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Antifungal activities of three supercritical fluid extracted cedar oils

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Abstract

Port-Orford cedar (*Chamaecyparis lawsoniana*), Alaska yellow cedar (*Chamaecyparis nootkatensis*), and Eastern red cedar (*Juniperus virginiana*) were submitted to supercritical fluid extraction with CO_2 (SCC) and Soxhlet extracted (SE) with hexane. The components in the extracted oils were identified by GC-MS. The oils were evaluated against two common wood decay fungi, brown-rot fungus (*Gloeophyllum trabeum*) and white-rot fungus (*Trametes versicolor*). The SCC extraction yields of *J. virginiana*, *C. nootkatensis*, and *C. lawsoniana* were 3.27%, 3.22%, and 3.29%, respectively. The SE yields of *J. virginiana*, *C. nootkatensis*, and *C. lawsoniana* were 0.80%, 0.71%, and 1.52%, respectively. The statistical analysis showed that SCC extracted cedar oils had higher antifungal activities than SE cedar oils against both fungi. *In vitro* studies showed that *C. nootkatensis* oils have the strongest antifungal activity, followed by *C. lawsoniana,* and *J. virginiana* oil.

Keywords: antifungal; cedar; supercritical fluid extraction; wood preservatives.

Introduction

The relationship between chemical composition and durability in wood was first reported by Hawley et al. (1924). Some heartwood has the inherent ability to resist biological degradation, often referred to as ''natural durability'' or ''decay resistance'' (Eaton and Hale 1993). Meanwhile, the relation between extractive content of heartwoods and their fungal tolerance is well established (see recent publications: Chedgy et al. 2007; Lim et al. 2007; Mburu et al. 2007; Kusuma and Tachibana 2008).

Three North American important commercial wood species, Port-Orford cedar (*Chamaecyparis lawsoniana*), Alaska yellow cedar (*Chamaecyparis nootkatensis*), and Eastern red cedar (*Juniperus virginiana*) are known to have significant natural durability. Cedar species have been reported to have special bioactivity against termites and wood decay fungi (Liu 2004; Gao et al. 2008). Evaluations on antifungal properties (Gao et al. 2008), biocidal application (Dolan et al. 2007), and termiticidal activities (Liu 2004) of *C. lawsoniana* extracts have been reported. A chemical ecological study of the components of the essential oil of *J. virginiana* from different habitats was performed by Setzer et al. (1992). Volatile oil from *J. virginiana*, consisting primarily of cedrene (a terpene) and cedral has been used in perfumery (Heide et al. 1988; Semen and Hiziroglu 2005) and as an insect repellent. *J. virginiana* oil has been widely used in a very broad range of products owing to its unique properties, such as odor and repellency or toxicity to many pests. In addition, the antibiotic activities of *C. nootkatensis* have been studied extensively. For example, antimicrobial activity of essential oil from *C. nootkatensis* has been tested against anaerobic bacteria and yeast (Johnston et al. 2001). Heartwood extractives from *C. nootkatensis* have been tested for resistance to termites and fungi (Taylor et al. 2006). The composition of the leaf oil from *C. nootkatensis* has also been reported (Andersen and Syrdal 1970; Cheng and Von 1970).

In most of these studies, conventional Soxhlet extraction (SE) was used, which is time consuming and requires organic solvents. Some bioactive components in cedar oils could be affected during SE. Supercritical CO_2 (SCC) extraction has several advantages in extracting non-polar components of complex mixtures of natural products. The low viscosity and high diffusivity of SCC can result in higher extraction efficiencies and CO_2 can be easily removed from the extract, leaving an extract that is uncontaminated by any solvent residue. However, SCC of these three cedar oils has been rarely reported (Eller and King 2000). The antifungal activities of these SCC-extracted oils has not been compared.

The objectives of this research are to compare the extraction efficiency between hexane SE and SCC extraction, identify the main chemical components by GC-MS, compare the chemical composition of the extracts obtained by the two methods, and evaluate the antifungal activities of the SCC extracts of the three cedar woods.

Materials and methods

Brown-rot fungus (*Gloeophyllum trabeum*) and white-rot fungus (*Trametes versicolor*) were cultured from existing laboratory stock. The air-dried heartwoods of *C. lawsoniana*, *C. nootkatensis*, and *J. virginiana*, were received from a sawmill in Oregon, USA. The samples were then cut into small strips with a razor blade. The strips were reduced to a 20–40 mesh size in a Wiley mill and then stored at -4°C until extraction.

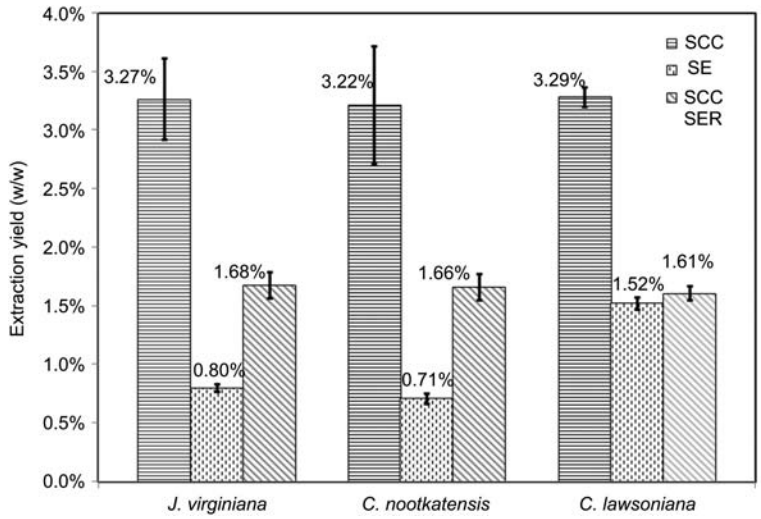

Figure 1 SCC extraction and SE yields of three cedar oils and residues.

Soxhlet extraction (SE)

The ground heartwood of *C. lawsoniana*, *C. nootkatensis*, and *J. virginiana* were Soxhlet extracted (SE) for 24 h with *n*-hexane (200 ml of *n*-hexane for 2 g of ground heartwood, one extraction cycle needed, approx. 1 h). The hexane extract was filtered to remove possible solid particles and concentrated by rotary evaporation, and the traces of solvent in residual oil were removed by nitrogen flushing. The extracted heartwood residue was air dried and stored for further supercritical fluid extraction. The weight of extracted oil was measured and the percentage SE yield (as an average of three independent extractions) was calculated based on unextracted dry matter.

Supercritical CO_2 extraction (SCC)

Apparatus: Applied Separations Spe-ed SFE model 7070 (Applied Separations Inc., Allentown, PA, USA). Heartwood of all three cedar species and the SE heartwood residue (SER) of the same species were extracted (at 60°C, 200 bar), with a restrictor of 80°C. Approximately 2-g samples were weighed and added to a 10-ml stainless steel extraction vessel sealed with polypropylene wool at the top and bottom of the extraction vessel. The extraction flow rate of industrial grade CO_2 was set as 1.5 l min^{-1} (expanded gas), and extracted oils were collected with 60 ml vials (neat collection). The extraction was performed by dynamic extraction until the vial weight no longer increased (approx. 60 min). The vials were weighed before and after the extraction to get the weight of the extracted oil, and % SCC extraction yields (a mean of three independent extractions) were calculated based on dry unextracted matter.

Gas chromatography-mass spectrometry (GC-MS)

Instrument: Varian GC-MS system (Varian, Inc., Santa Clara, CA, USA) equipped with a DB-5 column and He as the carrier gas. Temperature program: 40°C for 3 min → 40–280°C at a rate of 15°C min^{-1} → 280°C was held for 21 min. Injector temperature 250°C; inject volume 1 μl. The MSD (mass selective detector) conditions: EI of 70 eV at room temperature. The spectra were compared with a standard spectrum library (MAINLIB NIST). Relative percentages were obtained by integration and summation of peak areas.

Antifungal activity test against white-rot and brown-rot fungi

The antifungal activities were evaluated according to (Gao et al. 2008) with some modifications. Media were prepared with 2% malt extract, 1.5% agar, and 0.005% yeast extract and by sterilization for 20 min. The extracted oils were first dissolved in acetone to make a series of acetone solutions with different concentration. Two-milliliter acetone solutions were mixed with 98 ml culture medium to make final concentrations of 0.015, 0.03, 0.06, 0.13, 0.25, 0.50, and

Table 1 Summary of Duncan's multiple range test of the effect of extraction methods and species on yields and the antifungal indices for the fungi *Trametes versicolor* and *Gloeophyllum trabeum*.

	Extraction method			*Trametes versicolor*			*Gloeophyllum trabeum*		
	n	Mean of yield	Duncan grouping*	*n*	Mean of AI	Duncan grouping*	*n*	Mean of AI	Duncan grouping*
SCC	6	0.032690	A	45	0.67830	A	41	0.62344	A
SE	6	0.010167	B	45	0.56669	B	45	0.49722	B
C. nootkatensis	4	0.020160	A	30	0.77453	A	30	0.81921	A
C. lawsoniana	4	0.024108	A	30	0.66036	B	30	0.51781	B
J. virginiana	4	0.020018	A	30	0.43260	C	26	0.30100	C

*Means with the same letter are not significantly different at $\alpha = 0.05$.

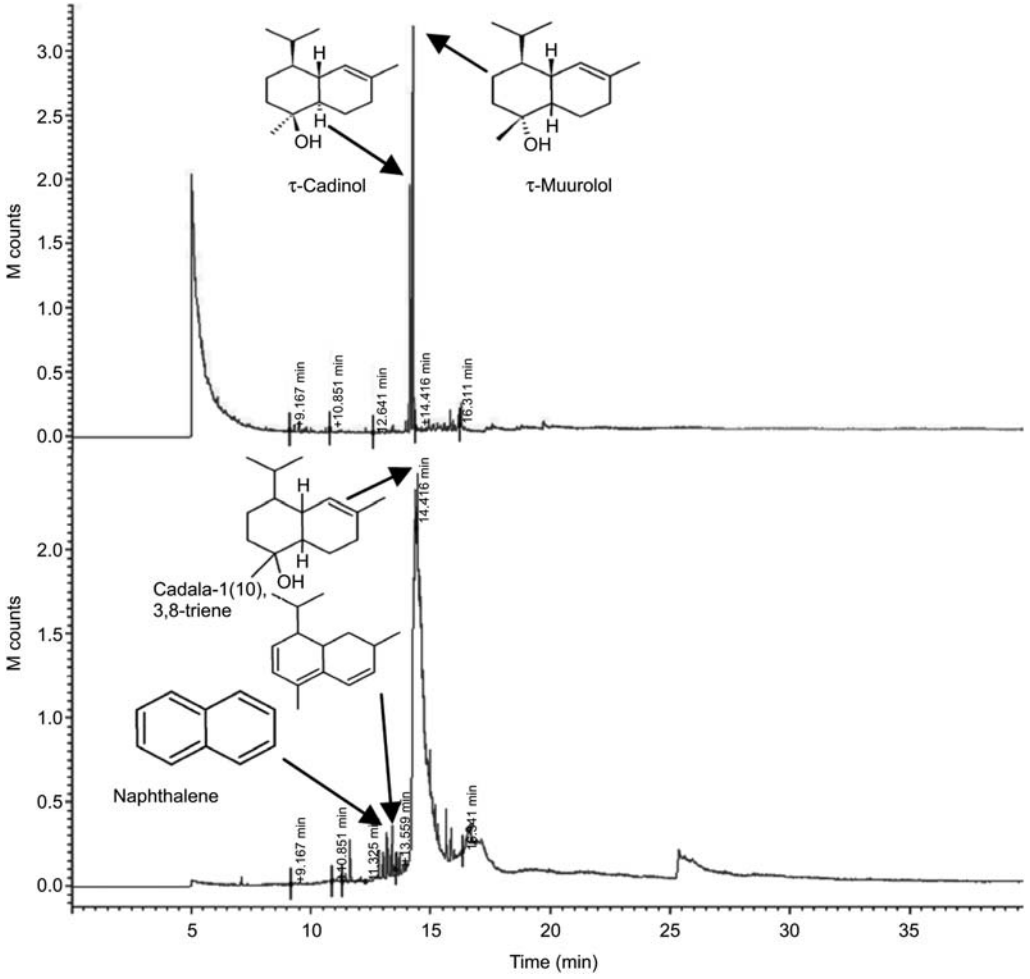

Figure 2 GC-MS spectra of SCC-extracted oils (top) and SE oils (bottom) of *C. lawsoniana*.

1.00 mg · ml^{-1} (oils weight to medium volume) and then transferred into Petri dishes (100 mm × 15 mm). Control Petri dishes were treated with 2 ml of acetone and 98 ml of medium. Either brown-rot fungus (*Gloeophyllum trabeum*) or white-rot fungus (*Trametes versicolor*) were inoculated to the center of the Petri dishes and incubated at room temperature. When the control fungus grew to the edges of the Petri dishes, the diameter of the fungus colony was measured and the antifungal index (AI) was expressed as percentage inhibition, which was calculated by $AI=((D_2-D_1):D_2)\times 100\%$. Where D_2 = diameter growth in the control Petri dishes (mm); D_1 = diameter growth in the experimental Petri dishes with extracts (mm). This estimation of antifungal activities was conducted in triplicate and the results were averaged.

Statistical analysis

The data were analyzed based on the SAS 9.0 software (SAS 2008). Analysis of variance (ANOVA) and Duncan multiple comparisons tests were performed. Comparisons were conducted at $\alpha = 0.05$. Yield differences and antifungal activities difference were analyzed.

Table 2 Summary of uncorrected peak areas of GC-MS of *Chamaecyparis lawsoniana* oils.

	RT (min)	Component	Peak area (%)	Molecular formula
SCC	14.21	τ-Cadinol	40.85	$C_{15}H_{26}O$
	14.25	1-Naphthalenol	5.54	$C_{15}H_{26}O$
	14.31	τ-Muurolol	42.15	$C_{15}H_{26}O$
SE	11.61	Bicyclo[4.1.0]heptane,3,7,7-trimethyl	0.86	$C_{10}H_{18}$
	13.20	Naphthalene	0.63	$C_{10}H_8$
	13.40	Cadala-1(10),3,8-triene	0.75	$C_{15}H_{22}$
	14.01–15.03	Broad peak could not be isolated	91.11	

SCC, supercritical CO_2 extraction; SE, Soxhlet extraction; RT, retention time.

Table 3 Summary of uncorrected peak areas of GC-MS of *Chamaecyparis nootkatensis* oils.

	RT (min)	Component	Peak area (%)	Molecular formula
SCC	12.84	Isolongifolene	8.14	$C_{15}H_{26}O$
	13.19	4,4-Dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptan	17.07	$C_{15}H_{22}$
	14.59	γ-Gurjunenepoxide-(2)	5.18	$C_{15}H_{24}O$
	15.44	Nootkatone	36.90	$C_{15}H_{22}O$
	16.33	Phthalic acid, butyl ester, ester with butyl glycolate	17.29	$C_{18}H_{24}O_6$
SE	13.21	4,4-Dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptan	5.22	$C_{15}H_{22}$
	14.61	τ-Cadinol	33.95	$C_{15}H_{26}O$
	15.46	Nootkatone	16.51	$C_{15}H_{22}O$
	16.30	Hexadecanoic acid	4.35	$C_{35}H_{68}O_5$
	17.45	Oleic acid	6.99	$C_{18}H_{34}O_6$
	19.43	Dotriacontane	1.59	$C_{32}H_{66}$
	20.07	Tritetracontane	5.74	$C_{43}H_{88}$
	20.80	Tritetracontane	7.89	$C_{43}H_{88}$

SCC, supercritical CO_2 extraction; SE, Soxhlet extraction; RT, retention time.

Results and discussion

Effect of extraction methods on extraction yield

The SCC yields for *C. lawsoniana*, *C. nootkatensis*, and *J. virginiana* oils were 3.29±0.08%, 3.22±0.50%, and 3.27±0.35%, respectively (Figure 1), whereas the corresponding SE yields were 1.52±0.05%, 0.80±0.03%, and 0.71±0.04%, respectively. The SCC extraction yields of the residues obtained after SE (SER) amounted to 1.61±0.06%, 1.66±0.11%, and 1.68±0.11%, respectively. The statistical analysis (Table 1) shows that there was a significant yield

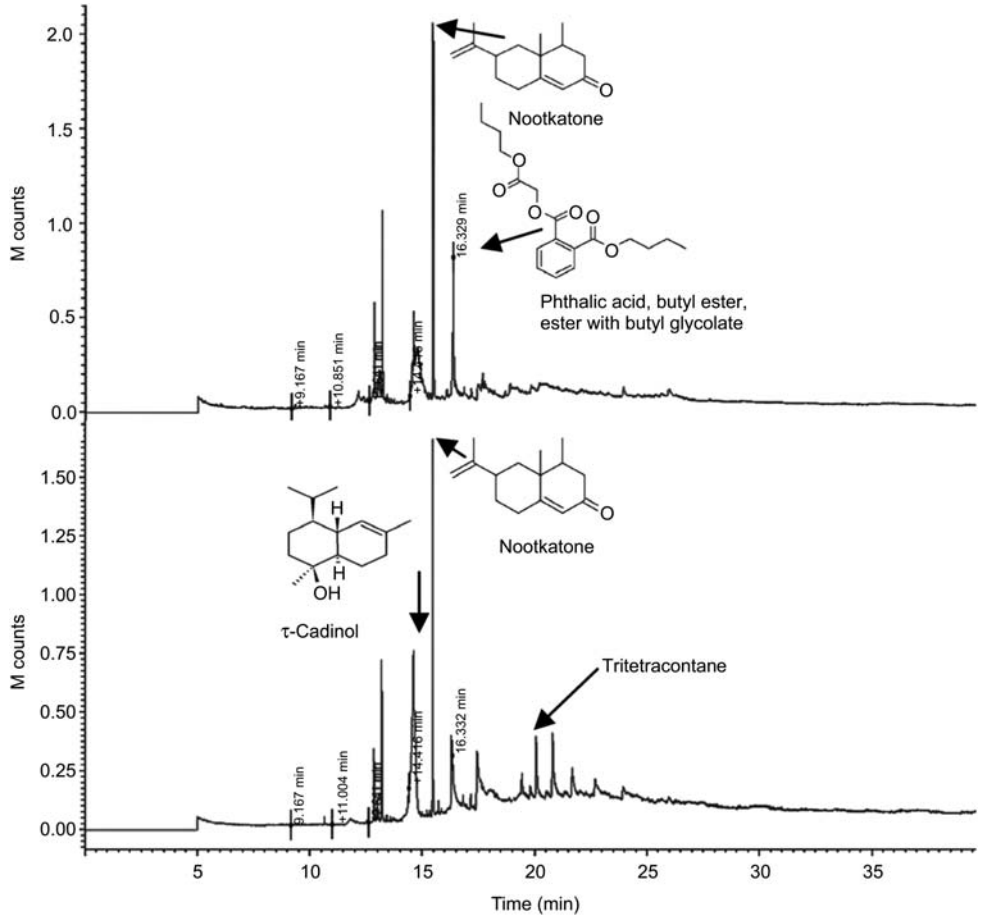

Figure 3 GC-MS spectra of SCC-extracted oils (top) and SE oils (bottom) of *C. nootkatensis*.

Table 4 Summary of uncorrected peak areas of GC-MS of *Juniperus virginiana* oils.

	RT (min)	Component	Peak area (%)	Molecular formula
SCC	13.99	Cedrol	78.60	$C_{15}H_{26}O$
	15.47	2(3H)-naphthalenone	6.21	$C_{15}H_{22}O$
SE	12.36	1H-3a,7-methanoazulene	7.18	$C_{10}H_8$
	12.44	1H-3a,7-methanoazulene	1.57	$C_{10}H_8$
	12.51	Thujopsene	1.50	$C_{15}H_{24}$
	14.10–14.82	Broad peak could not be isolated	63.38	$C_{15}H_{26}O$
	15.44	2(3H)-naphthalenone	2.43	$C_{15}H_{22}O$
	19.78	1,2-Benzenedicarboxylicacid, diisooctyl ester	2.45	$C_{24}H_{38}O_4$

SCC, supercritical CO_2 extraction; SE, Soxhlet extraction; RT, retention time.

difference between the extractions SCC (group A) and SE (group B) ($P < 0.05$). By contrast, it is remarkable that the residue after SE can be further extracted by SCC. The SCC yields from SER are greater than the corresponding SE yields. Obviously, the SCC extraction is more effective than the SE. In addition, the SCC extraction normally requires 1 h whereas SE requires nearly a full day. This is probably because SCC can diffuse through solid samples like a gas and dissolve materials like a liquid. SCC has high diffusivity and low viscosity and low surface tension and thus enhances mass transfer inside the solid matrix. By contrast, the sum of the yields of SE and SCC of SE residues is less than the SCC yield for all three cedar species. This implies that some volatile compounds might be lost during the long extraction period of hexane SE. In addition, and more probably, some compounds might be further lost during rotary evaporation and the nitrogen flushing process.

The moderate SCC pressure of 200 bar could be an issue for industrial applications. Generally, higher pressure results in more extraction power but along with this more sophisticated and expensive equipment is required. A moderate SCC extraction temperature was set to 60°C because if the

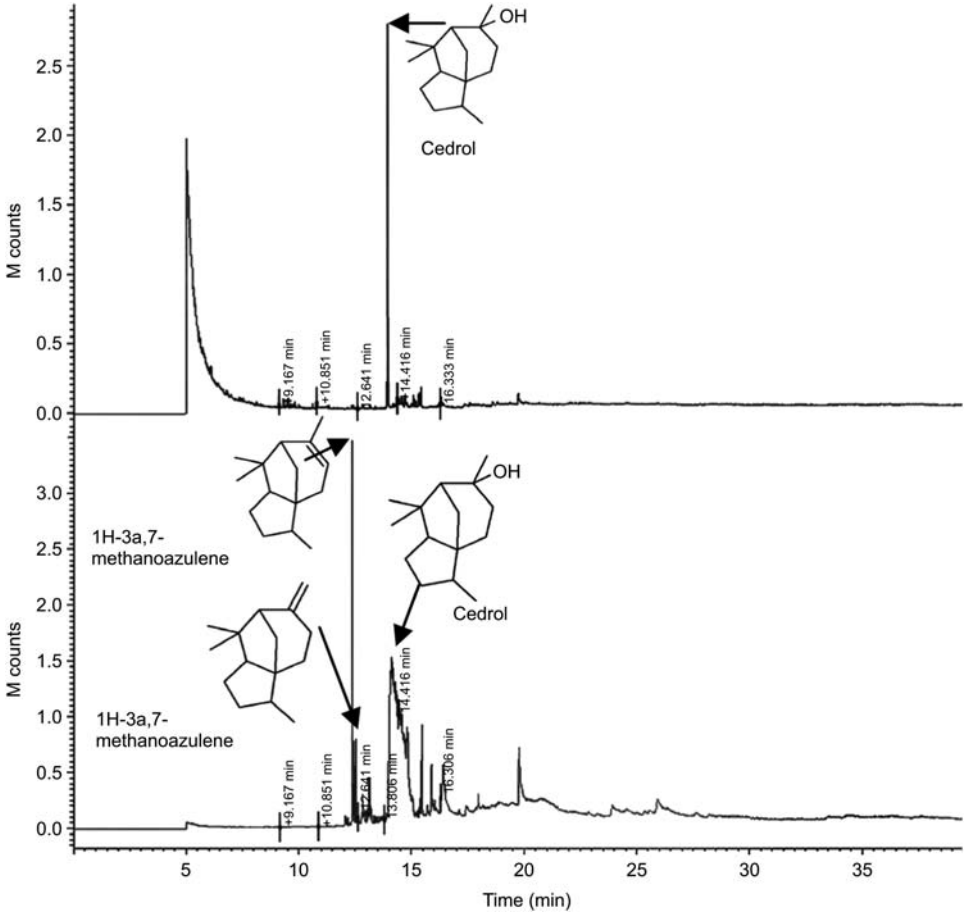

Figure 4 GC-MS spectra of SCC-extracted oils (top) and SE oils (bottom) of *J. virginiana*.

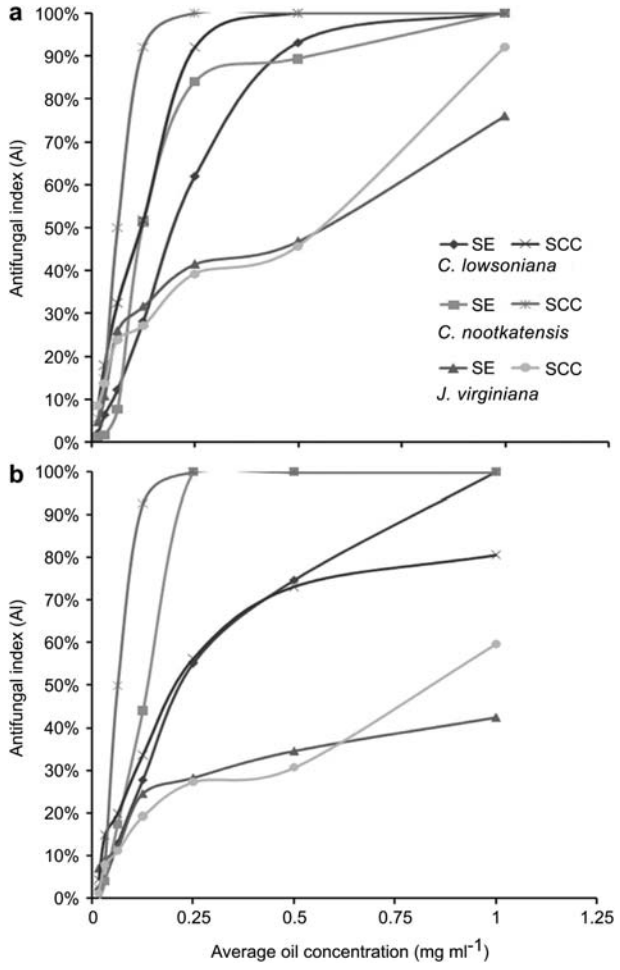

Figure 5 Antifungal indices of three different cedar oils against (a) *Trametes versicolor* fungus and (b) *Gloeophyllum trabeum* fungus.

extraction temperature was set too low, the temperature difference could become a main effect between SCC and SE (the boiling point of *n*-hexane is 69°C at standard conditions). By contrast, if the temperature was set too high, the SCC extraction power will greatly drop at relatively low extraction pressure.

Gas chromatography-mass spectrometry (GC-MS)

C. lawsoniana The GC-MS spectrum of SCC *C. lawsoniana* oil (Figure 2 top) shows only two main peaks. The two main components were determined to be τ-cadinol (14.21 min, 40.85%) and τ-muurolol (14.31 min, 42.15%). The peak areas in the spectrum of SE and SCC oils are summarized in Table 2. The GC-MS spectrum of SE oil (Figure 2 bottom) shows more small peaks than that of SCC of the same species. Accordingly, SE delivers a more complex oil. A broad peak (from 14.01 to 15.03 min) can be interpreted as a combination of cadinol, muurolol, and their derivatives because of the similarity of their retention times. In the SE oil, there might be more molecules with the formula $C_{15}H_{26}O$ as a result of the double-bond shift or other isomerization owing to the longer extraction time at higher temperature. The mixture of non-separated molecules with similar structures probably led to this broad peak.

Two more peaks were identified at lower retention times of 13.40 min (cadala-1(10),3,8-triene) and 13.20 min (naphthalene) in SE oil. Compared to τ-cadinol ($C_{15}H_{26}O$) and τ-muurolol ($C_{15}H_{26}O$) in SCC oil cadala-1(10),3,8-triene ($C_{15}H_{22}$) is a result of losing H_2O and molecular hydrogen from $C_{15}H_{26}O$, and naphthalene ($C_{10}H_8$) is a result of further decomposition of these molecules. It indicates that the high temperature and long extraction time of SE could cause the decomposition of some of oil components (Pickett et al. 1975; Eller and King 2000).

C. nootkatensis The peak areas in the spectra of SE and SCC oils of *C. nootkatensis* are summarized in Table 3. The GC-MS spectrum of SCC (Figure 3 top) shows the highest peak at 15.44 min (36.90%). This peak was identified to be nootkatone ($C_{15}H_{22}O$), which is thought to be the main antifungal component (Manter et al. 2006). The GC-MS spectrum of SE oil (Figure 3 bottom) also had the nootkatone peak at 15.46 min (16.51%). Figure 3 illustrates that the retention time of the main peaks were similar to that of SCC oils except that there were two more peaks in the spectrum of SE oil. Accordingly, SE oil contains more components as $C_{43}H_{88}$ which was identified as tritetracontane, and the nootkatone content was comparatively lower than in SCC oil.

J. virginiana The peak areas in the oil spectra of SE and SCC of *J. virginiana* are summarized in Table 4. The GC-MS spectrum of SCC oil (Figure 4 top) shows one main peak. It was identified as cedrol (13.99 min, 78.60%). The GC-MS spectrum of SE oil (Figure 4 bottom) shows a broad peak around 13.99 min, which can be a combination of cedrol and its derivatives because of the similar retention time to cedrol. As stated before, it is possible that the higher temperature and longer extraction time of SE that caused transformation of cedrol to its derivatives. In addition, there were two more peaks which could be preliminarily assigned to 1H-3a,7-methanoazulene at 12.36 min (7.18%) and 1H-3a,7-methanoazulene at 12.44 min (1.57%). Figure 4 illustrates that 1H-3a,7-methanoazulenes ($C_{10}H_8$) is a result of losing of one OH group and one H atom of cedrol. It is possible that the SE conditions caused cedrol to lose H_2O to generate 1H-3a,7-methanoazulenes.

Antifungal activity test against a white-rot fungus

All of the cedar oils reveal different degrees of inhibition on the growth of *T. versicolor* (Figure 5). One week was required for this white-rot fungus growth to reach the edges of the control dishes. The Duncan's multiple range comparisons for species and methods indicated that the antifungal index (AI) of SCC-extracted oils (group A) was significantly higher than that of SE oils (group B) (Table 1). One reason could be that SE decomposes some bioactive components, a hypothesis, which is supported by previous GC-MS analysis. SCC oil of *C. nootkatensis* (group A) shows the strongest white-rot resistance, followed by SCC oil of *C. lawsoniana*

Table 5 Summary of linear regression for the fungi *Trametes versicolor* and *Gloeophyllum trabeum*.

	Trametes versicolor			*Gloeophyllum trabeum*		
Oil obtained by	Equation	R^2	IC_{50}	Equation	R^2	IC_{50}
SCC of *C. nootkatensis*	$y=8.129x-0.073$	0.986	0.0704	$y=0.402x+1.623$	0.940	0.0613
SE of *C. nootkatensis*	$y=3.808x-0.077$	0.958	0.1514	$y=0.348x+1.296$	0.855	0.1018
SCC of *C. lawsoniana*	$y=3.460x+0.070$	0.992	0.1648	$y=0.176x+0.749$	0.936	0.2443
SE of *C. lawsoniana*	$y=2.553x-0.025$	0.998	0.2034	$y=0.187x+0.723$	0.878	
SCC of *J. virginiana*	$y=1.201x+0.109$	0.907		$y=0.092x+0.388$	0.981	
SE of *J. virginiana*	$y=1.451x+0.088$	0.838		$y=0.084x+0.394$	0.911	

SCC, supercritical CO_2 extraction; SE, Soxhlet extraction; IC_{50}, the half maximal inhibitory concentration.

(group B), and SCC oil of *J. virginiana* (group C). *C. nootkatensis* oils contain nootkatone, which has been reported to have strong antifungal and anti-insect activity (Manter et al. 2006). The nootkatone content in SCC oil of *C. nootkatensis* was higher than in its SE oil, and this could explain the stronger antifungal activity of the former. One of the more interesting results of the study is the extremely effective antifungal activity of *C. nootkatensis* and *C. lawsoniana* as compared with that of *J. virginiana*.

As shown in the evaluation of antifungal indices (AI), the best results were attained already below the concentration of 0.25 mg ml^{-1}. The AI data within the ranges of 0.25 mg ml^{-1} were therefore rectified also by the logarithmic evaluation $AI=a*\ln(c)+b$, where $\ln(c)$ is the natural logarithm of the concentration.

Table 5 summarizes the linear regressions of AI and natural logarithm of concentration. Based on the regression line, IC_{50} (the half maximal inhibitory concentration) is the concentration of an oil when its AI is 50%. The IC_{50} of SCC of *C. lawsoniana* and SCC of *C. nootkatensis* oils were estimated to be 0.165 and 0.070 mg ml^{-1}, respectively. The IC_{50} of SE of *C. lawsoniana* and SE *C. nootkatensis* were estimated to be 0.203 and 0.151 mg ml^{-1}, respectively. Because the antifungal properties of *J. virginiana* oils are weak, the SE oil concentration was out of the regression range when the AI is 50%. IC_{50} of *J. virginiana* oils could not be determined from the regression equations.

Antifungal activity test against brown-rot fungus

All the tested oils revealed different degrees of inhibition on the growth of *G. trabeum* (Figure 5). It took 2 weeks for the brown-rot fungus to reach the edges of the control dishes. The statistical analysis (Table 1) demonstrated that *C. nootkatensis* (group A) had the highest AI against the fungus, followed by *C. lawsoniana* (group B), and *J. virginiana* (group C). The Duncan's multiple comparisons indicate that the mean AI of SCC oils is significantly higher than those of SE oils. Table 5 also contains the summary of linear regressions of AI and natural logarithm of concentrations against *G. trabeum*. Also here, IC_{50} was calculated (when AI is 50%). The IC_{50} of SCC oil of *C. lawsoniana* and that of *C. nootkatensis* were estimated to be 0.244 and 0.061 mg ml^{-1}, respectively. The IC_{50} of SE oil of *C. nootkatensis* was estimated to be 0.102 mg ml^{-1}. The IC_{50} values of the oils of *J. virginiana* and SE oil of *C. lawsoniana* could not be determined owing to weak activity.

Conclusions

SCC was found to be more efficient than SE with *n*-hexane with regard to extraction yield and extraction time. The composition of SCC extracts was similar to that of the SE extracts, but contained fewer minor components. This finding was interpreted as decomposition of the major components of cedar oil under the more harsh conditions of SE. SCC of *C. lawsoniana* and SCC of *C. nootkatensis* oils have strong antifungal activities *in vitro*, which is encouraging for further studies. The wood-derived oils evaluated in this study have a high potential for developing environment-friendly wood preservatives.

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