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RESEARCH NOTE

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Antibacterial Activity of Essential Oils from *Zanthoxylum piperitum* A.P. DC. and *Zanthoxylum schinifolium*

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Abstract This study was carried out to investigate the potential use of *Zanthoxylum schinifolium* and *Zanthoxylum piperitum* A.P. DC. as a source of antimicrobial agents against foodborne pathogens. Essential oils of *Z. schinifolium* and *Z. piperitum* A.P. DC. were collected by steam distillation and analyzed by GC-MS. The antimicrobial activity of the essential oils was examined using the agar diffusion and micro-dilution assays. The effectiveness of *Z. schinifolium* essential oil was greater against *Bacillus cereus*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus* than other pathogens, and the minimal inhibitory concentration (MIC) values were 1.25, 2.5, and 1.25 μg/mL, respectively. *Z. piperitum* A.P. DC. essential oil was the most effective against all pathogens tested except for *Escherichia coli* O157:H7, and the MIC values against *B. cereus*, *Salmonella choleraesuis*, and *V. parahaemolyticus* were 1.25, 2.5, and 1.25 μg/mL, respectively. Limonene, the major component of *Z. piperitum* A.P. DC. essential oils, had the highest inhibitory activity toward *V. parahaemolyticus* with a MIC value of 0.15 μg/mL. Meanwhile, citronellal and geranyl acetate, major components of both essential oils, displayed antibacterial activity against only *B. cereus* with MIC values of 1.25 and 5 μg/mL, respectively. Therefore, these essential oils could be useful as antimicrobial agents against foodborne pathogens.

Keywords: *Zanthoxylum piperitum* A.P. DC., *Zanthoxylum schinifolium*, essential oil, foodborne pathogen, minimal inhibitory concentration (MIC)

Introduction

Because of increasing popular concern over food safety and the potential impact of synthetic additives on health, interest in plant products (phytochemicals) with various biological activities including antimicrobial activity has increased significantly (1-4). Some naturally occurring compounds found in edible and medicinal plants, herbs, and spices or essential oils are well known to possess antimicrobial activities against foodborne pathogenic bacteria, including *Vibrio parahaemolyticus* (5-7). In particular, the essential oils extracted from aromatic plants are highly enriched in terpene compounds that inhibit the growth of microorganisms through the destruction of the plasma membrane (8). Volatile essential oils are a complex mixture of compounds consisting mainly of monoterpenes, sesequiterpenes, and their oxygenated derivatives such as alcohols, aldehydes, esters, ethers, ketones, phenols, and oxides, which may also have antioxidant, antimicrobial, and anti-inflammatory activities (9,10).

Zanthoxylum schinifolium (*sancho*) and *Zanthoxylum piperitum* A.P. DC. (*chopi*) are aromatic medicinal plants belonging to the Rutaceae family and distributed in Korea, China, Japan, and Taiwan (11-15). They each have the same characteristic citrus-like flavor and are also used as traditional spices and plant medicines in Korea. The chemical constituents of *Z. schinifolium*, especially from the fruit, have been studied extensively. The known compounds in *Z. schinifolium* have been identified as aurapten, collinin, epoxyaurapten, hydrangetin, umbelliferone, acetoxycollinin, aesculetin dimethylether, norchelerythrine, dictamnine, skimmianine, and friedelin (11,12). *Z. piperitum* A.P. DC. essential oil contains myrcene, octanal, limonene, linalool, citronellal, geraniol, phellandral, geraniol, and geranyl acetate (13-15).

From this point of view, we have evaluated the growth inhibitory effects of the essential oils of *Z. schinifolium* and *Z. piperitum* A.P. DC. toward 7 foodborne pathogens.

Materials and Methods

Extraction of the essential oil Two plants, *Z. schinifolium* and *Z. piperitum* A.P. DC., were purchased from the Kyungdong herbal market (Seoul, Korea) in 2007. The samples were kept at -70°C in airtight bags until analysis was carried out. Essential oils from *Z. schinifolium* and *Z. piperitum* A.P. DC. were extracted by the stem distillation method. Briefly, air-dried and ground *Z. schinifolium* and *Z. piperitum* A.P. DC., were treated for 3 hr using a Clevenger-type apparatus (Hanil Labtech Ltd., Incheon, Korea). The obtained essential oils were dried over anhydrous sodium sulfate for 24 hr, and then stored in hermetically sealed dark glass containers in a freezer at –4°C until tested.

GC-MS analysis essential oil The essential oil constituents were analyzed using an Aglient 6890 GC/5973 mass selective detector (Aglient Co., Palo Alto, CA, USA) equipped with a HP-5MS capillary column (30 m length ×0.25 mm i.d.×0.25 mm film thickness; Aglient Co.).

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Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The column temperature was maintained at 40°C for 5 min and then programmed to increase as follows: rising from 40 to 150°C at a rate of 3°C/min, and the holding at 150°C for 5 min, and rising from 150 to 220°C at a rate of 7°C/min, and holding at 220°C for 5 min.

Identification of compounds The volatile components were identified by comparison of the mass spectra with those of an on-line computer library, Wiley 275 (Aglient Co.). Alkanes were used as reference points in the calculation of relative retention indices (RI). The RIs of compounds, determined using C_8-C_{22} as external references (16), were compared with published data (17,18). The quantification of each volatile component was carried out based on the ratio of the peaks obtained from a mass total ion chromatogram. Standard essential oils (limonene, citronellal, and geranyl acetate) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used as controls in the testing of antimicrobial activity.

Determination of antimicrobial activity

Microorganisms: The essential oils were analyzed for antimicrobial activity with the following bacterial strains: *Staphylococcus aureus* ATCC 6538P, *Listeria monocytogenes* KCTC 3710, *Salmonella choleraesuis* ATCC 13312, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* KTCT 3726, *Escherichia coli* O157:H7 KCCM 40406, and *V. parahaemolyticus* ATCC 17802. All bacterial strains were purchased from the Korean Culture Center of Microorganisms (Seoul, Korea) and the Biological Resource Center (Daejeon, Korea). *V. parahaemolyticus* was cultured at 37°C for 18 hr in nutrient broth (Difco, Detroit, MI, USA) with NaCl added to a final concentration of 3% (Na-NB broth). The other strains were cultured at 37°C in NB broth (Difco) for 18 hr. All bacterial cultures were incubated under aerobic conditions.

Disc diffusion assay: The disc diffusion assay was performed using cotton swabs to inoculate each bacterial suspension (0.1, OD 600 nm) uniformly onto an agar surface. Each oil to be tested was dissolved in 40% ethanol to a final concentration of 25%(v/v) and sterilized by filtration through 0.45 mm Millipore filters. There was no inhibition of bacterial growth with 5% ethanol. The 8 mm diameter discs were impregnated with 5 μg of essential oils/disc and placed on the inoculated agar. The plates were incubated at 37°C for 12 hr and then examined for inhibition. Antimicrobial activity was evaluated by measuring the zone of inhibition for each test organism. Each assay in this experiment was done in triplicate.

MIC assay: Minimal inhibitory concentration (MIC) was measured using the turbidity assay. All tested pathogens (0.1, OD 600 nm) were inoculated into 2 mL of NB media along with test essential oil, major essential oil (over the range of 0.0625-5 μg/mL) or antibiotic, and then incubated at 37°C for 24 hr. Ampicillin and tetracycline were used as positive controls, and appropriate negative controls with no essential oil were used. After incubation, the OD was measured at 600 nm. To determine the MIC, 50 μL of each culture broth was spread over sterile nutrient agar plates and incubated at 37°C for 24 hr after which bacterial growth was assessed.

Statistical analysis Results are the mean values of 3 replicates of the same sample. Statistical analysis was performed using analysis of variance.

Results and Discussion

The chemical composition of essential oils The main constituents (2%) of the essential oils of *Z. schinifolium* and *Z. piperitum* A.P. DC. used in the experiments are presented in Table 1. The main components of both oils were geranyl acetate and citronellal. *Z. schinifolium* also presented substantial amounts of β-phellandrene (22.54%) and citronellal (16.48%), whereas *Z. piperitum* A.P. DC. was dominated by high concentrations of limonene (18.00 %) and geranyl acetate (15.30%), in addition to the presence of cryptone, citronellal, cuminal, and phellandral.

Antimicrobial activity of essential oils The antimicrobial activities of essential oils, essential oil components (limonene, citronellal, and geranyl acetate), and antibiotics (ampicillin and tetracycline) were examined using the disc diffusion and micro-dilution susceptibility assays with 7 foodborne pathogens; Gram-positive (*L. monocytogenes*, *B. cereus*, *B. subtilis*, and *S. aureus*) and Gram-negative bacteria (*E. coli* O157:H7, *S. choleraesuis*, and *V. parahaemolyticus*). Antibiotics (ampicillin and tetracycline) were used as positive controls at a concentration of 3.125-50 μg/mL.

In the agar diffusion assay (Table 2), the effectiveness of *Z. schinifolium* essential oil (5 μL/disc) was greater against only *B. cereus* (20±0.3), *S. aureus* (12±0.3), and *V. parahaemolyticus* (23±0.5 mm, inhibition zone) than pathogens. Meanwhile, *Z. piperitum* A.P. DC. (5 μg/disc) showed strong inhibition of all tested pathogens except for *E. coli* O157:H7, wih the clear zone for *S. choleraesuis*, *B. cereus*, *S. aureus*, and *V. parahaemolyticus* being 19, 17, 12 and 20±0.3 mm, respectively. The 5% EtOH (negative control) did not show antibacterial activity against any of the bacteria tested. Also, the antibacterial properties of the essential oils were compared to the synthetic antibiotics,

Table 1. Major components (%) of *Z. schinifolium* and *Z. piperitum* A.P. DC. essential oils

Components	R.I.[1)]	Relative (%)	
		Z. schinifolium	*Z. pipperitum* A.P. DC.
β-Phellandrene	1,027	22.54	-
Limonene	1,025	-	18.0
Citronellal	1,143	16.48	7.1
Geranyl acetate	1,386	11.39	15.3
Cryptone	1,165	-	8.5
β-Myrcene	988	7.73	-
Cuminal	1,211	-	6.2
Phellandral	1,259	-	5.2
Citronellyl acetate	1,345	3.97	-
Citronellol	1,220	2.55	-
Linalool	1,090	2.52	-
Total		67.18	60.39

[1)]Retention indices (R.I.) were calculated using *n*-alkanes (C_8-C_{22}) as external references on an HP-5MS capillary column.

Table 2. Antimicrobial activities of *Z. schinifolium, Z. pipperitum* A.P. DC essential oils, major standard essential oils, and antibiotics by agar diffusion assay

Tested pathogens	Essential oil[1]		Standard[2]			Antibiotics[3]	
	Z. schinifolium	*Z. pipperitum*	Limonene	Citronellal	Geranyl acetate	Ampicillin	Tetracycline
L. monocytogenes	-[4]	-	-	-	-	15±0.3	23±0.5
B. cereus	20±0.3	17±0.3	-	17 ± 0.3	-	-	16±0.3
B. subtilis	-	-	-	-	-	12±0.3	19±0.3
S. choleraesuis	-	19±0.3	17±0.3	-	-	23±0.5	14±0.5
S. aureus	12±0.3	12±0.3	12±0.3	10 ± 0.1	-	13±0.5	17±0.3
E. coli O157:H7	-	-	-	-	-	15±0.3	16±0.3
V. parahaemolyticus	23±0.5	20±0.3	30±0.3	-	-	-	17±0.3

[1]Amount of essential oils and major components (standards) used was 5 μg/disc.
[2]Standards (limonene and citronellal) used for major essential oil components of *Z. schinifolium, Z. pipperitum* A.P. DC.
[3]Antibiotics (ampicillin and tetracycline) used as positive controls at 12.5 μg/disc.
[4]No inhibition; values are presented as the clear zone width (mm) and the results are means of 3 replications±SE.

ampicillin, and tetracycline (12.5 μg/disc). Ampicillin caused inhibition zones (12-23 mm) for all the pathogens except for *B. cereus* and *V. parahaemolyticus*, and tetracycline had strong antibacterial activity (14-23 mm) towards all tested pathogens. Roy *et al.* (19) assessed the susceptibility of *B. cereus* species to various antibiotics and reported that the cell wall synthesis in *B. cereus* was inhibited by treatment with ampicillin (10 μg/disc).

In micro-dilution susceptibility assays, as shown in Table 3, the essential oils of *Z. schinifolium* and *Z. piperitum* A.P. DC. exhibited the bacteriostatic activity toward *B. cereus* and *V. parahaemolyticus* at low concentration (1.25 μg/mL). *V. parahaemolyticus* is characterized as more acid-tolerant than most food-borne pathogens, although the sensitivity of this organism to organic acids varies with the nature of the acid (20), and it is recognized as an important seafood pathogen (21-24). Lin *et al.* (25) showed that *V. parahaemolyticus* could survive when lactic acid was used as the only antimicrobial substance at pH 6.0, but the sensitivity of *V. parahaemolyticus* to lactic acid increased in combination with phenolic phytochemicals which may create a lower pH microenvironment and cell membrane disruption due to stacking. Kim *et al.* (26) have reported that ethanolic extracts of *Z. schinifolium* had remarkable antimicrobial activities against *V. parahaemolyticus* and its active compound was estraglole (4-allyl anisole). *B. cereus* is easily spread to many types of food, especially those of plant origin, and causes food spoilage (27). However, the growth of *L. monocytogenes* and *B. subtilis* was not inhibited by the essential oils. The MIC values of *Z. schinifolium* essential oil against *B. subtilis*, *S. choleraesuis*, *E. coli* O157:H7, and *V. parahaemolyticus* were 5, 5, 5, and 1.25 μg/mL, respectively, and the corresponding MIC values of *Z. piperitum* A.P. DC. essential oils against *E. coli* O157:H7 and *V. parahaemolyticus* were 5 and 1.25 μg/mL, respectively. Of particular interest, concentrations as low as 2.5 μg/mL of the essential oil of *Z. piperitum* A.P. DC. dramatically inhibited the growth *S. choleraesuis*. The MIC (1.25 and 2.5 μg/mL, respectively) of *Z. schinifolium* and *Z. Pipperitum* A.D. against *B. cereus* and *V. parahaemolyticus* was the same as that of tetracycline (6.25 μg/mL).

We also tested the growth inhibitory activity of the essential oils constituents; limonene, citronellal, and geranyl acetate. Limonene, the major component of *Z. piperitum* A.P. DC. essential oils had the highest inhibitory activity toward *V. parahaemolyticus* with a MIC of 0.15 μg/mL. This is not surprising since limonene is commonly known

Table 3. MIC of *Z. schinifolium, Z. pipperitum* A.P. DC. essential oils, major standard essential oils, and antibiotics by micro-dilution assay

Tested pathogens	Essential oils[1]		Major essential oils constituents			Antibiotics[2]	
	Z. schinifolium	*Z. pipperitum*	Limonene	Citronellal	Geranyl acetate	Ampicillin	Tetracycline
	MIC[3]	MIC	MIC	MIC	MIC	MIC	MIC
L. monocytogenes	>5	>5	>5	>5	>5	25	12.5
B. cereus	1.25	1.25	5	1.25	5	>50	12.5
B. subtilis	5	>5	>5	>5	>5	25	12.5
S. choleraesuis	5	2.5	>5	>5	>5	12.5	25
S. aureus	2.5	2.5	2.5	>5	>5	>50	25
E. coli O157:H7	5	5	5	>5	>5	12.5	12.5
V. parahaemolyticus	1.25	1.25	0.15	>5	>5	50	6.25

[1]The concentration of essential oils and major components was 0.15-5 μg/mL(v/v).
[2]Antibiotics (ampicillin and tetracycline) used as positive controls at 3.125-50 μg/mL.
[3]Minimum inhibitory concentration as %(v/v).

to have the antibacterial activity (28). Meanwhile, citronellal and geranyl acetate, major components of both essential oils, have displayed antibacterial activity toward *B. cereus* only with MIC values of 1.25 and 5 μg/mL, respectively. These results indicate that the synergistic effects of the major and minor constituents present in these whole essential oils should be taken into consideration regarding their antibacterial activities, such that the whole oils are more potent than the individual components for various pathogens.

In conclusion, this is the first report of the growth inhibitory activity of essential oils from *Z. schinifolium* and *Z. piperitum* A.P. DC. toward foodborne pathogens including *V. parahaemolyticus*. Although further investigation addressing safety issues and activity relating to *Z. schinifolium* and *Z. piperitum* A.P. DC. as well as testing the flavoring activity in food systems are necessary, the present study indicates that essential oils from these plants are potential alternatives to phytochemical preserves, reducing the growth of important foodborne pathogens, and enhancing food safety and shelf life.

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