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Antifungal Activities of Three Supercritical Fluid Extracted Cedar Oils

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ABSTRACT

The antifungal activities of three supercritical CO_2 (SCC) extracted cedar oils, Port-Orford-cedar (POC) (*Chamaecyparis lawsoniana*), Alaska yellow cedar (AYC) (*Chamaecyparis nootkatensis*), and Eastern red cedar (ERC) (*Juniperus virginiana* L), were evaluated against two common wood decay fungi, brown-rot fungi (*Gloeophyllum trabeum*) and white-rot fungi (*Trametes versicolor*). The statistical analysis showed that SCC extracted cedar oils had higher antifungal activities othan hexane Soxhlet extracted cedar oils against both white-rot fungi and brown-rot fungi. *In vitro* studies showed that AYC oils showed the strongest antifungal activity among the three cedar wood oils, followed by POC oil and ERC oil.

Keywords: antifungal, cedar, environment friendly, supercritical fluid, wood preservatives

1. INTRODUCTION

Some heartwood has the inherent ability to resist biological degradation, often referred to as "natural durability" or "decay resistance" (Eaton and Hale 1993). Three important North American commercial wood species; Port-Orford-cedar (POC) (*Chamaecyparis lawsoniana*), Alaska yellow cedar (AYC) (*Chamaecyparis nootkatensis*), and Eastern red cedar (ERC) (*Juniperus virginiana* L) are known to have significant level of natural durability.

The relationship between chemical composition and durability in wood was first reported by Hawley et al. (1924). The chemical ecological study of the essential oil components of ERC (*Juniperus virginiana*) from different habitats was performed by Setzer et al. (1992). The antibiotic activities of AYC oil have been studied extensitively. For instance, antimirobial acitivity against anaerobic bacteria and yeast (Johnston 2001) and termites and fungi resistance (Taylor et al. 2006) of AYC have been reported. More recently, evaluations on antifungal properties (Gao et al. 2008), biocidal application (Dolan 2007), and termiticidal activities (Liu 2004) of POC extracts have been reported.

However, in most of these studies, conventional solvent extraction methods were used, which were often time consuming and involved organic solvents and high temperatures. Most bioactives in cedars are oils, which can be extracted by non-polar solvents (i.e. hexane). Supercritical carbon dioxide (SCC) has several advantages in extracting non-polar chemicals especially for natural products (Eller and King 2000). With the growing concern for environmental and human health risks associated with traditional, inorganic wood preservative systems, SCC extraction is being developed as a key step in the development of new, safe, and effective wood preservatives. The objectives of this research were to evaluate the antifungal activities of the SCC and SE extracts of three cedar woods and to compare antifungal activities with different concentrations, wood species, and extraction methods against different wood decay fungi.

2. MATERIAL AND METHODS

2.1 Sample Preparation

Brown-rot fungi (*Gloeophyllum trabeum*) and white-rot fungi (*Trametes versicolor*) were cultured from existing laboratory stock. Hexane Soxhlet (24 h) extracted oil from the heartwood of POC, AYC, and ERC was obtained from a previous experiment with yields of 0.80%, 0.71%, and 1.52%, respectively. Also, SCC extracted oil from the heartwood of POC, AYC, and ERC was obtained from a previous experiment with yields of 3.27%, 3.22%, and 3.29%, respectively.

2.2 Comparison of 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 by using 2% malt extract, 1.5% agar, and 0.005% yeast extract, and sterilized for 20 min. The extracted oils were first dissolved in acetone to make a series of acetone solutions with different concentration. Two mL acetone solutions were mixed with 98mL culture media to make final concentrations of 0.06, 0.13, 0.25, 0.50, and 1.00mgmL^{-1} (oils weight to medium volume). Control Petri dishes were treated with 2mL acetone and 98mL medium. Either brown-rot fungi (*Gloeophyllum trabeum*) or white-rot fungi (*Trametes versicolor*) fungi were inoculated to the center of the Petri dishes and incubated at room condition. When the control Petri dishes fungi grew to the edges of the control Petri dishes, the diameter of experimental fungi was measured and the antifungal index (AI) was expressed as % inhibition, which was calculated by the Eq. 1. This estimation of antifungal activities was carried out in triplicate and the results were averaged.

$$\%AI = \frac{D_2 - D_1}{D_1} \times 100\% \qquad (1)$$

Where D_2 = diameter growth in the control dishes (mm); D_1 = diameter growth in the experimental Petri dishes with extracts (mm).

2.3 Statistical Analysis

The data were analysed using SAS 9.0 software (SAS 2008). Analysis of variance (ANOVA) and Duncan multiple comparisons tests were performed. All tests were considered statistical significance at $p<0.05$ level.

3. RESULTS AND DISCUSSION

3.1 Antifungal Activity Test against White-rot Fungi

The mean AI and standard deviation of various concentrations of cedar oils extracted from POC, AYC, and ERC with SCC and hexane solvent extraction (SE) are summarized in Table 1. All of these oils showed a certain degree of inhibition on the growth of white-rot fungi. It required one week for the white-rot fungi to reach the edges of the control dishes. The analysis of variance (ANOVA) (Table 2) indicated that all three main experiment factors (wood species, methods of extraction, concentrations) had significant effects on AI. It is also noted that the interactions between species and concentration and species and methods had significant effects on AI.

Table 1 Antifungal Index (%) of various concentrations of cedar oils extracted with SCC and SE from POC, AYC, and ERC for white-rot fungi.

		Oil concentrations in medium				
Species	**Extraction Methods**	**0.06 [mgmL^{-1}]**	**0.13 [mgmL^{-1}]**	**0.25 [mgmL^{-1}]**	**0.50 [mgmL^{-1}]**	**1.00 [mgmL^{-1}]**
POC	**SCC**	20.8±0.6	51.3±0.8	92.1±0	100±0	100±0
	SE	12.2±1.1	28.2±3.0	62.0±4.6	93.2±0	100±0
AYC	**SCC**	50.0±6.6	92.1±0	100±0	100±0	100±0
	SE	7.7±1.1	51.4±1.7	84.0±1.1	89.4±0.7	100±0
ERC	**SCC**	10.0±0	24.3±2.0	39.3±1.0	45.7±0	92.1±0
	SE	25.9±0	31.6±3.0	41.5±0.6	46.8±1.7	76.7±22.4

Table 2 Analysis of variance of three experimental factors and their interactions for white-rot fungi.

Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr> F**
species	2	1.81827815	0.90913907	138.34	<.0001
methods	1	0.28030573	0.28030573	42.65	<.0001
concentration	4	6.00557525	1.50139381	228.46	<.0001
species*concentration	8	0.78369082	0.09796135	14.91	<.0001
concentration*methods	4	0.06146892	0.01536723	2.34	0.0639
species*methods	2	0.22691146	0.11345573	17.26	<.0001

The significant interaction effect of species and concentration on AI are shown in Fig. 1 On average, the AI of the three cedar oils increased as the oil concentration in the media increased. The mean AI of AYC and POC oils were significantly higher than that of ERC oil. The Duncan’s multiple range comparisons for species and methods indicated that the AI of SCC extracted oils was significantly higher than that of SE extracted oils (Table 3), suggesting that SCC extracted cedar oils has better antifungal activities than that of SE extracted oils against white-rot fungi. One possible reason is that SE, operating at higher temperatures and longer duration, decomposes some bioactive components.

Table 3 Summary of Duncan's multiple range test result on the effect of extraction methods and species on AI against white-rot fungi.

		N	**Mean**	**Duncan Grouping***
Methods	**SCC**	45	0.67830	A
	SE	45	0.56669	B
Species	**AYC**	30	0.77453	A
	POC	30	0.66036	B
	ERC	30	0.43260	C

**Means with the same letter are not significantly different.*

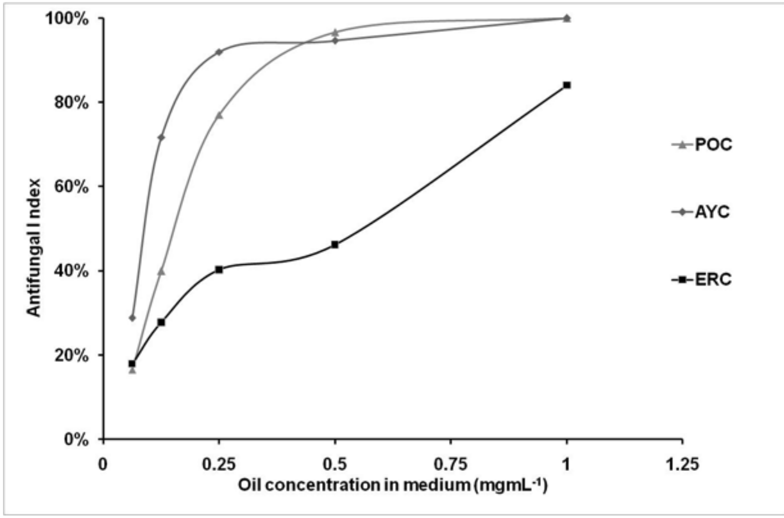
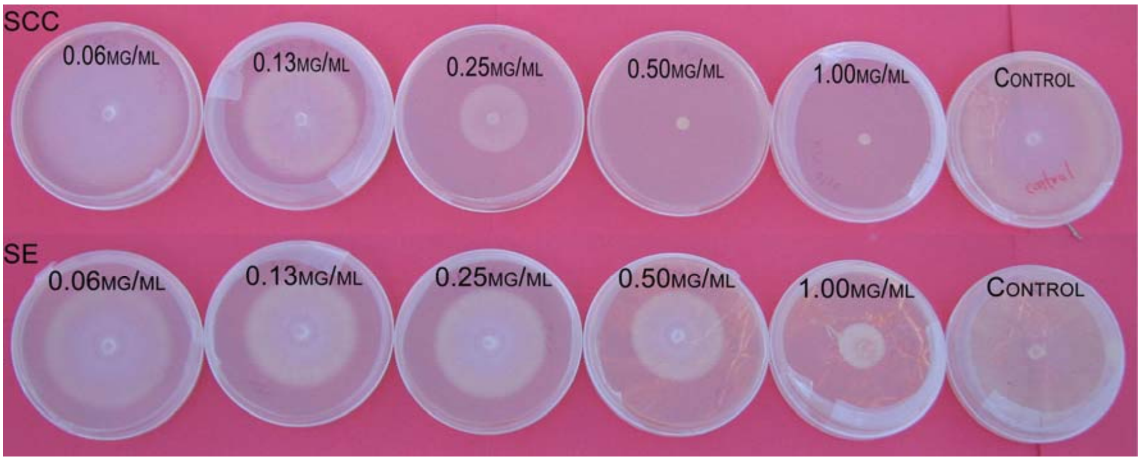


Figure 1 Concentration and species effect on AI for white-rot fungi.



The top row is SCC extracted samples, and the bottom row SE extracted samples

Figure 2 Image of antifungal test for white-rot fungi.

3.2 Antifungal Activity Test against Brown-rot Fungi

The mean AI and standard deviation of various concentrations of cedar oils extracted from POC, AYC, and ERC with SCC and hexane SE are summarized in Table 4. All of these oils showed a certain degree of inhibition on the growth of brown-rot fungi. It took two weeks for the brown-rot fungi to reach the edges of the control dishes. The analysis of variance (ANOVA) (Table 5) indicated that all three main experiment factors (wood species, method of extraction, and concentration) had significant effects on AI. It is also noted that the interactions of these three factors were significant ($p<0.05$). Again, we can see that SCC extracted AYC oil showed the strongest anti brown-rot fungi efftect. The AI of SCC extracted AYC oil is 100% for concentrations greater than 0.25mgmL^{-1}, indicating complete inhibitory effect on the growth of the brown-rot fungi.

Table 4 Antifungal Index (%) of various concentrations of cedar oils extracted from POC, AYC, and ERC with SCC and SE for brown-rot fungi.

		Oil concentrations in medium				
Species	**Extraction Methods**	**0.06 [mgmL^{-1}]**	**0.125 [mgmL^{-1}]**	**0.25 [mgmL^{-1}]**	**0.50 [mgmL^{-1}]**	**1.00 [mgmL^{-1}]**
POC	**SCC**	9.8±1.2	33.7±1.4	56.3±2.6	100±0	80.6±4.0
	SE	6.2±2.4	28.2±3.0	62.0±4.6	93.2±0	100±0
AYC	**SCC**	86.8±6.2	92.6±0	100±0	100±0	100±0
	SE	-4.53±2.7	44.1±1.8	100±0	100±0	100±0
ERC	**SCC**	11.2±0	19.3±0	27.35±0	32.7±2.9	59.7±1.9
	SE	12.0±2.4	24.6±0.9	28.3±1.8	34.6±1.8	42.6±3.9

Table 5 Analysis of variance of three experimental factors and their interactions for brown-rot fungi.

Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
species	2	3.81262712	1.90631356	164.49	<.0001
methods	1	0.22571798	0.22571798	19.48	<.0001
concentration	4	4.11450054	1.02862513	88.75	<.0001
species*concentration	8	0.63256838	0.07907105	6.82	<.0001
concentration*methods	4	0.41078239	0.10269560	8.86	<.0001
species*methods	2	0.38466728	0.19233364	16.60	<.0001

Figure 3 shows the concentration and species interaction effect against brown-rot fungi. With an increase of oil concentration in media, the AI of three cedar oils increased. This indicated the antifungal activity of the cedar oils is concentration reliable against brown-rot fungi. As shown in this figure, the AI of AYC oil is the highest, followed by POC and ERC oils, which is consistent with the results of the antifungal test against white-rot fungi. The Duncan's multiple comparisons for methods and species were tested (Table 6). It indicates that the mean AI of SCC extraction is significant higher than SE, suggesting SCC extracted cedar oils had better antifungal activities against brown-rot fungi. It also noted that AYC (group A) had the highest AI against brown-rot fungi, followed by POC (B), and then ERC (group C).

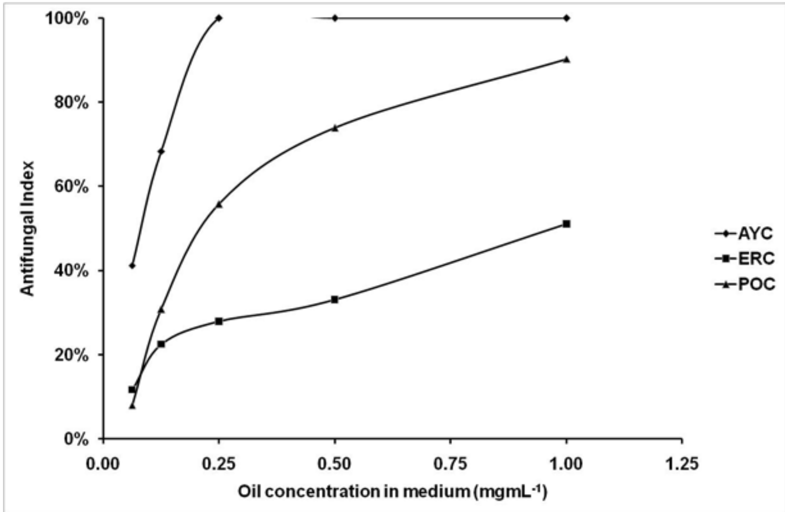
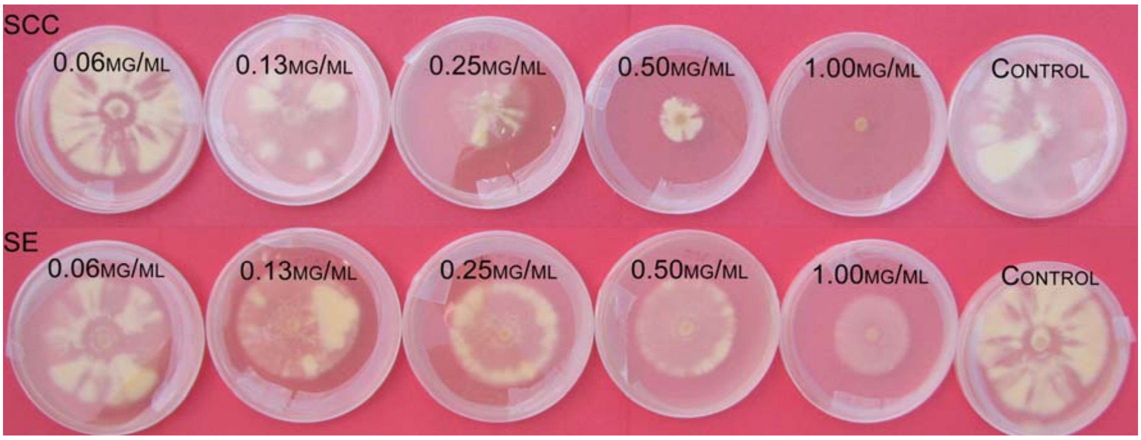


Figure 3 Concentration and species effect on the AI for brown-rot fungi

Table 6 Summary of Duncan's multiple range test result on the effect of extraction methods and species on AI against brown-rot fungi

		N	Mean	Duncan Grouping*
Methods	**SCC**	41	0.62344	A
	SE	45	0.49722	B
Species	**AYC**	30	0.81921	A
	POC	30	0.51781	B
	ERC	26	0.30100	C

**Means with the same letter are not significantly different.*



The top row is SCC extracted samples, and the bottom row is SE extracted samples

Figure 4 Image of antifungal test against brown-rot fungi

3.3 Discussion

One of the most interesting results of the study was the extremely effective antifungal activities of AYC and POC as compared to that of ERC, particularly the AYC. As shown in the evaluation of AI, a characteristic of the antifungal activities is most AI was attained below the concentration of 0.25mgmL^{-1}. The AI data within the ranges of 0.25 mgmL^{-1}were therefore rectified to use the natural logarithm instead of quantity itself to test for regression analysis by using the logarithm equation AI = a*ln(c) + b (the ln(c) is a natural logarithm of the concentration). The 12 correlation regression lines obtained between AI and ln(c) (3 species by two extraction methods by two fungi) had the correlation coefficient ranged from 0.909 to 0.999, suggesting that the correlation between AI and concentration (ln(c)) was exponential. Table 7 summarizes the regression coefficient (i.e., relative rate of increase of AI) and the intercept (i.e., b) from the regression analysis.

Table 7 Summary of the natural logarithm regression result.

		Intercept (b)		Relative rate increase (a)	
		SE	**SCC**	**SE**	**SCC**
AYC	**Brown-rot**	2.03	3.77	0.75	1.05
	White-rot	1.62	2.18	0.55	0.61
POC	**Brown-rot**	1.03	1.03	0.35	0.34
	White-rot	1.08	1.61	0.36	0.51
ERC	**Brown-rot**	0.46	0.43	0.12	0.12
	White-rot	0.56	0.68	0.11	0.21

It is interesting to note that all three cedar oils extracted with SCC consistently yielded higher relative rates of increase for AI and greater intercepts as compared to that of SE extracted samples exposed to white-rot fungi, but same result was shown only for AYC against brown-rot fungi.

With data of SCC and SE pooled, the mean values of relative rate of increase of AI for AYC were more than 2.6 and 7.5 times greater than that of POC and ERC, respectively, against brown-rot fungi; and 1.3 and 3.6 times, respectively, against white-rot fungi. These results strongly indicated that the natural durability of AYC is superior than that of POC and ERC.

4. CONCLUSIONS

This study furthers our understanding of why the heartwood of certain trees has considerable natural durability. The isolation and purification of the active constituents may result in a product that is many times more active. AYC oils showed superior antifungal activities of the three cedars *in vitro*, which is encouraging for further study. The SCC extracted oil has better antifungal activities than hexane SE extracted oil. The AYC and POC oils have the potential for developing environment-friendly wood preservatives. The wood of these trees used to prepare these oils is available as forest products residues and by-products.

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