Antibacterial and Antifungal Effects of Essential Oils from Coniferous Trees

Eui-Ju HONG,[a] Ki-Jeung NA,[b] In-Gyu CHOI,[c] Kyung-Chul CHOI,[d] and Eui-Bae JEUNG*,[a]

[a] *Laboratory of Veterinary Biochemistry and Molecular Biology, College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University; and* [b] *Laboratory of Veterinary Clinical Pathology, College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University; Cheongju, Chungbuk 361–763 Republic of Korea:* [c] *Department of Forest Products, College of Agriculture and Life Sciences, Seoul National University; Seoul 151–742 Republic of Korea: and* [d] *Department of Obstetrics and Gynecology, British Columbia Children's and Women's Hospital, British Columbia Research Institute for Children's and Women's Health, University of British Columbia; Vancouver, BC V6H 3V5, Canada.* Received January 26, 2004; accepted March 2, 2004

Essential oils have potential biological effects, *i.e.*, antibiotic, anticarcinogenic, and sedative effects during stress. In the present study, we investigated the antibacterial and antifungal effects of essential oils extracted from the coniferous species *Pinus densiflora*, *Pinus koraiensis*, and *Chamaecyparis obtusa*, because their biological activities have not been yet elucidated. The essential oils were quantified using gas chromatography and identified in gas chromatography-mass spectrometric analysis. Simultaneously, antibacterial and antifungal assays were performed using the essential oils distilled from the needles of coniferous trees. The major components and the percentage of each essential oil were: 19.33% β-thujene in *P. densiflora*; 10.49% α-pinene in *P. koraiensis*; 10.88% bornyl acetate in *C. obtusa*. The essential oils from *P. densiflora* and *C. obtusa* have antibacterial effects, whereas essential oils from *P. koraiensis* and *C. obtusa* have antifungal effects. These results indicate that the essential oils from the three coniferous trees, which have mild antimicrobial properties, can inhibit the growth of gram-positive and gram-negative bacteria and fungi.

Key words essential oil; antimicrobial effect; coniferous tree

Essential oils are generally not only antibiotic and anticarcinogenic,[1,2] but also have a sedative effect on stress. It has been shown that these essential oils contain ketone, terpene, and phenolic ether for sedation.[3] Although essential oils have been regarded as useful sedatives,[4] there is little information on the antimicrobial or antifungal activities of essential oils extracted from coniferous trees. Essential oils with antimicrobial properties from medicinal as well as other edible plants have been recognized since antiquity.[2] In addition, essential oils are used as food flavoring agents, and have a broad spectrum of *in vitro* antimicrobial activities attributed to the high content of phenolic derivatives.[5] More recently, plant extracts have been developed and proposed for use in foods as natural antioxidants.[6]

In the present study, we investigated the antimicrobial and antifungal activities of several essential oils extracted from the coniferous trees *Pinus densiflora*, *Pinus koraiensis*, and *Chamaecyparis obtusa* against bacteria and fungi that commonly cause foot rot and other diseases. The essential oils were quantified using gas chromatography (GC) and identified in gas chromatography-mass spectrometric (GC-MS) analysis. In addition, the antibacterial effects against gram-positive and gram-negative bacteria and antifungal effects against fungi were assayed using essential oils distilled from the needles of coniferous trees.

MATERIALS AND METHODS

Essential Oil Extraction The needles of the Japanese red pine (*P. densiflora*), Korean pine (*P. koraiensis*), and Japanese cypress (*C. obtusa*) were collected at the Reforestation Experiment Site of Chungbu Forest Experiment Station, Gyeonggi province, Korea. The essential oil from freshly cut needles of each species was obtained by steam distillation using a manufactured apparatus with a condenser. Distillation continued for 2—3 h at 100 °C, and the volatile compounds containing the water-soluble fraction were allowed to settle for 20 min. The essential oil layer was separated and finally purified through a microfiltering and dehydration process.

Measurement of Refractive Index The refractive index of chemical compounds is considered important because it indicates characteristic physical properties. We determined the index of the oils using an Abbe refractometer equipped with a sodium lamp (Bausch & Lomb, GD8804, U.S.A.).

Quantification and Identification Each essential oil compound was quantified using a gas chromatograph (GC, Shimadzu GC-14A, Japan) equipped with a Shimadzu CPB-20 capillary column (0.2 mm inner diameter×50 m length). First, the calibration curves for several standard essential oils were obtained, and the calibration equation of each compound was used for quantification. GC analysis was carried out using helium carrier gas with an FID detector, and the injection and detection temperatures were 150 and 200 °C, respectively. The oven temperature was increased from 50 to 200 °C at intervals of 2 °C/min over 75 min. Some other essential oils were identified using a gas chromatograph (Hewlet Packard, HP6890, U.S.A.)-mass spectrometer (JEOL, JMS-600W, Japan). The GC column was a 60-m (length)×0.25-mm (inner diameter) DB-WAX (0.25-μm film) fused silica capillary column (J&W Scientific, U.S.A.), and the spectra were obtained in the EI mode with 70-eV ionization energy. The compounds were identified by comparison with retention times and the mass spectra obtained from authentic standards on the GC-MS system used for analysis.

Comparative Antimicrobial and Antifungal Screening Antibacterial and antifungal assays were carried out of the

* To whom correspondence should be addressed. e-mail: ebjeung@chungbuk.ac.kr

essential oils from *P. densiflora*, *P. koraiensis*, and *C. obtusa* against *Salmonela typhimurium* (ATCC 14028), *Listeria monocytogen* (KCTC 3569), *Escherichia coli* (ATCC 27662), *E. coli* O157:H7 (KCTC 1039), *Staphylococcus aureus* (ATCC 25923) and *Klebsiella pneumoniae* (KCTC 2242), and against the fungus *Candida albicans*. The antibacterial and antifungal activities were determined using the agar diffusion method. Filter paper discs 10 mm in diameter (Advantec, Japan) were impregnated with 50 μl of essential oils at various dilutions. The bacterial strains were placed in tubes of trypticase soy agar (Difco, U.S.A.) and the fungus in tubes of Sabouraud dextrose agar (Difco). After 24-h incubation at 37 °C (bacteria) and 30 °C (fungus), four or five colonies were inoculated in 4 ml of Muller–Hinton broth (MHB) or Sabouraud dextrose broth and incubated for 2 h at 37 °C and 35 °C, respectively. These inocula were adjusted to the 0.5 MacFarland Standard (0.048 M $BaCl_2$ 0.5 ml + 0.18 M H_2SO_4 99.5 ml).

All assays were carried out in triplicate, and the control employed consisted of discs of chloramphenicol (30 μg) in the antibacterial and nystatin (100 units) in the antifungal assays. The Petri dishes were refrigerated (4 °C) for 30 min to facilitate diffusion of the essential oil in the medium before incubation. They were subsequently incubated at 37 °C for 24 h in the case of the bacteria, while the fungus was cultured at 30 °C for 24 and 48 h. After incubation, the growth inhibition rings were quantified by measuring the diameter for the zone of inhibition in millimeters (including the diameter of the disc) from the lower surface of the Petri dishes.

RESULTS

Qualitative Analysis of the Essential Oils The refractive index (nD^{20}) of essential oils extracted from three conifer needles is shown in Table 1, which ranged from 1.476 to 1.480 as determined by the D-line of a sodium lamp at 20 °C. No significant variation in the refractive index was observed among essential oils from the three coniferous trees. The chemical components of essential oils determined by GC-MS analysis are shown in Table 2. The major components and their percentage of each essential oil were: 19.33% β-thujene in *P. densiflora*; 10.49% α-pinene in *P. koraiensis*; and 10.88% bornyl acetate in *C. obtusa*.

Comparative Antimicrobial Screening The effects of three essential oils on antibacterial and antifungal activities are shown in Table 3. Treatment with essential oils inhibited the growth of gram-positive and gram-negative bacteria and the fungus in the antimicrobial and antifungal activity assays. Antimicrobial activity of essential oil from *P. densiflora* was observed in *L. monocytogenes* (12 mm), *S. aureus* (14 mm), and *K. pneumoniae* (12 mm) when treated with one disk containing the essential oil–Tween 20 (9 : 1; 5 μl/disk). Furthermore, 1/2 of a disk (2.5 μl/disk) inhibited the growth of *L. monocytogenes* (11 mm) and *S. aureus* (12 mm). However, the essential oil from *P. koraiensis* had only antifungal activity (one disk, 12 mm; 1/2 disk and 1/4 disk, 11 mm) against *C. albicans* (Tables 3A, B). In addition, treatment with essential oil from *C. obtusa* resulted in the growth inhibition of *L. monocytogenes* (one disk and 1/2 disk, 12 mm), *S. aureus* (one disk, 16 mm; 1/2 disk, 14 mm), *Legionella anisa* (one disk, 13 mm; 1/2 disk, 12 mm), and *C. albicans* (one disk, 12 mm; 1/2 disk, 11 mm) as demonstrated in Table 3C. Treatment with oils from *P. densiflora*, *P. koraiensis*, and *C. obtusa* exhibited an inhibitory range of 11—16 mm with some microorganisms tested. However, other concentrations of essential oils did not inhibit the growth of microorganisms. Interestingly, these results indicate that the essential oil from *P. densiflora* has antibacterial effects, and the essential oil from *P. koraiensis* has antifungal effects, whereas the essential oil from *C. obtusa* has both antibacterial and antifungal effects.

Table 1. Refractive Index of Essential Oils Extracted from Three Conifer Needles

Species	Index of refraction (nD^{20})
Pinus densiflora	1.4780
Pinus koraiensis	1.4768
Chamaecyparis obtusa	1.4746

Table 2. Chemical Components (%) of Tree Conifer Essential Oils by GC/MS

Component	*Pinus densiflora*	*Pinus koraiensis*	*Chamaecyparis obtusa*
Tricyclene	1.22	1.18	0.09
α-Pinene	14.44	10.49	5.69
Camphene	3.86	5.23	0.60
β-Pinene	9.82	2.81	0.42
Sabinene	0.35	0.18	13.62
Myrcene	12.19	7.22	5.98
Phellandrene	0.45	0.25	0.12
α-Terpinene	0.33	0.59	1.56
Limonene	4.34	6.55	6.99
β-Thujene	19.33	1.07	0.23
γ-Terpinene	0.33	0.30	4.11
p-Cymene	0.49	0.42	1.65
α-Terpinolene	2.87	5.64	1.66
α-Cubebene	0.16	0.33	—
α-Copaene	0.40	0.50	—
Camphore	0.28	0.32	—
Linalool	—	—	0.11
Linalyl acetate	—	—	0.13
Bornyl acetate	5.67	7.13	10.88
Thymol methyl ether	1.23	0.33	—
Caryophyllene	3.26	6.51	0.94
Terpinene-4-ol	0.30	0.22	5.05
Widdrene	—	—	2.06
α-Humulene	0.59	—	—
β-Selinene	—	1.60	—
γ-Muurolene	0.64	2.62	2.32
α-Terpineol	0.50	0.61	—
Borneol	0.35	0.52	—
α-Terpinyl acetate	—	—	13.77
γ-Elemene	—	—	0.9
α-Muurolene	0.36	1.21	—
α-Cadinene	—	—	0.84
δ-Cadinene	1.26	4.43	—
γ-Cadinene	0.30	1.59	—
p-Cymenen-3-ol	0.18	0.43	—
Caryophyllene oxid	0.28	0.38	—
Elemol	—	—	6.97
α-Cedrol	—	—	1.04
γ-Selinene	—	—	1.48
T-Muurolol	—	0.62	—
Stach-15-ene	—	—	3.77
β-Eudesmol	—	—	1.41
Globulol	—	—	1.62

Table 3. Antimicrobial Activities of Essential Oils from *Pinus densiflora* (A), *Pinus koraiensis* (B), and *Chamaecyparis obtuse* (C)

A. *Pinus densiflora*

Microorganism	Zone of inhibition (mm)					
	Oil–Tween 20 (9 : 1)	1/2	1/4	1/8	1/16	Control
Salmonella typhimurium	—	—	—	—	—	26[a]
Listeria monocytogenes	12	11	—	—	—	24[a]
E. coli	—	—	—	—	—	30[a]
E. coli O157:H7	—	—	—	—	—	22[a]
Staphylococcus aureus	14	12	—	—	—	18[a]
Klebsiella pneumoniae	12	—	—	—	—	30[a]
Candida albicans	—	—	—	—	—	38[b]

B. *Pinus koraiensis*

Microorganism	Zone of inhibition (mm)					
	Oil–Tween 20 (9 : 1)	1/2	1/4	1/8	1/16	Control
Salmonella typhimurium	—	—	—	—	—	26[a]
Listeria monocytogenes	—	—	—	—	—	24[a]
E. coli	—	—	—	—	—	30[a]
E. coli O157:H7	—	—	—	—	—	22[a]
Staphylococcus aureus	—	—	—	—	—	18[a]
Klebsiella pneumoniae	—	—	—	—	—	30[a]
Candida albicans	12	11	11	—	—	38[b]

C. *Chamaecyparis obtusa*

Microorganism	Zone of inhibition (mm)					
	Oil–Tween 20 (9 : 1)	1/2	1/4	1/8	1/16	Control
Salmonella typhimurium	—	—	—	—	—	26[a]
Listeria monocytogenes	12	12	Trace	—	—	24[a]
E. coli	—	—	—	—	—	30[a]
E. coli O157:H7	—	—	—	—	—	22[a]
Staphylococcus aureus	16	14	—	—	—	18[a]
Klebsiella pneumoniae	—	—	—	—	—	30[a]
Legionella anisa	13	12	—	—	—	10[a]
Candida albicans	12	11	—	—	—	38[b]

—: complete lack of activity. *a*) Chloramphenicol 30 μg. *b*) Nystatin 100 units.

DISCUSSION

In this study, the composition and antimicrobial properties of essential oils from *P. densiflora*, *P. koraiensis*, and *C. obtusa* were examined. The major components of each essential oil were β-thujene in *P. densiflora*, α-pinene in *P. koraiensis*, and bornyl acetate in *C. obtusa*. Essential oils, which are odorous and volatile products of plant secondary metabolism, have a wide application in folk medicine, food flavoring and preservation, and fragrance industries. The antimicrobial properties of essential oils have been known for many centuries. Many essential oils and their constituents have been investigated for their antimicrobial properties against bacteria and fungi and there are more than 500 reports.[7] The differences observed in the antimicrobial and antifungal activities of these essential oils suggest the susceptibility of microorganisms to various chemical components of the oils. The compositions of essential oils depend on the plant species, chemotypes, and climatic conditions.[8] These oils have been demonstrated to have antimicrobial activity, even though their effect against bacteria was not weak.

In the present study, treatment with the essential oil from *P. densiflora* inhibited the growth of *L. monocytogenes* (45.9—50% *vs*. control), *S. aureus* (66.7—77.8% *vs.* control), and *K. pneumoniae* (40% *vs.* control), while the essential oil from *P. koraiensis* only had antifungal activity against *C. albicans* (28.9—31.5% *vs.* control). In addition, treatment with essential oil from *C. obtusa* resulted in the growth inhibition of *L. monocytogenes* (50% *vs.* control), *S. aureus* (77.8—88.9% *vs.* control), *L. anisa* (120—130% *vs.* control), and *C. albicans* (28.9—31.5% *vs.* control). It is of interest that an antifungal effect on the growth inhibition of *C. albicans* was observed for all essential oils except that from *P. densiflora*, suggesting that the composition of each essential oil may vary in effectiveness against *C. albicans*.

The refractive index of liquid oil or fatty acids depends on the numbers of carbon and double bonds and the presence of ketone or hydroxyl groups. Therefore the variation in the re-

fractive index can provide the basis for simple measurements to predict antimicrobial activity and to discriminate between pure and mixed oils. This will also illustrate the variation of essential oil composition between sites, species, and even in individual plants.[9] Australian endemic plants produce a wide range of essential oils such as tea tree oil (oil of *Melaleuca alternifolia*), which is used in consumer health products including topical antiseptics, mouthwashes, and acne treatments.[10,11] The broad-spectrum antimicrobial activity, chemistry, and *in vitro* cytotoxicity of tea tree oil have been well documented.[12,13] Terpinen-4-ol is generally believed to be the antibacterial constituent of essential oils, and α-terpineol and α-pinene are also found to be active in inhibiting the growth of microorganisms.[14] In the present study, we demonstrated that the essential oil from *P. densiflora* has an antibacterial effect, whereas essential oil from *P. koraiensis* has an antifungal effect. Interestingly, the essential oil of *C. obtusa* had an antibacterial as well as antifungal activity. It is possible that these essential oils from coniferous trees can be used as antibacterial and/or antifungal agents in food or other ingredients. The mechanism of antibacterial and antifungal effects of these essential oils extracted from coniferous trees needs to be further examined for potential uses. In conclusion, these results indicate that the essential oils derived from coniferous trees, which have mild antimicrobial properties, can inhibit the growth of gram-positive and gram-negative bacteria and fungi.

Acknowledgments This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, and Enbite Co. Ltd., Republic of Korea.

E.-J. Hong and K.-J. Na contributed equally to this work and should be considered as first authors.

REFERENCES

1) Carson C. F., Cookson B. D., Farrelly H. D., Riley T. V., *J. Antimicrob. Chemother.*, **35**, 421—424 (1995).
2) Harkenthal M., Reichling J., Geiss H. K., Saller R., *Pharmazie*, **54**, 460—463 (1999).
3) Carvalho-Freitas M. I., Costa M., *Biol. Pharm. Bull.*, **25**, 1629—1633 (2002).
4) Na K. J., Kang H. Y., Oh J. H., Choi I. G., Yun Y. W., Jeung E. B., *Kor. J. Lab. Anim. Sci.*, **14**, 93—96 (1998).
5) Aziz N. H., Farag S. E., Mousa L. A., Abo-Zaid M. A., *Microbios*, **93**, 43—54 (1998).
6) Basaga H., Poli G., Tekkaya C., Aras I., *Cell. Biochem. Funct.*, **15**, 27—33 (1997).
7) Kalemba D., Kunicka A., *Curr. Med. Chem.*, **10**, 813—829 (2003).
8) Thompson J. D., Chalchat J. C., Michet A., Linhart Y. B., Ehlers B., *J. Chem. Ecol.*, **29**, 859—880 (2003).
9) Porter N. G., Wilkins A. L., *Phytochemistry*, **50**, 407—415 (1999).
10) Bassett I. B., Pannowitz D. L., Barnetson R. S., *Med. J. Aust.*, **153**, 455—458 (1990).
11) Schwarz K., Huang S. W., German J. B., Tiersch B., Hartmann J., Frankel E. N., *J. Agric. Food Chem.*, **48**, 4874—4882 (2000).
12) Carson C. F., Riley T. V., *Med. J. Aust.*, **160**, 236 (1994).
13) Soderberg T. A., Johansson A., Gref R., *Toxicology*, **107**, 99—109 (1996).
14) Raman A., Weir U., Bloomfield S. F., *Lett. Appl. Microbiol.*, **21**, 242—245 (1995).