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Anti-*Salmonella* Activity of Lemongrass Oil Alone and in Combination with Antibiotics

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Abstract – The effects of *Cymbopogon citratus* essential oil (Lemongrass oil) and its main component, citral (84.30%), on antibiotic-susceptible and -resistant strains of *Salmonella enteritidis* and *S. typhimurium* were assessed. *C. citratus* oil and citral significantly inhibited all strains of the two *Salmonella* species examined, with minimum inhibiting concentrations (MICs) ranging from 0.5 mg/ml to 8.0 mg/ml. The combined effects of *C. citratus* oil and citral (84.30%) were evaluated using a checkerboard microtiter assay. Essential oil fractions of *C. citratus* and citral exhibited strong synergistic or additive effects with streptomycin or kanamycin against *S. typhimurium* strains with fractional inhibitory concentration (FIC) indices in the range of 0.28 to 1.00. In conclusion, a combination of streptomycin and lemongrass oil or its main component, citral, may be useful for reducing the minimum effective dose of antibiotic required for the treatment of resistant *S. typhimurium* infections.
Keywords – *Cymbopogon citratus*, citral, *Salmonella enteritidis, S. typhimurium,* antibiotic-resistant, kanamycin, streptomycin, synergism

Introduction

Increased therapy and excessive application have accelerated the resistance of microorganisms against specific antibiotics, and in many cases, multiple drugs (Low *et al.*, 1996; Humphrey, 2001; Karlowsky and Sahm, 2002; Shin, 2004).

The *Salmonella* species comprises one of the common pathogenic bacterial groups causing food-borne diseases (Jung and Beuchat, 1999; Mastroeni and Sheppard, 2004; Fluit, 2005). The emergence of resistant strains has increased progressively, particularly due to the consumption of processed food and agricultural products in contact with antibiotics (Logue *et al.*, 2003; Schlegelov *et al.*, 2004; Khaschabi and Schopf, 2005).

Lemongrass, a perennial herb, is widely cultivated throughout the warm tropical climates of the world. It includes two different species, *Cymbopogon flexuosus* Stapf. and *C. citratus* Stapf. An infusion of this plant is used to induce sleep, loosen and reduce mucus, and treat fevers, cramps, and stress. The essential oil is used as food flavoring, and an ingredient in cosmetics and perfumes. Inhibitory activities of lemongrass oil against different microbial species and insects have been documented (Alam *et al.*, 1994; Oyedele *et al.*, 2002; Palhano *et al.*, 2004). The *C. citratus* plant is currently cultivated in the greenhouses of many herbal gardens in Korea. Moreover, its essential oil and various products utilizing the imported oil are commercially marketed.

In this study, we examined the effects of *C. citratus* essential oil (Lemongrass oil), its main component, citral, and limonene against antibiotic-susceptible and -resistant strains of *S. enteritidis* and *S. typhimurium*, with a view to developing safe and effective agents against antibiotic-resistant *Salmonella*. In addition, the combined effects of *C. citratus* oil and its main component, citral, were evaluated using a checkerboard microtiter assay. Our data reveal synergistic activity between the oil and antibiotics commonly used in therapy against *Salmonella* infections.

Materials and Methods

Analysis of essential oils from *C. citratus* – Essential oils were extracted by steam distillation from fresh leaves of *C. citratus* cultivated in Pochun, Gyeonggi, Korea, and analyzed for antibacterial activity. The oil (0.54%) obtained was analyzed using Hewlett-Packard 6890 GC and Hewlett-Packard 5973 MSD apparatus (Agilent 5973 network mass selective detector, 280°C) with an Ultra 2 (5% phenylmethylsiloxane, 50 m×200 μm×0.11 μm) fused silica capillary column. The injector was adjusted to

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250°C, and the oven temperature was regulated as follows: initial temperature at 60°C for 5 min, 2°C/min up to 230°C, and 30 min at 180°C.

Strains – *S. enteritidis* KCCM 12021, *S. enteritidis* CCARM 8010, *S. enteritidis* CCARM 8011, *S. typhimurium* KCCM 11862, *S. typhimurium* CCARM 8007, and *S. typhimurium* CCARM 8009 were subdivided from the Korean Culture Center of Microorganisms (KCCM) and Culture Collection of Antibiotic Microbes (CCARM). Organisms were subcultured in Müller Hinton Broth (YM, Difco, USA) for 28 h at 37°C. The turbidity of the cell suspension was measured at 600 nm, and adjusted with medium to match the 0.5 McFarland standard (10^5-10^6 colony forming units (CFU)/ml).

Compounds – Citral (a mixture of *trans* and *cis*) was isolated from the essential oil fraction with silicagel 60 (63~100 μm) and Sephadex LH-20 column chromatograhy and identified by comparison with the spectral data of the standard compound (98%, Aldrich, USA), Limonene (99%), streptomycin (99%), and kanamycin (98.8%) were purchased from Sigma Chemical Co.

Determination of minimal inhibitory concentration (MIC) – The MIC tests were performed as previously reported (Shin, 2005). The MIC value was defined as the lowest concentration that inhibited more than 50% of visible bacterial growth after 24 h. Each organism was additionally cultured with blank solution containing Tween 80 at concentrations equivalent to test solutions.

Checkerboard titer test – To evaluate the combined effects of essential oil compounds, ten series of two-fold dilutions of each standard or the essential oil fraction of *C. citratus* with culture medium containing Tween 80 and eight serial two-fold dilutions of streptomycin or kanamycin with DMSO (dimethyl sulfoxide) were prepared using the solvents used for MIC analyses. The next procedures were carried out as described by Shin (2005). Fractional inhibitory concentrations (FICs) were calculated by dividing the MIC values of the oil and streptomycin or kanamycin combinations by those of oil, streptomycin or kanamycin alone. The FIC index, obtained by adding both FIC values, was interpreted as a synergistic effect at $\leq$ 0.5, additive or indifferent at > 0.5 and $\leq$ 2.0, and antagonistic at > 2.0 (White *et al.*, 1996; Williamson, 2001). An isobologram was constructed from the checkerboard data to depict the synergism of citral or the essential oil fraction of *C. citratus* with streptomycin or kanamycin against the *Salmonella* species. DMSO and Tween 80 solvents were used at concentrations equivalent to those in the test solutions to certify that these vehicles did not affect bacterial growth.

Table 1. Compounds identified in the essential oil fraction of *C. citrates* by GC-MS

Compounds	*RI*	Area (%)
α-phellandrene	922	0.01
α-pinene	927	0.55
camphene	940	0.27
sabinene	967	0.31
β-pinene	968	0.26
myrcene	989	0.10
p-cymene	1021	0.01
limonene	1027	7.75
1,8-cineol	1032	0.50
δ-ocimene	1047	0.07
δ-3-carene	1055	0.01
terpinolene	1084	0.01
linallol	1098	0.50
trans-citral	1251	30.68
piperitone	1259	0.07
neryl acetate	1261	0.01
cis-citral	1280	53.62
eugenol	1359	0.03
isoeugenol	1360	0.02
nerol	1388	1.03
trans-methyl eugenol	1406	0.02
trans-caryophyllene	1406	0.46
α-farnesene	1433	0.01
α-gingiberene	1433	0.01
α-bisabolene	1448	0.02
α-humulene	1449	0.02
γ-cadinene	1492	0.02
δ-cadinene	1521	0.02
cis-isoelemycin	1559	0.01
caryophllene oxide	1577	0.09
In total		96.73

[a]*RI* : GC retention indices calculated against C_9 to C_{24} *n*-alkanes on an Ultra-2 capillary column.

Results and Discussion

The components of *C. citratus* essential oil identified with GC and GC-MS analyses are listed in Table 1. There have been a considerable number of reports on the composition of lemongrass oil (Singh *et al.*, 1989). The oil compositions documented vary tremendously, depending on the plant source. In this study, a Wiley 275 library search using GC-MS data, and GC analysis with standard compounds led to the identification of 30 compounds in the essential oil of *C. citratus*. The predominant compo-

Table 2. MICs (minimum inhibitory concentrations) and MBCs (minimum bactericidal concentrations) of the *C. citratus* oil fraction against antibiotic-susceptible and -resistant strains of *S. enteritidis* and *S. typhimurium*

Sample (mg/ml)		*S. enteritidis*			*S. typhimurium*		
		KCCM 12021	CCARM 8010	CCARM 8011	KCCM 11862	CCARM 8007	CCARM 8009
C. citratus oil	MIC	8.0	4.0	8.0	1.0	4.0	4.0
	MBC	8.0	4.0	8.0	2.0	4.0	4.0
Citral	MIC	2.0	2.0	1.0	0.5	2.0	1.0
	MBC	2.0	2.0	1.0	1.0	2.0	1.0
Limonene	MIC	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0
	MBC	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0
Streptomycin*	MIC	2.0	>128.0	>128.0	64.0	128.0	>128.0
	MBC	2.0	>128.0	>128.0	64.0	128.0	>128.0
Kanamycin *	MIC	2.0	4.0	1.0	8.0	8.0	>64.0
	MBC	2.0	4.0	1.0	8.0	8.0	>64.0

The values represent mean values from experiments performed in triplicate. μg/ml.

Table 3. FICs (fractional inhibitory concentrations) and FIC indices (FICI) in combination with antibiotics and citral or the essential oil fraction of *C. citratus* against *S. typhimurium* strains

Sample *S. typhimurium*		CC[a]-SM[b]		CT[c]-SM		CC-KM[d]		CT-KM	
KCCM 11862	FIC	0.50	0.12	0.25	0.06	0.25	0.03	0.25	0.12
	FICI	0.67		0.31		0.28		0.37	
CCARM 8007	FIC	0.50	0.06	0.50	0.125	0.25	0.50	0.50	0.50
	FICI	0.56		0.62		0.75		1.00	

[a] *C. citratus*, [b]streptomycin, [c]citral, [d]kanamycin
FIC of oil = MIC of oil in combination with antibiotics/ MIC of oil alone
FIC of antibiotics = MIC of antibiotics in combination with oil/ MIC of antibiotics alone
FICI = FIC of oil + FIC of antibiotics.

nents were *trans*-citral (30.68) and *cis*-citral (53.62%), which accounted for about 84.30% of the oil. Accordingly, the main component, citral, may contribute significantly to the antibacterial activity of lemongrass oil.

As depicted in Table 2, *C. citratus* oil and its components, citral and limonene, significantly inhibited the three strains of each *Salmonella* species in the broth dilution test, with MIC values in the range of 0.5-8.0 mg/ml. Citral (approximately 3 : 5 mixture of trans and cis form), the predominant compound of this oil, exhibited higher activity than the total essential oil fraction with MIC values that were two-fold or lower. Generally, no remarkable differences in MIC values were observed between antibiotic-susceptible and -resistant strains of *Salmonella* species.

In many cases, natural antimicrobial compounds from plants possess relatively weak activity, compared to synthetic antibiotics. As a strategy to enhance this activity, synergism of antibiotics in combination with natural products has been evaluated using the checkerboard titer test and construction of isobolograms (Shin and Kim, 2004; Sakagami *et al*, 2005).

In the checkerboard titer test, both lemongrass oil and citral exhibited significant synergism with streptomycin and kanamycin, particularly against *S. typhymurium* KCCM 11862, displaying fractional inhibitory concentration indices (FICI) between 0.28 and 0.67, which indicate significantly lower MICs of streptomycin or kanamycin in combination with oil (Table 3). Furthermore, results of the checkerboard titer test depicted in the isobologram (Fig. 1 and 2) confirm this synergism between *C. citratus* oil and streptomycin or kanamycin. Specifically, a combination of both compounds produced a curve to the left of the line (dotted) of the combination effects of the two compounds employed separately. Our results fulfill the criterion for synergism, which is defined as "the activity observed when a combination is greater than the sum of the effects observed with the two agents independently" (White *et al.*, 1996). Therefore, despite their relatively mild activity, essential oil from the leaves from *C. citratus* may facilitate

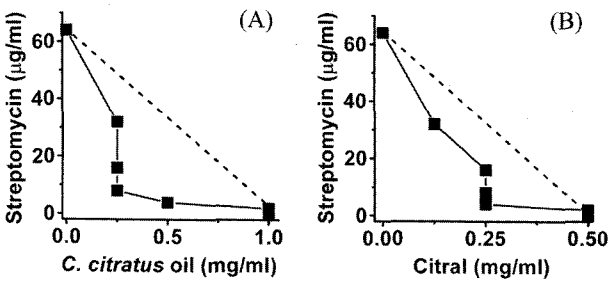

Fig. 1. Isobolograms of streptomycin in combination with *C.citratus* oil (A), or its main component, citral (B) against *S.typhimurium* KCCM 11862 (intermediate resistant strain to streptomycin). The curves were constructed by plotting with the concentrations in the wells which showed the most advantageous combination of the oil sample and streptomycin on checkerboard titer tests and compared with the control (additive line: dotted) which describes the sum of the effects with two samples independently.

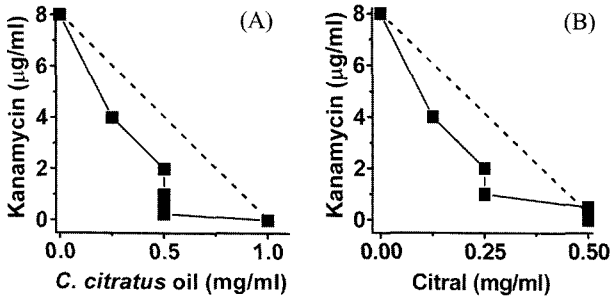

Fig. 2. Isobolograms of kanamycin in combination with *C. citratus* oil (A), or its main component, citral (B) against *S. typhimurium* CCARM 8007 (intermediate resistant strain to kanamycin). The curves were constructed by plotting with the concentrations in the wells which showed the most advantageous combination of the oil sample and kanamycin on checkerboard titer tests and compared with the control (additive line: dotted) which describes the sum of the effects with two samples independently.

antibacterial therapy, especially against *S. typhimurium*, by combination with streptomycin or kanamycin. However, further *in vivo* experiments are necessary to assess their potential for therapeutic application.

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