J. Korean Wood Sci. Technol. 42(5): 533~542, 2014 http://dx.doi.org/DOI : 10.5658/WOOD.2014.42.5.533

Antibacterial Activity of Essential Oil from *Abies holophylla* against Respiratory Tract Bacteria[1]

Su-Yeon Lee[2] · Seon-Hong kim[2] · Mi-Jin Park[3] · Sung-Suk Lee[3] · In-Gyu Choi[2,4,†]

ABSTRACT

We extracted essential oils from four species (*Pinus densiflora, Larix kaempferi, Pinus koraiensis, and Abies holophylla*) in the family *Pinaceae* to investigate their antibacterial activities against respiratory tract bacteria (*Klebsiella pneumoniae, Haemophillus influenzae, Streptococcus pyogenes, Streptococcus pneumonia, and Neisseria meningitides*). Among the tested oils, that from *A. holophylla* was showed strong activity based on disc diffusion and broth medium dilution (minimum inhibitory concentration, MIC) assays. Qualitative analysis of *A. holophylla* oil was carried out by GC-MS; α-pinene, camphene, β-pinene, 3-carene, limonene, bornyl acetate, borneol, β-caryophyllene, α-caryophyllene, caryophyllene oxide, and α-bisabolol were identified as its major constituents. Fractionation by silica gel chromatography was performed to analyze the active constituents of the crude oil. In particular, one fraction containing caryophyllene oxide as the major constituent showed stronger antibacterial activity than the crude oil of *A. holophylla*. Growth rates of bacterial strains exposed to fraction D were explored by optical density (OD600) measurements while morphology was examined by optical microscopy observations (× 1000). OD600 of *K. pneumoniae* decreased from 0.2582 to 0.005 in response to treatment with fraction D at a MIC value of 0.31 $\mu\ell$/mℓ.

Keywords : essential oil, *Abies holophylla*, respiratory tract bacteria, *Klebsiella pneumoniae*

1. INTRODUCTION

Essential oils are biosynthesized by plants as secondary metabolites for protection against viruses, bacteria, fungi, insects, and herbivores (Reichling 1999). The main constituents of essential oils are mixtures of terpene compounds such as monoterpenes, sesquiterpenes, and phenylpropanoids including carbohydrates, alcohols, ethers, aldehydes, and ketones (Hüsnü *et al*. 2007).

Essential oils have been shown to have antimicrobial activities against pathogenic bacteria and fungi. Respiratory tract infections are some of the most common bacterial diseases encountered in med-

[1] Date Received March 18, 2014, Date Accepted June 6, 2014

[2] Department of Forest Sciences, College of Agriculture and Life Sciences, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul, 151-921, South Korea

3 Division of Wood Chemistry & Microbiology, Department of Forest Resources Utilization, Korea Forest Research Institute, Seoul, 130-712, Republic of Korea

4 Research Institute for Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul, 151-921, South Korea

[†] Corresponding author : In-Gyu Choi (e-mail: cingyu@snu.ac.kr)

ical practice today (Mufson *et al*. 1967). Specific strains such as *Pseudomonas* spp., *Streptococcus* spp., *Klebsiella* spp., *Staphylococcus* spp., and *Haemophilus influenzae* (Fritsche *et al*. 2005) are common bacteria known to cause respiratory diseases. Studies carried out in Finland and the USA have shown that Streptococcus spp. are the most common bacterial pathogens in children aged less than 5 years, accounting for about 30% of all cases of pneumonia (Mccracken Jr 2000).

However, many antibiotic-resistant strains of these bacteria have been documented. The emergence of antibiotic resistance in respiratory tract infections is a serious public health concern in many countries.

Inhibitory effects of some essential oils against respiratory track bacteria have been investigated (Inouye *et al*. 2001; Fabio *et al*. 2007). In South Korea, conifer species in the plant families *Pinaceae* (e.g., *Pinus koraiensis, Pius densiflora, Larix kaempferi, Abies holophylla*) and *Cupressaceae* (e.g., *Chamaecyparis obtusa, Platycladus orientalis, Chamaecyparis pisifera*) are widely distributed (Shannon, 1956). However, antibacterial activities of essential oils of these species (Yang *et al.* 2007; Lee *et al.* 2008) were little known as literatures. We therefore investigated the antibacterial activities of essential oils from *P. densiflora, L. kaempferi, P. koraiensis* and *A. holophylla* against *K. pneumoniae, H. influenzae, S. pyogenes, S. pneumonia*, and *N. meningitides*, which are the bacterial species most often associated with respiratory tract infections (Bauernfeind *et al*. 1999; Corless *et al*. 2001).

2. MATERIALS and METHODS

2.1. Extraction and Fractionation of Essential Oils

Leaves of *P. koraiensis, P. densiflora, L. kaempferi, A. holophylla* were collected from the Korea National Arboretum in Gyeonggi-do, South Korea. Oil was immediately extracted from 500 g fresh leaves with 2 L distilled water by steam distillation using a Clevenger-type apparatus at 100°C.

Oil was initially fractionated into fractions using silica gel (70-230 mesh) chromatography columns with hexane and ethyl acetate mixes as the eluent (50:1, 32:1, 16:1, 8:1, 4:1, 2:1) at a flow rate of 30 mℓ/min. One hundred twenty-five fractionated solutions each containing 30 mℓ of solution were profiled by TLC (silica gel 60 pre-coated plates, Merck, Germany). Crude oil of A. holophylla was divided into seven fractions based on the retention fraction (RF): fraction A (RF value: 1~0.8), fraction B (RF value: 0.6), fraction C (RF value: 0.45), fraction D (RF value: 0.3) fraction E (RF value: 0.2), and fraction G (RF value: 0.1~0).

2.2. Standard Compounds

External standards of α-pienene, β-pinene, camphene, β-caryophyllene, α-caryophyllene, caryophyllene oxide, and α-bisabolol were purchased from Sigma–Aldrich Korea for quantitative and qualitative analysis.

2.3. Test Microorganisms

Five bacterial strains, namely *K. pneumoniae* CCARM 0015, *N. meningitidis* CCARM 0073, *H. influenzae* CCARM 9001, *S. pyogenes* CCARM 0032, and *S. pneumoniae* CCRAM 4001 were used in this study. These strains were provided by the Culture Collection of Antimicrobial Resistant Microbes (CCARM), which is a Korea National Research resource bank. Strains were maintained on 5% blood (horse and sheep) agar and chocolate agar at 37~38°C.

Table 1. History of isolation of respiratory tract bacterial pathogens

Gram	Strain	Isolation	Isolation date
Negative	*K. pneumoniae*	Korea Research Institute of Chemical Technology	2000.01
	N. meningitides	Korean Culture Center of Microorganisms	2002.01
	H. influenzae	Gangnam Severance Hospital	2004.12
Positive	*S. pyogenes*	Korean Culture Center of Microorganisms	2000.01
	S. pneumoniae	Asan Medical Center	2001.01

2.4. Antibacterial Activity

2.4.1. Paper Disc Diffusion Method

Modified disc diffusion and broth dilution methods were used to determine the susceptibility of bacteria to the essential oils. Cell suspensions of bacteria were collected by scraping them from pre-inoculated Mueller-Hinton agar (MHA) medium, and suspensions were standardized to a final concentration of McFarland 0.5 (1.5×10^8 CFU/mℓ). Cell suspensions were then applied to MHA medium (20 mℓ). Each oil sample (10 μℓ) was applied to a sterile filter paper disc (8 mm diameter) that was then placed on the surface of the inoculated MHA medium followed by a 24-h incubation at 37°C after which the zone of inhibition for each oil sample was measured. Each test was performed in triplicate.

2.4.2. Broth Dilution Method

Minimum inhibitory concentration (MIC) of the oils was determined by the broth dilution method. MHB supplemented with 3~5% lysed horse blood and sheep blood for *H. influenza*, *S. pneumonia*, and *N. meningitis* was used. Macro-dilution tray (24 well) containing 100 μℓ of HTM agar was used. Test suspensions were prepared in 0.9% saline and adjusted to a turbidity equivalent to a 0.5 McFarland standard. Each well of the plate was inoculated with a final cell density of 1.5×10^5 CFU/mℓ bacteria and incubated for 20 to 24 h in ambient air at 35°C. Serial two-fold dilutions of each oil component were prepared in a 24-well tray over the concentration range of 5~0.078 μℓ/mℓ. Trays were incubated at 37°C for 24 h. Wells were then examined for evidence of growth and the MIC value was determined as the lowest concentration that inhibited growth by visible observation. All samples were examined in duplicate in three separate experiments.

2.4.3. Optical Density (OD) and Microscopy Observations

Growth of strains exposed to *A. holophylla* oil was monitored using a UV-vis spectrophotometer by measuring the optical density at 600 nm. After OD600 measurement, bacteria in culture media were observed under a photomicroscope (Universal Research) equipped with Infinity color-corrected system lenses. Images were captured using a TV system camera.

2.5. Chemical Analysis

We used gas chromatography - mass spectrometry (GC-MS) to analyze the chemical nature of the essential oils. We used an Agilent model 6890A gas chromatograph equipped with a FID and mass detector. The stationary phase was a DB-5 column (dimensions 30 m × 0.25 mm, coating thickness of 0.25 μm) and the carrier gas was He flowed at a rate of 1 mℓ/min. Working conditions were as follows: injector 300°C, detector 250°C. Oven temper-

Table 2. Clear inhibition zones of bacteria by extracted essential oils using disc diffusion method

	S. pyogenes	*S. pneumoiae*	*H. influenzae*	*K. pneumoniae*	*N. meningitis*
P. densiflora[a]	-	-	-	-	-
L. kaempferi[a]	-	-	-	-	-
P. koraiensis[a]	-		-	-	-
A. holophylla[a]	12.59	11.50	11.60	15.72	12.08
Ampicillin[b]	25.47	38.46	28.96	20.04	25.04

[a] : diameter of zone of inhibition (mm) including disc diameter 8 mm by essential oil (10 ㎕)
[b] : diameter of zone of inhibition (mm) including disc diameter 8 mm by ampicillin (30 ㎍)
- : Complete lack of activity

Table 3. Clear inhibition zones of bacteria by crude oil and fractions of *A. holophylla*

	S. pyogenes	*S. pneumoiae*	*H. influenzae*	*K. pneumoniae*	*N. meningitis*
Crude oil[a]	9.50	-	-	10.57	10.75
Fraction A[a]	-	-	-	-	-
Fraction B[a]	-	-	-	-	-
Fraction C[a]	-	-	-	-	-
Fraction D[a]	12.04	12.30	12.56	11.59	12.52
Fraction E[a]	-	-	-	-	-
Fraction F[a]	-	-	-	-	-
NC[b]	-	-	-	-	-

[a] : Diameter of zone of inhibition (mm) including disc diameter 8 mm by essential oil (5 ㎕)
[b] : Negative control, each paper absorbed ethanol 10 ㎕

ature was increased from 40 to 280 at 5°C /min, with an initial holding time and a final holding time of 10 min. A split ratio of 5:1 and mass range from 50 to 800 m/z were used. Peak identification was based upon mass spectra comparison with the National Institute of Standards and Technology (NIST) 08 library and spectra of injected standards. Identification of the retention indices of compounds was based on comparison of relative retention times with an *n*-alkane (C8-C30) mixture on the DB-5 column, and by matching of mass spectra peaks with those in the NIST 08 library.

3. RESULTS and DISCUSSION

3.1. Antibacterial Activities of Essential Oils

Essential oils were extracted from the needles and twigs of four species by steam distillation for 6 h at 100°C and yields of between 0.25 and 2.15% (w/w) were obtained (Fig. 1). Yield from fresh leaves of *A. holophylla* (2.15%) was the greatest.

Antibacterial activities were first assayed using the disc diffusion method. *A. holophylla* oil (10 ㎕) exhibited noteworthy activity with a broad spectrum of activity against the five strains of bacteria with inhibition ranging from 11.50 to 15.72 mm (Table 2).

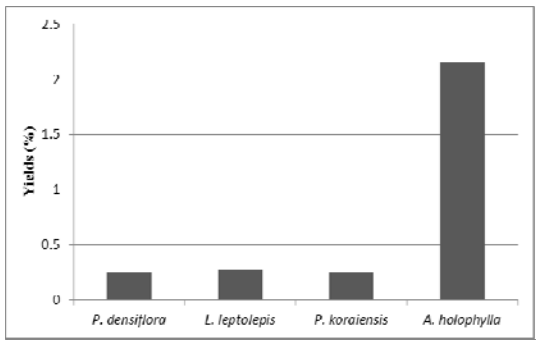

Fig. 1. Yield (%) of essential oils from four species by steam distillated extraction.

K. pneumoniae, *S. pyogenes*, and *N. meningitis* were more susceptible to *A. holophylla* oil than the other two bacterial strains.

A. holophylla (Manchurian fir, also called needle fir) is a fir species native to mountainous regions of northern Korea, China, and Russia. Antibacterial activities of *A. holophylla* oil against bacteria have been researched in a previous study. This oil is more effective at inhibiting the growth of Gram-negative bacteria, such as *E. coli* and *K. pneumonia* strains, than Gram-positive bacteria (Lee and Hong 2009). However, the effects of individual active constituents of *A. holophylla* oil have not been investigated. We performed open column chromatography using silica gel to separate out the complex constituents of *A. holophylla* oil. Solutions separated by open column chromatography and TLC assay were divided into seven fractions (A-G). Solvent was removed from these seven fractions by vacuum evaporation, and the antibacterial activities of these solvent-free fractions were examined by the disc diffusion method and compared with that of crude *A. holophylla* oil.

Crude oil and each fraction (5 $\mu\ell$) were placed on 8-mm paper discs. After 24 h, fraction D showed a stronger inhibitory effect than the other fractions and even the crude oil. These results indicated that important antibacterial constituents were present in fraction D.

3.2. Chemical Analysis

Constituents of *A. holophylla* oil and its fractions are presented in Table 5. Eleven constituents were identified as major constituents of the crude oil, accounting for 95.80% of the total crude oil content. Crude oil contained mostly monoterpenes (84.47%), followed by sesquiterpenes (11.43%). Major constituents of *A. holophylla* oil were the monoterpene hydrocarbons of α-pinene (10.74%), camphene (10.96%), β-pinene (5.31%), 3-carene (14.02%), and limonene (16.89%); oxygenated monoterpenes of borneol (4.89%), bornyl acetate (19.63%); sesquiterpene hydrocarbons of β-caryophyllene (1.52%), α-caryophyllene (0.83%); oxygenated sesquiterpenes of caryophyllene oxide (2.77%), and the sesquiterpene alcohol of α-bisabolol (5.42%). Limonene and bornyl acetate were the most abundant constituents in the crude oil.

Essential oils from plants in the genus *Pinus* are usually composed of monoterpene hydrocarbons such as α-pinene, β-pinene, limonene, β-caryophyllene, germacrene D, and 3-carene, and oxygenated monoterpenes such as borneol and bornyl acetate (Krauze-Baranowska *et al.* 2002).

Crude oil of *A. holophylla* was separated into seven fractions. Fraction A included α-pinene (94.37%). Fraction B was characterized by the presence of monoterpene hydrocarbons, namely β-pinene (29.45%) and limonene (70.55%). Fraction C contained α-pinene (9.16%), camphene (13.83%), 3-carene (19.51%), and limonene (55.17%). Fraction D contained caryophyllene-type sesquiterpenes such as β, α−caryophyllene and caryophyllene oxide. Borneol, bornyl acetate, and α-bisabolol were separated into fractions E, F, and G. the major constituents of *A. holophylla* crude oil were therefore hydrocarbon and oxygenated terpenes.

Table 4. MIC values of bacteria by crude oil and fractions

	S. pyogenes	*S. pneumoiae*	*H. influenzae*	*K. pneumoniae*	*N. meningitis*
Oil[a]	0.31	0.62 <	0.62 <	0.31	0.62
Fraction D[a]	0.15	0.62	0.62	0.31	0.62
Ampicillin[b]					

[a] : MIC Values given as $\mu\ell$/mℓ

Table 5. Chemical composition of the essential oil and fractions from *A. holophylla* by GC-MS analysis

RI[a]	Constituents	Crude oil	A	B	C	D	E	F	G
934	α-Pinene	10.74	94.37		9.16				
945	β-Pinene	5.31		29.45					
950	Camphene	10.96			13.83				
1009	3-Carene	14.02			19.51				
1138	Limonene	16.89		70.55	55.17				
1174	Borneol	4.89					81.17		
1285	Bornyl acetate	19.63						93.64	
1419	β-Caryophyllene	1.52				7.51			
1478	α-Caryophyllene	0.83				4.87			
1582	Caryophyllene oxide	2.77				53.30			
1684	α-Bisabolol	5.42							100

[a] : Retention index relative to n-alkanes (C_{10}~C_{25}) on DB-5 capillary column, RI identified based on comparison of retention index of the compounds compared with NIST 08

[b] : Relative portion (%)

3.3. MIC of The Crude Oil and Its Fractions

MIC values of *A. holophylla* oil ranged from 0.31~0.62 $\mu\ell$/mℓ against the five bacterial strains. The oil had the strongest inhibitory effect (MIC 0.31 $\mu\ell$/mℓ) against *K. pneumoniae* and *S. pyogenes*, as reported for the disc diffusion method (Table 4).

K. pneumoniae and *S. pyogenes* are important pathogens that produce beta-lactamases to protect against penicillins, cephalosporins, or monobactams (Appelbaum 1992; Livermore 1995). Hospitalized or elderly patients with *K. pneumoniae* infections are at great risk of dying, with reported mortality rates of between 25% and 60% (Lawlor *et al.* 2005). S. pyogenes is also a known cause of morbidity and mortality in humans and is responsible for both respiratory tract infections and invasive diseases (Musher 1992; Reinert *et al.* 2004).

A. holophylla essential oil fractions also showed considerable antibacterial activity. In particular, fraction D had MIC values ranging from 0.15~0.31 $\mu\ell$/mℓ against *K. pneumoniae* and *S. pyogenes*. These results indicated that fractionated constituents had higher antibacterial properties than the crude oil. This is consistent with a previous study that reported that active compounds isolated by fractionation had higher activity than the original extract (Nguefack *et al.* 2007). As can be seen from Table 5, fraction D consisted of caryophyllene oxide as a major com-

Table 6. Quantitative analysis (mg/mℓ) of fraction D by external standards

	1 × MIC	1/2 × MIC	1/4 × MIC	1/8 × MIC	1/16 × MIC	1/32 × MIC
β-caryophyllene	0.1915	0.0286	0.0122	0.0036	0.0016	-
α-caryophyllene	0.1196	0.0233	0.0078	0.0021	0.0008	-
Caryophyllene oxide	0.5629	0.1347	0.0059	0.0175	0.0023	0.0010
Total (R^2: 0.9219)	0.8740	0.1866	0.0429	0.0232	0.0047	0.0010

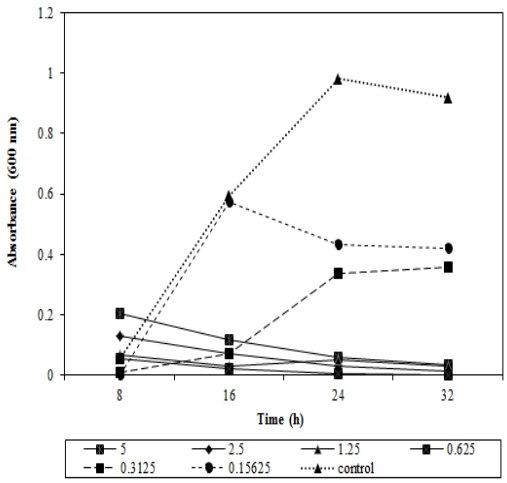

Fig. 2. Optical density of *K. pneumoniae* by two-serial diluted solutions of fraction D.

pound as well as β, α-caryophyllene. Caryophyllene oxide, an oxygenated terpenoid, is a well-known preservative in food, drugs, and cosmetics. Caryophyllene oxides are natural bicyclic sesquiterpenes present in some essential oils. However, there are only a few reports of the antimicrobial activities of caryophyllene-type sesquiterpenes against respiratory track bacteria in the literature. We suggest that the stronger activity of fraction D than the crude oil may be due to the presence of a higher concentration of caryophyllene oxide in fraction D than the crude oil. The data shown in Table 6 support this hypothesis. Qualitative analysis of two-fold serially diluted solutions of the fractions revealed some interesting findings. In the MIC values 0.31 $\mu\ell$/mℓ, caryophyllene oxide was only identified only because of their relative portion. Calibration curve of fraction D by quantitative analysis using external standard was above 0.9219. The broth dilution method was originally designed to test the antimicrobial activity of conventional antimicrobial agents such as antibiotics. Therefore, methods should be modified when testing the antimicrobial activity of essential oils.

3.4. Optical Density and Microscopy Findings

As described above, fraction D showed antibacterial activity against *K. pneumoniae* and *S. pyogenes*. We further explored the antibacterial activity of this fraction by optical density measurements. Growth of bacterial strains in serial two-fold dilutions of fraction D was monitored at 600 nm every 8 h. The OD_{600} of *K. pneumonia* decreased from 0.2582 to 0.005 when exposed to 0.31 $\mu\ell$/mℓ fraction D (Fig. 2). These results indicated that the growth of *K. pneumoniae* was inhibited by 90% by treatment with the MIC of fraction D. In contrast, fraction D only exhibited bacteriostatic activity against *S. pyogenes*. The OD_{600} of *S. pyogenes* increased from 0.033 to 0.123 at a fraction D concentration of 0.31 $\mu\ell$/mℓ (Fig. 3).

After measurement of OD_{600}, diluted solutions exposed to fraction D were observed under an optical microscope (Fig. 4). Untreated *K. pneumoniae* were smooth and regular in appearance with an intact cell

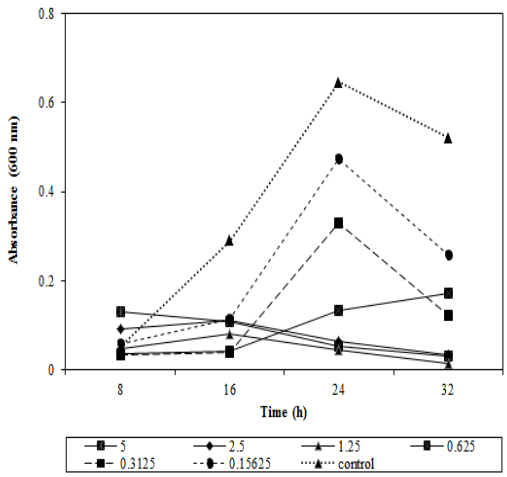

Fig. 3. Optical density of *S. pyogenes* by two-serial diluted solutions of fraction D.

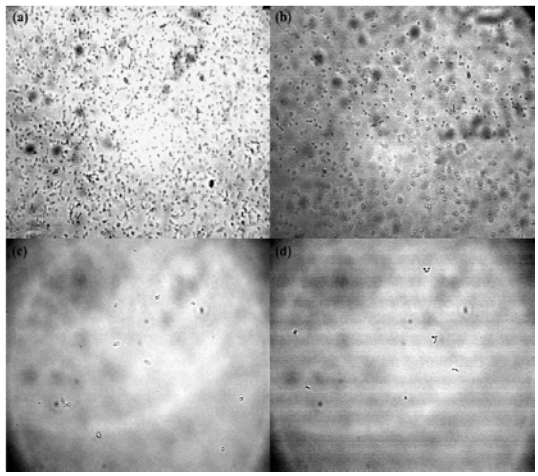

Fig. 4. Morphological observation of *K. pneumoniae* and *S. pyogenes* exposed to fraction D. (a) and (b): untreated cell of *K. pneumoniae* and *S. pyogenes*, (c) and (d): cells of *K. pneumoniae* and *S. pyogenes* exposed to fraction D (0.31 ㎕/mℓ) by microscopy (× 1000), after 16 h.

shape. However, cells exposed to fraction D appeared unfilled and cell density was decreased relative to that of the control. We therefore concluded that caryophyllene oxide, β−caryophyllene, and α−caryophyllene components of fraction D had a bactericidal rather than a bacteriostatic effect against *K. pneumoniae*. However, fraction D exhibited bacteriostatic activity against *S. pyogenes*. It has been reported that *S. pyogenes* cells are spherical, diplococcic, and lancet-shaped. However, we were not able to identify normally-shaped *S. pyogenes*. In conclusion, *A. holophylla* oil has the potential to inhibit bacterial cell growth. Although the mechanism of action of the oil constituents and their toxicities need to be evaluated, our results suggest that *A. holophylla* oil can potentially be used to inhibit the growth of respiratory bacteria.

4. CONCLUSION

We investigated the antibacterial activity of essential oils derived from four species against respiratory track bacteria that are known antibiotic-resistant pathogens. Among the tested oils, *A. holophylla* oil showed the strongest antibacterial effects against *K. pneumoniae* and *S. pyogenes* by in vitro assay. Bactericidal and bacteriostatic effects of *A. holophylla* oil were confirmed by optical density measurements and microscopy observations.

Our results suggest that a complex mixture of caryophyllene-type sesquiterpenes from *A. holophylla* oil can confer high resistance against bacteria. Therefore, essential oils of *A. holophylla* that contain caryophyllene oxides have the potential to treat respiratory track bacterial infections. The toxicity of the *A. holophylla* oil should be tested, and its antibacterial effects investigated in more detail before practical application.

ACKNOWLEDGEMENT

This study was totally funded by a grant from ‘Development of Integrated Medical Application

Technologies for establishing of basement the healing effects of forests (Project No. FM0400-2010-01) provided by Korea Forest Research Institute.

REFERENCES

Appelbaum, P.C. 1992. Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. Clinical Infectious Diseases, 15(1): 77～83.

Bauernfeind, A., Schneider, I., Jungwirth, R., Sahly H., Ullmann, U. 1999. A novel type of AmpC β-lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. Antimicrobial Agents and Chemotherapy 43(8): 1924～1931.

Corless, C.E., Guiver M., Borrow, R., Edwards-Jones, V., Fox, A.J., Kaczmarski, E.B. 2001. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of *meningitis* and *septicemia* using real-time PCR. Journal of Clinical Microbiology 39(4): 1553～1558.

Fabio, A., Cermelli, C., Fabio, G., Nicoletti, P., Quaglio, P. 2007. Screening of the antibacterial effects of a variety of essential oils on microorganisms responsible for respiratory infections. Phytotherapy Research 21(4): 374～377.

Fritsche, T.R., Sader, H.S., Stiwell, M.G., Dowzicky, M.J., Jones, R.N. 2005. Antimicrobial activity of tigecycline tested against organisms causing community-acquired respiratory tract infection and *nosocomial pneumonia*. Diagnostic Microbiology and Infectious Disease 52(3): 187～193.

Hüsnü, K., Demirci, F. 2007. Chemistry of essential oils. Flavours and Fragrances. Springer. Berlin Heidelberg. Germany.

Inouye, S., Yamaguchi, H., Takizawa, T. 2001. Screening of the antibacterial effects of a variety of essential oils on respiratory tract pathogens, using a modified dilution assay method. Journal of Infection and Chemotherapy 7(4): 251～254.

Krauze-Baranowska, M., Mardarowicz, M., Wiwart, M., Poblocka, L., Dynowska, M. 2002. Antifungal activity of the essential oils from some species of the genus *Pinus*. Zeitschrift Fur Naturforschung C57(5/6): 478～482.

Lawlor, M.S., James, H., Rick, P.D., Miller V.L. 2005. Identification of *Klebsiella pneumoniae* virulence determinants using an intranasal infection model. Molecular Microbiology 58(4): 1054～1073.

Lee, J.H., Hong, S.K. 2009. Comparative Analysis of Chemical Compositions and Antimicrobial Activities of Essential Oils from *Abies holophylla* and *Abies koreana*. Journal of Microbiology and Biotechnology, 19(4): 372～377.

Lee, J.H., Lee, H.S., Hong, S.K. 2008. Chemical composition and antimicrobial activity of essential oil from cones of Pinus koraiensis. Journal of Microbiology and Biotechnology 18(3): 487～821.

Livermore, D.M. 1995. beta-Lactamases in laboratory and clinical resistance. Clinical Microbiology Reviews 8(4): 557～584.

Mccracken Jr, G.H. 2000. Diagnosis and management of pneumonia in children. The Pediatric Infectious Disease Journal 19(9): 924～928.

Mufson, M.A., Chang, V., Gill, V., Wood, S.C., Romansky, M.J., Chanock, R.M. 1967. The role of viruses, mycoplasmas and bacteria in acute pneumonia in civilian adults. American Journal of Epidemiology 86(3): 526～544.

Musher, D.M. 1992. Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity, and treatment. Clinical Infectious Diseases 14(4): 801～807.

Nguefack, J., Nguikwie, S.K., Fotio, D., Dongmo, B., Amvam Zollo, P.H., Leth, V., Nkengfack,

A.E., Poll, L. 2007. Fungicidal potential of essential oils and fractions from Cymbopogon citratus, Ocimum gratissimum and Thymus vulgaris to control Alternaria padwickii and *Bipolaris oryzae*, two seed-borne fungi of rice (*Oryza Sativa L.*). Journal of Essential Oil Research 19(6): 581～587.

Reichling, J. 1999. Plant-microbe interactions and secondary metabolites with antiviral, antibacterial and antifungal properties. In Annual Plant Reviews Volume 39: Functions and Biotechnology of Plant Secondary Metabolites. Wiley-Blackwell, Oxford, UK.

Reinert, R.R., Franken, C., Liden, M., Luttichen, R., Cill, M., Lahham, A.A. 2004. Molecular characterisation of macrolide resistance mechanisms of *Streptococcus pneumoniae* and *Streptococcus pyogenes* isolated in Germany, 2002–2003. International Journal of Antimicrobial Agents 24(1): 43～47.

Shannon, F.A. 1956. The reptiles and amphibians of Korea. Herpetologica 12(1): 22～49.

Yang, J.K., Choi, M.S., Seo, W.T., Rinker, D.L., Hang, S.W., Cheong, G.W. 2007. Chemical composition and antimicrobial activity of Chamaecyparis obtuse leaf essential oil. Fitoterapia 78(2): 149～152.