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Antimicrobial activity of solvent fractions and bacterial isolates of Korean domestic honey from different floral sources

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Abstract Forty solvent fractions and 387 bacterial isolates of seven varieties of Korean domestic honey and manuka honey from New Zealand were screened for antimicrobial activity. The minimum inhibitory concentrations and minimum bactericidal concentrations of the honey fractions were determined; only *Bacillus cereus* ATCC 14579, ATCC 11778, and F4552 were inhibited by 11, 1, and 16, respectively, out of the 40 honey fractions. The bacterial isolates showed the highest incidence (30.2%) of antimicrobial activity against *Listeria monocytogenes* ATCC 15313. The growth of at least one of the five foodborne pathogens tested was inhibited by 109 of the 327 isolates (33.3%) from seven types of Korean domestic honey. The percentage of such isolates of manuka honey was significantly higher (76.7%). Solvent fractionation of honey could contribute to the detection of antimicrobial activity of the nonsugar compounds in honey. Moreover, the bacterial isolates from Korean domestic honey may be good sources for the natural antimicrobials used in the food industry and other related industries.

Keywords: antimicrobial activity, floral source, honey isolate, Korean domestic honey, solvent fraction

Introduction

Honey is a natural food and is present in beehives as condensed nectar, which is collected from the nectaries of different floral sources. Honey contains not only sugars, including fructose, glucose, sucrose, and other carbohydrates, but also phenolic compounds and enzymes, including glucose oxidase and catalase (1-3). In honey, these enzymes produce or degrade peroxide compounds (1,4). The characteristic color, flavor, and taste of individual honey may be attributable to the floral sources of plants, atmosphere, and other environmental conditions in different geographical areas (5-7).

One of the most significant biological properties of honey is the antimicrobial activity (1,8-11). Since ancient times, honey has been used as a folk medicine to treat infectious diseases (12). The antimicrobial activity of honey is considered to be originating from osmotic pressure due to high contents of sugar, low pH, hydrogen peroxide (H_2O_2) produced by glucose oxidase, and unidentified compounds (1,9). Honey with a very low water activity is a super-saturated solution; this inhibits microbial growth in honey (4). Osmotic pressure due to high sugar content, which attracts water, causes dehydration of microorganisms (1,4). High acidity due to organic acids, including gluconic acid, maintains the pH of honey in the range of 3.2–4.5, which is lower than the optimum pH required for the growth of most microorganisms (1). H_2O_2 is produced by glucose oxidase in honey when honey is diluted in water; this induces an antimicrobial activity against foodborne pathogens (7,13). In addition, volatile organic acids, flavonoids, pollen, beeswax, and propolis are associated with the antimicrobial activity of honey (13,14). It was reported that different varieties of honey from different floral sources affect gastrointestinal microbiota when cocultured with beneficial microorganisms such as *Bifidobacterium* spp. (15).

Manuka honey, which is indigenous to New Zealand, has been reported to show clear inhibitory activity against different pathogens, including *Pseudomonas aeruginosa*, an opportunistic pathogen (16); *Porphyromonas gingivalis*, a major causative agent of periodontal diseases (17); and *Helicobacter pylori*, which causes peptic ulcers (18). Nonperoxide compounds, including methylglyoxal in manuka honey, have been identified to show antimicrobial activity (19,20). Several varieties of Korean domestic and foreign honey, as well as artificial honey, were screened for antimicrobial activity against *Staphylococcus aureus*, depending on the addition of catalase to each sample (21). In addition, Korean domestic honey samples from different areas have been reported to show antimicrobial activity against several pathogens, including *P. aeruginosa* and methicillin-resistant *S. aureus* (8). However, these previous reports (8,21) have only shown that the characteristic properties of honey, including high contents of sugar, low pH, and peroxides, were associated with the antimicrobial activity of Korean domestic honey. Recently, a South African honey extract using chloroform and a manuka honey extract

using diethyl ether inhibited urease activity, which is crucial for the survival of *H. pylori* under harsh gastric conditions (22). However, there are no other reports that have investigated, via fractionation using organic solvents, the nonsugar or nonperoxide compounds in honey that show antimicrobial activity.

In this study, we attempted to fractionate the antimicrobial compounds of seven varieties of Korean domestic honey and manuka honey from New Zealand using organic solvents. To investigate the nature of the nonsugar compounds showing antimicrobial activity in honey, the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the honey fractions were determined. In addition, bacterial isolates from the eight varieties of honey used in this study were screened for the production of antimicrobial compounds against foodborne pathogens.

Materials and Methods

Honey Seven varieties of Korean domestic honey collected in 2011 from different floral sources were purchased: basswood, Korean raisin, chestnut, and acacia honey from Yangyang, Korea; acacia honey from Seosan, Korea; acacia and a multifloral honey from Mokpo, Korea. Manuka honey (UMF® 10+; Comvita New Zealand Ltd., Coominya, New Zealand) was purchased as a foreign honey for comparison with Korean domestic honey. Honey was stored at room temperature in a dark place before analysis.

Indicator microorganisms Seven foodborne pathogens were selected as the indicators of antimicrobial susceptibility tests. Three Gram-positive bacteria, *Listeria monocytogenes* ATCC 15313, *Bacillus cereus* ATCC 11778, *B. cereus* ATCC 14579, and two Gram-negative bacteria, *Escherichia coli* O157:H7 ATCC 43895 and *Salmonella enterica* serovar Typhimurium ATCC 4931, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *S. aureus* KCTC 1916 was purchased from the Korea Collection for Type Cultures (KCTC, Jeongeup, Korea). *B. cereus* F4552 was kindly provided by Dr. Randy W. Worobo from the Cornell University (Ithaca, NY, USA).

Organic-solvent fractionation of honey Honey was extracted and fractionated using organic solvents according to a method described in a previous study (23). First, 40 g of each type of honey was extracted with 300 mL of absolute methanol via sonication under N_2 purging. The mixture was then homogenized using a Polytron homogenizer (PT 10/35; Kinematica, Kriens-Luzern, Switzerland) at 15,000 rpm for 2 min. The methanolic extract was filtered through a Whatman No. 2 filter paper (Whatman International Ltd., Kent, England) on a chilled Büchner funnel under reduced pressure. The filtrate was subsequently concentrated using a rotary evaporator at 40°C under reduced pressure. The solid concentrate of the methanolic extract after evaporation was dissolved in 1-L deionized water. The dissolved concentrate was extracted using four organic solvents: *n*-hexane, chloroform, ethyl acetate (EtOAc), and *n*-butanol (BuOH), sequentially. All the solvent fractions were concentrated and dried using a rotary evaporator. The solution was separated from the BuOH fraction, and the water fraction was lyophilized. All the fractions were kept at −20°C prior to analysis.

MIC determination Forty solvent fractions of honey (SFH) were assayed in order to determine their MICs. Firstly, 160 μL of Tryptic Soy Broth (TSB, BD, Sparks, MD, USA) was loaded onto a 96-well microplate (SPL, Seoul, Korea). Subsequently, 20 μL of SFH was added to the medium to make final concentrations equal to 50, 100, 250, 300, and 500 μg/mL. Finally, appropriately diluted culture broths (20 μL) of the seven indicator microorganisms grown under optimum conditions were added onto the wells to make final inoculum size equal to 5.0×10^5 CFU/mL. As a blank, 20 μL of TSB was substituted for the culture broth. The microplate in the assay was incubated using a microplate shaker (Confido-S202; FinePCR, Gunpo, Korea) at 37°C for 10 h. The optical density of the incubated mixture in the microplate was measured at 655 nm using a microplate reader (iMark; Bio-Rad, Hercules, CA, USA). MIC was defined as the lowest concentration of SFH that inhibited the growth of the indicator strains compared with the blank.

MBC determination MBC was determined using the same method employed in the MIC determination assay, followed by spreading of the incubated mixture at the MIC and at a concentration higher or lower than the MIC. After incubation in a 96-well microplate for MIC determination, 100 μL of each mixture at the MIC and at a concentration higher or lower than the MIC were spread onto TSB containing 1.5% (w/v) agar (TSA). After 24 h of incubation, the number of colonies that survived the antagonistic activity of the SFH was counted. MBC was defined as the lowest concentration of SFH showing a bactericidal activity at least higher than 99.9% against the initial cell number of the inoculum (5.0×10^5 CFU/mL).

Isolation of bacterial strains from honey To isolate bacterial strains from the eight varieties of honey used in this study, 3 g of each type of honey was mixed with 3 g of distilled water to make 50% (w/w) honey. After agitation of the mixture using a vortex mixer, 100 μL of 50% (w/w) honey was spread onto TSA. The plates were incubated at 30°C for 24–48 h under aerobic conditions. The grown bacterial colonies were streaked on fresh TSA. The bacterial isolates from honey were stocked in 15% (v/v) glycerol at −70°C prior to use.

Antimicrobial activity of honey isolates Antimicrobial activity of the bacterial isolates from honey was assayed using the spot-on-lawn test (24). First, colonies of the honey isolates were inoculated onto the TSA plates using toothpicks and incubated at 30°C for 12 h. After incubation, the plates were overlaid with 6 mL of TSA soft agar [0.75% (w/v) agar] inoculated with 100 μL of the overnight culture

broths of five foodborne pathogens except *B. cereus* ATCC 11778 and *B. cereus* F4552 among the seven indicator microorganisms listed in the previous section. After a 12-h incubation of the overlaid plates, the size of the inhibition zone was measured to determine the antimicrobial activity of the bacterial isolates of honey.

Results and Discussion

MICs and MBCs of the organic-solvent fractions of honey Forty fractions of eight different varieties of honey were initially screened for MIC determination against five foodborne pathogens: *L. monocytogenes* ATCC 15313, *B. cereus* ATCC 14579, *S. aureus* KCTC 1916, *E. coli* O157:H7 ATCC 43895, and *Sal.* Typhimurium ATCC 4931 were used as the indicator strains. None of the indicators except *B. cereus* ATCC 14579 was susceptible to SFH (data not shown). Subsequently, another two *B. cereus* strains (ATCC 11778 and F4552) were subjected to MIC determination (Table 1). The growth of *B. cereus* ATCC 14579 and *B. cereus* F4552 was inhibited by 11 and 16, respectively, of the 40 fractions. However, only the *n*-hexane fraction of manuka honey, whose MIC was 100 μg/mL, showed antimicrobial activity against *B. cereus* ATCC 11778. The chloroform fraction of Korean raisin honey showed the lowest MIC (50 μg/mL) against both *B. cereus* ATCC 14579 and *B. cereus* F4552.

MBCs of the 16 fractions active against at least one of three *B. cereus* strains were determined (Table 1). MBC of the *n*-hexane fraction of manuka honey was 100 μg/mL, which is equal to MIC of the fraction against *B. cereus* ATCC 11778. Of the 11 fractions active against *B. cereus* ATCC 14579, the chloroform fraction of Korean raisin honey showed the lowest MBC (100 μg/mL); however, this value was higher than MIC (50 μg/mL) of the fraction. Conversely, MBCs of the other 10 fractions active against *B. cereus* ATCC 14579 were equal to the MIC of each fraction. Seven out of the 16 fractions active against *B. cereus* F4552 exhibited higher MBC than MIC. In the case of the *n*-hexane fractions of basswood honey from Yangyang and multifloral honey from Mokpo, less than 50 colonies of *B. cereus* F4552 grown on each TSA plate for MBC determination, indicating that a bactericidal effect higher than 99.9%, were observed at 150 and 250 ppm, respectively (Table 1). However, the other five *n*-hexane fractions completely inhibited the growth of *B. cereus* F4552; thus, no colony was observed on the TSA plates.

As shown in Table 1, *n*-hexane and chloroform, which are the most nonpolar solvents used in this study, extracted antimicrobial compounds from five varieties of honey except basswood (EtOAc fraction), Korean raisin (BuOH fraction), and the acacia (EtOAc fraction) honey from Yangyang. Conversely, all the water fractions of the eight honey samples showed no antimicrobial activity against the five foodborne pathogens tested. These results imply that the antimicrobial compounds extracted from the honey tested in this study might be of low polarity. Recently, the total phenolic contents (TPC) and antioxidant capacities of the eight varieties of honey used herein were reported (23). In the present study, the same honey fractions used in the abovementioned previous study were screened for antimicrobial activities against foodborne pathogens. The EtOAc fractions of the eight honey samples showing the highest TPC and antioxidant capacities did not show antimicrobial activity except for the EtOAc fractions of basswood and acacia honey from Yangyang (Table 1); therefore, it was assumed that the majority of the antimicrobial compounds extracted from Korean domestic and foreign honey herein might be less polar than the phenolic compounds responsible for the majority of antioxidant capacities. A low polarity of the antimicrobial compounds in honey tested in this study was consistent with a report in which honey fractions using diethyl ether or chloroform inhibited urease activity, which is indispensable to the growth of *H. pylori* under acidic conditions (22). Currently, it has been found that several EtOAc fractions of the honey samples that were tested in this study showed high levels of hepatoprotective and anti-tyrosinase activities (unpublished results). Consequently, it was suggested that the antimicrobial compounds in the tested SFH might be different from the compounds that show other biological activities.

In our study, it was found that the *n*-hexane and chloroform fractions of manuka honey inhibited the growth of three strains of *B. cereus,* a Gram-positive pathogen. Compared with a previous study that determined both MIC and MBC of manuka honey against *Clostridium difficile*, a pathogenic bacterium (25), we could fractionate Korean domestic honey as well as manuka honey using four organic solvents sequentially. This enabled us to further separate the antimicrobial compounds in honey on the basis of polarity. Therefore, it was expected that the sugar in honey fractionated by more polar solvents such as BuOH or water could be excluded to discover unidentified nonsugar antimicrobial compounds via solvent fractionation.

Antimicrobial activity of honey isolates To isolate the bacterial strains, all the eight honey samples were diluted to make 50% (w/v) honey. In total, 387 isolates of honey were selected on the basis of colony morphology. Most of the isolates were rough in shape and viscose. Conversely, some colonies of the isolates appeared to be small or ivory in color. The highest number of isolates was 71 (from the acacia and multifloral honey from Mokpo), and the lowest number was 10 (from the acacia honey from Yangyang).

The bacterial isolates from seven types of Korean domestic honey and manuka honey were screened for the production of antimicrobial compounds (Table 2). Five foodborne pathogens, including three Gram-positive and two Gram-negative bacteria (listed in Table 2) were selected as indicator strains. The highest incidence of antimicrobial activity was 30.2% (117 isolates) against *L. monocytogenes* ATCC 15313. In particular, 41 isolates (78.8%) of basswood honey and 45 isolates (75.0%) of manuka honey were the two highest incidences of anti-listeria activity. The growth of the other two Gram-positive pathogens, *B. cereus* ATCC 14579 and *S. aureus* KCTC 1916, was

Table 1. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the solvent fractions of Korean domestic and foreign honey active against three strains of *Bacillus cereus*

Honey		Solvent fraction	MIC (μg/mL)			MBC (μg/mL)		
			B. cereus ATCC 14579	*B. cereus* ATCC 11778	*B. cereus* F4552	*B. cereus* ATCC 14579	*B. cereus* ATCC 11778	*B. cereus* F4552
Basswood	Yangyang	*n*-Hexane	150	-[1)]	100	150	-	150
		Chloroform	250	-	250	250	-	250
		Ethyl acetate	-	-	300	-	-	300
		n-Butanol	-	-	-	-	-	-
		Water	-	-	-	-	-	-
Korean raisin		*n*-Hexane	250	-	300	250	-	300
		Chloroform	50	-	50	100	-	250
		Ethyl acetate	-	-	-	-	-	-
		n-Butanol	-	-	300	-	-	ND[2)]
		Water	-	-	-	-	-	-
Chestnut		*n*-Hexane	-	-	-	-	-	-
		Chloroform	250	-	250	250	-	ND
		Ethyl acetate	-	-	-	-	-	-
		n-Butanol	-	-	-	-	-	-
		Water	-	-	-	-	-	-
Acacia		*n*-Hexane	250	-	250	250	-	250
		Chloroform	-	-	250	-	-	ND
		Ethyl acetate	-	-	300	-	-	ND
		n-Butanol	-	-	-	-	-	-
		Water	-	-	-	-	-	-
Acacia	Seosan	*n*-Hexane	250	-	250	250	-	250
		Chloroform	-	-	250	-	-	250
		Ethyl acetate	-	-	-	-	-	-
		n-Butanol	-	-	-	-	-	-
		Water	-	-	-	-	-	-
Acacia	Mokpo	*n*-Hexane	250	-	250	250	-	250
		Chloroform	-	-	-	-	-	-
		Ethyl acetate	-	-	-	-	-	-
		n-Butanol	-	-	-	-	-	-
		Water	-	-	-	-	-	-
Multifloral		*n*-Hexane	250	-	250	250	-	250
		Chloroform	-	-	-	-	-	-
		Ethyl acetate	-	-	-	-	-	-
		n-Butanol	-	-	-	-	-	-
		Water	-	-	-	-	-	-
Manuka	New Zealand	*n*-Hexane	150	100	150	150	100	150
		Chloroform	250	-	100	250	-	250
		Ethyl acetate	-	-	-	-	-	-
		n-Butanol	-	-	-	-	-	-
		Water	-	-	-	-	-	-

[1)]Minus sign (-) indicates that the MIC or MBC of the fraction was not determined because of no antimicrobial activity of the fraction.
[2)]ND stands for not determined, indicating that MBC of the fraction showing antimicrobial activity was not determined.

inhibited by 91 (23.5%) and 96 isolates (24.8%) out of the 387 isolates, respectively. However, only 5.7% (22 isolates) and 3.4% (13 isolates) of the 387 isolates showed an antagonistic effect against two Gram-negative bacteria, *E. coli* O157:H7 ATCC 43895 and *Sal.* Typhimurium ATCC 4931, respectively (Table 2). *E. coli* O157:H7 ATCC 43895 was inhibited by 15 isolates (4.6%) out of the 327 isolates from seven types of Korean domestic honey, compared with seven isolates (11.7%) out of 60 isolates from manuka honey. However, none of the manuka honey isolates was found to inhibit *Sal.* Typhimurium ATCC 4931 (Table 2). According to a previous study (26), the bacterial isolates from six varieties of honey from the United States (US) and two varieties of manuka honey showed a higher incidence of

Table 2. Total number and percentage of the bacterial isolates from Korean domestic and foreign honey against five foodborne pathogens

Honey		No.[1)]	Indicator microorganism				
			L. monocytogenes ATCC 15313	*B. cereus* ATCC 14579	*S. aureus* KCTC 1916	*E. coli* O157:H7 ATCC 43895	*Sal.* Typhimurium ATCC 4931
			The number of isolates exhibiting antimicrobial activity (% of total active isolates)[2)]				
Basswood	Yangyang	52	41 (78.8)	29 (55.8)	22 (42.3)	9 (17.3)	5 (9.6)
Korean raisin	Yangyang	40	11 (27.5)	6 (15.0)	7 (17.5)	2 (5.0)	3 (7.5)
Chestnut	Yangyang	54	5 (9.3)	3 (5.6)	5 (9.3)	2 (3.7)	2 (3.7)
Acacia	Yangyang	10	3 (30.0)	4 (40.0)	2 (20.0)	1 (10.0)	2 (20.0)
Acacia	Seosan	29	3 (10.3)	2 (6.9)	1 (3.4)	1 (3.4)	0 (0.0)
Acacia	Mokpo	71	12 (16.9)	5 (7.0)	7 (9.9)	0 (0.0)	1 (1.4)
Multifloral	Mokpo	71	20 (28.2)	3 (4.2)	15 (21.1)	0 (0.0)	0 (0.0)
Sub total		327	95 (29.1)	52 (15.9)	59 (18.0)	15 (4.6)	13 (4.0)
Manuka	New Zealand	60	45 (75.0)	39 (65.0)	37 (61.7)	7 (11.7)	0 (0.0)
Total		387	117 (30.2)	91 (23.5)	96 (24.8)	22 (5.7)	13 (3.4)

[1)]No. is the number of isolates from each type of honey.
[2)]% represents the percentage of isolates exhibiting antagonistic activity against the indicator microorganisms.

antimicrobial activity against Gram-positive pathogens rather than Gram-negative bacteria. Moreover, *L. monocytogenes* was the most sensitive among the eight indicators tested in the study (26). Consequently, it was evident that the tendency of indicators' susceptibility to the bacterial isolates of Korean domestic honey used in this study was consistent with the previous study (26) that used US domestic honey, i.e., a high susceptibility of Gram-positive foodborne pathogens to honey isolates, among which *L. monocytogenes* was the most sensitive and low or no antimicrobial activity of honey isolates against Gram-negative foodborne pathogens, was observed. In general, the microbiota of indigenous foods such as natural and fermented foods may be affected by environmental factors such as soil, climate, and food sources in the local areas where the ingredients are produced. Therefore, it is interesting and meaningful that each group of the bacterial isolates of honey from different floral sources in geographically distant locations (Korea vs. the US) show the same tendency in the incidence of antimicrobial activity.

Significance of the antagonistic effects of Korean domestic honey

Table 3 shows that 109 isolates (33.3%) from seven varieties of Korean domestic honey showed antimicrobial activity against at least one out of the five foodborne pathogens listed in Table 2. In contrast, 46 isolates (76.7%) out of the 60 isolates from manuka honey exhibited antimicrobial activity against at least one out of the five indicator microorganisms. The highest number of bacterial isolates (44 isolates, 84.6%) from basswood honey showed antimicrobial activity against at least one out of the five pathogens. According to a

Table 3. Incidence of honey isolates exhibiting antimicrobial activity against five foodborne pathogens

Honey		No.[1)]	Antimicrobial activity	
			No.	Percentage (%)[2)]
Basswood	Yangyang	52	44	84.6
Korean raisin	Yangyang	40	11	27.5
Chestnut	Yangyang	54	9	16.7
Acacia	Yangyang	10	3	30
Acacia	Seosan	29	4	13.8
Acacia	Mokpo	71	15	21.1
Multifloral	Mokpo	71	23	32.4
Subtotal		327	109	33.3
Manuka	New Zealand	60	46	76.7
Total		387	155	40.1

[1)]No. is the number of isolates from each type of honey.
[2)]% represents the percentage of isolates exhibiting antagonistic activity against at least one of the five food pathogens which are listed in Table 2.

previous study (26), 1,991 isolates (83.0%) from eight varieties of US and manuka honey exhibited antagonistic activity against at least one of the bacterial indicator strains tested in the study. Interestingly, incidence of the bacterial isolates (76.7%) of manuka honey used in this study and the incidence (99.6%) of a variety of manuka honey (MH1) in the previous study (26) were elucidated to be higher than the average incidence of seven Korean domestic (33.3%) and six American domestic honey (78.3%), respectively. An isolate from the previous study (26) was selected to purify and characterize an antimicrobial peptide; the isolate designated as *B. thuringiensis* SF361 produced thurincin H, a bacteriocin, and the genetic locus of thurincin H was determined (27). Moreover, it was reported that a honey isolate, *B. subtilis* H215, showed a broad range of antifungal spectra using peptide antibiotics, bacillomycin F, and its derivatives (28). Currently, several bacterial isolates from the Korean domestic honey used in this study that showed high levels of antimicrobial activity were selected to purify and characterize antimicrobial compounds. These compounds may be applicable in the food industry to control major foodborne pathogens.

This study was performed to investigate the antimicrobial activity of Korean domestic honey collected from different floral sources and their bacterial isolates. To the best of our knowledge, this is the first report that has characterized antimicrobial compounds in honey using solvent fractionation, which enables the separation of biologically active compounds in honey on the basis of polarity. A further study on unknown antimicrobial compounds existing in honey except sugars, low pH, and peroxides yet to be identified remains to be performed on the basis of this study. Furthermore, the bacterial isolates from Korean domestic honey may be valuable as new sources of food-grade antimicrobial compounds for use in the food, agricultural, and pharmaceutical industries for controlling pathogenic microorganisms.

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