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Activity of Essential Oil from *Mentha piperita* against Some Antibiotic-Resistant *Streptococcus pneumoniae* Strains and Its Combination Effects with Antibiotics

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Abstract – To investigate natural antibiotics from plant essential oils and to evaluate their synergism with current antimicrobial drugs in inhibiting antibiotic-resistant strains of *Streptococcus pneumoniae*. The minimal inhibitory concentrations (MICs) of eleven plant essential oils and their main components were established for two antibiotic-susceptible and two antibiotic-resistant strains of *S. pneumoniae,* using broth microdilution tests. Potential synergism with oxacillin, norfloxacin, or erythromycin was evaluated using a checkerboard microtitre assay. Among the tested oils, *Mentha piperita* oil and its main component, menthol, exhibited the strongest inhibitory activities against all of the tested strains. The activity of antibiotics against antibiotic-resistant strains of *S. pneumoniae* was enhanced significantly by combination with *Mentha piperita* oils and its main component, menthol. In conclusion, the combination of *Mentha piperita* essential oil or menthol with antibiotics could be used to reduce the effective dose of antibiotic and to modulate the resistance of *S. pneumoniae* strains.
Keywords – *Mentha piperita*, menthol, antibiotic-resistance, *Streptococcus pneumoniae* synergism.

Introduction

The emergence of antibiotic-resistant bacterial strains that cause respiratory infections, especially community-acquired pneumonia, is a serious problem worldwide. Of particular concern, treatment of *Streptococcus pneumonia* infection is currently hampered by increasing incidence of antibiotic resistance (Esposito and Principi, 2002).

Streptococcus pneumoniae is one of the most common causes of invasive bacterial infections. Pneumococcus is also a frequent cause of acute otitis media and sinusitis (Ho *et al.*, 2004). It is commonly treated with penicillin (Koneman *et al.*, 1992; Baron *et al.*, 1994). In recent years, a major issue in pneumococcal infection has been the emergence and global dissemination of penicillin-resistant and multiple-resistant strains. 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 (Appelbaum, 1992; Hesueh, 2005; Ardanuy *et al.*, 2006).

It is well established that many herbal essential oils possess antimicrobial activity. To investigate the possibility that these oils could yield effective and safe, natural antibiotics or could be used in therapeutic cocktails with commonly used drugs, we selected eleven plant essential oils. All selected oils have been used in aromatherapy preparations for either the prevention or alleviation of respiratory infections in Korea. The minimal inhibitory concentrations (MICs) of each essential oil fraction, and their main components, against antibiotic-susceptible and antibiotic-resistant strains of *S. pneumoniae* were investigated by broth microdilution tests. Combination effects with oxacillin or erythromycin were also evaluated using a checkerboard microtitre assay.

Experimental

Oils and chemicals – *Mentha piperita* (leaf, Labiatae) is cultivated in the herbal garden of Duksung Women's University and harvested in September 2006. The essential oil fraction was extracted by steam distillation from its fresh leaves. The essential oils from *Cedrus atlantica* (wood, Pinaceae), *Citrus bergamia* (fruit, Rutaceae), *Citrus mandurensis* (fruit, Rutaceae), *Commiphora molmol* (resin, Burseraceae), *Coriandrum sativum, Eucalyptus golbulus* (leaf, Myrtaceae), *Juniperus communis* (fruit, Cupressaceae), *Lavandula angustifolia* (flower), *Pseudotsuga menziesii* (wood, Pinaceae), and

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Styrax tonkinensis (wood, Styraceae) were purchased from Neumond Co, Raistings, Germany. The compositions of the essential oils were analyzed by GC-MS on a Hewlett-Packard 6890 GC and Hewlett-Packard 5973 MSD apparatus using an HP-5 capillary column (Shin and Kim, 2005). Menthol was isolated by column chromatography and re-crystallization from the essential oil of *M. piperita*. Camphor, 1,8-cineol, limonene, menthone, linalool, oxacillin, and erythromycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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 (resistant strains against oxacillinand erythromycin) were obtained from the Culture Collection of Antibiotic Resistant Microbes (CCARM).

MIC (Minimum Inhibitory Concentration) test – MIC values of the oils and the antibiotics were determined using the broth microdilution method. A range of two-fold dilutions of essential oils in medium containing 2% Tween-80 was prepared. 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 equivalent to those in test solutions to verify that the vehicle used did not affect growth. The MICs of the oils were compared with those of the antibiotics, oxacillin and erythromycin.

Checkerboard-titre tests – For checkerboard-titre tests, 50 µL aliquots 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 norfloxacin divided by the MIC of the oil or norfloxacin 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 erythromycin.

Results

Composition of the herbal essential oils – GC-MS analysis, which was employed to confirm the composition of the herbal essential oils, resulted in the identification of more than fifty compounds which included the following major components of oils: junipene (21.2%) in *C. atlantica*, linalyl acetate (35.5%) in *C. bergamia*, limonene (58.8%) in *C. mandurensis*, linalool (55.9%) in *C. sativum*, 1,8-cineol (81.3%) in *E. golbulus*, widdrene (33.2%) in *J. communis*, linalool (30.9%) in *L. angustifolia*, *l*-menthol (28.8%) in *M. piperita*, and benzoic acid (67.7%) in *S. tonkinensis*.

MICs of the essential oil fractions and their main components – As demonstrated in Table 1, we used two

Table 1. MICs (mg/mL) of several essential oils against antibiotic-susceptible and antibiotic–resistant strains of *S. pneumoniae*

Sample	S. pneumoniae strain			
	Sp410	Sp33	Sp09	Sp10
C. atlantica	8.00	> 16.0	> 16.0	> 16.0
C. bergamia	> 16.0	> 16.0	> 16.0	> 16.0
C. mandurensis	4.00	2.00	> 16.0	> 16.0
C. molmol	0.50	0.50	> 16.0	> 16.0
C. sativum	4.00	8.00	2.00	4.00
E. globulus	4.00	4.00	16.0	8.00
J. communis	4.00	4.00	8.00	8.00
L. angustifolia	4.00	2.00	4.00	4.00
M. piperita	4.00	2.00	2.00	4.00
P. menziesii	4.00	2.00	2.00	1.00
S. tonkinensis	2.00	4.00	> 16.0	> 16.0
Benzoic acid	1.00	2.00	> 16.0	> 16.0
Camphor	8.00	8.00	8.00	8.00
1,8-cineol	2.00	1.00	4.00	4.00
Limonene	> 16.0	> 16.0	> 16.0	> 16.0
Linalool	4.00	8.00	4.00	4.00
l-Menthol	1.00	1.00	1.00	2.00
l-Menthone	4.00	8.00	8.00	4.00
Erythromycin*	0.06	0.06	1.00	1.00
Oxacillin*	8.00	8.00	128.00	64.00

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

susceptible and two resistant strains of *S. pneumoniae*, which showed distinct differences in sensitivity to oxacillin and erythromycin.

Most of the tested oils, with the exception of *C. atlantica* and *C. bergamia* oils, significantly inhibited the antibiotic-susceptible strains of *S. pneumoniae* (KCCM 40410 and KCCM 4033). The oil fractions of *C. sativum*, *E. globules* and *J. communis*, and also their main components, showed relatively high MICs ranging from 2 mg/mL to 16 mg/mL. It is noteworthy that most of the oils exhibited lower activity against the resistant strains than against the susceptible strains. There were significant differences in sensitivity to *C. molmol* and *C. mandurensis* oil between antibiotic-susceptible and antibiotic-resistant strains (CCARM 4009 and CCARM 4010). Notably, the MICs of *C. molmol* against the resistant strains were more than four times higher than for the susceptible strains. Among the examined substances, menthol, the main component of *M. piperita*, was the most potent inhibitor with an MIC of 1 mg/mL against all tested strains. The relatively weak activity of the total oil fraction of *M. piperita* compared with its main component may reflect the presence of non-oxygenated hydrocarbons and/or other mildly active compounds within it (Table 2).

Table 2. Components (> 1%) of the essential oil fraction of *M. piperita*

Compound	RI		Peak Area (%)
	HP-5[a]	HP-IW[b]	
2-β-Pinene	923	940	1.47
1,8-Cineole	962	990	6.27
l-Menthone	1051	1089	20.57
Menthofurane	1056	1094	14.49
l-Menthol	1067	1115	28.81
α-Neioisomenthol	1070	1131	1.15
4-trimethyl-α,α-3-cyclohexene-1-methanol	1075	1152	1.19
Pulegone	1107	1196	5.03
Neomenthol	1110	1203	6.05
3,7,7-Trimethyl-bicyclo [4.1.0] heptane	1145	1297	5.43

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

Combination effects of *M. piperita* oil and menthol with antibiotics to inhibit the growth of antibiotic-resistant *S. pneumoniae* – As demonstrated in Table 3, the MICs of antibiotics combined with oil samples were markedly decreased, resulting in FICs of 0.03 to 0.25. Combination of *M. piperita* oil or menthol with antibiotics produced mainly additive or indifferent effects, resulting in FICIs ranging from 0.53 to 0.75. However, with *S .pneumoniae* CCARM 4010, one of the resistant strains, a high degree of synergism was observed when *M. piperita* oil was combined with erythromycin (FICI = 0.26).

Table 3. Fractional Inhibitory Concentrations (FICs) and FIC Indices (FICIs) of essential oils from *M. piperita* in combination with oxacillin or erythromycin against *S. pneumoniae* strains

Sample	Sp410		Sp33		Sp09		Sp10	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
M. piperita	0.50	0.52	0.50	0.53	0.50	0.56	0.50	0.53
Oxacillin	0.02		0.03		0.06		0.03	
Menthol	0.50	0.52	0.50	0.53	0.50	0.53	0.50	0.53
Oxacillin	0.02		0.03		0.03		0.03	
M. piperita	0.50	0.53	0.50	0.75	0.50	0.56	0.02	0.26
Erythromycin	0.03		0.25		0.06		0.25	
Menthol	0.50	0.56	0.50	0.53	0.50	0.56	0.25	0.38
Erythromycin	0.06		0.03		0.06		0.13	

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 lone), FICI = FIC index; (MIC a combined with b / MIC a alone) +(MIC b combined with a / MIC b alone)

Discussion

M. piperita (peppermint) plants are cultivated worldwide for various uses, including as a herbal medicine, tea and an ingredient for various foodstuffs. Along with other species of *Mentha*, it is also an important source of menthol. (Mimica-Dukic *et al.*, 2003). In the current study, both *M. piperita* oil and its major component, menthol, potently inhibited antibiotic-resistant bacterial strains in comparison to other tested oils (Schelz *et al.*, 2006). There are many varieties of *M. piperita* and various different methods of cultivation, which can affect the production and composition of essential oil and influence its menthol content (Aflatuni *et al.*, 2005). This might account for the relatively marked variation in the antibacterial activities of *M. piperita* oil, which were previously reported to depend on the plant source (Marcum and Hanson, 2006). Menthone (20.57%), the second most abundant component of *M. piperita* oil possesses much milder antibacterial activity. This might be another reason why the essential oil fraction of *M. piperita* displayed higher MICs than menthol itself. The mechanism underlying the antimicrobial activity of plant essential oils has not been clarified in detail. However, in experiments with three essential oil compounds, including menthol, Trombetta *et al.* (2005) reported that their efficacy might be related to alteration of membrane permeability and to leakage of intracellular materials, factors that depend largely on lipophilicity and water solubility. 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). Although the antimicrobial activity of menthol is relatively modest compared with thymol, carbacrol, or eugenol, all of which contain phenolic hydroxyl groups, the aliphatic alcohol group on menthol does increase its hydrophilicity. This property is especially important in the development of liquid drug preparations (Ben Arfa *et al.*, 2006) Moreover, given that the cultivation of *Mentha* species is possible in most regions of the world and that relatively large doses of it have been used by the human race as foodstuffs etc, its development as a drug source could be both valuable and feasible (Jay and Rivers, 1984).

Though many of the plant essential oils tested here possess relatively strong antibacterial activity, they generally had considerably higher MICs than commonly used antibiotics. For this reason, their therapeutic application may be limited to complementary treatments or for the alleviation of symptoms. However, since the antibacterial mechanisms of essential oils appear to be substantially different from currently used antibiotics, they could be considered a promising source of new drugs for the inhibition of pathogenic, antibiotic-resistant strains of bacteria (Shin and Pyun 2006; Filoche *et al.*, 2005). On this basis, we investigated their ability to synergize with traditional antibiotics in the inhibition of both antibiotic-sensitive and antibiotic-resistant *S. pneumoniae* strains. Checkerboard microtiter tests were constructed with *M. piperita* oil, and menthol, as these had exhibited the highest antibiotic activity among the tested samples in this study. Notably, the resistant S. pneumoniae CCARM 4010 was the only strain for which significant synergism was observed. This synergism was especially pronounced when erythromycin was combined with *Mentha piperita* oil or menthol, with FICIs ranging between 0.26 and 0.38. Although the other combinations only produced additive results, it is notable that the MICs of all three tested antibiotics were markedly decreased by combination with *Mentha* oil or with menthol. Moreover, this effect was observed with both the antibiotic-susceptible and the antibiotic-resistant strains, contrary to the strong resistance observed with traditional antibiotics alone.

In conclusion, we here evaluated the antibacterial activity of plant essential oils against antibiotic-susceptible and antibiotic-resistant strains of *S. pneumoniae*, which are one of the most common causes of invasive bacterial infections in humans and animals. We demonstrated that the antibacterial potency of oxacillin and erythromycin is significantly enhanced by combination with menthol and *Mentha piperita* oil. These results may present a new strategy in antimicrobial development-the therapeutic application of an essential oil for the treatment of antibiotic-resistant *S. pneumoniae* infection. However, additional *in vivo* experiments are required to assess the true therapeutic potential of essential oils and/or their components.

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