J. Microbiol. Biotechnol. (2008), **18**(3), 497–502

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Chemical Composition and Antimicrobial Activity of Essential Oil from Cones of *Pinus koraiensis*

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Received: October 8, 2007 / Accepted: November 29, 2007

The essential oil from the cones of *Pinus koraiensis* was prepared after removing the seeds, and its chemical composition analyzed using gas chromatography-mass spectrometry (GC-MS). Hydrodistillation of the *P. koraiensis* cones yielded 1.07% (v/w) of essential oil, which was almost three times the amount of essential oil extracted from the needles of the same plant. Moreover, the antimicrobial activities of the oil against the growth of Gram-positive bacteria, Gram-negative bacteria, and fungi were evaluated using the agar disc diffusion method and broth microdilution method. Eighty-seven components, comprising about 96.8% of the total oil, were identified. The most abundant oil components were limonene (27.90%), α-pinene (23.89%), β-pinene (12.02%), 3-carene (4.95%), β-myrcene (4.53%), isolongifolene (3.35%), (−)-bornyl acetate (2.02%), caryophyllene (1.71%), and camphene (1.54%). The essential oil was confirmed to have significant antimicrobial activities, especially against pathogenic fungal strains such as *Candida glabrata* YFCC 062 and *Cryptococcus neoformans* B 42419. Therefore, the present results indicate that the essential oil from the cones of *Pinus koraiensis* can be used in various ways as a nontoxic and environmentally friendly disinfectant.

Keywords: Essential oil, antimicrobial activity, *Pinus koraiensis*

Pinus koraiensis, a large conifer, is found mainly across Korea, Japan, and the north-eastern part of China. It only grows in locations higher than 1,000 m above sea level and can reach 1 m in diameter and 20–30 m in height. The seeds of *P. koraiensis* have been used as a food supplement and the plant has been used in oriental medicine for thousands of years. It has also been reported that *Pinus* bark extract, including that from *P. koraiensis*, exhibits antitumor, antioxidant, antiaging, and antimutation activities based on removing superfluous free radicals and enhancing immunity [1, 2, 14].

Essential oils are volatile oils from various parts of fragrant plants, such as the flower, flower bud, leaf, root, and stalk [5, 15]. These oils are known to be antibiotic, anticarcinogenic, and helpful for sedation in the case of stress [1, 2, 14]. Essential oils are also known to contain ketone, terpene, and phenolic ether, which have antitumor, antioxidant, antiaging, antimutation, and sedative effects, plus the high phenolic derivative content of essential oils contributes to their antimicrobial properties [8]. Thus, essential oils from medicinal and other edible plants have been recognized as safe food flavoring agents and aromatic disinfectants with antimicrobial and antioxidizing activities [17]. Recently, the antibacterial and antifungal activities of essential oils extracted from the needles of three coniferous trees, *Pinus densiflora*, *P. koraiensis*, and *Chamaecyparis obtusa*, were reported [12]. However, the antimicrobial activities of these three essential oils were found to be very weak, and moreover, the oil from *P. koraiensis* exhibited no antibacterial activity.

Accordingly, the present study extracted the essential oil from the cones of *P. koraiensis* after removing the seeds, and then investigated the chemical composition and antimicrobial and antifungal activities of the essential oil.

First, the essential oil was prepared using the hydrodistillation method [12]. *P. koraiensis* cones without seeds were collected in November 2005 from San-99, Gapyong-gun, Gyeonggi Province, South Korea. The cones (1,000 g) were immersed in 9 l of distilled water and submitted to steam distillation using an apparatus with a condenser manufactured by Hanil Labtech (Korea). The distillation continued for 2 h at 100°C, and then the volatile compounds contained in the water-soluble fraction were allowed to settle for 20 min. Next, the essential oil layer was separated and purified through microfiltration [12]. The temperature

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of the cooling water used in the condenser was 4°C. As a result, the hydrodistillation of the *P. koraiensis* cones yielded 1.07% (v/w) essential oil, which was almost three times the amount of essential oil extracted from the needles of the same plant.

The essential oil was analyzed 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

Table 1. Major chemical composition (>0.1%) of essential oil from *Pinus koraiensis* cones.

Retention time	Area (%)	Compounds
7.746	0.237	Tricyclene
7.903	0.101	α-Phellandrene
8.426	23.887	α-Pinene
8.934	1.540	Camphene
9.076	0.440	Verbenene
9.876	0.281	*m*-Cymene
9.974	0.458	Sabinene
10.332	12.020	β-Pinene
10.886	4.530	β-Myrcene
11.820	4.950	3-Carene
12.642	0.688	*p*-Cymene
13.225	27.904	Limonene
16.103	0.362	α-Terpinolene
16.388	0.298	Paracymene
17.172	0.181	Undecane
18.144	0.100	Fenchyl alcohol
18.668	0.550	2,2,3-Trimethyl-3-cyclopentene-1-acetaldehyde
19.490	0.733	[1S-(1α,3α,5α)]-6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptan-3-ol
19.834	0.444	4,5-Epoxy-1-isopropyl-4-methyl-1-cyclohexene
20.828	0.245	6,6-Dimethyl-2-methylenebicyclo[2.2.1]heptan-3-one
21.486	0.264	Camphol
22.061	0.183	4-Terpineol
22.555	0.283	3-Methyl-6-hydroxybenzo[c]-dihydrofuran
23.086	1.135	1,3-Cycloheptadien-1-ylmethylketone
23.713	0.175	Verbenone
24.611	0.415	*trans*-(+)-Carveol
25.455	0.155	*cis*-2-Methyl-5-(1-methylethenyl)-2-cyclohexen-1-ol
26.158	0.325	1-Carvone
28.887	2.022	(−)-Bornyl acetate
32.841	1.183	2,6,6,9-Tetramethyltricyclo[5.4.0.0.2]undec-9-ene
34.097	0.105	δ-Cadinene
34.276	0.125	(+)-Longicyclene
34.560	0.721	α-Copaene
34.994	0.108	β-Bourbonene
35.465	0.108	[1S-(1α,3aβ,4α,7α,7aβ)]-Octahydro-4-methyl-8-methylene-7-(1-methylethyl)-1,4-methano-1H-indene
36.549	3.348	Isolongifolene
37.229	1.714	Caryophyllene
39.360	0.293	α-Humulene
39.629	0.159	*trans*-β-Farnesene
40.944	0.297	Germacrene D
42.111	0.252	1,2,4a,5,6,8a-Hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene
43.284	0.148	δ-Cadinene
46.768	0.635	(−)-Caryophyllene oxide
56.060	0.228	Pentyl cinnamate
69.022	0.185	4b,5,6,7,8,8a,9,10-Octahydro-4b,8-dimethyl-2-isopropylphenanthrene
79.114	0.119	[1R-(1α,4aβ,10aα)]-1,2,3,4,4a,9,10,10a-Octahydro-1,4a-dimethyl-7-(1-methylethyl)-1-phenanthrenecarboxaldehyde
Total (%)	94.515	

for each compound was used for quantitation. The GC analysis was carried out with helium as the carrier gas using an FID detector, and the injection and detection temperatures were increased from 150 to 350°C. The oven temperature was increased from 50 to 350°C at intervals of 4°C per min over 75 min. The compounds were identified by comparison with the retention times of the authentic standards.

The GC analysis revealed that 46 major compounds (above 0.1% of the total composition) comprised 94.52% of the essential oil (Table 1), whereas 41 minor compounds (below 0.1% of the total composition) comprised 2.1% of the oil (Table 2). The main components were limonene (27.90%), α-pinene (23.89%), β-pinene (12.02%), 3-carene (4.95%), β-myrcene (4.53%), isolongifolene (3.35%), (−)-bornyl acetate (2.02%), caryophyllene (1.71%), camphene (1.54%), 2,6,6,9-tetramethyltricyclo[5.4.0.0.2]undec-9-ene (1.18%), and 1,3-cycloheptadien-1-ylmethylketone (1.14%). These data showed little difference with the chemical composition of the essential oil prepared from the needles as the control (data not shown). However, according to the previous report, the main components of the essential oil from the needles of *P. koraiensis* were α-pinene (10.49%),

Table 2. Minor chemical composition (<0.1%) of essential oil from *Pinus koraiensis* cones.

Retention time	Area (%)	Compounds
2.600	0.011	Heptane
3.500	0.057	Toluene
4.920	0.009	2-Ethylidene-1,1-dimethylcyclopentane
6.250	0.054	Santene
6.410	0.010	1,1'-(1,1,2,2-Tetramethyl-1,2-ethanediyl)bisbenzene
6.870	0.032	*n*-Nonane
11.570	0.097	*m*-Cymene
12.200	0.082	α-Terpinene
13.910	0.044	3,7-Dimethyl-1,3,7-octatriene
14.530	0.079	γ-Terpinene
15.860	0.043	2,4-Dimethylbicyclo[4.2.0]octa-1,3,5-triene
16.260	0.057	Fenchone
16.860	0.021	α-Pinene oxide
18.330	0.098	*p*-Mentha-trans-2,8-dien-1-ol
18.880	0.069	4-Acetyl-1-methylcyclohexene
18.990	0.039	α-Pinene oxide
20.120	0.098	β-Phellandren-8-ol
21.700	0.076	3-Pinanone
25.360	0.094	2-Methoxy-4-methyl-1-(1-methylethyl)benzene
26.450	0.027	Carvotanacetone
28.139	0.068	Perilla
29.784	0.074	Perilla alcohol
35.981	0.040	Caryophyllene
38.231	0.065	2,6-Dimethyl-6-(4-methyl-3-pentenyl)bicyclo[3.1.1]hept-2-ene
40.100	0.042	*trans*-Caryophyllene
40.668	0.085	α-Amorphene
41.542	0.028	(+)-Epi-bicyclosesquiphellandrene
42.806	0.067	(*S*)-1-Methyl-4-(5-methyl-1-methylene-4-hexenyl)Cyclohexene
43.426	0.042	ι-Calamenene
46.005	0.035	(*E*)-3,7,11-Trimethyl1,6,10-dodecatrien-3-ol
47.844	0.052	Longiborneol
51.029	0.041	γ-Gurjunene
52.950	0.035	α-Bisabolol
56.381	0.009	Isoamyl cinnamate
63.423	0.022	Butyl ester-3-phenyl-2-propenoic acid
64.724	0.036	14-Isoprop-1,3,6,10-cyclotetradecatetraene
67.961	0.073	3R-(3α,4aβ,6aα,10aβ,10bα)]-3-Ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-1H-naphtho[2,1-b]pyran
68.192	0.028	1-[2-(Trimethylsilyl)ethynyl]-3-phenylcyclohexene
70.316	0.091	β-Elemene
70.801	0.085	13-Isopropodocarpa-8,11,13-triene
72.177	0.033	2',3'-Dimethyl-2,3,4,5,6-pentafluorobiphenyl
Total (%)	2.148	

myrcene (7.22%), bornyl acetate (7.13%), limonene (6.55%), α-terpinolene (5.64%), camphene (5.23%), δ-cadinene (4.43%), caryophyllene (6.51%), β-pinene (2.81%), γ-muurolene (2.62%), β-selinene (1.60%), γ-cadinene (1.59%), α-muurolene (1.21%), tricyclene (1.18%), and β-thugene (1.07%). Although difficult to explain, the differences in the analytical data may have been caused by climate and collection differences between the sampling areas.

The essential oil was tested for antibacterial and antifungal activities against various nonpathogenic and pathogenic strains (Tables 3, 4, and 5). The agar disc diffusion method was employed to determine the antimicrobial activity of the essential oil [12, 18]. Briefly, a 100-μl suspension containing 10^5 CFU/ml of bacteria was spread on a Mueller Hinton (MH) agar. Discs (6 mm in diameter) impregnated with 10 μl of the essential oil diluted with 5% dimethylsulfoxide (DMSO) under aseptic conditions were then placed on the inoculated agar. Negative controls were prepared using the same solvent spread on the agar plates, whereas chloramphenicol (30 μg per disc) was used as the positive reference standard to determine the sensitivity of each bacterial strain tested. The inoculated plates were incubated at 37°C for 24 h and the antimicrobial activity was evaluated by measuring the zone of growth inhibition against the test organisms. According to the results given in Tables 3 and 4, the essential oil of *P. koraiensis* exhibited potential antimicrobial activity against various kinds of bacteria. For example, 2.2 μg of *P. koraiensis* oil inhibited the growth of *B. subtilis* ATCC 6633 (12 mm), *E. coli* ATCC 33312 (13 mm), and *K. oxytoca* ATCC 10031 (14 mm), whereas *S. aureus* ATCC 25923 (10 mm) and *S. aureus* 503 (10 mm) were sensitive to a higher concentration (8.8 mg) of oil, and *S. aureus* ATCC 6538P was insensitive to the oil under the test conditions (Table 3).

Minimal inhibitory concentration (MIC) values were determined for bacterial strains and unicellular fungi using a broth microdilution method [10, 18]. For the bacterial strains, all the tests were performed in a Mueller Hinton (MH) broth. Overnight broth cultures of each strain were prepared and the final concentration in each well was adjusted to 1×10^4 CFU/ml. The essential oil was dissolved in nine volumes of dimethylsulfoxide (DMSO), and then a 2-fold serial doubling dilution using the MH broth was prepared in a 96-well microtiter plate. The oil was tested against all the bacterial cultures, standards, and controls (wells containing MH broth only, each type of bacteria with no oil, and MH broth containing oil). The plates were covered with a sterile plate sealer, the well contents mixed on a plate shaker for 20 sec, and the plates incubated at 37°C for 18 h. The MIC was defined as the lowest concentration of the sample at which the microorganism did not demonstrate visible growth, as indicated by the turbidity. The MIC values of the essential oil for the bacterial strains *B. subtilis* ATCC 6633, *E. coli* ATCC 33312, and *S. aureus* ATCC 25923 were 21.8 μg/ml, whereas other strains gave higher MIC values than 21.8 mg/ml (Table 4).

The fungicidal activity of the essential oil from the *P. koraiensis* cones was tested on various fungal strains that commonly cause foot rot and other diseases. The tests were all performed in the same manner as those for the bacterial strains, except for the use of RPMI 1640 broth. The inoculum size of each well was adjusted to 5×10^3 CFU/

Table 4. Minimal inhibitory concentrations of essential oil from *Pinus koraiensis* cones against bacterial strains tested.

Microorganisms	MIC (mg/ml)
Bacillus subtilis ATCC 6633	21.8
Acinetobacter calcoaceticus ATCC 19606	>21.8
Citrobacter freundii ATCC 6750	>21.8
Enterobacter aerogenes ATCC 13048	>21.8
Enterobacter cloacae ATCC 13047	>21.8
Escherichia coli ATCC 10536	>21.8
Escherichia coli ATCC 25922	>21.8
Escherichia coli ATCC 33312	21.8
Klebsiella pneumoniae ATCC 10031	>21.8
Pseudomonas aeruginosa NCTC 10490	>21.8
Serratia marcescens ATCC 25419	>21.8
Staphylococcus aureus ATCC 25923	21.8
Staphylococcus aureus ATCC 6538P	>21.8

Table 3. Antimicrobial activity of essential oil from *Pinus koraiensis* cones against bacterial strains tested.

Microorganisms	Zone of inhibition (mm)			
	Essential oil			Chloramphenicol
	8.8 μg	4. 4 μg	2. 2 μg	30 μg
Bacillus subtilis ATCC 6633	16	14	12	30
Escherichia coli ATCC 33312	18	16	13	28
Klebsiella oxytoca ATCC 10031	16	15	14	34
Staphylococcus aureus ATCC 6538P	-	-	-	25
Staphylococcus aureus ATCC 25923	10	-	-	24
Staphylococcus aureus 503	10	-	-	25

-; Complete lack of activity.

Table 5. Minimal inhibitory concentrations of essential oil from *Pinus koraiensis* cones against fungal strains tested.

Microorganisms	MIC (mg/ml)
Candida albicans B02630	>2.18
Candida krusei ATCC 6258	2.18
Candida glabrata YFCC 062	>0.545
Candida tropicalis ATCC 13803	>2.18
Candida pseudotropicalis KCCM 11658	1.09
Candida parapsilosis ATCC 34136	1.09
Cryptococcus neoformans B42149	0.136
Aspergillua fumigatus B19119	1.09

ml, and the plates were incubated at 37°C for 18 h. The MIC values for the fungal strains that were sensitive to the essential oil were extremely low, within the range of 0.136–2.18 mg/ml (Table 5). The most sensitive strain was *Cryp. neoformans* B42149, which showed an MIC value of 0.136 mg/ml. *C. glabrata* YFCC 062 was also sensitive to the oil (MIC, 0.545–1.09 mg/ml), whereas *C. pseudotropicalis* KCCM 11658, *C. parapsilosis* ATCC 34136, and *A. fumigatus* B19119 showed an MIC value as low as 1.09 mg/ml. Growth inhibition of *C. albicans* B02630 and *C. tropicalis* ATCC 13803 was also observed; however, the MIC values for these strains were estimated to be above 2.18 mg/ml.

Essential oils, as odorous and volatile products of the secondary metabolism of plants, have a wide application in folk medicine, food flavoring and preservation, and fragrance industries. However, their applicability has recently expanded because of their antioxidant, antiaging, antimutation, and sedative effects [5, 16]. The antimicrobial properties of essential oils have also been known for many centuries, and various essential oils have already been studied for their antimicrobial properties against bacteria and fungi [10, 17].

The antimicrobial activities of the essential oils prepared from the needles of three coniferous trees, *P. densiflora*, *P. koraiensis*, and *C. obtuse*, were previously reported [12]. Whereas the essential oils from *P. densiflora* and *C. obtuse* showed some degree of antibacterial activity, the essential oil from *P. koraiensis* only exhibited antifungal activity against *C. albicans* when using the agar diffusion method. Therefore, the present study examined the antimicrobial activity of the essential oil obtained from the cones of *P. koraiensis* using the agar diffusion method and determined the MIC values using a microwell dilution assay. Unexpectedly, the present results showed that the *P. koraiensis* oil effectively inhibited the growth of *B. subtilis* ATCC 6633, *E. coli* 33312, and *K. oxytoca* ATCC 10031 (Table 3) with MIC values of 21.8 mg/ml (Table 4), which were similar to the MICs for other essential oils [3, 18].

The MIC values for various pathogenic fungal strains were extremely low, within a range of 0.136–2.18 mg/ml (Table 5). Recently, it was reported that the essential oil of *Illicium verum* and *Schizonepeta tenuifolia* strongly inhibited the mycelial growth of *Botrytis cinerea* by over 90% at 10 μg/disc [13]. Thus, based on the MIC values for various fungal strains, the antifungal activity of the essential oil from the *P. koraiensis* cones would appear to be quite outstanding, highlighting the need for further studies on the identification of the active fungicidal compounds.

Tea tree oil is an essential oil produced by the Australian endemic plant *Melaleuca alternifolia* and used in consumer health products, including topical antiseptics, mouthwashes, and acne treatments [16]. The broad-spectrum antimicrobial activity, chemistry, and *in vitro* cytotoxicity of tea tree oil have already been well studied [6, 7, 9]. As a result, the cell binding affinity of tea tree oil has been found to be about two-fold higher than that of other essential oils, suggesting that the effective antibacterial activity of tea tree oil is essentially derived from its strong cell adhesion ability [4]. In general, terpinen-4-ol is believed to be the main antibacterial constituent of essential oils, plus α-terpineol and α-pinene are thought to be active in inhibiting the growth of microorganisms [6, 11]. However, the essential oil from *P. koraiensis* cones included a relatively lower amount of terpinen-4-ol and α-terpineol, yet a higher amount of the monoterpene α-pinene (23.89%), which may have produced the strong antifungal activity. In contrast to the previous report [12], the present study clearly showed strong antibacterial and antifungal activities without any difference in the components and MIC values between the essential oils from the needles and cones of *P. koraiensis* (data not shown). Therefore, the differences in results between the current and previous reports may have been due to the natural circumstances of the sampling areas, including the soil conditions, climate, and sampling times. In particular, the samples used in the present study were collected during the harvest period for pine nuts in November, which may have influenced the composition of the essential oil. Although the mechanism of the antibacterial and antifungal effects of the essential oil from *P. koraiensis* still needs to be further examined, broad application of this essential oil as an antibacterial and/or antifungal agent for food and other products is expected.

Acknowledgment

This research was supported by the Driving Force Project for the Next Generation, Gyeonggi Provincial Government, Republic of Korea.

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