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ARTICLE

2-Isopropyl-5-methylphenol Isolated from *Ruta graveolens* and Its Structural Analogs Show Antibacterial Activity against Food-borne Bacteria

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Abstract The antimicrobial activities of the essential oil obtained from the aerial parts of *Ruta graveolens* and 2-isopropyl-5-methylphenol analogs were evaluated against six food-borne bacteria. The essential oil of *R. graveolens* aerial parts exhibited potent antimicrobial activity against six food-borne bacteria. 2-Isopropyl-5-methylphenol was isolated by chromatographic analyses. The structure-activity relationships of the 2-isopropyl-5-methylphenol analogs, 2-isopropyl-5-methylphenol, and its structural analogs (2-isopropylphenol, 2-methylphenol, phenol, and 2-isopropyl-5-methylbenzene) were determined against six food-borne bacteria. When employing the agar diffusion method, 2-isopropyl-5-methylphenol and 2-isopropylphenol had potent antimicrobial activities against the six food-borne bacteria. The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) values of 2-isopropyl-5-methylphenol and its structural analogs were determined against the six food-borne bacteria. 2-Isopropyl-5-methylphenol exhibited the strongest activity (MIC, 5–6.25 μg/mL; MBC, 6.25–12.5 μg/mL) against the six food-borne bacteria. Therefore, the essential oil of *R. graveolens* and 2-isopropyl-5-methylphenol analogs should be useful as natural food preservatives.

Keywords 2-isopropyl-5-methylphenol · antimicrobial food-borne bacteria *R. graveolens*

Introduction

Various illnesses are caused by consuming food contaminated with bacteria (Oussalah et al, 2007). Thus, antimicrobial drugs are widely used to control food-borne diseases. However, conventional use of antimicrobial drugs leads to the emergence of antibiotic-resistant pathogenic strains (Yang et al., 2013). The principle mechanisms of bacterial resistance to antimicrobials include active drug efflux systems, mutations that result in altered cell permeability, cellular degradation of antimicrobials, and alterations in their cellular targets (Nikaido, 1994; Walsh, 2000). Increasing concern about this problem has led to the development of safe alternatives, such as natural products against food-borne bacteria (Lee and Ahn, 1998; Lim et al., 2007).

Essential oils are aromatic oily liquids obtained from plant materials (fruits, flowers, buds, seeds, twigs, leaves, bark, wood, herbs, and roots), and these essential oils can be obtained by expression, fermentation or extraction; however, the steam distillation method is the most commonly used method for commercial production (Prabuseenivasan et al., 2006; Yang et al., 2002). In addition, plant-derived oils have been used as flavoring agents in food for a long time and as natural agents for food preservation (Helander et al., 1998; Lee, 2002). Bioactive constituents with antimicrobial activity found in plants, spices, and herbs include phenolic compounds, terpenes, aldehydes, aliphatic alcohols, ketones, and isoflavonoids (Katayama and Nagai, 1960; Farag et al., 1989; Dorman and Deans, 2000; Lambert et al., 2001; Burt and Foegeding, 2003; Kim et al., 2003; Kim et al., 2004). Above all, the modes of action of phenolic compounds as antimicrobial agents have been reported (Lopez et al., 2007). The effect of phenolic compounds on microbial growth could be the result of their ability to alter microbial cell permeability, and permit the loss of macromolecules from the interior (Santiesteban-Lopez et al., 2007). *Ruta graveolens* L. (Rutaceae) is a traditional medicinal plant that prevents attacks by fleas and other noxious insects (Vincenzo et al., 2002). In addition, *R. graveolens* is used as a flavoring agent in foods and spirits (Vincenzo et al., 2002) and has been recommended as an herbal medicine for treatment of

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headache, insomnia, nervousness, abdominal cramps, and renal troubles (Malik et al., 2013). The objective of the present study was to isolate the active components of *R. graveolens* oil and test them against six foodborne bacteria. We also discuss the structure-activity relationships of the analogs.

Materials and Methods

Chemicals. 2-Isopropylphenol, 1-isopropyl-4-methylbenzene, 2-methylphenol, and phenol were purchased from Sigma-Aldrich (USA). All other compounds were of reagent grade.

Plant materials. Aerial parts of *R. graveolens* from air-dried plants were purchased from a local market (Korea). The essential oil of *R. graveolens* was extracted by the steam distillation extraction method. The extracted oil (yield, 0.06%) was dried with anhydrous sodium sulfate and stored in a sealed vial at 4°C prior to isolation.

Isolation and identification. The oil of *R. graveolens* aerial parts (20 g) was sequentially fractionated by chromatography on a silica gel column (Merck 70–230 mesh, 600 g, 6.0 cm i.d.×80 cm; USA), and then continuously eluted with a step gradient of hexane-ethyl acetate (10:0 to 0:10, v/v), which gave five fractions (R1–5). These fractions were analyzed by thin-layer chromatography (TLC), and fractions with similar patterns were combined. The active R3 (14.5 g) fraction was subjected to silica gel column chromatography (6.0×80 cm) using hexane-ethyl acetate (9:1 and 8:2, v/v) as the mobile phase to provide four fractions (R31–4). Fraction R33 (8.4 g) was isolated by preparative high-performance liquid chromatography (prep. HPLC, Japan Analytical Industry Co. Ltd, Japan) using a Jai gel W series column (W 253 50 cm+W 252 50 cm), with 100% chloroform as the mobile phase at a flow rate of 3.0 mL/min, which produced three fractions (R331–3). Fraction R331 was subjected to further chromatography to acquire a refined active compound on a Jai gel GS series column (GS 310 50+GS 310 30 cm) using hexane-ethyl acetate (1:9, v/v), which gave three fractions (R3311–3). Fraction R3312 was subjected to a Jai gel GS series column, with 100% chloroform as the mobile phase under the same conditions, providing two fractions (R33121 and R33122). Finally, fraction R33121 (1.2 g) was identified by spectroscopic method. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) were conducted using a JNM-EX 600 spectrometer (JEOL, Japan) in deuterochloroform ($CDCl_3$) with trimethylsilane as the internal standard at 600 and 150 MHz, respectively. Additionally, electron-impact-mass spectroscopy (EI-MS) spectra were obtained with a JEOL JMS-DX 30 spectrometer.

Gas chromatography-mass spectrometry (GC-MS). Aerial parts of *R. graveolens* were analyzed on a gas chromatograph (6890, Agilent, Technologies, USA)-mass spectrometer (5973 IV, Agilent). A 30 m×0.25 mm inside diameter DB-5 (0.25 mm film) fused silica capillary column (J&W Scientific, USA) was used as the GC column. The GC conditions were as follows: injector temperature, 210°C; column temperature, isothermal at 50°C for 15 min, then programmed to rise to 200°C at 2°C/min and held at this temperature for 15 min; ion source temperature, 230°C. Helium gas was used as the carrier gas at a flow rate of 0.8 mL/min. The GC effluent directly entered the MS. Spectra were obtained in the EI mode with 70 eV. The mass analyzer was set to scan from 50 to 600 amu for 2 s. Components were identified by comparison of their retention time, retention index, and mass spectra with those in a mass spectra library (The Wiley Registry of Mass Spectral Data, 8th edition).

Bacterial strains and culture conditions. The food-borne bacteria used in the experiments included the Gram-positive bacteria *Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 15313, and *Staphylococcus intermedius* ATCC 29663, and the Gram negative bacteria *Salmonella enterica* ATCC 43971, *Salmonella typhimurium* IFO 14193, and *Shigella sonnei* ATCC 25931. The bacterial strains were obtained from the Korean Culture Center of Microorganisms (Korea). The bacteria were aerobically cultured at 37°C for 24 h in nutrient broth (Difco, USA), with the exception of *Escherichia coli*, which was cultured in brain heart infusion broth (Difco).

Antimicrobial activity The paper disc agar diffusion method was used to determine the antimicrobial activities of 2-isopropyl-5-methylphenol and its structural analogs such as 2-isopropylphenol, 2-methylphenol, phenol, and 1-isopropyl-4-methylbenzene. To assay the antimicrobial activity of the test microorganisms used, one loopful of bacteria was suspended in 1 mL of sterilized physiological saline. An aliquot (0.1 mL) of the test bacterial suspension was seeded on a Mueller-Hinton agar (MHA, Difco) plate. Each test sample was then dissolved in 100 μL methanol solution and applied to a paper disc using a Drummond glass microcapillary (8 mm diameter and 1 mm thickness; Advantec Roshi, Japan). After evaporating the solvents, the discs were placed on an agar surface preincubated with test bacteria. All plates were then incubated anaerobically at 37°C for 2 days. The control discs received 100 μL methanol. All growth inhibition tests were replicated three times. The range of antimicrobial activity was: potent activity, >30 mm; strong activity, 21–30 mm; moderate activity, 16–20 mm; weak activity, 10–5 mm; and little or no activity, <10 mm.

Minimum bactericidal concentration (MBC)/minimum inhibitory concentration (MIC). The MBC and MIC values of the essential oil and the 2-isopropyl-5-methylphenol analogs were determined by the broth micro-dilution technique, as described previously (NCCLS, 2003). Each sample (10 mg) was dissolved in methanol (10 mL) as a stock solution and was consecutively diluted from 100 to 0.1 μg/mL using methanol as the solvent. Each dilution (50 μL) was dispensed into a 96-well microplate that had been injected with 100 μL Mueller-Hinton broth and was inoculated with 50 μL of 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 defined as the lowest concentration of a substance that inhibited visible growth of the microorganisms. The MBC

was the lowest concentration at which no colony formulation was observed on the agar plates, determined by spreading 100 μL of each microorganism on a MHA plate. These plates were incubated at 37°C for 24 h, and all experiments were performed in triplicate.

Results and Discussion

Antimicrobial activity of the essential oil derived from *R. graveolens* aerial parts was tested against six food-borne bacteria via the paper disc agar diffusion method. The essential oil of *R. graveolens* at 5.0 mg/disc exhibited potent antimicrobial activity against *B. cereus*, *L. monocytogenes*, *S. intermedius*, *S. enterica*, *S. typhimurium*, and *S. sonnei* (Table 1). The essential oil from *R. graveolens* was analyzed to determine the active compounds.

The components identified in the essential oil from the aerial parts of *R. graveolens* by GC-MS are presented in Table 2. The analysis led to the identification of seven constituents from the essential oil. The main constituents were 2-acetoxytridecane (5.64%), 2,4-dimethylundecane (2.42%), 4-hexadecanyl pivalate (6.12%), *p*-isopropyltoluene (1.85%), 2-isopropyl-5-methylphenol (31.13%), 1-methyltridecyl pentanoate (12.13%), and pentadecanol (18.52%). The main constituents made up 77.81% of the oil. Soleimani et al. (2009) reported the main compounds of the oil from *R. graveolens* aerial parts as 2-nonanone, geyrene, 2-heptanol acetate, 2-undecanone, and 1-dodecanol. In this regard, natural components isolated from plants are influenced by plant species, plant parts (flower, root, and leaves), and geographical location where the plants were grown (Oribe and Miyazzaki, 1997).

Silica gel column chromatography, TLC, and prep HPLC were performed to isolate the active component of the *R. graveolens* oil, using single or mixed organic solvents. As a result, the R33121 fraction was successfully isolated and analyzed by spectroscopic analyses, including EI-MS, ^{1}H-NMR, ^{13}C-NMR, and distortionless enhancement by polarization transfer-NMR spectra. The isolated R33121 fraction was characterized as 2-isopropyl-5-methylphenol ($C_{10}H_{14}O$, MW 150); EI-MS (70 eV) *m/z* (% relative intensity) M^+ 150 (34), 135 (100), 107 (19), 91 (25), 71 (8), 58 (26), 50 (4); ^{1}H-NMR ($CDCl_3$, 600 MHz, δ ppm) 6.99-7.02 (1H, d, J=7.9 Hz), 6.72-6.73 (1H, d, J=7.9 Hz), 6.56 (1H, s), 3.12-3.19 (1H, t, J=6.9 Hz), 1.23-1.24 (3H, d, J=6.9 Hz), 1.23-1.24 (3H, d, J=6.9 Hz), and 2.26 (3H, s); ^{13}C-NMR ($CDCl_3$, 150 MHz, δ ppm) 152.6, 136.7, 131.4, 126.3 (CH), 121.7 (CH), 116.1 (CH), 26.8 (CH), 22.7 (CH3), 22.7 (CH3) and 20.9 (CH3). The spectroscopic data of 2-isopropl-5-methylphenol were compared with those of previous studies (Ezoubeiri et al., 2005; Jeong et al., 2008).

The antimicrobial activity of the *R. graveolens* essential oil against several micro-organisms has been reported (Alzoreky and Nakahara, 2003; Ivanova et al., 2005). Thymol (2-isopropyl-5-methylphenol) has also been tested against multiple food-borne

Table 1 Antibacterial activities of essential oil of *Ruta graveolens* against food-borne bacteria, as determined by the paper disk agar diffusion method[a]

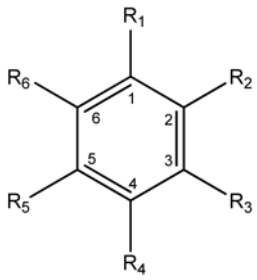
Microorganisms[c]	Strain source	Clean zone[b] (mean±SD)	MIC[d] (μg/mL)	MBC[d] (μg/mL)
Gram positive				
B. cereus	ATCC14579	14.5±0.9	75	100<
L. monocytogenes	ATCC15313	13.0±1.1	75	100
S. intermedius	ATCC29663	14.3±1.4	25	50
Gram negative				
S. enterica	ATCC43971	21.5±1.7	25	75
S. typhimurium	IFO14193	15.2±1.3	25	50
S. sonnei	ATCC25931	14.1±1.6	25	50

[a]Exposed to 5 mg per disc.
[b]Values (mm) are expressed as mean±SD of three parallel measurements, p <0.05.
[c]Bc, *Bacillus cereus* ATCC14579; Lm, *Listeria monocytogenes* ATCC 15313; Si, *Staphylococcus intermedius* ATCC29663; Sae, *Salmonella enterica* ATCC 43971; St, *Salmonella typhimurium* IFO 14193; *Ss, Shigella sonnei* ATCC 25931.
[d]MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MIC and MBC values <100 μg/mL.

Table 2 Chemical composition of volatile oil isolated by steam distillation from *R. graveolens*

Retention time (min)	Library search	Retention index[a]	Mass spectral data	Relative (%)
6.92	*p*-Isopropyltoluene	1042	50,65,77,91,103,119,134	1.85
11.52	2-Isopropyl-5-methylphenol	1262	58,71,91,107,135,150	31.13
11.65	Pentadecanol	1715	55,69,83,97,111,125,125	18.52
13.52	2-Acetoxytridecane	1755	55,70,87,111,125,154	5.64
13.77	2,4-Dimethylundecane	1913	57,71,85,103,120,126	2.42
16.33	4-Hexadecanyl pivalate	2013	55,57,71,85,103,125,154	6.12
16.38	1-Methyltridecyl pentanoate	2127	55,57,71,85,103,120,155	12.13

[a]Kovats indices were determined on a DB-5 capillary column.



Compounds	R_1	R_2	R_3	R_4	R_5	R_6
Phenol	OH	H	H	H	H	H
2-Methylphenol	OH	CH_3	H	H	H	H
2-Isopropylphenol	OH	$CH(CH_3)_2$	H	H	H	H
2-Isopropyl-5-methylphenol	OH	$CH(CH_3)_2$	H	H	CH_3	H
1-Isopropyl-4-methylbenzene	$CH(CH_3)_2$	H	H	CH_3	H	H

Fig. 1 Structures of 2-isopropyl-5-methylphenol and its derivatives.

Table 3 Antimicrobial activities of 2-isopropyl-5-methylphenol and its derivatives against food-borne bacteria, as determined by the paper disc agar diffusion method

Compounds[a]	Clean zone (mm)[b]					
	Microorganisms[c]					
	Bc	Lm	Si	Sae	St	Ss
2-Isopropyl-5-methylphenol	20.2±1.3	18.6±1.5	18.2±0.9	17.5±0.8	20.1±1.2	20.0±1.3
2-Isopropylphenol	15.0±1.1	14.3±0.9	17.0±1.3	15.5±1.1	15.3±0.9	15.6±1.6
2-Methylphenol	nd[e]	nd	nd	nd	nd	nd
Phenol	nd	nd	nd	nd	nd	nd
1-Isopropyl-4-methylbenzene	nd	nd	nd	nd	nd	nd
Chloramphenicol[d]	20.5±1.4	20.0±1.3	23.1±1.1	21.5±1.4	18.4±1.2	23.1±1.4
Tetracycline[d]	23.4±1.7	20.1±0.8	22.3±1.4	24.2±1.4	22.1±1.5	20.3±1.3

[a]Exposed to 0.5 mg per disc.
[b]Values (mm) are expressed as mean ± SD of three parallel measurements, $p < 0.05$.
[c]Bc, *Bacillus cereus* ATCC14579; Lm, *Listeria monocytogenes* ATCC 15313; Si, *Staphylococcus intermedius* ATCC29663; Sae, *Salmonella enterica* ATCC 43971; St, *Salmonella typhimurium* IFO 14193; *Ss, Shigella sonnei* ATCC 25931.
[d]Chloramphenicol and tetracycline served as positive controls (Exposed to 0.01 mg per disc).
[e]nd, not detected.

bacteria in previous studies (Juven et al., 1994; Friedman et al., 2002). Although the antimicrobial activities of the *R. graveolens* oil and 2-isopropyl-5-methylphenol against *Bacillus subtilis, Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, Salmonella enterica Staphylococcus aureus, Staphylococcus pyogenes,* and *Streptococcus pyogenes* have been reported (Juven et al., 1994; Friedman et al., Alzoreky and Nakahara, 2003; Ivanova et al., 2005; Liolios et al., 2009), 2-isopropyl-5-methylphenol isolated from the aerial parts of *R. graveolens* has not been reported. Therefore, the present study was conducted to assay the antimicrobial activities of 2-isopropyl-5-methylphenol and its analogs isolated from the aerial parts of *R. graveolens* against food-borne bacterial. The antimicrobial activities of 2-isopropyl-5-methylphenol and its structural analogs (2-isopropylphenol, 2-methylphenol, phenol, and 1-isopropyl-4-methylbenzene) were evaluated by the paper disc agar diffusion method at 0.5 mg/disc (Fig. 1, Table 3). The antimicrobial values of 2-isopropyl-5-methylphenol isolated from the *R. graveolens* aerial parts had moderate activity against *B. cereus* (clean zone diameter, 20.2 mm), *S. typhimurium* (20.1 mm), *S. sonnei* (20.0 mm), *L. monocytogenes* (18.6 mm), *S. intermedius* (18.2 mm), and *S. enterica* (17.5 mm). These results indicate that the antimicrobial activity of the *R. graveolens* aerial parts could be attributed to 2-isopropyl-5-methylphenol. 2-Isopropylphenol exhibited weak antimicrobial activity against *S. intermedius* (clean zone diameter, 17.0 mm), *S. sonnei* (15.6 mm), *S. enterica* (15.5 mm), *S. typhimurium* (15.3 mm), *B. cereus* (15.0 mm), and *L. monocytogenes* (14.3 mm). However, 2-methylphenol, phenol, and 1-isopropyl-4-methylbenzene exhibited no growth inhibitory effects against the six food-borne bacteria. Treatment with commercially available antibiotic tetracycline and chloramphenicol resulted in strong activity against the six food-borne bacteria at 0.01 mg/disc (Table 3).

The MBC and MIC values of 2-isopropyl-5-metylphenol, 2-

Table 4 MIC and MBC values[a] of the 2-isopropyl-5-methylphenol derivatives

Compounds	Microorganisms[b]											
	Bc		Lm		Si		Sae		St		Ss	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
2-Isopropyl-5-methylphenol	5	12.5	5	6.25	5	10	6.25	10	5	6.25	6.25	12.5
2-Isopropylphenol	5	10	6.25	12.5	5	6.25	6.25	12.5	12.5	25	12.5	25
2-Methylphenol	50	100<	50	100	50	100	25	75	50	100	50	100
Phenol	100<	100<	100<	100<	100<	100<	100<	100<	100<	100<	100<	100<
1-Isopropyl-4-methylbenzene	100<	100<	100<	100<	100<	100<	100<	100<	100<	100<	100<	100<

[a]MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MIC and MBC values <100 μg/mL.
[b]Bc, *Bacillus cereus* ATCC14579; Lm, *Listeria monocytogenes* ATCC 15313; Si, *Staphylococcus intermedius* ATCC29663; Sae, *Salmonella enterica* ATCC 43971; St, *Salmonella typhimurium* IFO 14193; *Ss, Shigella sonnei* ATCC 25931.

isopropylphenol, 2-methylphenol, phenol, and 1-isopropyl-4-methylbenzene were determined against the six food-borne bacteria (Table 4). 2-Isopropyl-5-methylphenol exhibited the strongest activity (MIC 5–6.25 μg/mL, MBC 6.25–12.5 μg/mL) followed by 2-isopropylphenol (MIC 5–12.5 μg/mL, MBC 6.25–25 μg/mL), 2-methylphenol (MIC 25–50 μg/mL, MBC 75 to >100 μg/mL), and phenol and 1-isopropyl-4-methylbenzene (MIC >100 μg/mL, MBC >100 μg/mL) against the six food-borne bacteria.

2-Isoproyl-5-methylphenol contains isopropyl and methyl functional groups on the phenol skeleton. 2-Isopropyl-5-methylphenol, which is conjugated with an isopropyl functional group and a methyl functional group, showed the strongest antimicrobial activity against the six food-borne bacteria, whereas 2-isopropylphenol, conjugated with an isopropyl functional group, showed lower antimicrobial activity against the six food-borne bacteria than that of 2-isopropyl-5-methylphenol. 2-Methylphenol, which is conjugated with a methyl functional group, showed no antimicrobial activity against the six food-borne bacteria. Similarly, phenol, which is the skeleton of 2-isopropyl-5methylphenol, showed no antimicrobial activity against the six food-borne bacteria. The position of the hydroxyl functional group in the phenol ring is the effective component against Gram-positive and Gram-negative bacteria (Dorman and Deans, 2000; Gallucci et al., 2009). Interestingly, 1-isopropyl-4-methylbenzene, which contains isopropyl and methyl functional groups on the benzene skeleton, showed no antimicrobial activity against the six food-borne bacteria. Previous studies have reported that biological activities are influenced by the presence and position of various functional groups (e.g., isopropyl and methyl functional groups) (Ilkay et al., 2004; Yang and Lee, 2013). These results indicate that the isopropyl functional group in the phenol ring is more sensitive than the methyl functional group to food-borne bacteria. Furthermore antimicrobial activity due to the isopropyl and methyl functional groups was shown only in the phenol ring.

Based on the Material Safety Data sheet provided by Sigma-Aldrich, the oral lethal dose of 2-isopropyl-5-methylphenol (980 mg/kg) indicated low acute toxicity to mammals (Sigma-Aldrich, USA). This result indicates that the essential oil of *R. graveolens* aerial parts and 2-isopropyl-5-methylphenol analogs would be useful as natural bactericides and potentially suitable as alternative chemical preservatives.

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