J. Microbiol. Biotechnol. (2009), **19**(4), 391–396
doi: 10.4014/jmb.0803.191
First published online 24 January 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Comparison of Chemical Compositions and Antimicrobial Activities of Essential Oils from Three Conifer Trees; *Pinus densiflora, Cryptomeria japonica*, and *Chamaecyparis obtusa*

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Received: March 9, 2008 / Revised: August 26, 2008 / Accepted: September 8, 2008

The chemical compositions, and antibacterial and antifungal effects of essential oils extracted from three coniferous species, *Pinus densiflora, Cryptomeria japonica*, and *Chamaecyparis obtusa*, were investigated. Gas chromatography mass analysis of the essential oils revealed that the major components and the percentage of each essential oil were 16.66% β-phellandrene and 14.85% α-pinene in *P. densiflora;* 31.45% kaur-16-ene and 11.06% sabinene in *C. japonica;* and 18.75% bicyclo [2, 2, 1] heptan-2-ol and 17.41% 2-carene in *Ch. obtusa*. The antimicrobial assay by agar disc diffusion method showed that 2.2 μg of *Ch. obtusa* oil inhibited most effectively the growth of *Escherichia coli* ATCC 33312 and *Klebsiella oxytoca* ATCC 10031, whereas the *C. japonica* oil gave weak antimicrobial activity. The minimal inhibitory concentration (MIC) values for bacterial strains were in the range of 5.45–21.8 mg/ml depending on essential oils, but most Gram-negative bacteria were resistant even at 21.8 mg oil/ml. *P. densiflora* oil showed the most effective antifungal activity and the MIC values for *Cryptococcus neoformans* B42419 and *Candida glabrata* YFCC 062CCM 11658 were as low as 0.545 and 2.18 mg/ml, respectively. *Cryp. neoformans* B42419 was the most sensitive to all essential oils in the range of 0.545–2.18 mg/ml. Our data clearly showed that the essential oils from the three conifers had effective antimicrobial activity, especially against fungi.

Keywords: Essential oil, *Pinus densiflora, Cryptomeria japonica, Chamaecyparis obtusa,* antimicrobial activity

Essential oils are mixtures of volatile oils from various parts of plants, and are considered as important antimicrobial, antifungal, and insecticidal or insect-repelling agents present in plants [11, 13], and some have been reported to endow antioxidant, anti-inflammatory, antitumor, antiaging, antimutation, and sedative effects [2, 3, 19, 21, 22]. Monoterpenes, sesquiterpenes, and their oxygenated derivatives such as alcohols, aldehydes, esters, ethers, ketones, and phenols, are the main components present in essential oils, which may be involved in its physiological and biological activities [14, 26]. However, their actual compositions and biological activities are quite different among the plants, even in the same species, depending on their environmental and genetic variations. All the more, the chemical and biological properties are quite different from the essential oils depending on the part of the same plant, which makes it difficult to understand systematically the effectiveness of essential oils [5, 10, 15, 20].

Essential oils from medicinal as well as other edible plants have been recognized as safe food flavoring agents and aromatic disinfectants with antimicrobial and antioxidizing activities. *P. densiflora, C. japonica*, and *Ch. obtusa* are coniferous trees and mainly distributed in Korea, Japan, and the north eastern part of China. Their seeds and pollens have been used as food supplement and the plants have been used in oriental medicine for thousands of years. Additionally, they can emit monoterpenes that could be helpful for a therapeutic walk in the forest [4]. Thus, the essential oils from conifers were recognized as safe natural disinfectant for various applications, and studies on their chemical components and antimicrobial activities have been extensively performed worldwide.

The 29 components and the mild antimicrobial activities of the essential oils from *P. densiflora* and *Ch. obtusa* against seven bacteria were previously reported by Hong *et al.* [15]. The 42 components of the *Ch. obtusa* essential oils with strong antibacterial activity against Gram-positive

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bacteria and a good antifungal activity were also reported by Yang *et al.* [25]. The 68 compounds were also identified in the Japanese cedar (*C. japonica* D. Don) essential oil [5]. In addition to its antibacterial activity, excellent antitermite [7, 8] and larvicidal effects against mosquito larvae [9], of the essential oil from *C. japonica* leaf, were demonstrated.

However, most of these efforts have been focused on only one kind of essential oil in different experimental conditions, which makes it difficult to compare the components and relative antimicrobial activities among the essential oils. Thus, we prepared the essential oils from three coniferous trees, *P. densiflora, C. japonica*, and *Ch. obtusa*, and investigated their chemical compositions, and antimicrobial and antifungal activities against 15 bacterial and 8 pathogenic fungal strains under the same condition.

Materials and Methods

Plant Material

The needles and twigs of *P. densiflora* were collected from Gwangneung, Gyeonggi province of Korea and those of *C. japonica* and *Ch. obtusa* were picked at Nonsan-si and Puyeo-gun, Chungnam province of Korea, respectively, in November 2003. Voucher specimens (No. KNA20031156 for *P. densiflora,* No. KNA20031157 for *C. japonica,* and No. KNA20031158 for *Ch. obtusa*) have been deposited at the Korea National Arboretum, Gyeonggido, Korea.

Preparation of the Essential Oil

Each sample (1,000 g) of plants was immersed in 9 l of distilled water and submitted to steam distillation using a manufactured apparatus with a condenser by Hanil Labtech (Korea). Distillation continued for 2 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 purified through microfiltration. The temperature of cooling water used for the condenser was 4°C.

GC Analysis

Gas chromatograph (GC) analysis was performed on a Hewlett-Packard (HP) model 6890 gas chromatograph equipped with an apolar capillary DB-5MS 1127 column (30 m×0.32 mm i.d., 0.25 μm film thickness) and a split-splitless injection port (split mode). The oven temperature was programmed from 50°C to 250°C at 2°C/min and held at this temperature for 30 min. Injector and FID detector temperatures were 250°C, respectively. The carrier gas was helium at a flow rate of 1.5 ml/min. The GC-MS was carried out on an HP model 5973 mass spectrometer operating in the EI mode at 70 eV, combined with the GC described above. Most constituents were identified by gas chromatography by comparison of their GC retention indices with those of the literature or with those of standards available in our laboratories. Further identification was made by comparison of their mass spectra with those stored in the Wiley 7N Library or with mass spectra from the literature [1, 17].

Microbial Strains

The essential oils were tested for antibacterial and antifungal activities against *Candida albicans* B02630, *Candida krusei* ATCC 6258, *Candida glabrata* YFCC 062, *Candida tropicalis* ATCC 13803, *Candida pseudotropicalis* KCCM 11658, *Candida parapsilosis* ATCC 34136, *Cryptococcus neoformans* B.42419, *Aspergillus fumigatus* B.19119, *Bacillus subtilis* ATCC 6633, *Acinetobacter calcoaceticus* ATCC 19606, *Citrobacter freundii* ATCC 6750, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* ATCC 13047, *Escherichia coli* ATCC 10536, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 33312, *Klebsiella pneumoniae* ATCC 10031, *Klebsiella oxytoca* ATCC 10031, *Pseudomonas aeruginosa* NCTC 10490, *Serratia marcescens* ATCC 25419, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538P, and *Staphylococcus aureus 503.*

Antimicrobial Assay

The agar disc diffusion method was employed for the determination of antimicrobial activity of the essential oil [15], Briefly, 100 μl of suspension containing 10^5 CFU/ml of bacteria was spread on MH agar. The discs (6 mm in diameter) were impregnated with 10 μl of essential oil diluted with 5% DMSO under aseptic conditions and placed on the inoculated agar. Negative controls were prepared using the same solvent that was spread on the agar plates. Chloramphenicol (30 μg per disc) was served as positive reference standards to determine the sensitivity of each bacterial strain tested. The inoculated plates were incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the zone of growth inhibition against the test organisms.

MIC Evaluation by a Micro-Well Dilution Assay

The minimal inhibitory concentration (MIC) values were studied for the bacterial strains and unicellular fungi by a broth microdilution method [14]. For bacterial strains, all tests were performed in 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 oils were dissolved in nine volumes of dimethylsulfoxide (DMSO), and then a 2-fold serial doubling dilution of the oil using MH broth was prepared in a 96-well microtiter plate. The oil was tested against all the bacterial cultures, standards, and controls made: wells containing MH broth only; each type of bacteria but with no oil; MH broth containing oil. The plate was covered with a sterile plate sealer. Contents of each well were mixed on a plate shaker for 20 sec and plates were incubated at 37°C for 18 h. The MIC is defined as the lowest concentration of the sample at which the microorganism does not demonstrate visible growth. The microorganism growth was indicated by the turbidity. For unicellular fungal strains, all tests were performed in the same way as that for bacterial strains, except using RPMI 1640 broth. The inoculum size of each well was adjusted to 5×10^3 CFU/ml and the plates were incubated at 37°C for 18 h.

Results and Discussion

Chemical Composition of the Essential Oils

The essential oil was prepared from the needles and twigs of three coniferous plants by distillation for 2 h at 100°C. As a result, the hydrodistillation of *P. densiflora, C. japonica,* and *Ch. obtusa* yielded 0.09%, 0.28%, and 0.79% (v/w) essential oils, respectively.

Gas chromatography mass spectrometry analysis (GC/MS) of the essential oil from *P. densiflora* resulted in that 48

Table 1. Chemical composition of the essential oils from *Pinus densiflora, Cryptomeria japonica,* and *Chamaecyparis obtusa.*

Components	Peak area (%)		
	P. densiflora	*C. japonica*	*Ch. obtusa*
Tricyclene	0.570	0.103	0.232
Thujene	-	0.539	0.454
(−)-α-Pinene	14.850	4.209	5.144
Camphene	2.672	0.973	0.966
Sabinene	0.422	11.061	12.839
1-β-Pinene	10.488	-	-
β-Myrcene	8.992	2.259	6.143
δ3-Carene	0.815	1.614	0.384
α-Terpinene	-	0.895	0.758
β-Phellandrene	16.660	-	-
dl-Limonene	4.551	3.554	7.562
γ-Terpinene	-	1.783	2.727
α-Terpinolene	1.360	0.882	1.089
2-Ethenyl-2-butenal	0.701	-	-
3-Cyclohexen-1-ol	-	1.680	3.318
Thymyl methyl ether	1.103	-	-
α-Fenchyl acetate	10.339	1.455	0.069
Bicyclo [2.2.1] heptan-2-ol	-	-	18.754
(+)-2-Carene	-	-	17.411
α-Copaene	0.572	-	-
1,4-Methanoazulene	1.148	-	-
trans-Caryophyllene	5.007	0.174	0.189
Widdrene	-	-	0.679
α-Caryophyllene	0.801	0.070	-
Germacrene D	5.118	0.598	1.339
Germacrene B (CAS)	0.599	-	-
β-Cubebene	0.206	0.066	0.501
α-Amorphene	0.795	0.596	0.311
α-Muurolene	0.926	0.466	-
δ-Cadinene	1.790	1.611	0.381
Hedycaryol	-	-	3.888
Elemol	-	8.602	-
(−)-Spathulenol	1.094	-	-
Caryophyllene oxide	1.181	-	-
1-Hydroxy-1,7-dimethyl-4-isopropyl-2,7-cyclodecadiene	-	1.310	-
2-Naphthalenemethanol	-	-	0.503
γ-Eudesmol	-	2.875	-
t-Muurolol	0.729	-	-
β-Eudesmol	-	4.824	0.561
α-Eudesmol	-	3.531	0.850
α-Cadinol	1.023	1.482	-
o-Menth-8-ene-4-methanol	-	4.013	-
Rimuene	-	0.288	0.674
Beyerene	-	0.318	4.713
Kaur-16-ene	-	31.451	-
β-Elemene	0.607	-	-
Dexetimide	-	0.709	-
Sum of components occupying less than 0.5%	3.259	4.548	5.586
Sum total	98.378	98.539	98.025

Compounds occupying more than 0.5% are listed in order of their elution on the DB-5MS 1127 column.
- Not detected.

compounds were taking 98.38% of the oil (Table 1). The main components were β-phellandrene (16.66%), α-pinene (14.85%), β-pinene (10.49%), α-fenchyl acetate (10.4%), β-myrcene (8.99%), germacrene D (5.02%), *trans*-caryophyllene (5.01%), dl-limonene (4.55%), and camphene (2.67%). In a previous report [15], the major components and the percentage of *P. densiflora* oil were β-thujene (19.33%), α-pinene (14.44%), myrcene (12.19%), and β-pinene (9.82%).

The essential oil from *C. obtusa* had mainly 51 compounds that were holding 98.03% of the oil (Table 1). The main oils were bicyclo [2, 2, 1] heptan-2-ol (18.75%), (+)-2-carene (17.41%), sabinene (12.84%), dl-limonene (7.56%), β-myrcene (6.14%), α-pinene (5.14%), beyerene (4.71%), hedycaryol (3.89%), 3-cyclohexen-1-ol (3.32%), and γ-terpinene (2.73%). In Yang *et al.* [25], the chemical composition of essential oil obtained from the leaves of *Ch. obtusa* was mainly α-terpinyl acetate (13.71%), sabinene (10.97%), isobornyl acetate (8.85%), and limonene (6.89%).

Sixty-three compounds were occupying 98.54% of the essential oil from *C. japonica*. The main oils were kaur-16-ene (31.45%), sabinene (11.06%), elemol (8.60%), β-eudesmol (4.82%), α-pinene (4.21%), *o*-menth-8-ene-4-methanol (4.01%), dl-limonene (3.55%), α-eudesmol (3.53%), γ-eudesmol (2.88%), and β-myrcene (2.26%). The essential oil from the leaf of *C. japonica* was previously reported to have 16-kaurene (20.44%), elemol (19.05%), eudesmol (11.80%), sabinene (10.21%), and terpinen-4-ol (6.19%) as the main components [9].

Our data were significantly different from the previous data. Accroding to Chang *et al.* [6], the compositions of oils prepared from the bark, leaf, and twig of the same plant (*C. japonica)* were quite different. Thus, it may be natural that the essential oils prepared from the twig and leaf together have different compositions from those prepared with the leaf of the three conifers. In addition, many reports emphasized the influence of geographic circumstance, climate, harvesting period, and age of plant on the content of essential oil of the plant [6, 9, 10, 23]. These variations could be a plausible explanation for the differences of the oil compositions.

Antimicrobial Activity

The antimicrobial properties of essential oils have been known for many centuries and various essential oils have been studied for their antimicrobial properties against bacteria and fungi. Therefore, the *in vitro* antimicrobial activities of essential oils against various microorganisms and their activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters, and MIC values. According to the results given in Tables 2 and 3, the essential oil of *Ch. obtusa* had great potential of antimicrobial activity against most kinds of bacteria tested. Specifically, the antimicrobial assay by agar disc diffusion method showed that 2.2 μg of *Ch. obtusa* oil inhibited most effectively the growth of *E. coli* ATCC 33312 (11 mm) and *K. oxytoca* ATCC 10031 (10 mm). The essential oils from *P. densiflora* showed relatively lower antimicrobial activity than those of *Ch. obtusa* in the range of 4.4–8.8 μg per disc against most microorganisms. Contrasting to the essential oils from *Ch. obtusa* and *P. densiflora,* the *C. japonica* oil gave weak antimicrobial activity only to *E. coli* ATCC 33312 (10 mm at 4.4 μg per disc) and *K. oxytoca* ATCC 10031 (10 mm at 8.8 μg per disc). *S. aureus* ATCC 6538P and *E. coli* 1507E were insensitive to the oil even at 8.8 μg per disc.

The MIC values for bacterial strains *B. subtilis* ATCC 6633, *A. calcoaceticus* ATCC 19606, *E. coli* ATCC 33312, *K. pneumoniae* ATCC 10031, *S. aureus* ATCC 25923, and *S. aureus* ATCC 6538P were in the range of 5.45–21.8 mg/ml depending on essential oils, but other strains gave higher MIC values than 21.8 mg/ml (Table 4).

The mild antimicrobial activities of the essential oils prepared from the needles of three conifers, *P. densiflora, P. koraiensis,* and *Ch. obtusa,* were previously reported [15]. Contrasting to this, our recent investigation revealed

Table 2. Antimicrobial activity of the essential oils from *Pinus densiflora, Cryptomeria japonica,* and *Chamaecyparis obtusa.*

Microorganisms	Zone of inhibition (mm)[a]									
	P. densiflora			*C. japonica*			*Ch. obtusa*			CAM[b]
	8.8 μg	4.4 μg	2.2 μg	8.8 μg	4.4 μg	2.2 μg	8.8 μg	4.4 μg	2.2 μg	30 μg
Bacillus subtilis ATCC 6633	9.5	-	-	-	-	-	-	-	-	30
Escherichia coli ATCC 33312	10	-	-	11	10	-	14	13	11	28
Klebsiella oxytoca ATCC 10031	11	10	-	10	-	-	16	13	10	34
Staphylococcus aureus ATCC 6538P	-	-	-	-	-	-	-	-	-	25
Staphylococcus aureus ATCC 25923	10	-	-	-	-	-	10	-	-	24
Staphylococcus aureus 503	11	-	-	-	-	-	10	-	-	25
Escherichia coli 1507 E	-	-	-	-	-	-	-	-	-	25

[a]Diameter of zone of inhibition (mm) including disc diameter 6 mm.
[b]Chloramphenicol as the positive control.
-; Complete lack of activity.

Table 3. Minimal inhibitory concentrations of the essential oils from *Pinus densiflora, Cryptomeria japonica,* and *Chamaecyparis obtusa.*

Microorganisms	MIC (mg/ml)		
	P. densiflora	*C. japonica*	*Ch. obtusa*
Bacillus subtilis ATCC 6633	10.9	>21.8	>21.8
Acinetobacter calcoaceticus ATCC 19606	>21.8	10.9	>21.8
Citrobacter freundii ATCC 6750	>21.8	>21.8	>21.8
Enterobacter aerogenes ATCC 13048	>21.8	>21.8	>21.8
Enterobacter cloacae ATCC 13047	>21.8	>21.8	>21.8
Escherichia coli ATCC 10536	>21.8	>21.8	>21.8
Escherichia coli ATCC 25922	>21.8	>21.8	>21.8
Escherichia coli ATCC 33312	21.8	>21.8	10.9
Klebsiella pneumoniae ATCC 10031	>21.8	>21.8	>21.8
Pseudomonas aeruginosa NCTC 10490	>21.8	>21.8	>21.8
Serratia marcescens ATCC 25419	>21.8	>21.8	>21.8
Staphylococcus aureus ATCC 25923	5.45	10.9	>21.8
Staphylococcus aureus ATCC 6538P	10.9	10.9	>21.8

that the essential oil from *P. koraiensis* cone could effectively inhibit the growth of many bacteria and pathogenic fungal strains [20]. Similar to this, our present data clearly show that the essential oils from the three conifers can effectively inhibit the growth of several microorganisms tested. However, the MIC values (5.45–21.8 mg/ml) of the essential oils were comparatively higher than those of other reports. For example, the essential oil obtained from the leaves of *Ch. obtusa* showed relatively strong antibacterial activities against Gram-positive bacteria in the MIC value range 0.025–5 μl/ml, and above 10 μl/ml against Gram-negative bacteria (Lee *et al.*, 2008). The essential oil of *C. japonica* exhibited considerable inhibitory effects against many bacteria tested (MICs, 0.025–0.05 mg/ml; Cha *et al.* [5]). This difference seems to be attributed to the difference of assay conditions, especially media used for cultivating bacteria. Our condition for antimicrobial assay was the same as the condition performed by Hong *et al.* [15], and our data showed similar but stronger antimicrobial activity than by Hong *et al.* in spite of the quite different oil compositions.

Among the components of the essential oils, terpinen-4-ol is believed to be the antibacterial constituent of essential oils, and α-terpineol and α-pinene are also thought to be active in inhibiting the growth of microorganisms [12]. The essential oils prepared in this work do not have terpinen-4-ol, and only *Ch. obtusa* oil has 0.35% α-terpineol. However, the α-pinene, a monoterpene, was detected in all the essential oils, such as *P. densiflora* (14.85%), *C. japonica* (4.21%), and *Ch. obtusa* (5.14%), which may deliver the antibacterial activity.

Antifungal Activity

The fungicidal activities of the essential oils were tested on various fungal strains that commonly cause foot rot and other diseases (Table 4). Among the three essential oils tested, *P. densiflora* oil was the most effective and the MIC values against *Cryp. neoformans* B42419 and *C. glabrata* YFCC 062 were as low as 0.545 and 2.18 mg/ml, respectively. Interestingly, *Cryp. neoformans* B42419 was the most sensitive to all the essential oils tested in the range 0.545–2.18 mg/ml.

It was reported that the *C. japonica* oil had strong antifungal activities at 0.5 mg/ml against various fungi, which coincides with our data [10]. Although the major components of essential oils showing the profound antifungal

Table 4. Minimal inhibitory concentrations of the essential oils from *Pinus densiflora, Cryptomeria japonica,* and *Chamaecyparis obtusa.*

Microorganisms	MIC (mg/ml)		
	P. densiflora	*C. japonica*	*Ch. obtusa*
Candida albicans B02630	>2.18	>2.18	>2.18
Candida krusei ATCC 6258	>2.18	>2.18	>2.18
Candida glabrata YFCC 062	2.18	>2.18	>2.18
Candida tropicalis ATCC 13803	>2.18	>2.18	>2.18
Candida pseudotropicalis KCCM 11658	>2.18	>2.18	>2.18
Candida parapsilosis ATCC 34136	>2.18	>2.18	>2.18
Cryptococcus neoformans B 42419	0.545	2.18	2.18
Aspergillus fumigatus B 19119	>2.18	>2.18	>2.18

effect need to be further identified, it was suggested that phenolic alcohols or aldehydes in the essential oils can interfere with membrane-associated enzyme proteins and finally cause damage to biological membranes. Specifically, essential oils may disrupt the permeability of cell membranes and inhibit respiration, and its efficiency depends on the rate of monoterpene penetration through the fungal cell wall and cell membrane structure [18, 12]. Several compounds, such as ascaridoles [16] and terpinen-4-ol [24], have been reported as the effective antifungal components; further investigation on the active components and their fungicidal mechanism would be necessary to apply the essential oils as safe natural disinfectants.

Acknowledgment

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

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