Antibacterial characteristics of citrus peel essential oils against food-borne pathogens

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Summary

The antibacterial activity against food-borne pathogens was investigated using essential oils of the peels of Hallabong (*Citrus reticulata* ‚Shiranui'), Redhyang (*Citrus* hybrid 'Kanpei'), Cheonhyehyang (*Citrus* hybrid 'Setoka') and orange (*Citrus sinensis)*. In the agar well diffusion assay, citrus peel essential oils showed antibacterial activity against *Bacillus subtilis* and *Vibrio vulnificus*. In particular, Cheonhyehyang peel essential oil (10 mg per well) had the highest antibacterial activity against *V. vulnificus* (a zone of 6.62 mm in diameter). Additionally, octanal showed strong antimicrobial activity against *V. vulnificus*. The essential oils of the peels of Redhyang, Cheonhyehyang and orange had minimum inhibitory concentrations (*MIC*) of 10 mg·ml^{-1}, 20 mg·ml^{-1} and 30 mg·ml^{-1} for *V. vulnificus*, respectively. Octanal showed *MIC* of approximately 5–10 mg·ml^{-1} for all tested food-borne pathogenic bacteria. However, the citrus peel essential oils used in this study did not show the minimum bactericidal concentration (*MBC*) for food-borne pathogens in the 0–30 mg·ml^{-1} range.

Keywords

agar well diffusion; minimum inhibitory concentration; minimum bactericidal concentration; food-borne pathogen; citrus peel essential oil

Environmental changes due to climate change, such as global warming, and an increase in fine dust, are constantly increasing concerns about microbial infections [1]. Infection and addiction to toxin by food-borne pathogens in an unsanitary environment cause various health risks. Food-borne pathogens, which are present in unhygienic or contaminated foods, might have a fatal effect on the health of the elderly, children, patients and pregnant women with a weak immunity. Thus, the importance of hygiene management has been emphasized [2]. Various antibiotics and preservatives are used in food processing to control contamination by food-borne pathogens and putrefactive bacteria to ensure consumer safety. The concern regarding the exposure risk to chemicals in processed foods and their side effects has led to a demand for the development of safer natural substances with antibacterial activity to replace the chemical preservatives [3].

Various natural substances derived from aromatic plants may be employed to extend the storage of processed foods [4, 5]. Volatile essential oils are aromatic plant-derived secondary metabolites that are organic substances with biological activities, such as antibacterial, anti-inflammatory, antioxidant or anticancer activities. Essential oils in foods are expected to replace chemical preservatives by inhibiting food-borne pathogens and food spoilage as natural preservatives [6, 7].

In Korea, more than 30 % of citrus fruits are produced in Jeju Island [8]. The annual production of citrus fruits is 560 000 tons, of which 80–85 % is consumed as fruit on the table and 15–20 % is processed into jams or juices [9]. A large amount of citrus peel is produced as a by-product and its economical treatment is required. Essential oils obtained from the peel of citrus fruits are used as a flavouring in cosmetics, perfumes, aroma oils, foods and medicines. Moreover, citrus peel essential oils have various biological activities, such as antibacterial, anti-inflammatory or antioxidant activities [10], so they have attracted attention as a useful resource for manufacturing foods, cosmetics and pharmaceuticals [6].

Citrus hybrids Hallabong (Bujiwha, Shiranui),

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Redhyang (Kampei) and Cheonhyehyang (Setoka) are widely grown in Korea, but only little research has been done on the antibacterial activity of their peel essential oils [9]. The purpose of this study was to provide experimental data on the possibility of using citrus peel essential oils possessing antibacterial activity as substitutes for chemical preservatives and antibacterial agents in foods and hygiene products.

MATERIALS AND METHODS

Materials and reagents

For the extraction of essential oils, Hallabong [(*C. unshiu* Marc. × *C. sinensis* Osb.) × (*C. reticulata* Blanco)], Cheonhyehyang [((*C. unshiu* × *C. sinensis*) × *C. reticulata*) × (*C. reticulate* × *C. sinensis*)], Redhyang (*Citrus* hybrid Kanpei) and the navel orange (*C. sinensis* Osb.) were purchased from Jeju Island (South Korea). The essential oil from the citrus peels was extracted using an essential oil extractor (EssenLab - PLUS, Hanil Labtech, Yangju, South Korea) by hydrodistillation and stored at –20 °C. Linalool, limonene, γ-terpinene, and octanal, reported as major constituents in a previous study [11], were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and Tokyo Chemical Industry (Tokyo, Japan). Streptomycin (Sigma-Aldrich) was used as a positive control for the antibacterial activity assay.

Microorganisms and culture conditions

Gram-positive bacteria *Bacillus cereus*, *B. subtilis*, *Listeria monocytogenes*, and *Staphylococcus aureus*, together with Gram-negative bacteria *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Choleraesuis, *S. enterica* serovar Typhimurium, *Shigella sonnei*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* were obtained from the Korean Culture Center of Microorganisms (KCCM, Seoul, South Korea) and the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Guri, South Korea) (Tab. 1).

The liquid culture media used were nutrient broth (beef extract 3.0 $g \cdot l^{-1}$, peptone 5.0 $g \cdot l^{-1}$); brain heart infusion (calf brains 7.7 $g \cdot l^{-1}$, beef heart 9.8 $g \cdot l^{-1}$, proteose peptone 10.0 $g \cdot l^{-1}$, dextrose 2.0 $g \cdot l^{-1}$, sodium chloride 5.0 $g \cdot l^{-1}$, disodium phosphate 2.5 $g \cdot l^{-1}$); trypticase soy broth (pancreatic digest of casein 17.0 $g \cdot l^{-1}$, papaic digest of soybean 3.0 $g \cdot l^{-1}$, sodium chloride 5.0 $g \cdot l^{-1}$, dipotassium phosphate 2.5 $g \cdot l^{-1}$, dextrose 2.5 $g \cdot l^{-1}$) from BD Biosciences (Sparks, Maryland, USA).

Culturing took place in a shaking incubator SIF 600R (Lab companion, Billerica, Massachusetts, USA) for 12 h at 37 °C and 2.5 Hz.

Agar well diffusion assay

The antibacterial activity of citrus peel essential oils and their constituents against food-borne pathogens was investigated priorly using an agar well diffusion assay [12]. A well was cut in agar plate using a sterile cork-borer (5 mm diameter) and then, the cultured bacteria (100 μl, optical density at 600 nm (OD_{600}) 0.1–0.2, approximately 1×10^7 $CFU \cdot ml^{-1}$) was spread homogeneously using a sterile spreader on the agar plate. Citrus peel essential oil (10 mg), constituents (10 mg) or streptomycin (10 μl, 10 $mg \cdot ml^{-1}$) were added to the

Tab. 1. Food-borne pathogens used in this study.

Strains	Catalog No.	Culture medium
Gram-positive bacteria		
Bacillus cereus	KCCM 11204	Nutrient broth
Bacillus subtilis	KCCM 11316	Nutrient broth
Listeria monocytogenes	KCCM 40307	Brain heart infusion + NaCl 5 $g \cdot l^{-1}$
Staphylococcus aureus	KCCM 12214	Nutrient broth
Gram-negative bacteria		
Pseudomonas aeruginosa	KCCM 11266	Nutrient broth
Salmonella enterica serovar Choleraesuis	KCCM 13096	Nutrient broth
Salmonella enterica serovar Typhimurium	CCARM 8009	Nutrient broth
Shigella sonnei	KCCM 41282	Nutrient broth
Vibrio parahaemolyticus	KCCM 11965	Nutrient broth + NaCl 30 $g \cdot l^{-1}$
Vibrio vulnificus	KCCM 41665	Trypticase soy broth + NaCl 15 $g \cdot l^{-1}$

KCCM – Korean Culture Center of Microorganisms (Seoul, South Korea), CCARM – Culture Collection of Antimicrobial Resistant Microbes (Guri, South Korea).

wells and the agar plate was incubated for 12 h at 37 °C in an incubator HB-101M (Hanbaek Scientific, Bucheon, South Korea). The antibacterial activity of each sample was evaluated as the diameter of a clear zone around the well on the plate.

Minimum inhibitory concentration

The minimum inhibitory concentration (*MIC*) against food-borne pathogens was determined by observing the growth of each food-borne pathogen in a liquid medium (Tab. 1) containing citrus peel essential oil or constituents (0–30 mg·ml^{-1}) [13]. The cultures were incubated at 37 °C and 2500 mHz for 24 h in a shaking incubator SIF 600R and bacterial growth was evaluated by measuring the absorbance on 600 nm using a spectrophotometer V-1100D (Labinno, Seoul, South Korea). *MIC* was determined as the minimum concentration that inhibited the growth of foodborne pathogens.

Minimum bactericidal concentration

The cultured broth (50 μl), in which the growth of food-borne pathogens was not observed in the *MIC* assay, was spread evenly on a solid medium plate with 15 g·l^{-1} of agar (Tab. 1) and cultured at 37 °C. After 48 h of incubation, the minimum concentration of citrus peel essential oils or their components, at which food-borne pathogens did not grow, was determined to be the minimum bactericidal concentration (*MBC*) [14].

Statistical analysis

Antibacterial activity values were expressed as mean and standard deviation of at least three results, using SPSS Statistics software version 24.0 (IBM, Chicago, Illinois, USA). Results were analyzed using the one-way analysis of variance (ANOVA) and Duncan's multiple comparison test for individual comparisons, and were considered statistically significant when *p*-values were below 0.05.

RESULTS AND DISCUSSION

Antibacterial activity

The antibacterial activity of the citrus peel essentials oils (10 mg per well) and the constituents (10 mg per well) were analysed using an agar well diffusion assay (Tab. 2). Citrus peel essential oils exhibited antibacterial activity against *B. cereus*, *B. subtilis*, *V. parahaemolyticu*, and *V. vulnificus.* Cheonhyehyang peel essential oil showed the highest antibacterial activity (6.62 mm) against *V. vulnificus*, and *B. subtilis* was

Tab. 2. Antimicrobial activity of citrus peel essential oils and constituent against food-borne pathogens.

Foodborne pathogens	Inhibition zone diameter [mm]									Antibiotics (100 μg per well)
	Essential oil (10 mg per well)				Constituent (10 mg per well)					
	Hallabong	Redhyang	Cheonhyehyang	Orange	Limonene	Linalool	γ-Terpinene	Octanal*	Streptomycin
Bacillus cereus	2.13 ± 1.35^{c}	+	3.52 ± 0.76^{c}	–	14.56 ± 1.08^{a}	7.15 ± 1.21^{b}	2.95 ± 0.79^{d}	4.94 ± 0.49^{c}	12.78 ± 0.57^{a}
Bacillus subtilis	3.66 ± 0.49^{cd}	3.15 ± 0.17^{cd}	4.06 ± 0.56^{c}	2.91 ± 1.54^{cd}	10.44 ± 1.39^{a}	8.01 ± 0.35^{b}	4.93 ± 0.86^{c}	3.93 ± 0.75^{d}	11.18 ± 0.31^{b}
Listeria monocytogenes	+	+	+	+	7.66 ± 0.90	+	+	5.23 ± 1.83	7.46 ± 0.46
Staphylococcus aureus	+	+	+	+	11.95 ± 1.69^{a}	8.99 ± 1.85^{b}	2.31 ± 0.35^{d}	5.83 ± 0.57^{c}	9.60 ± 0.28
Pseudomonas aeruginosa	–	–	–	–	–	7.66 ± 2.19	–	2.53 ± 0.65	10.20 ± 1.29
Salmonella enterica Choleraesuis	–	–	–	–	10.12 ± 0.38^{a}	10.13 ± 0.85^{a}	–	3.86 ± 0.52^{b}	10.04 ± 0.63
Salmonella enterica Typhimurium	+	+	+	+	14.85 ± 0.62^{a}	10.79 ± 0.62^{b}	–	3.62 ± 0.81^{c}	10.21 ± 0.29
Shigella sonnei	+	+	+	+	10.78 ± 0.22^{a}	5.15 ± 0.85^{c}	–	6.92 ± 1.03^{b}	10.39 ± 0.56
Vibrio parahaemolyticus	2.43 ± 0.42^{c}	2.14 ± 0.14^{c}	2.22 ± 0.35^{c}	+	15.51 ± 1.35^{a}	5.32 ± 0.20^{b}	–	2.72 ± 0.44^{c}	4.56 ± 0.25^{b}
Vibrio vulnificus	2.60 ± 0.68^{c}	2.46 ± 0.82^{c}	6.62 ± 2.18^{b}	2.76 ± 0.83^{c}	14.39 ± 1.36^{b}	10.87 ± 2.35^{c}	+	24.86 ± 1.53^{a}	7.68 ± 0.22^{b}

Each value represents mean ± standard deviation. Superscript letters in the same column indicate significant differences at $p < 0.05$ in Duncan's multiple range test. Antibiotics (10 μl, 10 mg·ml^{-1}) served as a positive control.
* – the antibacterial activity of octanal was measured at 5 mg per well. (+) – faint clear zone 0–2 mm of diameter, (–) – clear zone not detected.

Tab. 3. Minimum inhibitory concentration of citrus peel essential oils and their constituents against food-borne pathogens.

Foodborne pathogens	Minimum inhibitory concentration [mg·ml^{-1}]								
	Essential oil				Constituent				Antibiotics
	Hallabong	Redhyang	Cheonhyehyang	Orange	Limonene	Linalool	γ-Terpinene	Octanal	Streptomycin
Bacillus cereus	–	–	–	–	–	20	–	5	5
Bacillus subtilis	–	30	–	–	–	20	–	5	5
Listeria monocytogenes	–	–	–	–	–	20	–	10	–
Staphylococcus aureus	–	–	–	–	–	20	–	5	5
Pseudomonas aeruginosa	–	–	–	–	–	–	–	10	5
Salmonella enterica Choleraesuis	–	–	–	–	–	20	–	5	5
Salmonella enterica Typhimurium	–	–	–	–	–	10	–	5	5
Shigella sonnei	–	–	–	–	–	10	–	5	5
Vibrio parahaemolyticus	–	–	–	–	–	20	–	5	–
Vibrio vulnificus	–	10	20	30	30	10	–	5	–

(–) – the minimum inhibitory concentration was not observed in the range of 0–30 mg·ml^{-1}.

Tab. 4. Minimum bactericidal concentration of citrus peel essential oils and their constituents against food-borne pathogens.

Foodborne pathogens	Minimum bactericidal concentration [mg·ml^{-1}]								
	Essential oil				Constituent				Antibiotics
	Hallabong	Redhyang	Cheonhyehyang	Orange	Limonene	Linalool	γ–Terpinene	Octanal	Streptomycin
Bacillus cereus	–	–	–	–	–	30	–	5	5
Bacillus subtilis	–	–	–	–	–	30	–	10	5
Listeria monocytogenes	–	–	–	–	–	–	–	–	–
Staphylococcus aureus	–	–	–	–	–	–	–	–	5
Pseudomonas aeruginosa	–	–	–	–	–	–	–	30	5
Salmonella enterica Choleraesuis	–	–	–	–	–	20	–	30	5
Salmonella enterica Typhimurium	–	–	–	–	–	20	–	10	5
Shigella sonnei	–	–	–	–	–	20	–	30	5
Vibrio parahaemolyticus	–	–	–	–	–	20	–	20	–
Vibrio vulnificus	–	–	–	–	30	10	–	10	–

(–) – the minimum bactericidal concentration was not observed in the range of 0–30 mg·ml^{-1}.

sensitive (3.15–4.06 mm) to all citrus peel essential oils. *Citrus lemon* essential oils were previously shown to possess antibacterial activity against *B. cereus*, *B. subtilis*, *Staph. aureus* and *S. enterica* serovar Typhimurium with a broad antimicrobial spectrum against food-borne pathogens [15]. OBIDI et al. [16] reported that the essential oils of orange fruit (*Citrus sinensis*) peel possesses antibacterial activity against food-borne pathogens and fungi, such as *Staph. aureus*, *P. aeruginosa*, *Escherichia coli*, *Enterobacter faecalis* and *Candida albicans*, in the agar well diffusion test. In addition, orange, lemon and their constituent, linalool, exhibited antibacterial activity against *L. monocytogenes*, *B. cereus*, *Staph. aureus*, *E. coli* O157 and *Campylobacter jejuni* [17]. However, there are few reports on the antibacterial activity of orange hybrids Hallabong, Cheonhyehyang, and Redhyang peel-essential oils.

Limonene showed antibacterial activity against all food-borne pathogens except *P. aeruginosa* and showed strong antibacterial activity (zones of 14.39–15.51 mm) against *S. enterica* serovar Typhimurium, *B. cereus*, *V. parahaemolyticus* and *V. vulnificus.* Linalool showed a clear zone of more than 10 mm in diameter against *V. vulnificus*, *S. enterica* serovar Typhimurium and *S. enterica* serovar Choleraesuis, but showed relatively low antibacterial activity against other food-borne pathogens. Octanal exhibited the strongest antibacterial activity against *V. vulnificus* (a zone of 24.86 mm in diameter) and against other food-borne pathogens (zones of 2.53–6.92 mm in diameter). In addition, γ-terpinene exhibited antibacterial activity against *B. cereus*, *B. subtilis* and *Staph. aureus*. The antibacterial activity of limonene and linalool were previously reported against *P. aeruginosa*, *L. monocytogenes*, *B. cereus*, *Staph. aureus*, *E. coli* and *Micrococcus luteus* using the agar well diffusion assay [18–20] and were similar to the antibacterial results except *P. aeruginosa* in this study.

The agar diffusion assay is suitable for the primary screening of large numbers of potential antimicrobial compounds, but does not accurately reflect the antimicrobial activity of compounds in liquid cultures [21]. The size of the inhibition zone around agar well is affected not only by the strength of the antimicrobial activity of the tested compound, but also by the solubility of compound and degree of diffusion in the agar medium. Moreover, essential oil is a mixture of highly volatile compounds and do not accurately reveal the antimicrobial activity by agar diffusion assays [21–23].

Minimum inhibitory concentration

The *MIC* values of citrus peel essential oils and their constituents against food-borne pathogens are shown in Tab. 3. Redhyang peel essential oil showed strong inhibitory activity against *V. vulnificus* and *B. subtilis*, with *MIC* of 10 mg·ml^{-1} and 30 mg·ml^{-1}, respectively. Additionally, Cheonhyehyang and orange peel essential oils had *MIC* values of 20 mg·ml^{-1} and 30 mg·ml^{-1} against *V. vulnificus*, respectively. On the other hand, Hallabong peel essential oil exhibited the *MIC* value against food-borne pathogens in the range of 0–30 mg·ml^{-1}. Octanal had the highest antibacterial activity with *MIC* of 5–10 mg·ml^{-1} against all tested food-borne pathogens. Linalool exhibited *MIC* values of 10 mg·ml^{-1} and 20 mg·ml^{-1} against all food-borne pathogens except *P. aeruginosa*, and limonene showed *MIC* of 30 mg·ml^{-1} only against *V. vulnificus*. Streptomycin showed strong antibacterial activity with *MIC* of 5 mg·ml^{-1} against various food-borne pathogens, but did not inhibit the growth of *L. monocytogenes*, *V. parahaemolyticus* and *V. vulnificus* at concentrations in the range of 0–30 mg·ml^{-1}.

Minimum bactericidal concentration

The *MBC* values of Hallabong, Redhyang, Cheonhyehyang, and orange peel essential oils and their constituents against food-borne pathogens are shown in Tab. 4. Citrus peel essential oils did not show *MBC* in the range of 0–30 mg·ml^{-1} against the tested food-borne pathogens. *MBC* of linalool was 10 mg·ml^{-1} against *V. vulnificus* and 20 mg·ml^{-1} against *S. enterica* serovar Choleraesuis, *S. enterica* serovar Typhimurium, *V. parahaemolyticus* and *Shi. sonnei*, and 30 mg·ml^{-1} for *B. cereus* and *B. subtilis*, respectively. Octanal exhibited *MBC* of 5–30 mg·ml^{-1} for all tested food-borne pathogens, except *L. monocytogenes* and *Staph. aureus*. Limonene and γ-terpinene did not possess a remarkable *MBC* for the food-borne pathogens used in this study. Additionally, linalool had almost the same *MIC* and *MBC* values, which means that the bacteriostatic process for food-borne pathogens progressed rapidly to the bactericidal one. Against *L. monocytogenes* and *Staph. aureus*, it is judged that a linalool concentration of 30 mg·ml^{-1} or higher is required to be bactericidal.

CONCLUSION

Redhyang, Cheonhyehyang and orange peel essential oils showed antibacterial activity against some food-borne pathogens in agar well diffusion assay and remarkable *MIC* values, but did not show *MBC* in the concentration range of

0–30 mg·ml^{-1}. The *MIC* and *MBC* assays are the most common predictive assays for antimicrobial activity. However, they do not accurately reflect the antimicrobial activity against foodborne pathogens during culture period, because they do not consider the kinetics of antimicrobial effects and incubation time. In order to accurately analyse the antimicrobial activity of citrus peel essential oils against food-borne pathogenic bacteria, it will be necessary to evaluate the growth inhibitory activity and bactericidal activity according to the incubation time.

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