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Combined Effects of the Essential Oil from *Pelargonium graveolens* with Antibiotics against *Streptococcus pneumoniae*

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Abstract – The antimicrobial activity of the essential oil from *Pelargonium graveolens* and its effects when it was combined with current antibiotics against antibiotic-resistant strains of *Streptococcus pneumoniae* were evaluated. The minimal inhibitory concentrations (MICs) of the essential oil fraction and the main components of this plant were determined for two antibiotic-susceptible and two antibiotic-resistant strains of *S. pneumoniae* using broth microdilution tests. The combined effects of the oil with erythromycin, norfloxacin, or oxacillin were evaluated using a checkerboard microtitre assay. The combination of the oil fraction of *P. graveolens*, or its main component, together with the antibiotics tested significantly lowered the MICs of the antibiotics against all of the tested strains with fraction inhibiting concentration indices (FICs) ranging from 0.16 to 1.50. In particular, the activity of norfloxacin against all of the tested strains of *S. pneumoniae* was enhanced significantly by combination with citronellol. In conclusion, the combination of *P. graveolens* oil with antibiotics could be used to reduce the effective dose of antibiotic and to modulate the resistance of *S. pneumoniae* strains.

Keywords – *Pelargonium graveolens*, citronellol, geraniol, linalool, combination effects, antibiotic-resistance, *Streptococcus pneumoniae*

Introduction

Pelargonium graveolens (Geraniaceae), commonly known as the rose geranium, is an undershrub that is native to southern Africa. It grows throughout the world and is cultivated in the herb gardens of many countries including Korea. The essential oil fraction of this plant is widely and extensively used in the cosmetic industry, in aromatherapy, and for flavoring food due to its unique strong rose-like fragrance. Its mosquito repellent property is also well known. The oil has a relatively strong hydrophilic property and contains a high percentage of the acyclic monoterpene alcohols, geraniol, citronellol and linalool, which provides advantages in many corresponding preparations (Lis-Balchin and Deans, 1996). There are significant variations in the composition of the oil depending on cultivars and culture conditions (Rajeswara Rao, *et al.,* 1996; 2002); intra-clonal variation was also identified by Kulkarni, *et al.* (1997).

Streptococcus pneumoniae is one of the most common causes of invasive bacterial infections. In recent years, a major issue in pneumococcal infection has been the emergence and global dissemination of penicillin-resistant and multiple-resistant strains (Appelbaum, 1992; Subramanian, *et al.,* 2003; File, *et al.,* 2006; Mera, *et al.,* 2006). For penicillin-allergic patients, erythromycin or other antibiotics, such as norfloxacin, are generally used. Approximately 5% of all *S. pneumoniae* strains are relatively resistant to penicillin (Teele, 2002). Pneumococcal strains with resistance to erythromycin and fluoro-quinolones have also been found in recent decades (Bruinsma, *et al.,* 2004; Hesueh, 2005).

Choi and Shin (2007) reported the anti-streptococcal activity of the essential oil from *Mentha piperita,* however, the combined effects of this oil with antibiotics were not significant enough to develop an effective treatment against antibiotic-resistant strains of *S. pneumoniae.*

In order to develop effective and safe natural antibiotics for either the treatment or alleviation of respiratory infections, in particular for the antibiotic resistant strains of *S. pneumoniae* in Korea, the composition of the essential oil fraction from *P. graveolens* was analyzed by gas chromatography-mass spectrometry and it main components were isolated by column choromatography. The minimal inhibitory concentrations (MICs) of the essential oil fraction and the main components of this plant were

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determined for antibiotic-susceptible and -resistant strains of *S. pneumoniae* using broth microdilution tests. On the basis of the MIC results, the combined effects of the oil with erythromycin, norfloxacin, or oxacillin were evaluated using a checkerboard microtitre assay.

Experimental

Extraction of oils and analysis – *P. graveolens* is cultivated in the herbal garden of Duksung Women's University and harvested in August. A voucher specimen was deposited at the herbarium of Duksung Women's University (No. GERPG1). The essential oil fraction was extracted by steam distillation from its fresh leaves and flowers. The composition of the essential oils was analyzed by GC-MS on a Hewlett-Packard 6890 GC and Hewlett-Packard 5973 MSD apparatus using an HP-5 and HP-Innowax capillary column (Shin and Kim, 2005). The injector was adjusted to 260 °C, and the oven temperature was regulated as follows: HP-5 column; initial temperature at 50 °C for 5 min, 2 °C/min up to 180 °C, 3 °C/min up to 280 °C., HP-Innowax column; initial temperature, 50 °C, 2 °C/min up to 170 °C, 3 °C/min up to 260 °C and 20 min at 260 °C. Citronellol, geraniol and linalool were isolated by column chromatography (silicagel, toluene:ethyl acetate = 93 : 7) and re-crystallization from the essential oil of *P. graveolens*. The MS, IR, ^{1}H- and ^{13}C-NMR spectrum of the isolated compounds were compared with those of their corresponding standards.

Citronellol – Colorless oil, EI-MS m/z: 156 $[M]^+$, ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.04 (1H, t, J = 7.2, H-6), 3.59 (2H, m, H-1), 2.09 (1H, s, -OH), 1.95 (2H, m, H-5), 1.62 (3H, s, H-9), 1.54 (3H, s, H-8), 1.30 (2H, m, H-2a, H-4a), 1.12 (2H, m, H-2b, H-4b). 0.85 (3H, m, H-10); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 131.1 (C-7), 125.1 (C-6), 60.75 (C-1), 42.9 (C-2), 38.3 (C-4), 29.5 (C-3), 25.9 (C-5), 25.7 (C-9), 19.7 (C-8), 17.8 (C-10).

Geraniol – Colorless oil, EI-MS m/z: 154 $[M]^+$, ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.40 (1H, t, J = 6.6, H-2), 5.02 (1H t, J = 6.6, H-6), 4.14 (2H, d, H-1), 2.04 (4H, m, H-4, H-5), 1.67 (6H, s, H-9, 10), 1.60 (3H, s, H-8); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 137.9 (C-3), 129.1 (C-7), 122.3 (C-2), 121.7 (C-6), 56.8 (C-1), 37.8 (C-4), 24.4 (C-5), 23.7 (C-9), 15.7 (C-8), 14.2 (C-10).

Linalool – Colorless oil, EI-MS m/z: 154 $[M]^+$, ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.94 (1H, dd, J = 28.2, J = 10.8, H-2), 5.21 (1H, dd, J = 16.4, J = 1.1, H-1a), 5.06 (1H, dd, J = 10.7, J = 1.1, H-1b), 5.12 (1H, t, J = 5.4, H-6), 2.02 (2H, q, J = 7.5, H-5), 1.67 (3H, s, H-8), 1.60 (3H, s, H-9), 1.27 (3H, s, H-10); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 145.1 (C-2), 132.2 (C-7), 124.9 (C-6), 112.4 (C-1), 73.8 (C-3), 42.4 (C-4), 28.2 (C-5), 26.1 (C-9), 22.9 (C-10), 18.04.2 (C-8).

Strains – *S. pneumoniae* KCCM 40410 and *S. pneumoniae* KCCM 4033 were subdivided by the Korean Culture Center of Microoganisms (KCCM). *S. pneumoniae* CCARM 4009 and *S. pneumoniae* CCARM 4010 were obtained from the Culture Collection of Antibiotic Resistant Microbes (CCARM).

MIC (Minimum Inhibitory Concentration) test – The MIC values of the oils and antibiotics were determined using the broth microdilution method. A range of two-fold dilutions of essential oils was prepared in medium containing 2% Tween-80. The oil suspensions (100 μL) were added to 96-well plates. The turbidity of the bacterial suspensions was measured at 600 nm, and adjusted with medium to match the 0.5 McFarland standard (10^5 - 10^6 colony forming units/mL). Next, a 190- μL bacterial culture was inoculated into each well, and plates were incubated at 36 °C for 24 hours. Antibiotics were similarly diluted in DMSO to generate a series of concentrations, ranging from 128 to 0.03 μg/mL per well. MIC values were determined in duplicate and re-examined where appropriate. Each organism was also cultured with a blank solution containing Tween-80 and DMSO at concentrations that were equivalent to those in the test solutions in order to verify that the vehicle used did not affect growth. The MICs of the oils were compared with those of the antibiotics, erythromycin, norfloxacin, and erythromycin. Erythromycin, norfloxacin and oxacillin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Checkerboard-titer tests – Aliquots (50-μL) of individual oil dilutions were added to the wells of 96-well plates in a vertical orientation, 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 four *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 erythromycin divided by the MIC of the oil or erythromycin alone. The FIC index (FICI) was calculated by adding both of the FICs and was interpreted as a synergistic effect when it was ≤ 0.5, as an additive or indifferent effect 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 using norfloxacin and oxacillin.

Table 1. Constituents of the essential oil from *P. graveolens* by GC-MS

Compounds	RI		Peak Area (%)
	HP-5[a]	HP-IW[b]	
Limonene	1022	1193	0.10
Linalool oxide	1065	1434	0.45
Linalool	1100	1553	8.78
trans-Rose oxide	1106	1338	1.05
l-Menthone	1146	1445	6.60
cis-Ocimene	1166	1228	0.04
α-Terpineol	1191	1690	0.00
Citronellol	1236	1774	20.84
Geraniol	1265	1852	12.93
Citronellyl propionate	1279	1660	5.52
Geraniol formate	1305	1704	2.80
β-Bourbonene	1375	1490	1.44
Geranyl acetate	1391	1755	0.02
α-Gurjenene	1398	1508	0.09
β-Caryophyllene	1407	1575	1.43
β-Cubebene	1419	1569	1.23
Aromadendrene	1428	1602	2.59
3,7-Guaiadiene	1435	1588	0.48
α-Humulene	1442	1644	0.31
Germacrene-D	1456	1650	1.37
δ-Cadinene	1466	1609	2.57
α-Amorphene	1469	1736	0.54
Geranyl Propionate	1479	1817	0.59
Bicyclogermacrene	1488	1708	1.07
α-Muurolene	1494	1707	0.32
Cadina-1,4-diene	1525	1758	0.20
δ-Selinene	1530	2070	13.93
Geranyl isobutyrate	1562	1891	1.23
Caryophyllene oxide	1571	1941	0.36
Viridiflorol	1580	2044	0.22
Phenylethyl tiglate	1588	2165	1.77
γ-Selinene	1618	2119	0.50
γ-Eudesmol	1622	2141	0.49
Valencene	1626	2147	0.73
β-Eudesmol	1640	2192	0.88
T-Cadinol	1647	2200	0.54
Geranyl butyrate	1662	2079	0.21
Geranyl tiglate	1703	2099	1.86
total			96.79

* Compounds are listed in order of their elution on the HP-5MS column.
[a] GC retention indices (RI) was calculated against C_9 to C_{24} *n*-alkanes on an HP-5MS column.
[b] GC retention indices (RI) were calculated against C_9 to C_{24} *n*-alkanes on an HP-INNOWAX column.

Table 2. MICs of several essential oils against antibiotic-susceptible and -resistant strains of *Streptococcus pneumoniae*

Sample (mg/ml)	*S. pneumoniae*			
	Sp410	Sp33	Sp09	Sp10
P. graveolens	1.00	2.00	2.00	1.00
Citronellol	1.00	1.00	1.00	1.00
Geraniol	1.00	1.00	1.00	1.00
Linalool	4.00	8.00	4.00	4.00
Menthone	4.00	8.00	8.00	4.00
Erythromycin*	0.06	0.06	0.50	0.50
Norfloxacin*	16.0	16.0	32.0	32.0
Oxacillin*	8.00	8.00	128	64.0

Sp410: *S. pneumoniae* KCCM 40410, Sp33: *S. pneumoniae* KCCM 4033,
Sp09: *S. pneumoniae* CCARM 4009, Sp10: *S. pneumoniae* CCARM 4010.
* μg/ml.

Results and Discussion

Thirty eight compounds were identified in the essential oil fraction of *P. graveolens* by GC-MS. As listed in Table 1. *P. graveolens* oil contained a high percentage of the acyclic monoterpene alcohols, citronellol (28.84%), geraniol (12.93%), and linalool (8.78%). The non-oxygenated hydrocarbon, δ-selinene was also present at high concentrations (13.93%). The components *l*-menthone, a monoterpene ketone, citronellyl propionate and geranyl formate were also detected in relatively high concentrations.

As demonstrated in Table 2, we used two susceptible and two resistant strains of *S. pneumoniae,* which showed distinct differences in sensitivity to oxacillin and erythromycin. All of the tested strains were resistant to norfloxacin. The standards that were suggested by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (2006) were used to determine whether the strains were susceptible or resistant to the antibiotics.

The total oil fraction of *P. graveolens*, and its main components, citronellol, geraniol, and linalool (Fig. 1) exhibited significant inhibiting activity against antibiotic-susceptible as well as -resistant strains of *S. pneumonia.* Among the main components of the oil that we examined, geraniol and citronellol, the unsaturated primary alcohols, were found to be the most potent inhibitors of all of the strains tested, with an MIC of 1 mg/ml. In contrast linalool, the secondary alcohol, showed relatively weak activity resulting in an MIC between 4 mg/ml and 8 mg/ml. *P. graveolens* oil contains 6.60% menthone; this compound possesses a mild antibacterial activity. Thus, those active ingredients that contain at least one free

hydroxyl group in their structure are more potent than their ketone derivatives (Imai, *et al.*, 2001). The mechanism underlying the antimicrobial activity of plant essential oils has not been clarified in detail. Trombetta, *et al.* (2005) reported that their efficacy might be related to alterations in membrane permeability and to leakage of intracellular materials, factors that depend largely on lipophilicity and water solubility. Nidiry (1998) has confirmed that acyclic, unsaturated and primary alcohol structure is advantageous for antifungal activity against *Collectotrichum gloeosporioides*. The results of our study imply that a similar mechanism might also occur in bacteria in spite of the structural differences in the membranes of fungi and bacteria.

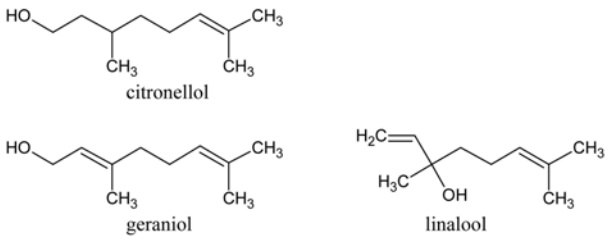

Fig. 1. Structures of the main components that were isolated from *P. graveolens*.

In experiments to test the combined effects of *P. graveolens* oil and its main components, citronellol, geraniol, or linalool with erythromycin, norfloxacin, or oxacillin to inhibit the growth of *S. pneumoniae,* additive or synergistic effects were observed against the antibiotic-susceptible and -resistant strains with fraction inhibiting concentration indices (FICIs) ranging from 0.16 to 1.50 (Table 3). In particular, the activity of norfloxacin combined

Table 3. Fractional Inhibiting Concentrations (FICs) and FIC Indices (FICIs) of essential oils from *P. graveolens* in combination with erythromycin against *S. pneumoniae*

Sample	Sp410		Sp33		Sp09		Sp10	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
P. graveolens - erythromycin	0.50 0.13	0.63	0.50 0.06	0.56	0.50 0.13	0.63	0.50 0.06	0.56
Citronellol - erythromycin	0.25 0.13	0.38	0.50 0.06	0.56	0.50 0.03	0.53	0.50 0.06	0.56
Geraniol - erythromycin	0.50 0.03	0.53	0.25 0.25	0.50	0.50 0.03	0.53	0.13 0.13	0.26
Linalool - erythromycin	0.50 0.13	0.63	0.50 0.25	0.75	0.50 0.03	0.53	0.50 0.03	0.53
P. graveolens - norfloxacin	0.50 0.13	0.63	0.50 0.06	0.56	0.25 0.13	0.38	0.50 0.13	0.63
Citronellol - norfloxacin	0.25 0.13	0.38	0.25 0.06	0.31	0.13 0.03	0.16	0.25 0.03	0.28
Geraniol - norfloxacin	0.50 0.06	0.56	0.50 0.06	0.56	0.50 0.06	0.56	0.50 0.03	0.53
Linalool - norfloxacin	0.02 0.50	0.52	0.13 0.5	0.63	0.5 0.03	0.53	0.50 0.03	0.53
P. graveolens - oxacillin	0.50 0.13	0.63	0.50 0.06	0.56	0.50 0.06	0.56	0.50 0.02	0.52
Citronellol - oxacillin	0.50 0.06	0.56	0.50 0.06	0.56	0.50 0.06	0.56	0.50 0.03	0.53
Geraniol - oxacillin	0.50 0.03	0.53	0.50 0.25	0.75	0.50 0.13	0.63	0.50 0.03	0.53
Linalool - oxacillin	0.50 1.00	1.50	0.50 0.03	0.53	0.50 0.03	0.52	0.50 0.03	0.53

Sp410: *S. pneumoniae* KCCM 40410, Sp33: *S. pneumoniae* KCCM 4033, Sp09: *S. pneumoniae* CCARM 4009, Sp10*: S. pneumoniae* CCARM 4010.

FIC = Fractional inhibitory concentration (MIC of the sample in combination / MIC of the sample alone), FICI = FIC index; (MIC a combined with b / MIC a alone) + (MIC b combined with a / MIC b alone)

with citronellol (FICI of 0.16) was significantly enhanced against *S. pneumoniae* CCARM 4009, one of the norfloxacin resistant strains; this result was the strongest synergism that was detected in this study. The MIC of norfloxacin was lowered from 32.0 mg/ml to 1 mg/mL resulting in an FIC of 0.03. The MIC of citronellol was reduced from 32.0 mg/mL to 1 mg/ml when it is combined with norfloxacin resulting an FIC of 0.13

In conclusion, faced with the rapid increase of antibiotic-resistant *S. pneumonia* strains, in order to develop an effective and safe natural product, we studied the activity of the essential oil components in *P. graveolens* that is easily cultivated in most places of the world including Korea. The combination of the oil fraction of *P. graveolens*, or its main component, together with the antibiotics tested significantly enhanced the activity against all of the tested antibiotic-resistant strains of *S. pnumoniae*. These results may provide a new strategy for the development of new natural antibiotics from *P. graveolens* oil or citronellol for the treatment of antibiotic-resistant *S. pneumoniae* infections.

References

Appelbaum, P.C., Antimicrobial resistance in *Streptococcus pneumoniae* : An overview. *Clin. Infec. Di.* **15**, 77-83 (1992).

Bruinsma, N., Kristinsson, K.G., Bronzwaer, S., Schrijnemakers, P., Degener, J., Tiemersma, E., Hryniewicz, W., Monen, J., and Grundmann, H., Trends of penicillin and erythromycin resistance among invasive *Streptococcus pneumoniae* in Europe. *J. Antimicrob. Chemother.* **54**, 1045-1050 (2004).

Choi, S.H. and Shin, S., Activity of essential oil from *Mentha piperita* against some antibiotic-resistant *Streptococcus pneumoniae* strains and its combination effects with antibiotics. *Nat. Prod. Sci.* **13**, 164-168 (2007).

Clinical and Laboratory Standard Institute, Performance standards for antimicrobial susceptibility testing; 17th informational supplement. M100-S17, Wayne, p. 98, 2007.

File, T.M., Tan, J.S., and Boex, J.R., The clinical relevance of penicillin-resistant Streptococcus pneumoniae: a new perspective. *Clin. Infec. Dis.* **42**, 798-800 (2006).

Husueh, O.-R., Decreasing rates of resistance to penicillin, but not *erythromycin*, in *Streptococcus pneumoniae* after introduction of a policy to restrict antibiotic usage in Taiwan. *Clin. Microbiol. Infect.* **11**, 925-927 (2005).

Imai, H., Osawa, K., Yasuda, H., Hamashima, H., Arai, T., and Sasatsu, M., Inhibition by the essential oils of peppermint and spearmint of the growth of pathogenic bacteria. *Microbes* **106**, 31-39 (2001).

Kulkarni, R.N., Baskaran, K., Ramesh, S., and Kumar, S., Intra-clonal variation for essential oil content and composition in plants derived from leaf cuttings of rose-scented geranium (*Pelargonium* sp.). *Ind. Crop. Prod.* **6**, 133-144 (1997).

Lis-Balchin, M., Deans, S.G., and Hart, S., Bioactive geranium oils from different commercial sources. *J. Essent. Oil Res.* **8**, 281-290, (1996).

Mera, R.M., Miller, L.A., and White, A., Antibacterial use and Streptococcus pneumoniae penicillin resistance: A temporal relationship model. *Microb. Drug Resist.* **12**, 158-163 (2006).

Nidiry, E.S., Structure-fungitoxicity relationships of the monoterpenoids of the essential oils of Peppermint (*Mentha piperita*) and scented geranium (*Pelargonium graveolens*). *J. Essent. Oil Res.* **10**, 628-632 (1998).

Rajaeswara Rao, B.R., Kaul, P.N., Mallavarapu, G.R., and Ramesh, S., Effects of Seasonal climatic changes on Biomass Yield and Terpenoid composition of rose-scented geranium (*Pelargonium* species). *Biochem. Syst. Ecol.* **24**, 627-635 (1996).

Rajaeswara Rao, B.R., Biomass yield, essential oil yield and essential oil composition of rose-scented geranium (*Pelargonium* species) as influenced by row spacings and intercropping with cornmint (*Mentha arvensis* L, f. *piperascens* Malinv. Ex Holmes). *Ind. Crop. Prod.* **16**, 133-144 (2002).

Shin, S. and Lim, S., Antifungal activities of herbal essential oils alone and in combination with ketoconazole against *Trichophyton* spp. *J. Appl. Microbiol.* **97**, 289-1296 (2004).

Shin, S. and Kim J.H., In vitro inhibitory activities of essential oils from two Korean *Thymus* species against antibiotic-resistant pathogens. *Arch. Pharm. Res.* **28**, 897-901 (2005).

Subramanian, D, Sandoe, J.A.T., Keer, V., and Wilcox, M.H., Rapid spread of penicillin-resistant Streptococcus pneumoniae among high-risk hospital inpatients and the role of molecular typing in outbreak confirmation. *J. Hosp. Infec.* **54**, 99-103 (2003).

Teale, C.J., Antimicrobial resistance and the food chain. *J. Appl. Microbiol.* **92**, 85-89 (2002).

Trombetta, D., Castelli, F., Sarpietro, M.G.S., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G., and Bisigano G., Mechanism of antibacterial action of three monoterpenes. *Antimicrob. Agents Chemother.* **49**, 2474-2478 (2005).

White, R.L., Burgess, D.S., Manduru, M., and Bosso, J.A., Comparison of three diffenent in vitro methods of detecting synergy: time–kill, checkerboard, and E test. *Antimicrob. Agents Chemother.* **40**, 1914-1918 (1996).

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