitted by green leaves has been known by the name of "green-odor" (*1*). This was found to be biosynthesized in green leaves from α-linolenic and linoleic acids via their respective hydroperoxides, and composed of eight volatile compounds of C6-aliphatic alcohols [*n*-hexanol and (2*E*)-, (3*Z*)-, and (3*E*)-hexenol] and the corresponding aldehydes [*n*-hexanal and (2*E*)-, (3*Z*)-, and (3*E*)-hexenal] (*1*−*6*). It is assumed that the compounds of plant origin play important roles in various physiological actions, such as allelopathy (plant−plant), pheromone (plant−insect), phytoncide (plant−microorganism), and aromatherapy (plant−human) (*6*). Recently, plant-derived fragrances, including leaf alcohol [(3*Z*)-hexenol] and aldehyde [(2*E*)-hexenal], were demonstrated to have calming effects against the autonomic stress response (*7*). A current study also showed that event-related pleasantness (ERP-300) of healthy women was significantly affected by the green-leaf-derived C6-aroma compounds (*8*). In addition to these aromachological investigations, a phytoncide effect can be expected to be observed for the "green-odor" compounds. The antiseptic qualities of a number of aromatic plants have been recognized since antiquity (*9*). It has been reported that some volatile compounds released from spices and herbs and extracts thereof showed wide antimicrobial activities against fungi (*10*−*14*) and/or bacteria (*15*−*22*). The green-leaf-derived C6-aroma compounds are the naturally occurring odor chemicals from green leaves. Therefore, there is growing interest in the possible use of alternative food additives with bacteriostatic and bactericidal activities. The aroma compounds could be of great value as safe antimicrobial agents of plant origin.

In recent years, there has been a growing awareness of the need for the development of new and safe antimicrobial agents against increasing food poisoning incidences, including *Escherichia coli* O157:H7 and *Salmonella enteritidis* (*23*, *24*). The use of antibiotics to control bacterial infection is a concern because it promotes the growth of drug-resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (*25*). In contrast to the action of chemical antibiotics, the physical antimicrobial action of aroma compounds is acceptable because of the high volatility of such compounds at room temperature. It may provide an effective approach to prevent bacterial infection without any concern about residual antibiotics in foods. Thus, in this study, the antimicrobial effects of eight green-leaf-derived C6-aroma compounds against *S. aureus* IFO 12732, MRSA, *E. coli* IFO 3301, *E. coli* O157:H7, and *Sal.*

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enteritidis were investigated by direct contact under both liquid and gaseous conditions. In addition, we investigated the effects of the complex formation between C6-aroma compounds and α-cyclodextrin, which has GRAS ("generally recognized as safe") status, on bactericidal activities for further application.

MATERIALS AND METHODS

Chemicals. Eight C6-aroma compounds {*n*-hexanol, *n*-hexanal, (2*E*)-hexenol [(*E*)-2-hexen-1-ol], (2*E*)-hexenal [(*E*)-2-hexen-1-al], (3*Z*)-hexenol [(*Z*)-3-hexen-1-ol], (3*Z*)-hexenal [(*Z*)-3-hexen-1-al], (3*E*)-hexenol [(*E*)-3-hexen-1-ol], and (3*E*)-hexenal [(*E*)-3-hexen-1-al]} were prepared with more than 99% purity as previously described (*3*, *5*). α-Cyclodextrin was obtained from Dr. K. Matsui, Shimane University, Matsue, Japan. Dimethyl sulfoxide (DMSO) was purchased from Sigma/Aldrich (Tokyo, Japan). Bactotrypton, bactoyeast extract, and bactoagar were from Difco Laboratories (Detroit, MI). Nutrient broth No. 2 was from OXOID (Hampshire, England). MacConkey agar and Mannitol-salt agar were from Nissui Pharmaceutical Co. Ltd (Tokyo, Japan). Other chemicals used were all of analytical grade.

Bacterial Strains. *S. aureus* IFO 12732 and *E. coli* IFO 3301 were purchased from the Institute for Fermentation (Osaka, Japan). Clinically isolated *E. coli* O157:H7 (*evt-1/2*) and *Sal. enteritidis* (*invA*) were provided courtesy of Shimane Prefectural Institute of Public Health and Environmental Science (Matsue, Japan). Methicillin-resistant *S. aureus* (MRSA) was obtained from the culture collection in our laboratory (*26*).

Growth Inhibitory Effects of Green-Leaf-Derived C6-Aroma Compounds. Each bacterial strain was incubated in nutrient broth No. 2 at 37 °C overnight (14 h), and test bacterial solutions were prepared with the same broth to give a concentration of 10^6 cells mL^{-1} by using a hemacytometer (Neubauer, LO-Laboroptik GmbH, Friedrichsdorf, Germany). In parallel, a serial 2-fold dilution of green-leaf-derived C6-aroma compounds (1000, 500, 250, 125, 62.5, 31.3, 15.6, and 7.8 μg mL^{-1}) was prepared using 50% DMSO, which showed no effects against any bacterial strain tested. Twenty microliters of each was added to 160 μL of nutrient broth No. 2 in a 96-well plate with 300 μL volume wells (Millipore, Tokyo, Japan). Finally, 20 μL aliquots of 10^6 cells mL^{-1} bacterial solution were inoculated into the wells and incubated at 37 °C for 24 h. Bacteriostatic activities of C6-aroma compounds were determined as the lowest concentration of growth inhibition that demonstrated no visible growth. The strength of the growth inhibition activities of the compounds was also confirmed by using a hemacytometer with the detection limit of 10^5 cells mL^{-1}.

Bactericidal Effects by Direct Contact under Liquid Conditions. Bactericidal effects of eight C6-aroma compounds were assessed on a typical Gram-positive bacteria, *S. aureus* IFO 12732, and a typical Gram-negative bacteria, *E. coli* IFO 3301. The test strains were harvested from cultures held overnight in nutrient broth No. 2 by centrifugation and were resuspended in sterile 50 mM potassium phosphate buffer (pH 7.0) after being washed twice with the same buffer. Washed cell preparations were diluted to 10^6 cells mL^{-1} by using a hemacytometer. The eight aroma compounds were diluted with 50% DMSO to prepare 100, 20, 10, 1, and 0.1 μg mL^{-1} solutions. Twenty microliters of the diluted chemicals was mixed well with 160 μL of phosphate buffer (pH 7.0) in a sterile microtube with a volume of 1.5 mL. Next, 20 μL of the 10^6 cells mL^{-1} bacterial cell suspension was added into the tube and subsequently incubated at 37 °C for 1 h. After incubation, decimal dilutions of the sample were carried out up to $\times 10^4$ using physiological saline adjusted to pH 7.2. One hundred microliters of diluted cell suspension was spread onto a Mannitol-salt agar plate for *S. aureus* IFO 12732 and a MacConkey agar plate for *E. coli* IFO 3301. All plates were incubated at 37 °C for 24 h, and then surviving cells were counted according to the colonies appearing on the plate. The percentage of survivors was presented with respect to the control mixture. The experiment was performed in triplicate.

Bactericidal Effects by Gaseous Contact. Bacterial cell suspensions with 10^6 cells mL^{-1} were prepared as described above using 50 mM potassium phosphate buffer (pH 7.0), and a 100 μL volume of the cell suspension was put into the interior room of a Conwey's unit (Iwaki Glass Co., Ltd., Tokyo, Japan) with 33 mL cubic capacity. The unit was sealed with petroleum jelly, and then 100 μL of 1000, 100, and 10 μg mL^{-1} C6-aroma compounds was spread on the exterior room of the unit. The unit was immediately and tightly resealed using a clasp and subsequently incubated at 37 °C for 1 h. After incubation, 900 μL of physiological saline (pH 7.2) was added and mixed well with the cell suspension in the interior room. The surviving bacterial cells were measured by using Mannitol-salt agar plates and MacConkey agar plates for *S. aureus* IFO 12732 and *E. coli* IFO 3301, respectively. This experiment was also performed in triplicate.

Bactericidal Effects of Inclusion Complex with α-Cyclodextrin. One and a half grams of α-cyclodextrin (α-CD) was mixed well with 500 μL of 10 mg mL^{-1} C6-aroma compounds for 1 min, and then 0.4 g of the inclusion complex with α-CD (equivalent to 1000 μg of C6-aroma compounds) was immediately placed into the exterior room of a Conwey's unit, in which 100 μL of 10^6 cells mL^{-1} bacterial cell suspension had been spread in the interior room. All surviving bacteria in the interior room of the unit were counted using agar plates after incubation at 37 °C for 1 h as described above. The remaining inclusion complex was subsequently incubated at 20 °C under open conditions for 1 week. At the given time, 0.4 g of the α-CD complex was withdrawn and measured for bactericidal effects as described above.

Statistical Analysis. The experiment was replicated three times with five treatments. The results of the experiments were pooled and analyzed by the Statistical Analysis System (SAS Institute Inc., Cary, NC, 1988) by using the general linear model procedure, and then comparisons were done by using least significant differences multiple range test and least-squares means.

RESULTS

Bacteriostatic Activities. All C6-aroma compounds examined exhibited potent antimicrobial activities against all bacterial strains: *S. aureus* IFO 12732, methicillin-resistant *S. aureus* (MRSA), *E. coli* IFO 3301, *E. coli* O157:H7 (O157:H7), and *Sal. enteritidis*, as shown in **Table 1**. The bacteriostatic activity of aldehyde compounds on the susceptibility of Gram-positive bacteria was observed to be greater than that of Gram-negative bacteria, while no difference was observed for alcohol compounds. Also, no difference in susceptibility was observed between MRSA and *S. aureus* IFO 12732, as well as between *E. coli* IFO 3301 and O157:H7. Among the eight compounds, (3*E*)-hexenal showed the highest bacteriostatic activity with the growth inhibitory concentration of 1.56 μg mL^{-1} for *S. aureus* IFO 12732 and MRSA, and 3.13 μg mL^{-1} for *Sal. enteritidis*, O157:H7, and *E. coli* IFO 3301. The growth inhibitory concentration of (2*E*)-hexenal and (3*Z*)-hexenal against *Sal. enteritidis*, O157:H7, and *E. coli* IFO 3301 was 6.25 μg mL^{-1}, while that against *S. aureus* IFO 12732 and MRSA was 3.13 μg mL^{-1}. Four aliphatic alcohol compounds and *n*-hexanal showed low activity, inhibiting all test strains at 12.5 μg mL^{-1}.

Bactericidal Effects under Liquid Conditions. Since no difference in susceptibility was identified between MRSA and *S. aureus* IFO 12732, classified as Gram-positive bacteria, and among *E. coli* IFO 3301 and O157:H7 and *Sal. enteritidis*, classified as Gram-negative bacteria, *S. aureus* IFO 12732 and *E. coli* IFO 3301 were used as typical Gram-positive and Gram-negative bacteria, respectively, in further studies. Bactericidal effects of green-leaf-derived C6-aroma compounds against *S. aureus* IFO 12732 and *E. coli* IFO 3301 are shown in **Table 2**. A 10^5 cells mL^{-1} bacterial cell suspension was directly exposed to the test compounds diluted with 50% DMSO at 37 °C for 1 h, and the number of surviving bacteria was measured. Bactericidal effects of (3*E*)-hexenal and (3*Z*)-hexenal were the greatest against *S. aureus* IFO 12732, followed by (2*E*)-hexenal. No living cell was observed in sample solution containing 2.1×10^5 cfu mL^{-1} (5.32 $\log_{10}$ cfu mL^{-1}) *S. aureus* after the treatments with 0.1 μg mL^{-1} each of (3*E*)-hexenal and (3*Z*)-

Table 1. Bacteriostatic Effects[a] of Green-Leaf-Derived C6-Aroma Compounds against Five Bacterial Strains

C6-aroma compound	bacterial strain[b]	concentration (μg mL^{-1})								
		100	50	25	12.5	6.25	3.13	1.56	0.78	0
n-hexanal	*E. coli*	–	–	–	–	+	+	++	++	++
	O-157:H7	–	–	–	–	+	+	++	++	++
	S. aureus	–	–	–	–	+/–	+	+	++	++
	MRSA	–	–	–	–	+/–	+	+	++	++
	Sal. enteritidis	–	–	–	–	+/–	+	++	++	++
(2*E*)-hexenal	*E. coli*	–	–	–	–	–	+	+	++	++
	O-157:H7	–	–	–	–	–	+/–	+	++	++
	S. aureus	–	–	–	–	–	–	+	++	++
	MRSA	–	–	–	–	–	–	+/–	++	++
	Sal. enteritidis	–	–	–	–	–	+/–	+/–	++	++
(3*Z*)-hexenal	*E. coli*	–	–	–	–	–	+	+	++	++
	O-157:H7	–	–	–	–	–	+/–	+	++	++
	S. aureus	–	–	–	–	–	–	+	+	++
	MRSA	–	–	–	–	–	–	+/–	+	++
	Sal. enteritidis	–	–	–	–	–	+/–	+/–	+	++
(3*E*)-hexenal	*E. coli*	–	–	–	–	–	–	+	++	++
	O-157:H7	–	–	–	–	–	–	+	++	++
	S. aureus	–	–	–	–	–	–	–	+	++
	MRSA	–	–	–	–	–	–	–	+	++
	Sal. enteritidis	–	–	–	–	–	–	+/–	+	++
n-hexanol	*E. coli*	–	–	–	–	+/–	+	++	++	++
	O-157:H7	–	–	–	–	+/–	+	++	++	++
	S. aureus	–	–	–	–	+	+	++	++	++
	MRSA	–	–	–	–	+	+	++	++	++
	Sal. enteritidis	–	–	–	–	+/–	+	++	++	++
(2*E*)-hexenol	*E. coli*	–	–	–	–	+/–	+	++	++	++
	O-157:H7	–	–	–	–	+/–	+	+	++	++
	S. aureus	–	–	–	–	+	+	++	++	++
	MRSA	–	–	–	–	+	+	++	++	++
	Sal. enteritidis	–	–	–	–	+/–	+/–	+	++	++
(3*Z*)-hexenol	*E. coli*	–	–	–	–	+/–	+	++	++	++
	O-157:H7	–	–	–	–	+/–	+	+	++	++
	S. aureus	–	–	–	–	+	+	++	++	++
	MRSA	–	–	–	–	+	+	++	++	++
	Sal. enteritidis	–	–	–	–	+/–	+	+	++	++
(3*E*)-hexenol	*E. coli*	–	–	–	–	+/–	+	++	++	++
	O-157:H7	–	–	–	–	+/–	+/–	+	++	++
	S. aureus	–	–	–	–	+	+	++	++	++
	MRSA	–	–	–	–	+	+	++	++	++
	Sal. enteritidis	–	–	–	–	+/–	+/–	+	++	++

[a] –, no growth (<10^5 cells mL^{-1}); +/–, weak growth (10^6–10^7 cells mL^{-1}); +, growth (10^8–10^9 cells mL^{-1}); ++, high growth (>10^{10} cells mL^{-1}). [b] *E. coli*, *Escherichia coli* IFO 3301; O-157:H7, *Escherichia coli* O-157:H7; *S. aureus*, *Staphylococcus aureus* IFO 12732, MRSA, methicillin-resistant *Staphylococcus aureus*; *Sal. enteritidis*, *Salmonella enteritidis*.

Table 2. Bactericidal Effects of Green-Leaf-Derived C6-Aroma Compounds against *S. aureus* IFO 12732 and *E. coli* IFO 3301 by Direct Contact under Liquid Conditions

	survivors ($\log_{10}$ cfu mL^{-1}, means of three experiments) at the given concentration[a]					
	0 μg mL^{-1}	0.01 μg mL^{-1}	0.1 μg mL^{-1}	1 μg mL^{-1}	2 μg mL^{-1}	10 μg mL^{-1}
			S. aureus IFO 12732			
n-hexanal	5.32 (100)a	5.29 (92.8)a	5.29 (92.8)a	4.87 (35.3)b	4.79 (29.3)b	nd
(2*E*)-hexenal	5.32 (100)a	4.61 (19.4)b	1.72 (0.02)d	nd	nd	nd
(3*Z*)-hexenal	5.32 (100)a	4.20 (7.5)c	nd	nd	nd	nd
(3*E*)-hexenal	5.32 (100)a	4.08 (5.7)c	nd	nd	nd	nd
n-hexanol	5.32 (100)a	5.32 (100)a	5.30 (95.0)a	4.63 (20.3)b	4.58 (18.1)b	nd
(2*E*)-hexenol	5.32 (100)a	5.32 (100)a	5.31 (97.2)a	4.45 (13.4)b	4.39 (11.7)b	nd
(3*Z*)-hexenol	5.32 (100)a	5.30 (95.0)a	5.29 (92.8)a	4.60 (19.0)b	4.47 (14.1)b	nd
(3*E*)-hexenol	5.32 (100)a	5.31 (97.2)a	5.29 (92.8)a	4.81 (30.7)b	4.76 (27.4)b	nd
			E. coli IFO 3301			
n-hexanal	5.15 (100)a	5.14 (98.6)a	5.10 (89.9)a	4.50 (22.6)b	4.39 (17.5)b	nd
(2*E*)-hexenal	5.15 (100)a	4.61 (29.1)b	4.28 (13.6)b	nd	nd	nd
(3*Z*)-hexenal	5.15 (100)a	4.75 (40.2)b	4.53 (24.2)b	nd	nd	nd
(3*E*)-hexenal	5.15 (100)a	4.69 (35.0)b	4.41 (18.4)b	nd	nd	nd
n-hexanol	5.15 (100)a	5.13 (96.4)a	5.13 (96.4)a	4.70 (35.8)b	4.63 (30.5)b	nd
(2*E*)-hexenol	5.15 (100)a	5.15 (100)a	5.15 (100)a	5.11 (92.0)a	5.10 (89.9)a	nd
(3*Z*)-hexenol	5.15 (100)a	5.15 (100)a	5.15 (100)a	5.09 (87.9)a	5.09 (87.9)a	nd
(3*E*)-hexenol	5.15 (100)a	5.14 (98.6)a	5.14 (98.6)a	4.78 (43.0)b	4.51 (23.1)b	nd

[a] Values in parentheses indicate survival ratio (%) based on the real number. Survival ratios with deferent letters within each bacteria tested are significantly different ($P < 0.05$). nd, not detected. The detection limit was 1.0 cfu mL^{-1}.

Table 3. Bactericidal Effects of Green-Leaf-Derived C6-Aroma Compounds against *S. aureus* IFO 12732 and *E. coli* IFO 3301 by Gaseous Contact

	survivors ($\log_{10}$ cfu plate^{-1}, means of three experiments) at the given concentration			
	0 μg mL^{-1}	0.3 μg mL^{-1}	3 μg mL^{-1}	30 μg mL^{-1}
	S. aureus IFO 12732			
n-hexanal	5.11 (100)a	5.11 (100)a	5.10 (96.8)a	4.00 (7.7)c
(2*E*)-hexenal	5.11 (100)a	5.10 (96.8)a	2.28 (0.15)d	nd
(3*Z*)-hexenal	5.11 (100)a	5.11 (100)a	2.00 (0.08)d	nd
(3*E*)-hexenal	5.11 (100)a	4.22 (12.7)b	nd	nd
n-hexanol	5.11 (100)a	5.10 (96.8)a	5.10 (96.8)a	4.31 (15.7)b
(2*E*)-hexenol	5.11 (100)a	5.11 (100)a	5.08 (92.3)a	4.25 (13.7)b
(3*Z*)-hexenol	5.11 (100)a	5.10 (96.8)a	5.08 (92.3)a	4.56 (27.9)b
(3*E*)-hexenol	5.11 (100)a	5.09 (94.6)a	5.09 (94.6)a	4.45 (21.7)b
	E. coli IFO 3301			
n-hexanal	5.34 (100)a	5.34 (100)a	4.68 (21.8)b	0.60 (0.002)d
(2*E*)-hexenal	5.34 (100)a	5.34 (100)a	4.24 (7.9)c	nd
(3*Z*)-hexenal	5.34 (100)a	5.30 (90.7)a	4.09 (5.6)c	nd
(3*E*)-hexenal	5.34 (100)a	4.86 (32.9)b	3.81 (2.9)c	nd
n-hexanol	5.34 (100)a	5.33 (97.2)a	5.33 (97.2)a	4.01 (4.7)c
(2*E*)-hexenol	5.34 (100)a	5.31 (92.8)a	5.31 (92.8)a	4.24 (7.9)c
(3*Z*)-hexenol	5.34 (100)a	5.32 (94.9)a	5.31 (92.8)a	4.51 (14.7)b
(3*E*)-hexenol	5.34 (100)a	5.30 (90.7)a	5.30 (90.7)a	4.11 (5.9)c

[a] Values in parentheses indicate survival ratio (%) based on the real number. Survival ratios with deferent letters within each bacteria tested are significantly different ($P < 0.05$). nd, not detected. The detection limit was 0.3 cfu plate^{-1}.

hexenal and 1 μg mL^{-1} of (2*E*)-hexenal at 37 °C for 1 h, although the detection limit was 1.0×10^1 cfu mL^{-1}. All *S. aureus* cells were also killed by direct contact with 10 μg mL^{-1} of *n*-hexanal, *n*-hexanol, (2*E*)-hexenol, (3*Z*)-hexenol, and (3*E*)-hexenol. On the other hand, (2*E*)-hexenal, (3*Z*)-hexenal, and (3*E*)-hexenal showed strong bactericidal effects against *E. coli* IFO 3391, in which there are no big discrepancies in these three aliphatic aldehyde compounds. A 1 μg mL^{-1} concentration of (2*E*)-hexenal, (3*E*)-hexenal, and (3*Z*)-hexenal showed lethal effects for 1.4×10^5 cfu mL^{-1} (5.15 $\log_{10}$ cfu mL^{-1}) *E. coli,* while the other five C6-aroma compounds required 10 μg mL^{-1}. The order of killing effects for *E. coli* IFO 3301 was (2*E*)-hexenal, (3*E*)-hexenal, and (3*Z*)-hexenal > *n*-hexanal, (3*E*)-hexenol, and *n*-hexanol > (2*E*)-hexenol and (3*Z*)-hexenol.

Bactericidal Effects under Gaseous Conditions. Bactericidal actions of green-leaf-derived C6-aroma compounds were investigated under gaseous contact conditions in air. As shown in **Table 3**, among the C6-aroma compounds, (3*E*)-hexenal was noted to be the most effective against *S. aureus* IFO 12732, in which 1.3×10^5 cfu plate^{-1} (5.11 $\log_{10}$ cfu plate^{-1}, cfu per plate of Conwey's unit) of bacterial cells completely disappeared upon exposure to 3 μg mL^{-1} of (3*E*)-hexenal at 37 °C for 1 h, although the detection limit was 2 cfu plate^{-1}. Overall, the bactericidal effects of aldehyde compounds were much stronger than those of alcohol compounds. Exposure to 30 μg mL^{-1} of (3*Z*)-hexenal and (2*E*)-hexenal had a lethal effect on *S. aureus* IFO 12732, and that of *n*-hexanal led to 92.3% death of the cell suspension. Bactericidal effects of aldehyde compounds against *E. coli* IFO 3301 were obviously weak compared to those against *S. aureus* IFO 12732, except for *n*-hexanal. No living cells were detected from the sample in which 2.2×10^5 cfu plate^{-1} (5.34 $\log_{10}$ cfu plate^{-1}) *E. coli* were exposed to 30 μg mL^{-1} of (3*E*)-hexenal, (3*Z*)-hexenal, and (2*E*)-hexenal, whereas exposure to *n*-hexanal killed more than 99.99% of the tested cells. On the other hand, alcohol compounds seem to be more effective against *E. coli* cells, as mentioned before. Survival ratios of *E. coli* IFO 3301 were 4.7, 5.9, 7.9, and 14.7% with the exposure to 30 μg mL^{-1} of *n*-hexenol, (3*E*)-hexenol, (2*E*)-hexenol, and (3*Z*)-hexenol, respectively, while those of *S. aureus* IFO 12732 were 13.7, 15.7, 21.7, and 27.9% from the same amount of exposure to (2*E*)-hexenol, *n*-hexenol, (3*E*)-hexenol, and (3*Z*)-hexenol, respectively.

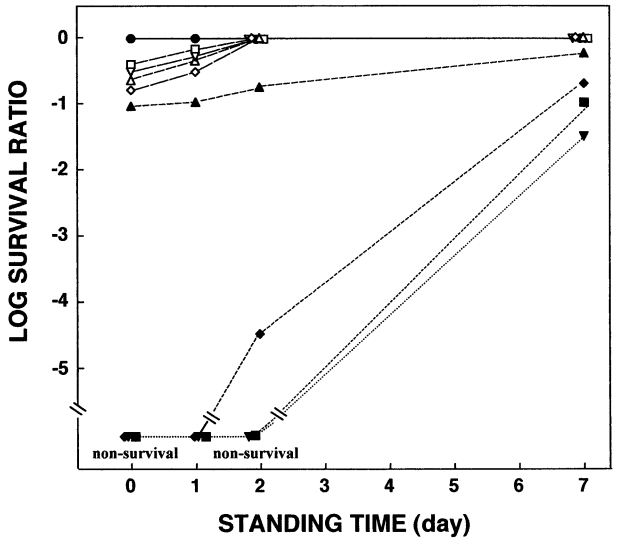

Figure 1. Durability of bactericidal effects of 30 μg mL^{-1} green-leaf-derived C6-aroma compounds against *S. aureus* IFO 12732 by the complex formation with α-cyclodextrin. Log survival ratio was calculated on the basis of the real numbers of survivors. The bacterial numbers in the control systems were 1.7×10^5, 1.3×10^5, 1.5×10^5, and 1.2×10^5 cfu plate^{-1} for samples after 0, 1, 2, and 7 days of incubation, respectively. Data are from a representative experiment repeated three times with similar results. △, *n*-hexenol; ▲, *n*-hexenal; ◇, (2*E*)-hexenol; ◆, (2*E*)-hexenal; □, (3*Z*)-hexenol; ■, (3*Z*)-hexenal; ▽, (3*E*)-hexenol; ▼, (3*E*)-hexenal; ●, control (50% DMSO).

Duration of Bactericidal Effects by Inclusion Complex Formation with α-Cyclodextrin. The antimicrobial effects of inclusion complexes of green-leaf-derived C6-aroma compounds with α-cyclodextrin and their durability were investigated at the concentration of 30 μg mL^{-1}. By complex formation with α-cyclodextrin (α-CD), thorough bactericidal action of (3*E*)-hexenal and (3*Z*)-hexenal against *S. aureus* IFO 12732 was extended by 2 days at 20 °C under open conditions, while that of (2*E*)-hexenal was 1 day, as shown in **Figure 1**. However, these effects continued to decrease rapidly after 1 week, where the bacterial number in the control was 1.2×10^5 cfu plate^{-1} (100%), while those in α-CD complexes with (3*E*)-hexenal, (3*Z*)-hexenal, and (2*E*)-hexenal were 3.7×10^3 cfu plate^{-1} (3.1%), 1.3×10^4 cfu plate^{-1} (10.8%), and 2.3×10^5 cfu plate^{-1} (19.2%), respectively. The bactericidal effect of the *n*-hexanal complex also remained against *S. aureus* IFO 12732 even after 1 week of incubation, whereas the bactericidal effects of the other four alcohol compounds were completely diminished. **Figure 2** shows the durability of antimicrobial effects of the aroma compound inclusion complexes with α-CD against *E. coli* IFO 3301. The (3*E*)-hexenal complex was the strongest in activity against *E. coli* IFO 3301, in which living cells completely disappeared from the unit following exposure after 1 day of incubation. The bactericidal effects of aldehyde compounds were clearly stronger than those of alcohol compounds, even in the case of the complex formation with α-CD. Although the effects of aldehyde compound complexes decreased with incubation time, considerable bactericidal activities remained in the complexes with (3*E*)-hexenal, (3*Z*)-hexenal, (2*E*)-hexenal, and *n*-hexanal for 1 week of incubation, whereas the bactericidal effects of the four alcohol compound complexes

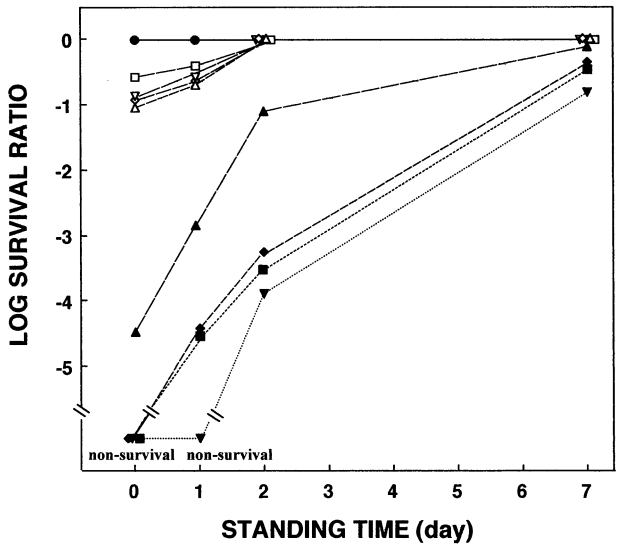

Figure 2. Durability of bactericidal effects of 30 μg mL^{-1} green-leaf-derived C6-aroma compounds against *E. coli* IFO 12732 by the complex formation with α-cyclodextrin. Log survival ratio was calculated on the basis of the real numbers of survivors. The bacterial numbers in the control systems were 1.5×10^5, 1.3×10^5, 1.5×10^5, and 1.8×10^5 cfu plate^{-1} for samples after 0, 1, 2, and 7 days of incubation, respectively. Data are from a representative experiment repeated three times with similar results. △, *n*-hexenol; ▲, *n*-hexenal; ◇, (2*E*)-hexenol; ◆, (2*E*)-hexenal; □, (3*Z*)-hexenol; ■, (3*Z*)-hexenal; ▽, (3*E*)-hexenol; ▼, (3*E*)-hexenal; ●, control (50% DMSO).

were eliminated entirely. The survival ratios of *E. coli* IFO 3301 in α-CD complexes with (3*E*)-hexenal, (3*Z*)-hexenal, (2*E*)-hexenal, and *n*-hexanal after 1 week of incubation were 15.1, 30.9, 40.7, and 64.6%, respectively, in comparison with the control system with the bacterial number of 1.8×10^5 cfu plate^{-1}.

DISCUSSION

This study describes the antibacterial effects of eight green-leaf-derived C6-aroma compounds against two Gram-positive strains and three Gram-negative strains including food-borne pathogens. Since most of the data published on the antimicrobial properties of plant essential oils and their volatile compounds employ basic screening techniques (*10*−*18*), this paper intends to take the work a stage further to establish precise bacteriostatic and bactericidal concentrations of the aroma compounds. As a result, all compounds showed bacteriostatic effects against all test strains of *S. aureus* IFO 12732, methicillin-resistant *S. aureus*, *E. coli* IFO 3301, *E. coli* O157:H7, and *Sal. enteritidis* at the diluted concentration of less than 12.5 μg/mL^{-1}. The bacteriostatic effect against *Sal. enteritidis* of (2*E*)-hexenal in this paper was 6.25 μg/mL^{-1}, which is 16 times stronger than that reported in the previous paper (*27*), in which the minimum inhibitory concentration (MIC) against *S. choleraesuis* was 100 μg mL^{-1}. This may arise from the differences in the test strains and/or the procedure to determine the antimicrobial activity. In this study, the inoculated bacterial number was correctly adjusted using a hemacytometer to 10^5 cells mL^{-1} as a final concentration, and the growth inhibitory value was microscopically determined after 24 h of incubation at 37 °C, where the bacterial number was less than 10^5 cells mL^{-1}, in addition to the observation based on visible bacterial growth. The determination of the bacteriostatic concentration using a hemacytometer is a more sensitive technique than the visibility technique, which showed no or very slight inhibitory properties against the five strains. Thus, generally lower bacteriostatic concentrations were required for all eight C6-aroma compounds.

This study revealed that both Gram-positive and Gram-negative bacteria were susceptible to the green-leaf-derived C6-aroma compounds. However, bacteriostatic actions of aldehyde compounds on Gram-positive bacteria were observed to be obviously greater than those on Gram-negative bacteria. The difference in sensitivity between Gram-positive and Gram-negative bacteria to inhibition has been supported by other papers, in that generally Gram-positive bacteria are more susceptible than Gram-negative bacteria against plant essential oils (*16*−*18*, *22*, *28*, *29*), although the mechanism is not still clearly demonstrated. It is assumed that the comparatively weak activity against Gram-negative bacteria was due to the presence of the outer membrane, which endows the bacterial surface with strong hydrophilicity and acts as a strong permeability barrier to hydrophobic chemicals (*28*). Polysaccharide chains surrounding the surface of Gram-negative bacteria may contribute to preventing interaction between hydrophobic chemicals and the outer membrane. Indeed, the antimicrobial activity was considerably different between aldehyde compounds and alcohol compounds, in that Gram-negative bacteria were more sensitive than Gram-positive bacteria to alcohol compounds. This may be one of the strongest pieces of evidence in support of the above hypothesis.

Antimicrobial effects of the green-leaf-derived C6-aroma compounds were further investigated under liquid and gaseous conditions without any effects of culture substances. The bactericidal actions of the C6-aroma compounds revealed that those of aldehyde compounds were much stronger than those of the alcohol ones resulting from both liquid and gaseous contacts. A typical Gram-negative bacteria, *E. coli* IFO 3301, and a typical Gram-positive bacteria, *S. aureus* IFO 12732, were both more susceptible to C6-aroma compounds by direct contact in this study, compared to the data on the bacteriostatic activity. The concentrations of the most effective compound, (3*E*)-hexenal, needed to kill 10^5 cfu mL^{-1} of *S. aureus* and *E. coli* were 0.1 and 1 μg mL^{-1}, respectively, whereas the lowest growth-inhibitory concentrations of (3*E*)-hexenal were 1.56 and 3.13 μg mL^{-1} to inhibit the growth of *S. aureus* IFO 12732 and *E. coli* IFO 3301, respectively. Furthermore, the bactericidal actions were found to be effective by gaseous contact, where 3 and 30 μg mL^{-1} of the most effective compound, (3*E*)-hexenal, killed 10^5 cfu plate^{-1} of *S. aureus* IFO 12732 and *E. coli* IFO 3301, respectively. The bactericidal effects by gaseous contact would depend on the permeability and/or interaction with the cell membrane (*22*). Alcohol compounds were more effective against *E. coli* IFO 3301 than *S. aureus* IFO 12732 due to the hydrophilicity of the surface of the Gram-negative bacteria (*28*). It is also revealed that the most effective bactericidal chemical against *S. aureus* IFO 12732 and *E. coli* IFO 3301 of all C6-aroma compounds was (3*E*)-hexenal in both the liquid and gaseous contact systems. This effect may be attributed to the position of the double bond, resulting in structural stability as well as cis structural formation (*5*).

The durability of bactericidal effects in the gaseous phase was also studied. The bactericidal effects of 30 μg mL^{-1} of the most effective compound, (3*E*)-hexenal, were thoroughly maintained throughout a 2 day and 1 day period against *E. coli* IFO 3301 and *S. aureus* IFO 12732, respectively, by the formation of an inclusion complex with α-cyclodextrin. Because the loss in activity could be negligible during complex formation, the durability of antimicrobial effects can be expected for a wide

range of applications in the industry. In addition, we have developed a large-scale preparation of green-leaf-derived C6-aroma compounds for industry applications. Leaf alcohols were prepared through a three-step reaction scheme by carbon chain elongation using sodium acetyl liquid ammonia with over 99% geometrical purity and an overall yield of 40% (*5*). Leaf aldehydes were prepared according to a four-step reaction scheme using butyric acid chloride and acetylene with an overall yield of 50% (*5*). [Note: Presently, leaf alcohols and leaf aldehydes are annually synthesized in amounts of ca. 650 000 and 70 000 kg, respectively, in Japan.] By modifying the methods, the entire series of positional and geometrical isomers was systematically obtained with good results. Thus, in conclusion, all eight C6-aroma compounds constituting "green-odor" were found to be have potent antimicrobial effects against five Gram-negative and Gram-positive bacteria, including food poisoning bacteria and a drug-resistant bacteria, at the low concentrations used in this study. Since it is well recognized that higher concentrations of essential oils of plant origin are required in food than in laboratory media (*30*), the low bacteriostatic and bactericidal concentrations against pathogens can provide a prospective future for green-leaf-derived C6-aroma compounds as a more "natural" alternative to antibiotics, with a wide spectra of antimicrobial effectiveness.

NOTE ADDED AFTER ASAP POSTING

Units in Table 2 were erroneously given in the original ASAP posting of November 21, 2002. These errors have been corrected in this posting.

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