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Analysis of Composition and Activity of Essential Oil from *Chrysanthemum zawadskii* var. *latilobum* and *C. indicum* against Antibiotic-Resistant Pathogenic Bacteria

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Abstract – The composition of essential oils from *Chrysanthemum zawadskii* var. *latilobum* and *C. indicum* were analyzed and compared. The results of gas chromatography-mass spectrometry revealed there were distinctly different compositional patterns between *C. zawadskii var. latilobum* and *C. indicum* essential oils. The combinatorial effect of the oil of *C. zawadskii* var. *latilobum* and *C. indicum,* with various antibiotics was assessed against antibiotic-susceptible and -resistant strains of *Staphylococcus aureus* and *Streptococcus pneumoniae.* The essential oil fraction significantly inhibited most of the tested antibiotic-susceptible and -resistant strains of *S. pneumoniae*, with minimum inhibiting concentrations (MICs) ranging from 0.5 to 4.0 mg/ml. The fractional inhibiting concentration indices (FICIs) of the oils when combined with antibiotics against *S. aureus* and *S. pneumoniae* ranged from 0.26 to 0.75, and showed synergistic or additive effects.
Keywords – *Chrysanthemum zawadskii* var. *latilobum*, essential oil, antibiotic-resistant, combination effects

Introduction

Among *Chrysanthemum* species widely distributed throughout the fields of Korea, *C. indicum* and *C. zawadskii* var. *latilobum* are regarded as important source materials for traditional herbal medicine. The flower of *C. indicum* has been indicated for pneumonia, colitis, stomatitis, boils, carbuncles and inflammation of the eyes, and also for headache and dizziness (Cheng *et al.,* 2005; Shunying *et al.*, 2005). The aerial parts of *C. zawadskii* var. *latilobum* are harvested primarily in September and used for traditional medicine treatment of various women's diseases because of its activity of warming the body, especially the uterus. As the important active components of *C. indicum* and *C. zawadskii* var. *latilobum,* essential oils, flavonoids and other phenolic derivatives are studied (Lee and Lee, 2007). Plant essential oils are prevalent natural antimicrobial agents and have been suggested for use as a countermeasure to confront the accelerated resistance of microorganisms against specific antibiotics and drugs (Humphrey, 2001; Karlowsky and Sahm, 2002; Shin, 2004).

Despite the introduction of new antibiotics, especially oxacillin and methicillin, the spread of antibiotic-resistant strains has been rapid. *S. aureus* is the most common pathogen among the Staphylococci, and it represents a serious problem in therapy (Chang *et al*., 1995; Dillard *et al.,* 1996; Ito *et al.,* 2003). Moreover, the emerging resistance of strains causing respiratory infections, especially community-acquired pneumonia, is a serious problem worldwide. In particular, the treatment of *Streptococcus pneumonia* infection is currently hampered by increasing antibiotic resistance (Esposito, and Principi, 2002).

In this study, we analyzed and compared the composition of essential oil fractions from the flower parts of *C. indicum* and the aerial parts of *C. zawadskii* var. *latilobum.* The growth inhibiting activity of the oil fractions and main components were evaluated against antibiotic-susceptible and -resistant strains of *S. aureus* and *S. pneumoniae* with the aim of developing safe and effective agents against antibiotic-resistant pathogenic bacteria. Moreover the combinatorial effects of certain antibiotics with these oils were evaluated by checkerboard-titer test (Shin and Kim, 2005).

Materials and Methods

Analysis of essential oils – Essential oils were extracted by steam distillation from the flower parts of *C.*

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indicum and the aerial parts of *C. zawadskii* var. *latilobum,* cultivated in Hongchun, Gangwondo and Pochun, Gyeonggido, Korea, respectively. The composition of the extracted oils was determined by GC-MS. Voucher specimens were deposited in the herbarium of Duksung Women's University (CHR1 and CHR2). The oil (0.54%) obtained was analyzed using a 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 250 °C, and the oven temperature was regulated as follows: initial temperature at 60 °C for 5 min, increasing temperature of 2 °C/min up to 230 °C, and a final temperature of 180 °C for 30 min.

Strains – *S. aureus* ATCC 29213, *S. aureus* CCARM 3511, *S. aureus* CCARM 3523, S. *pneumoniae* KCCM 40410, *S. pneumonia* KCCM 40339, *S. pneumoniae* CCARM 4009, and *S. pneumoniae* CCARM 4010, 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's standard (10^5 - 10^6 colony forming units (CFU)/mL).

Compounds – Borneol and camphor were isolated from the essential oil fraction with silicagel 60 (63~100 μm) column chromatography and identified by comparison with the spectral data of the standard compounds (98%, Aldrich, USA). Oxacillin, norfloxacin and erythromycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Determination of minimal inhibitory concentration (MIC) – MIC tests were performed as previously reported (Shin and Kim, 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 tests – For checkerboard-titer tests, 50-μL aliquots of individual oil dilutions were added in a vertical orientation to the wells of 96-well plates, and 10-μL aliquots of oxacillin dilutions were added in a horizontal orientation, so that the plate contained various concentration combinations of the two compounds. A 100-μL suspension of three *S. pneumoniae* strains was added to each well, and plates were cultured at 36 °C for 24 hours. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of the oil and oxacillin divided by the MIC of the oil or oxacillin alone. The FIC index (FICI) was calculated by adding both FICs, and was interpreted as a synergistic effect when it was ≤ 0.5, as additive or indifferent when it was > 0.5 to 2.0, and as antagonistic when it was > 2.0 (White *et al.*, 1996; Shin and Lim, 2004). Similar experiments were also performed with norfloxacin.

Results and Discussion

Components of essential oil fractions were obtained by steam distillation from the flower parts of *C. indicum* and the aerial parts of *C. zawadskii* var. *latilobum* and identified using GC and GC-MS analyses (Table 1). A Wiley 275 library search using GC-MS data, and GC analysis with standard compounds led to the identification of 47 and 34 compounds in the essential oil of *C. indicum* and *C. zawadskii* var. *latilobum,* respectively. The predominant components of *C. zawadskii* var. *latilobum* were camphor (14.70%) and borneol (compound, 13.58%), which accounted for about 28.28% of the oil. Accordingly, these main components may contribute significantly to the antibacterial activity of the total oil fraction of this plant. However, such a high percent contribution to the activity of the total oil fraction could not be expected from the first and second most abundant components of *C. indicum,* germacrene B (10.86%) and α-pinene (8.17%), which are non-oxygenated hydrocarbons. The compounds 1,8-cineol, chrysanthenone, trans-pinocarveol, camphor, pinocarvone, eugenol, β-caryophyllene, trans-β-farnesne, (+)-spatulenol, caryophyllene oxide, and nor-copanone were identified in both of the oils. However, in a previous report a substantially higher content of 1,8-cineol (30.41%) and camphor (23.52%) were identified in *C. indicum* essential oil (Shunying *et al.,* 2005). This discrepancy might be related to a different growth status of the plant material, as well as the plant culture conditions (Choi *et al.,* 2004).

To determine MIC values for oils from *C. zawadskii* var. *latilobum* and *C. indicum*, three *S. aureus* and four *S. pneumonia* antibiotic-susceptible or -resistant strains were used. Antibiotic susceptibilities of the tested *S. aureus* and *S. pneumonia* strains were determined by the recommended protocols of the Clinical and Laboratory Standard Institute (CLSI, 2007) (Table 2).

C. zawadskii var. *latilobum* essential oil fraction and its main components, borneol and camphor, significantly inhibited most of the antibiotic-susceptible and -resistant strains of *S. pneumonia* in the broth dilution test. MICs

Table 1. Constituents of essential oils from *C. zawadskii* and *C. indicum* analyzed by GC-MS

Compounds	RI[a]	Area (%)	
		C. zawadskii	*C. indicum*
α-pinene	932	–	8.17
yomogi alcohol	1001	0.64	–
1,8-cineol	1027	1.63	7.59
benzeneacetaldehyde	1038	0.31	–
linalool oxide	1067	3.95	–
linalool	1096	0.78	–
β-thujone	1099	2.87	–
ρ-mentha-2-en-1-ol	1115	0.53	–
filifolone	1116	–	–
isocyclocitral	1117	–	0.50
chrysanthenone	1119	0.96	7.29
trans-pinocarveol	1134	0.55	0.78
camphor	1140	14.70	1.12
isoborneol	1151	0.72	–
pinocarvone	1157	0.29	0.78
borneol	1163	13.58	–
Epoxylinalool	1174	10.38	–
γ-camphorenol	1179	–	0.60
Terpinene-4-ol	1842	–	3.10
ρ-cymene-8-ol	1184	0.35	–
α-terpineol	1189	0.76	2.88
nopol	1191	–	0.68
myrtenol	1194	0.62	–
trans-(+)-carveol	1218	0.21	–
2,3-dihydro-benzofuran	1221	0.31	–
cis-carveol	1229	0.27	–
methyl ρ-toluate	1270	0.26	–
6,7-dehydrotropine	1290	6.14	–
(–)-bornyl acetate	1291	–	5.31
γ-pyronene	1292	–	0.54
thymol	1294	0.42	–
eugenol	1357	0.49	0.68
β-caryophyllene	1416	0.28	0.97
coumarin	1431	0.68	–
trans-β-farnesene	1459	0.28	2.57
germacrene-D	1480	0.73	5.46
Ar-curcumene	1483	–	4.86
germacrene-A	1485	–	3.77
β-sesquiphellandrene	1488	–	3.98
hotrienol	1521	–	0.51
(+)-spathulenol	1576	2.57	1.07
caryophyllene oxide	1580	2.00	1.49
salvial-4(14)-en-1-one	1590	0.60	–
vulgarone A	1596	0.38	–
nor-copaanone	1619	0.20	0.61
γ-eudesmol	1631	6.74	–
δ-cadinene	1641	1.14	–
vulgarone B	1648	4.82	–
γ-gurjunene	1653	2.74	–
widdrene	1669	–	0.90
sesquisabinene hydrate	1672	–	0.52
methyl ρ-methoxycinnamate	1673	0.61	–
α-guaiene	1677	0.20	–
2-propyl-5-vinylthiophene	1681	0.41	–
6-butyl-1,4-cycloheptadiene	1691	2.07	–
α-cadinol	1713	–	0.78
4,5-dihydro-3-n-butylphthalide	1719	0.43	–
(–)-elema-1,3,11(13)-trien-12-ol	1731	–	1.60
germacrone	1733	–	1.59
butylidene dihydro-phthalide	1734	0.59	–
N-butylpyrrole	1753	0.34	–
valerenol	1756	0.46	–
trans-chsanthemal	1772	0.75	–
germacrene B	1790	–	10.86
9-isopropylene bicyclononae	1800	–	–
α-bisabolol	1819	–	5.52
2,4-bis-1,1-dimethylethyl phenol	1824	–	2.67
oplopenone	1849	–	0.81
isospathulenol	1855	–	3.18
δ-fenchane	1901	0.31	–
In total		91.03	93.74

[a] GC retention indices (RI) were calculated against C_9 to C_{24} *n*-alkanes on a HP-5MS column.

Table 2. Antibiotic susceptibility of *S. aureus* and *S. pneumoniae* strains

Strains	Antibiotics[a]		
	oxacillin	norfloxacin	Erythromycin
S. aureus			
CCARM 29213	resistant	susceptible	susceptible
CCARM 3511	resistant	resistant	resistant
CCARM 3523	resistant	resistant	resistant
S. pneumonia			
KCCM 40410	susceptible	susceptible	susceptible
KCCM 4033	susceptible	susceptible	susceptible
CCARM 4009	resistant	resistant	resistant
CCARM 4010	resistant	resistant	resistant

[a]The susceptibility and resistance of bacterial strains to antibiotics were determined by the recommended protocols of the Clinical and Laboratory Standard Institute (CLSI), USA.

Table 3. MICs of essential oils against antibiotic-susceptible and -resistant strains of *S. aureus* and *S. pneumoniae*

Sample(mg/ml)	*S. aureus*			*S. pneumoniae*			
	Sa13[c]	*Sa11*[d]	*Sa23*[e]	*Sp410*[f]	*Sp33*[g]	*Sp09*[h]	*Sp10*[i]
Cz[a]	8	8	4	2	1	0.5	1
Ci[b]	8	8	4	2	2	0.5	1
Borneol	4	4	2	1	1	1	1
Camphor	> 8	> 8	> 8	4	4	4	4
Oxacillin*	128	128	256	0.5	0.5	64	64
Norfloxacin*	1	256	256	2	2	16	16
Erythromycin *	0.25	> 128	> 128	0.13	0.06	1	1

* μg/mL.
[a]Essential oil fraction from the aerial parts of *C. zawadskii* var. *latilobum*; [b]Essential oil fraction from flowers of *C. indicum*; [c]*S. aureus* CCARM 29213; [d]*S. aureus* CCARM 3511; [e]*S. aureus* CCARM 3523; [f]*S. pneumoniae* KCCM 40410; [g]*S. pneumoniae* KCCM 4033; [h]*S. pneumoniae* CCARM 4009; [i]*S. pneumoniae* CCARM 4010.

Table 4. FICs (fractional inhibitory concentrations) and FICI (FIC indices) for the combinatorial effects of norfloxacin or oxacillin with *Chrysanthemum* essential oils or borneol against *S. aureus* and *S. pneumoniae*

Sample (mg/ml)	*S. aureus*				*S. pneumoniae*			
	Sa13[a]		*Sa23*[b]		*Sp410*[c]		*Sp33*[d]	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
Cz	0.50	0.75	0.25	0.50	0.25	0.38	0.25	0.50
norfloxacin*	0.25		0.25		0.13		0.25	
Ci	0.50	0.53	0.50	0.63	0.50	0.56	0.25	0.75
Norfloxacin*	0.03		0.13		0.06		0.50	
Borneol	0.50	0.56	0.25	0.28	0.13	0.26	0.13	0.38
Norfloxacin*	0.06		0.03		0.13		0.25	

Sample (mg/ml)	*S. aureus*				*S. pneumoniae*			
	Sa13		*Sa23*		*Sp410*		*Sp33*	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
Cz	0.25	0.27	0.50	0.63	0.50	0.56	0.50	0.62
Oxacillin*	0.02		0.13		0.06		0.12	
Ci	0.25	0.28	0.50	0.53	0.50	0.53	0.50	0.56
Oxacillin*	0.03		0.03		0.03		0.06	
Borneol	0.25	0.28	0.50	0.75	0.25	0.28	0.50	0.56
Oxacillin*	0.03		0.25		0.03		0.06	

* μg/mL.
FIC = (MIC a combined with b / MIC a alone) or (MIC b combined with a / MIC b alone).
FICI = (MIC a combined with b / MIC a alone) + (MIC b combined with a / MIC b alone).
[a]*S. aureus* CCARM 29213; [b]*S. aureus* CCARM 3523; [c]*S. pneumoniae* KCCM 40410; [d]*S. pneumoniae* KCCM 4033.

ranged from 0.5 - 4 mg/mL (Table 3). Camphor, the most abundant component in this oil, demonstrated MICs four times greater than those of borneol, the second most abundant component. Accordingly, borneol may contribute more significantly than camphor to the antibacterial activity of the total oil fraction. Tested strains of *S .aureus* strains exhibited lower susceptibility than *S. pneumonia* strains to *C. zawadskii* var. *latilobum* essential oil, and no remarkable differences in MIC values were observed between strains of the same species. Both *Chrysanthemum* oil fractions showed the greatest growth-inhibitory activity against *S. pneumonia* CCARM 4009 strain, with an MIC of 0.5 mg/mL.

Though plant essential oils represent a promising

source of novel, natural drugs for overcoming the problem of antibiotic-resistant bacteria strains, in most cases the antibacterial activities of essential oils are much milder than those of antibiotics used in current therapies. Consequently, to enhance the potency of essential oil therapies the efficacy of combining essential oils with antibiotics has been studied.

To evaluate the combinatorial effects of *Chrysanthemum* essential oil fractions and borneol with oxacillin or norfloxacin, checkerboard titer tests were performed. The fractional inhibiting concentration indices (FICIs) of the oil and antibiotic combinations ranged from 0.26 to 0.75, and synergistic or additive effects against the tested strains of *S. aureus* and *S. pneumoniae* were observed (Table 4). No distinct differences in FICIs were observed between the strains. The most significant synergism was observed against *S. pneumoniae* KCCM40410 by the combination of borneol and norfloxacin, with an FICI of 0.26. In most cases FICs of antibiotics ranged from 0.02 to 0.5, values indicating that the MICs of antibiotics were significantly lowered by combination with the essential oil or essential oil component.

In summary, we demonstrated significant anti-bacterial activity in the essential oil of *C. zawadskii* var. *latilobum* and borneol, one of its primary components, against antibiotic-susceptible and -resistant strains of *S. pneumoniae*. The activity of *C. zawadskii* var. *latilobum* essential oil revealed as nearly equivalent to those of *C. indicum* oil, a well known bacteriostatic agent. In addition, we showed that the combination of *C. zawadskii* var. *latilobum* essential oil and antibiotics produced greater potency against strains of *S. pneumonia* and *S. aurea*.

In conclusion, the essential oil of *C. indicym* and *C. zawadskii* var. *latilobum* or borneol as well, may be useful as an anti-*Streptococcus* agent, especially in the case of infection by antibiotic-resistant strains of *S. pneumoniae*. However, further experiments are necessary to assess their potential for therapeutic application.

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