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ARTICLE

1,2-Benzendiol Isolated from Persimmon Roots and Its Structural Analogues Show Antimicrobial Activities against Food-borne Bacteria

Min-Gi Kim · Hoi-Seon Lee

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Abstract Antimicrobial activities of the five fractions obtained from the methanol extract of persimmon (*Diospyros kaki*) roots were evaluated against eight food-borne bacteria using the agar diffusion method. The chloroform fraction possessed strong antimicrobial activity against eight food-borne bacteria. 1,2-Benzenediol was isolated by chromatographic analyses. The structure-activity relationships of two isomers (1,3-benzendiol and 1,4-benzenediol) and seven structural analogs (3-methy-, 4-methyl-, 3-methoxy-, 4-chloro-, 4-nitro-, 4-*tert*-butyl-, and tetrabromo-1,2-benzenediol) were tested against food-borne bacteria. When various functional groups were added to 1,2-benzenediol, the 1,2-benzendiol analogs exerted potent activities against the eight food-borne bacteria. In the case of minimum inhibitory concentration, 1,2-benzendiol and its structural analogs showed significantly potent antimicrobial activity against the tested bacteria. Taken together, these findings indicate that *D. kaki* root-isolated 1,2-benzendiol and its structural analogs could be useful as eco-food supplemental agents.

Keywords 1,2-benzendiol · antimicrobial activity · *Diospyros kaki* roots · food-borne bacteria

Introduction

Despite modern-day food production techniques, food-borne diseases remain a major concern in developed/developing countries (Nedorostova et al., 2009). Thus, the food industry widely uses chemical food preservatives to prevent growth of food-borne pathogens (Sahin et al., 2004). However, some chemical preservatives may cause side effects or strain resistance (Shahmohamadi et al., 2011; Yang et al., 2013). Due to the increased awareness of chemical preservatives, the food industry is now reflecting consumer opinions for safer food and is focusing on generally recognized as safe preservatives (Dillon and Board, 1994; Fazeli et al., 2007).

Natural substances from edible and medicinal plants have excellent antimicrobial properties including phenolic compounds and their subclasses, such as carvacrol, coumarins, eugenol, and flavonoids (Lee and Ahn, 1998; Kim et al., 2003; Sandri et al., 2007). Persimmon (*Diospyros kaki* L.) is a deciduous fruit cultivated throughout China, Japan, and Korea, with more than 1,000 local varieties estimated to be grown worldwide (Veberic et al., 2010). It is used traditionally for medicinal purposes, including treatment of burns, cough, paralysis, as well as to stop bleeding (Sun et al., 2011). Persimmon contains various active compounds such as carotenoids, flavonoids, tannins, kaempferol, quercetin, and vitamin A (Sun et al., 2011). Previous studies have shown that *D. kaki* has beneficial effects on apoplexy, atherosclerosis, constipation, hypertension, and allergic inflammation and is a good source of antioxidants, polyphenols, and dietary fiber (Kim et al., 2013). Despite these pharmacological effects, relatively little work has been conducted to evaluate the effects of *D. kaki* materials on food-borne bacteria. Therefore, we evaluated the antimicrobial activities of *D. kaki* materials and assessed the structure-activity relationships of two isomers and seven structural analogs against food-borne bacteria.

Materials and Methods

Chemicals. 1,3-Benzenediol, 1,4-benzenediol, 3-methyl-1,2-benzenediol, 4-methyl-1,2-benzenediol, 3-methoxy-1,2-benzenediol, 4-chloro-1,2-benzenediol, 4-nitro-1,2-benzenediol, 4-*tert*-butyl-1,2-benzenediol, and tetrabromo-1,2-benzenediol were purchased from Sigma-Aldrich (USA) and Fluka (USA). All other chemicals used were of reagent grade.

Isolation and identification of the compound. *D. kaki* roots

M.-G. Kim · H.-S. Lee (✉)
Department of Bioenvironmental Chemistry and Institute of Agricultural Science & Technology, College of Agriculture & Life Science, Chonbuk National University, Jeonju 561-756, Republic of Korea
E-mail: hoiseon@jbnu.ac.kr

were collected during the fall of 2010 in Korea. The roots (1 kg) were air-dried, ground to a fine powder, and extracted twice with methanol (2 L) at room temperature for 2 days, after which they were filtered (Toyo filter paper No. 2, Toyo Roshi, Japan) under vacuum. The extract was concentrated and combined *in vacuo* at 45°C using a rotary vacuum evaporator (EYELA autojack NAJ-100, Japan). The methanol extract (100 g) of *D. kaki* roots was sequentially partitioned into hexane (6.5 g), chloroform (25.0 g), ethyl acetate (15.5 g), butanol (21.0 g), and water-soluble (31.5 g) fractions for bioassay. The organic solvent fractions were concentrated to dryness by rotary evaporation at 45°C, whereas the water soluble fraction was freeze-dried (Bondiro; Ilshin Biobase, Korea).

To purify the active compound, the chloroform (20 g) fraction was chromatographed on a silica gel column (Merck 70–230 mesh, 600 g, 550 mm i.d.×700 mm;, USA) and was continuously eluted using a stepwise gradient of chloroform : methanol (3:7, 2:8, 1:9, and 0:10, v:v) into five fractions (D1-D5). Each fraction was analyzed by thin layer chromatography (TLC) to identify similar TLC patterns. D4 fraction (3.5 g) showed the strongest antimicrobial activity against the tested bacteria; hence, this fraction was applied to further chromatography on a column (Merck 70–230 mesh, 600 g, 550 mm i.d.×700 mm), and eluted with chloroform:methanol (2:8, v:v) to yield the D42 active fraction. The D42 (2.0 g) fraction was isolated by preparative high performance liquid chromatography (HPLC) (Recycling Preparative HPLC, Japan Analytical Industry Co., Ltd., Japan) using a JAI GS Series column (GS310 500 mm×2). The mobile phase was 100% methanol, which was applied at a flow rate of 3.5 mL/min, and UV detection was performed at 232 nm. The resulting active D421 fraction (246 mg) was further separated using a JAI W Series column (W252 500+W253 500 mm, Japan) and 100% methanol under the same conditions. The final active compound D4211 (99 mg) was isolated, and its structure was determined by various instrumental analyses. All nuclear magnetic resonance (NMR) spectra ^{1}H-NMR, ^{13}C-NMR, distortionless enhancement by polarization transfer (DEPT), correlation spectroscopy (COSY), and heteronuclear multiple quantum correlation (HMQC) were obtained with a JNM-ECA 600 spectrometer (JEOL Ltd., Japan; 600 and 150 MHz) using $CDCl_3$ as the internal standard. The UV visible absorption spectra were obtained with a UV spectrometer (DR 4000 spectrophotometer, HACH, Loveland, CO., USA).

Microorganisms. The food-borne bacteria used in this study were *Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* KCCM 11335, *S. epidermidis* ATCC 12228, *S. intermedius* ATCC 29663, *Salmonella enterica* ATCC 43971, *S. typhimurium* IFO 14193, and *Shigella sonnei* ATCC 25931. These strains were obtained from the Korean Culture Center of Microorganisms (Korea). Bacterial strains were aerobically cultured at 37°C for 24 h in nutrient broth (NB) and tryptic soy broth (TSB).

Agar diffusion method. Paper disc agar diffusion method was used to assess the antimicrobial activities of the samples. Briefly, microorganisms were incubated in NB and TSB at 37°C for 24 h to yield approximately 1.0×10^7 CFU/mL compared to the turbidity of the McFarland turbidity standard. An aliquot (0.1 mL of 1.0×10^7 CFU/mL) of the bacterial suspension was seeded onto Mueller-Hinton agar (MHA) plates. Sterilized paper discs (Advantec, diameter 8 mm and thickness 1 mm, Toyo Roshi, Japan) were impregnated with 40 μL of sample and placed on inoculated MHA plates. All plates were incubated under aerobic conditions at 37°C for 24 h. All tests were replicated three times, and antimicrobial activity was expressed as the diameter of the inhibition zone (mm). Values are presented as means ± standard deviation (SD) of three parallel measurements.

Minimum inhibitory concentration (MIC). The MICs of the samples derived from the *D. kaki* extract were determined by the broth microdilution technique as described by Natioanl Committee for Clinical Laboratory Standards (NCCLS, 2003). The samples were serially two-fold diluted ranging from 0.1-500 μg/mL. Each dilution (50 μL) was dispensed into a 96-well plate with an additional 100 μL of Mueller-Hinton broth, and inoculated with 50 μL of each bacterial suspension. The final concentration of each strain was adjusted to 10^7 CFU/mL (absorbance values of 0.08–0.10 at 625 nm, according to McFarland turbidity standards). The MIC was the lowest concentration of sample at which visible growth of microorganisms was not demonstrated. These plates were incubated for 24 h at 37°C.

Statistical analysis. All experiments were replicated three times. Results are presented as mean ± SD and were analyzed by analysis of variance. Statistical significance was accepted at $p<0.05$ (SAS Institute, 2004).

Results and Discussion

The yield of *D. kaki* root methanol extract was 33.89%. Five fractions were partitioned from the methanol extract of the *D. kaki* roots. The highest yield was obtained from the water-soluble (31.5%), followed by the chloroform (25.0%), butanol (21.0%), ethyl acetate (15.5%), and hexane fractions (6.5%). The antimicrobial activities of the methanol extract and the five fractions were tested against five Gram-positive (*B. cereus*, *L. monocytogenes*, *S. aureus*, *S. epidermidis*, and *S. intermedius*) and three Gram-negative (*S. enterica*, *S. typhimurium*, and *S. sonnei*) bacteria using the paper disc agar diffusion method (Table 1). Methanol extract and chloroform fraction derived from *D. kaki* roots exhibited antimicrobial activities against all tested microorganisms at 10 mg/disc. Antimicrobial activity of the hexane fraction was exhibited against *S. sonnei*, whereas the other fractions had no antimicrobial activity at 10 mg/disc. Therefore, the chloroform portion was selected to isolate the active component in *D. kaki* root.

Due to the strong activity of the chloroform fraction against food-borne bacteria, isolation of the active component was pursued using a silica gel column and HPLC. Bioassay-guided

Table 1 Antimicrobial activities of materials derived from *D. kaki* root extract

Materials[1)]	Clean zone							
	Microorganisms[2)]							
	Bc	Lm	Sae	St	Ss	Sa	Se	Si
Methanol	11.0±1.4[3)]	11.5±0.0	10.5±1.4	9.5±0.0	10.0±0.0	9.0±1.4	11.0±1.4	12.5±0.7
Hexane	-[4)]	-	-	-	-	-	-	11.0±0.7
Chloroform	12.5±0.7	13.0±1.4	12.5±2.1	11.0±0.7	11.5±1.4	10.5±0.7	12.5±2.1	13.5±1.4
Ethyl acetate	-	-	-	-	-	-	-	-
Butanol	-	-	-	-	-	-	-	-
Water	-	-	-	-	-	-	-	-

[1)]Exposed to 10 mg/disc.
[2)]BC, *Bacillus cereus* ATCC 14579; Lm, *Listeria monocytogenes* ATCC 15313; Sae, *Salmonella enterica* ATCC 43971; St, *S. typhimurium* IFO 14193; Ss, *Shigella sonnei* ATCC 25931; Sa, *Staphylococcus aureus* KCCM 11335; Se, *S. epidermidis* ATCC 1228; Si, *S. intermedius* ATCC 29663.
[3)]Values (mm) are mean ± SD of three parallel measurements, p <0.05.
[4)]-, no activity.

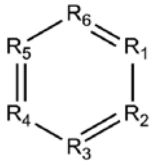
fractionation of the chloroform fraction yielded an active component that was identified by various spectroscopic analyses, including UV, electron impact-mass spectrometry (EI-MS), ^{1}H NMR, ^{13}C NMR, DEPT, COSY, and HMQC using a direct comparison with an authentic reference compound. The active compound was characterized as 1,2-benzenediol ($C_6H_6O_2$, MW: 110.11 g/mol); EI-MS (70 eV) *m/z*: M^+ 110, 92, 81, 64, 50; ^{1}H NMR ($CDCl_3$, 600 MHz, δ ppm) =6.307 (OH, *s*), 4.221-4.240 (1H, *m*, *J*=17.4), 4.082-4.112 (1H, *m*, *J*=18.0); ^{13}C NMR ($CDCl_3$, 150 MHz, δ ppm) =145.787, 119.801, 116.201. These findings are similar to those of Jeong et al. (2009). 1,2-Benzendiol is a phenolic compound synthesized by the shikimate pathway in plants. This compound is present in beech, birch, oak, hazelnut, poplar, and willow (Kuiters and Sarink, 1986; Kocaçalişkan et al., 2006).

The antimicrobial activity of 1,2-benzenediol isolated from *D. kaki* roots was evaluated using the agar diffusion method at 1.0 mg/disc and compared with that of tetracycline serving as the positive control (Table 2). 1,2-Benzenediol isolated from *D. kaki* roots had antimicrobial activities against the eight food-borne bacteria (13.0–17.5 mm; inhibitory zone diameter). These results indicate that the antimicrobial activity of *D. kaki* roots against the eight food-borne bacteria could be attributed to 1,2-benzenediol. The structure-activity relationships of the two isomers (1,3-benzenediol and 1,4-benzenediol) and seven structural analogs (3-methyl-, 4-methyl-, 3-methoxy-, 4-chloro-, 4-nitro-, 4-*tert*-butyl-, and tetrabromo-1,2-benzenediol) were evaluated by the agar diffusion method at 1.0 mg/disc (Table 2 and Fig. 1). 4-Nitro-1,2-

Table 2 Antimicrobial activities of the isolated compound and its derivatives

Compounds[1)]	Clean zone							
	Microorganisms[2)]							
	Bc	Lm	Sae	St	Ss	Sa	Se	Si
1,2-Benzenediol	15.5±1.4[3)]	16.5±1.4	16.0±0.7	14.5±2.1	15.0±1.4	13.0±0.7	16.5±1.4	17.5±1.4
1,3-Benzenediol	-[4)]	-	-	-	-	-	-	-
1,4-Benzenediol	11.5±0.7	12.0±1.4	11.0±0.7	11.0±0.7	11.5±1.4	-	11.5±0.7	12.0±0.0
3-Methyl-1,2-benezenediol	16.0±0.0	16.5±0.7	15.5±1.4	16.5±0.0	15.0±0.7	16.5±0.7	17.0±2.1	15.0±0.0
4-Mehtyl-1,2-benezenediol	15.0±0.0	15.0±1.4	15.5±0.0	13.5±0.0	13.5±0.7	13.0±0.0	15.5±0.7	16.0±0.7
3-Methoxy-1,2-benezenediol	10.5±0.0	11.5±0.7	11.0±0.0	11.5±0.7	11.0±0.0	10.0±0.7	11.5±0.0	13.5±0.0
4-Chloro-1,2-benezenediol	21.0±0.7	23.5±0.7	22.0±0.7	23.0±0.0	22.5±0.0	22.0±0.0	23.0±0.0	22.0±1.4
4-Nitro-1,2-benezenediol	23.0±0.0	26.5±0.0	25.5±0.0	27.5±1.4	27.0±0.0	27.5±0.7	27.0±0.0	36.0±0.0
4-*tert*-Butyl-1,2-benezenediol	18.5±0.7	17.5±0.0	17.0±0.7	16.5±0.7	16.0±0.0	19.0±0.0	19.5±0.0	20.0±1.4
Tetrabromo-1,2-benezenediol	23.5±0.0	22.5±0.0	22.5±0.0	22.5±0.7	23.0±0.0	23.5±0.0	23.0±0.7	31.5±0.0
Tetracycline	27.0±0.0	24.5±0.7	26.5±0.0	24.5±0.0	23.0±0.0	22.5±0.0	20.5±2.1	34.0±0.7

[1)]Exposed to 1 mg/disc, tetracycline =0.1 mg/disc.
[2)]BC, *Bacillus cereus* ATCC 14579; Lm, *Listeria monocytogenes* ATCC 15313; Sae, *Salmonella enterica* ATCC 43971; St, *S. typhimurium* IFO 14193; Ss, *Shigella sonnei* ATCC 25931; Sa, *Staphylococcus aureus* KCCM 11335; Se, *S. epidermidis* ATCC 1228; Si, *S. intermedius* ATCC 29663.
[3)]Values (mm) are mean ± SD of three parallel measurements, p <0.05.
[4)]-, no activity.



Compounds	R_1	R_2	R_3	R_4	R_5	R_6
1,2-Benzenediol	**OH**	**OH**	C	C	C	C
1,3-Benzenediol	**OH**	C	**OH**	C	C	C
1,4-Benzenediol	**OH**	C	C	**OH**	C	C
3-Methyl-1,2-benezenediol	**OH**	**OH**	**CH_3**	C	C	C
4-Mehtyl-1,2-benezenediol	**OH**	**OH**	C	**CH_3**	C	C
3-Methoxy-1,2-benezenediol	**OH**	**OH**	**OCH_3**	**C**	C	C
4-Chloro-1,2-benezenediol	**OH**	**OH**	C	**Cl**	C	C
4-Nitro-1,2-benezenediol	**OH**	**OH**	C	**NO_2**	C	C
4-*tert*-Butyl-1,2-benezenediol	**OH**	**OH**	C	**C_4H_9**	C	C
Tetrabromo-1,2-benezenediol	**OH**	**OH**	**Br**	**Br**	**Br**	**Br**

Fig. 1 Structures of 1,2-benzenediol and its structural analogs.

benzenediol (26.5 mm) had potent antimicrobial activity against *L. monocytogenes* at 1 mg/disk, followed by 4-chloro-1,2-benzenediol (23.5 mm), tetrabromo-1,2-benzenediol (22.5 mm), 4-*tert*-butyl-1,2-benzenediol (17.5 mm), 1,2-benzenediol (16.5 mm), 3-methyl-1,2-benzenediol (16.5 mm), 4-methyl-1,2-benzenediol (15.0 mm), 1,4-benzenediol (12.0 mm), and 3-methoxy-1,2-benzenediol (11.5 mm); however, 1,3-benzenediol showed no antimicrobial activity. 1,4-Benzenediol (*para* isomer form of 1,2-benzenediol) exhibited similar or slightly decreased antimicrobial activity. Moreover, 1,3-benzenediol (*meta* isomer form of 1,2-benzenediol) showed no antimicrobial activity against all tested microorganisms. These results indicate that changing the position of the hydroxyl radical in the benzene ring changed the antimicrobial activity (*ortho*→*para*→*meta*-positions). The number of hydroxyl groups and their positions on the benzene ring are important for phenols. Finotti and Di Majo (2003) reported that *ortho*- and *para*-substitutions of the radicals are more stable compared to that of *meta*-substitution. Antimicrobial activity and the functional radicals of 1,2-benzendiol (seven structural analogs) with a methyl group on the 1,2-benzenediol (3-methyl- and 4-methyl-1,2-benzenediol) had similar antimicrobial activity as that of 1,2-benzenediol against the eight food-borne bacteria. 1,2-Benzenediol combined with a methoxy group (3-methoxy-1,2-benzenediol) showed decreased antimicrobial activity relative to that of 1,2-benenediol against the eight food poisoning bacteria. The antimicrobial activities of 4-chloro-, 4-nitro-, 4-*tert*-butyl-, and tetrabromo- groups on 1,2-benzenediol were greater than that of 1,2-benzenediol against the eight tested bacteria.

The MIC values of 1,2-benzendiol and its derivatives were revealed by determining antimicrobial activities (Table 3). The MIC values of 1,2-benzendiol were 62.25 μg/mL against *S. typhimurium* and *S. sonnei*, 125 μg/mL against *B. cereus*, *L.*

Table 3 Minimum inhibitory concentration of 1,2-benezendiol and its derivatives

Materials[1)]	Microorganisms[2)]							
	Bc	Lm	Sae	St	Ss	Sa	Se	Si
1,2-Benzenediol	125	125	250	62.25	62.25	500	125	250
1,3-Benzenediol	500<	500<	500<	500<	500<	500<	500<	500<
1,4-Benzenediol	500	500	500	500	500	500<	500<	500<
3-Methyl-1,2-benezenediol	125	125	125	125	125	125	125	500
4-Mehtyl-1,2-benezenediol	125	125	125	500	500	500	500	500
3-Methoxy-1,2-benezenediol	500	500	500	500	500	500	500	500
4-Chloro-1,2-benezenediol	7.81	15.63	15.63	3.91	15.63	7.81	7.81	7.81
4-Nitro-1,2-benezenediol	3.91	7.81	7.81	3.91	7.81	3.91	7.81	7.81
4-*tert*-Butyl-1,2-benezenediol	1.95	3.91	1.95	7.81	15.63	3.91	15.63	15.63
Tetrabromo-1,2-benezenediol	7.81	3.91	3.91	7.81	7.81	3.91	7.81	3.91

[1)]MIC values <0.5 μg/mL.
[2)]BC, *Bacillus cereus* ATCC 14579; Lm, *Listeria monocytogenes* ATCC 15313; Sae, *Salmonella enterica* ATCC 43971; St, *S. typhimurium* IFO 14193; Ss, *Shigella sonnei* ATCC 25931; Sa, *Staphylococcus aureus* KCCM 11335; Se, *S. epidermidis* ATCC 1228; Si, *S. intermedius* ATCC 29663.

monocytogenes, and *S. epidermidis*, 250 μg/mL against *S. enteria* and *S. intermedius*, and 500 μg/mL against *S. aureus*. Furthermore, 4-chloro-, 4-nitro-, 4-*tert*-butyl-, and tetrabromo-1,2-benzenediol possessed excellent antimicrobial effects (MICs, 1.95-15.63 μg/mL) against the eight food poisoning bacteria. Hassan Shah et al. (2012) reported that MIC values of *D. kaki* bark extract were 10 μg/mL against *B. subtilis*, *B. cereus*, and *E. coli* and 20 μg/mL against *S. aureus*, *P. aeruginosa*, and *S. typhi*. Furthermore, these results were similar to those of previous studies reporting that 1,2-benzenediol structures substituted at the C-4 position with a functional group (e.g., 4-chlro-, 4-nitro-, and 4-*tert*-butyl-1,2-benzenediol) were more active toward *Clostridium* and *E. coli* than 1,2-benzenediol structures substituted at the C-3 position (e.g. 3-methyl- and 3-methoxy-1,2-benzendiol) (Jeong et al., 2009).

Based on the Material Safety Data Sheet provided by Sigma-Aldrich (2013), the oral LD_{50} values of 1,2-benzenediol (260 mg/kg), 1,3-benzenediol (301 mg/kg), 1,4-benzenediol (302 mg/kg), and 4-*tert*-butyl-1,2-benzenediol (815 mg/kg) indicated moderate or low acute toxicity to mammals. Moreover, the results of previous studies indicate that 1,2-benzenediol and its derivatives act as antioxidants in eukaryotic cells, preventing degenerative diseases caused by free radicals, including immune system decline, heart disease, and cancer (Berberian et al., 2006; Jeong et al., 2009). These results indicate that *D. kaki* roots and 1,2-benzenediol analogs could be used as a source of natural antimicrobial agents potentially suitable to replace chemical preservatives.

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