Antifungal Activity of Organic Extracts from *Juniperus virginiana* Heartwood against Wood Decay Fungi

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Abstract

Easter red cedar (*Juniperus virginiana*) is a valuable source of heartwood extractives that provide decay resistance against termites and wood decay fungi. This study sought to determine the antifungal activity of heartwood extracts obtained using solvents with increasing polarity (hexane, chloroform, ethyl acetate, and methanol) against two wood decay fungi. The heartwood was extracted with methanol, and the methanol extract was sequentially extracted with hexane, chloroform, and ethyl acetate. The yield of the methanol extractives was 5.26 percent based on dry wood and the percentages of the hexane, chloroform, and ethyl acetate soluble fractions from the methanol extract were 46.4, 8.3, and 28.7 percent, respectively. Hexane and chloroform soluble fractions showed a high inhibitory effect on the growth of the wood decay fungi *Trametes versicolor* and *Gloeophyllum trabeum*. Gas chromatography–mass spectrometry analysis identified skeletons of sesquiterpenes and sesquiterpene alcohols in both extracts and the most abundant compounds identified, cedrol, cedrenes, and thujopsenes, were individually screened for antifungal activity. Among the three major sesquiterpenes, cedrol and thujopsene showed the highest inhibitory effects against *G. trabeum* and *T. versicolor,* respectively.

The demand for environmentally benign chemicals as a substitute for toxic metal–based wood preservatives such as chromated copper arsenate, banned in Denmark, Sweden, Germany, and other countries, has increased worldwide (Schultz and Nicholas 2000). Therefore, there is a research emphasis around the world on finding alternative, environmentally friendly wood preservatives.

Tree heartwood, in particular cedar, contains extractives that may be effective against insects and microorganisms that cause wood decay (Schultz et al. 1995, Chang et al. 1999, Onuorah 2000, Johnston et al. 2001, Mihara et al. 2005, Watanabe et al. 2005). Alaska cedar (*Chamaecyparis nootkatensis*) and western red cedar (*Thuja plicata*) heartwoods are known to have considerable natural resistance to insects and microorganisms (Adams et al. 1988, Grace and Yamamoto 1994, DeBell et al. 1997, Gao et al. 2008, Kang et al. 2010). Nootkatone is a major termiticidal compound occurring in *C. nootkatensis* (Grace and Yamamoto 1994, Gao et al. 2008). Kang et al. (2010) recently reported that *T. plicata* wood showed decay resistance compared with pine wood in a soil bed decay test. Port-Orford cedar (*Chamaecyparis lawsoniana*) also has shown excellent termite resistance and antifungal activity against brown and white rot fungi (Gao et al. 2008).

Some *Juniperus* species also have strong antitermite, antibacterial, antifungal, and antitumor activities. Western juniper (*Juniperus occidentalis*) heartwood has a strong resistance to termites. The antitermite compounds are reported to be cedrenes and sesquiterpenes of a 15-carbon skeleton (Karchesy 1998). Chinese juniper (*Juniperus chinensis*) is used as a folk remedy in Korea and is known to have various bioactivities, such as antitumor, antibacterial, antifungal, antiviral, and abortifacient activities (Kwon et al. 2010a, 2010b). Eastern red cedar (*Juniperus virginiana*) is not a true cedar. It is a juniper and the most widely distributed native conifer in the eastern United States. *J. virginiana* is known for its aromatic smell, toxicity, and ability to repel moths, flour beetles, cockroaches, and ants (Eller et al. 2010). The heartwood of *J. virginiana* has resistance to termite attack, and the effectiveness is attributed to sesquiterpene alcohols, such as cedrol and

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widdrol (Liu 2004, Eller et al. 2010). Cedar wood oils obtained by supercritical fluid extraction (CO_2) and ethanol extraction of *J. virginiana* have been tested against wood decay fungi, and the results indicated that the CO_2 extract showed resistance to decay, while the ethanol extract showed moderate resistance to decay. According to the American Society for Testing and Materials (1998) moderate resistance to decay is represented by an average weight loss of 25 to 44 percent and resistance to decay is represented by an average weight loss of 11 to 24 percent. However, to our knowledge the individual solvent fractions, the effective concentration, and the individual compounds of *J. virginiana* heartwood extracts responsible for antifungal activities against wood decay fungi have not been previously reported. Therefore, the objectives of this research were to determine the effective organic solvent fractions and their effective concentrations and to identify compounds of *J. virginiana* heartwood extract responsible for antifungal activity against brown- and white-rot fungi.

Materials and Methods

Materials

An 80-year-old *J. virginiana* tree located on the campus of Mississippi State University was used in this study. The tree was cut into small sections with a chain saw and then subjected to a chipper. The heartwood chips were ground into wood meal with a Wiley mill containing a 1-mm screen. The wood meal was immediately packed in a zipper-lock plastic bag and stored at 4°C until extracted.

Thujopsene, α-cedrene, cedrol, methanol, hexane, chloroform, and ethyl acetate were purchased from Sigma Aldrich Chemical (St. Louis, Missouri, USA) and used without further purification.

Preparation of solvent extracts

Approximately 1 kg (air-dried weight) of *J. virginiana* heartwood meal was placed in a 6-liter Erlenmeyer flask. Four liters of methanol was added to the flask and let stand for 24 hours at ambient temperature with occasional shaking. The supernatant was then collected and filtered using a Büchner funnel lined with No. 1 filter paper (Whatman, Thermo Fisher Scientific, Hanover Park, Illinois, USA). The filtrate was transferred to a 1-liter round bottom flask and evaporated to dryness at 60°C. The methanol extraction was repeated three times. All filtrates were combined, evaporated to remove solvent, and weighed to calculate the methanol extract yield.

The methanol extract (15.274 g) was placed in a 1-liter Erlenmeyer flask and suspended in 200 mL of deionized water. The suspension was transferred into a 1-liter separatory funnel and successively extracted with hexane, chloroform, and ethyl acetate, as shown in Figure 1. Each solvent extract was evaporated to dryness at 50°C to 60°C and weighed to determine the yield of extractives for each solvent.

Antifungal assays

Antifungal assays using potato dextrose agar media (Difco, Fisher Scientific, Pittsburgh, Pennsylvania, USA) were performed according to Gao et al. (2008), using the white-rot fungus *Trametes versicolor* ATCC 12679 and the brown-rot fungus *Gloeophyllum trabeum* ATCC 13021. The medium was sterilized for 20 minutes at 120°C and cooled to 55°C, and all extracts (methanol, hexane, chloroform, ethyl acetate, and methanol soluble) dissolved in ethanol were added to the medium to yield final concentrations of 0.125, 0.5, and 2.5 mg/mL. The amended media were dispensed into petri dishes (100-mm outside diameter by 15-mm height). A 5-mm plug from the edge of actively growing fungal culture was transferred to the center of the amended media and incubated at 28°C.

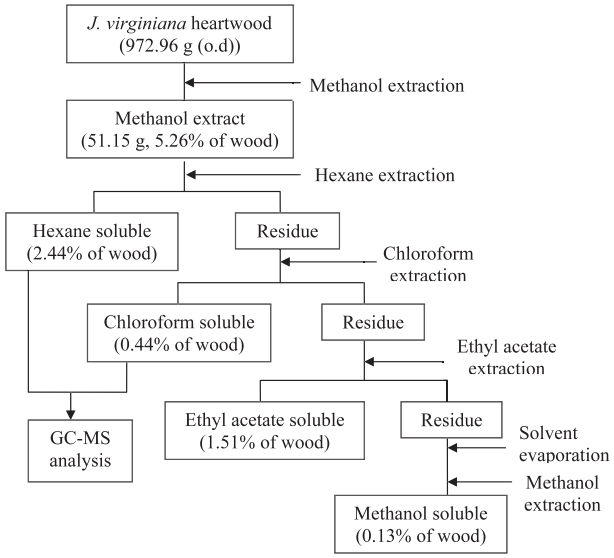


Figure 1.—Solvent extraction and fractionation scheme of Juniperus virginiana *heartwood. Dry weights for each fraction are indicated in parentheses.*

Pure sesquiterpenes (thujopsenes, α-cedrene, and cedrol) identified from *J. virginiana* heartwood were also investigated for antifungal activity in the same manner as the extracts. Cedrol was not soluble in ethanol at 2.5 mg/mL; therefore, a 1-mg/mL concentration was the highest concentration of cedrol used in this study.

All antifungal assays were carried out in triplicate with the exception of thujopsene, and the data obtained were averaged. Potato dextrose media with and without ethanol were used as controls. The fungal growth was measured when the mycelia diameter in the control treatments nearly reach the perimeter of the petri dish (7 to 10 days). The antifungal index (AI) was calculated as follows:

$$\mathrm{AI} = [1 - (D_1/D_2)] \times 100$$

where D_1 is the radial growth of the mycelium on the media amended with solvent extract or pure sesquiterpene, and D_2 is the radial growth of the mycelium on the ethanol amended media.

GC-MS analysis

Hexane and chloroform soluble fractions were analyzed by gas chromatography–mass spectrometry (GC-MS; Hewlett Packard 5890 Series II gas chromatograph equipped with a Hewlett Packard 5971A mass selective detector). For the GC-MS analysis, hexane and chloroform soluble fractions were dissolved in each extraction solvent. Separation of the compounds in the extract was achieved using a DB-5 column (25 m by 0.25 mm, 0.25-μm film thickness; Agilent Technologies, Santa Clara, California, USA). The oven temperature was maintained at 50°C for 1

minute and then ramped at 3°C/min to 270°C and held for 10 minutes. Helium flow rate was held at 1.0 mL/min and the split ratio was adjusted to 10. The mass selective detector was operated in electron ionization mode at 70 eV with an interface temperature of 230°C. Compounds were identified by comparison with commercially available standards, literature data, and Wiley 139 library data of the GC-MS system.

Results

Solvent extracts

J. virginiana heartwood was exhaustively extracted with methanol, which is able to extract a wide range of compounds from wood. The methanol extract was then extracted with hexane, chloroform, ethyl acetate, and methanol successively in order to extract compounds with increasing polarity contained in the methanol extract. The yield of methanol extract was 5.26 percent, based on the oven dried heartwood. The hexane, chloroform, ethyl acetate, and methanol soluble fractions in the methanol extractives comprised 46.4, 8.3, 28.7, and 15.7 percent, respectively (Fig. 1).

Antifungal activities of solvent extracts on wood decay fungi

The antifungal activities of all solvent extracts prepared from *J. virginiana* heartwood for this experiment were evaluated by the growth inhibition of the brown-rot fungus *G. trabeum* and the white-rot fungus *T. versicolor*. The results are presented in Figure 2 in which the antifungal activity was expressed as the AI, with a higher AI correlating to a higher inhibition. The AI of the methanol extract revealed concentration dependency, and at a concentration of 2.5 mg/mL, growth of both fungi was completely inhibited (Fig. 2a). The AI of the hexane and chloroform soluble fractions of the methanol extract also showed a similar tendency for both brown rot and white rot fungi (Figs. 2b and 2c). The ethyl acetate soluble fraction showed a lower AI compared with the methanol extract and the hexane and chloroform soluble fractions (Fig. 2d). At 2.5 mg/mL, the AI of the ethyl acetate soluble fraction was 77 percent for *G. trabeum*, while the AI for *T. versicolor* showed a lower value of 48 percent. After successive solvent extractions of the methanol extract, the remaining methanol soluble faction had little or no antifungal activity on the wood decay fungi (Fig. 2e).

GC-MS analysis of hexane and chloroform soluble fractions

The hexane and chloroform soluble fractions were subjected to GC-MS analysis because they showed a high antifungal activity against the two wood decay fungi selected for this study. The total ion chromatograms (TICs) of each solvent fraction after GC-MS analysis were very similar to each other (Fig. 3) and also similar to those of cedar wood oils prepared from steam distillation and supercritical carbon dioxide extraction of the same wood (Eller and King 2000). Peak 10 (Table 1; Fig. 4) was the most abundant component and was identified as cedrol. Peaks 1 and 2 were identified as α- and β-cedrene, respectively, and were the second-most abundant of the identified components in the hexane and chloroform soluble fractions, while thujopsenes, Peak 3 and a seven-membered ring structure, were the third-most abundant components (Fig. 4; Table 1). The sum of the areas of these three main components, cedrol, cedrenes, and thujopsenes, in the hexane soluble fraction accounted for more than 74 percent of the total TIC area compared with 50 percent in the chloroform fraction. This suggests that while hexane removed a majority of the low polarity extractives, significant residual cedrol, cedrenes, and thujopsenes remained and were extracted by the chloroform. In the chloroform soluble fraction, cedrol was again the major compound. Cedrol, α-cedrene, and thujopsenes were individually screened for antifungal activity against *G. trabeum* and *T. versicolor* at concentration ranges of 0.125 to 2.5 mg/mL (Table 2). Results indicated that cedrol and thujopsenes showed the highest antifungal activity against *G. trabeum* (81% and 47%, respectively), while thujopsene showed the highest antifungal activity (44%) against *T. versicolor*.

Discussion

Large-diameter *J. virginiana* wood is used in the furniture industry to manufacture chests and cabinets, and the sawdust and other waste wood from lumber mills are a source of cedar wood oil (Eller and Taylor 2004). The amount of cedar wood oil reported to be present in this wood varies widely from 0.97 to 4.6 percent (Eller and King 2000). Since *J. virginiana* wood contains low polarity extractives such as cedar wood oil, nonpolar or relatively low polar solvents such as hexane, chloroform, and ethyl acetate can be used for extraction. In our results, as shown in Figure 1, the hexane soluble fraction was the major component of the total extract. After solvents were removed, the hexane and chloroform soluble fractions were viscous oils with a light brown or an amber color, respectively, and all had a pleasant odor similar to the original *J. virginiana* wood. This indicates that these fractions contain many volatile compounds of *J. virginiana* heartwood origin. The total amounts of these fractions accounted for 2.88 percent of *J. virginiana* heartwood by weight, and the value was in the range of the cedar wood oil content previously reported by Eller et al. (2010). The ethyl acetate soluble fraction, the second highest component, seemed to contain the compounds that contributed to the color of *J. virginiana* heartwood because it had a distinctive red purple color that was similar to the color of *J. virginiana* heartwood.

All solvent extracts prepared from *J. virginiana* heartwood were tested for antifungal activity against the brown-rot fungus *G. trabeum* and the white-rot fungus *T. versicolor* in agar medium. We confirmed that the components having antifungal activity against both wood decay fungi were extracted by methanol because the methanol extract showed complete inhibition of the growth of the two fungi at a concentration of 2.5 mg/mL. Because a wide variety of wood components having different polarities are extracted by methanol, we conducted successive extractions of the methanol extract using solvents with increasing polarities to find the solvent fraction responsible for antifungal activity. It was revealed that the major antifungal components of the methanol extract were extracted by hexane and chloroform and only a small portion of antifungal components was extracted by ethyl acetate. This result indicates that the low polarity components of *J. virginiana* heartwood have antifungal activity against wood decay fungi.

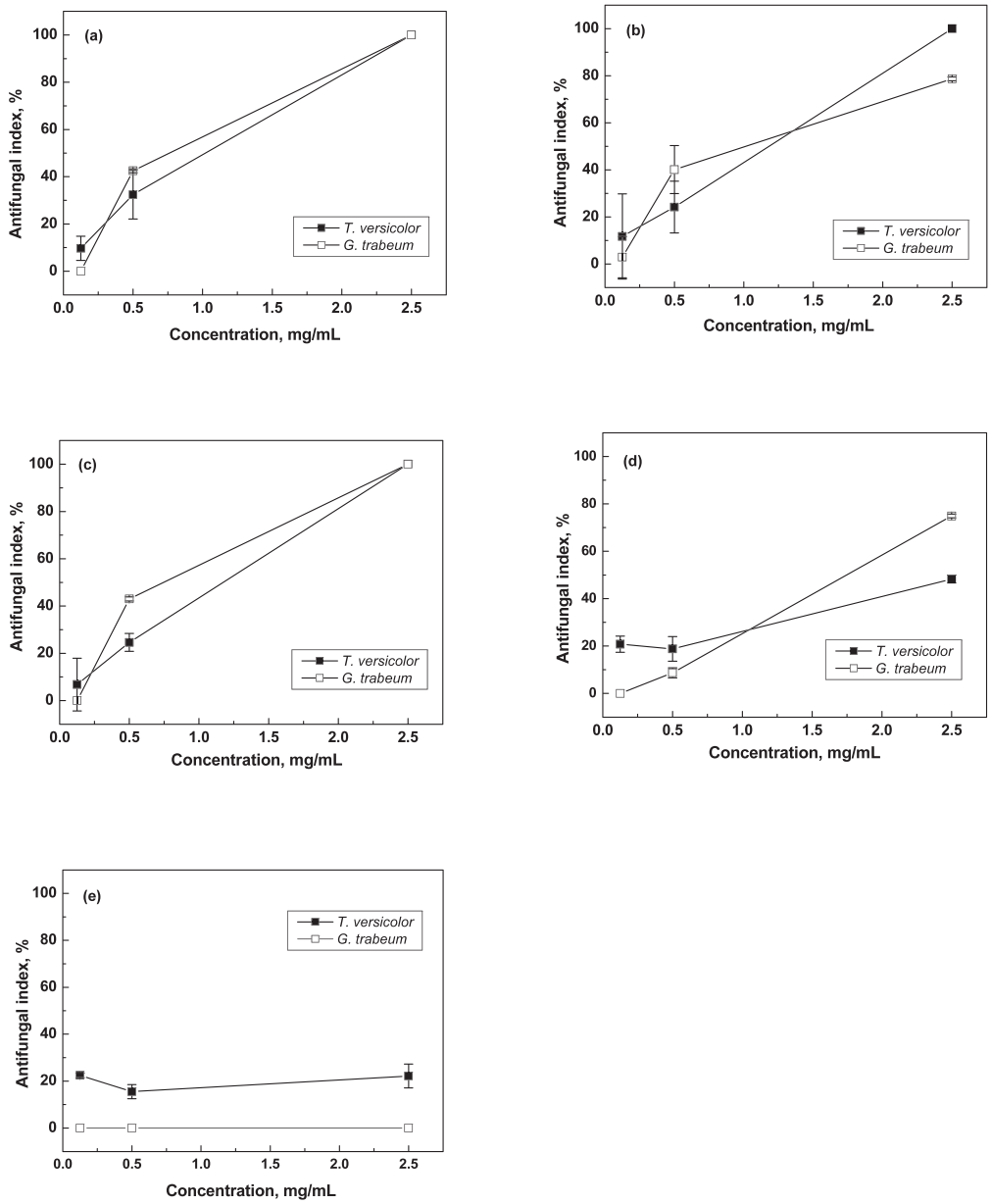


Figure 2.—Antifungal activity of organic solvent extracts prepared from Juniperus virginiana *heartwood against white rot and brown rot fungi: (a) methanol extract, (b) hexane soluble fraction, (c) chloroform soluble fraction, (d) ethyl acetate soluble fraction, (e) methanol soluble fraction.*

The sesquiterpene alcohol cedrol was the most abundant component in both hexane and chloroform fractions. Eller and King (2000) also reported that cedrol was a major compound in the cedar wood oil, but another sesquiterpene alcohol, widdrol, also existed in smaller amounts and appeared next to the cedrol peak on their gas chromatogram. However, widdrol was not found in our GC-MS analysis. Very small amounts of widdrol may exist in Peak 10 on the TIC, but poor resolution of our GC column (25 m in length) could not further separate the compounds in this peak. Cedrol and widdrol in the cedarwood oil were separated using a 60-m SP-2380 column by Eller and King (2000). These sesquiterpene alcohols, cedrol and widdrol, in *J. virginiana* heartwood are known to contribute to termiticidal toxicity (Chang et al. 2003, Liu 2004). In addition, cedrol has been reported to have moderate antifungal activity against wood decay fungi (Cheng et al. 2011).

Cedrenes and thujopsenes were the second and the third highest components, respectively, in the hexane and chloroform fractions as shown in Table 1. These compounds are reported to have antimicrobial activity against the bacterium *Propionibacterium,* the pathogenic fungus *Phytophthora ramorum,* and yeast (Johnston et al. 2001, Manter et al. 2007) and therefore may account for antifungal activity in this study. The chloroform soluble fraction had less (49.8%) cedrol, cedrenes, and thujopsenes than the hexane soluble fraction (74.1%), although these were predominant components in the chloroform fraction. This lower percentage was mainly due to the partial removal of the two main compounds, cedrenes and thujopsenes, by hexane and thereby the residual concentrations were

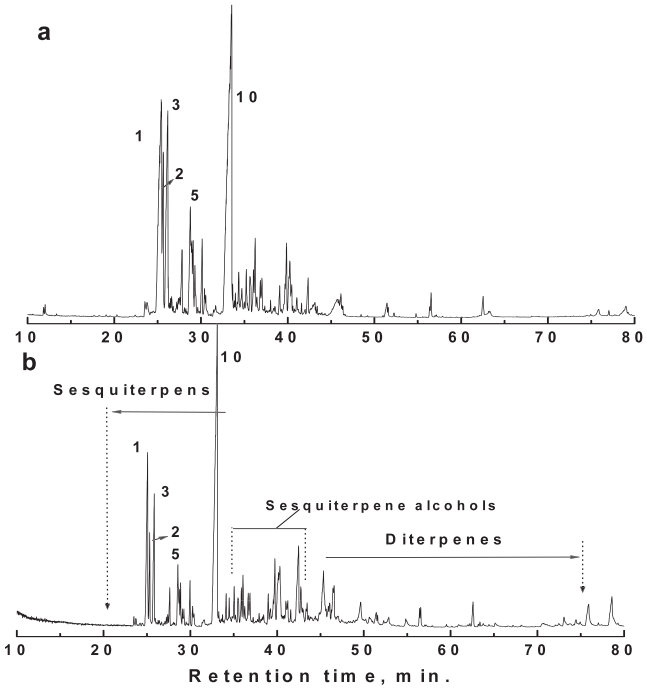


Figure 3.—Gas chromatography–mass spectrometry total ion chromatograms of (a) hexane and (b) chloroform soluble fractions of methanol extract from Juniperus virginiana *heartwood.*

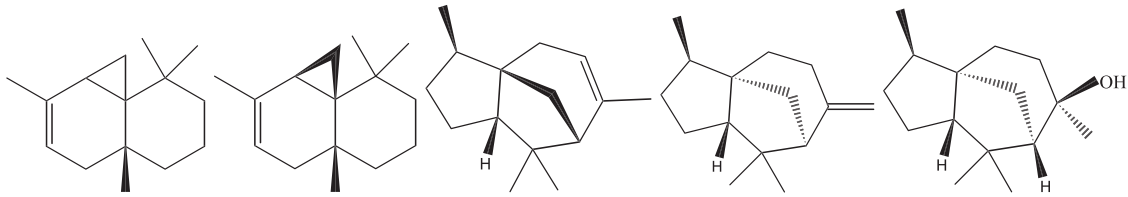
reduced. Some sesquiterpene alcohols (e.g., cedrene alcohols and clovan diol) increased or appeared for the first time in the chloroform extract. In addition, there were many unknown peaks in retention times from 39.87 to 42.78 minutes and from 45.69 to 53.17 minutes (Table 1). These peak areas accounted for 27.5 percent in the chloroform fraction, which was greater than in the hexane fraction (5.5%). Although the sum of the three major components was smaller in the chloroform soluble fraction compared with the hexane soluble fraction, the chloroform soluble fraction also showed a strong antifungal activity against wood decay fungi, as shown in Figure 2. This indicates that the antifungal activity was not only affected by the three major components but may also have been affected by unknown compounds and sesquiterpene alcohols. The many unknown peaks in the TIC were assumed to have originated from sesquiterpene alcohols from their mass fragmentation patterns and molecular weight of their parent ions. Therefore, it was thought that the strong antifungal activity of the chloroform soluble fraction was also attributed to these unknown sesquiterpene alcohols, but further study is needed. Several sesquiterpene alcohols such as cedrene alcohol and widdrol have already been reported to have very strong antifungal activity (Chang et al. 2003). Consequently, the high durability of *J. virginiana* heartwood is most likely due to three main components, cedrol, cedrenes, and thujopsenes, as well as other sesquiterpene alcohols.

Screening the individual major components of the hexane and chloroform fractions for antifungal activities against the two wood decay fungi revealed a lower inhibition observed than when the components were combined in the hexane and chloroform extracts. Cedrol, the most abundant compound in the chloroform and hexane extracts of *J. virginiana* heartwood showed the highest inhibition of 80 percent for *G. trabeum* but only 25 percent for *T. versicolor* at a concentration of 1.0 mg/mL. Because of its poor solubility in ethanol, higher concentrations of cedrol could not be prepared. If concentrations of cedrol above 1.0 mg/mL in ethanol could have been prepared, the fungal growth of *G. trabeum* might have been completely inhibited.

Table 1.—Composition of the hexane and chloroform soluble fractions of methanol extract from Juniperus virginiana *heartwood.*

Peak	RT (min)[a]	Molecular formula	Components	TIC area (%)[b]	
				Hexane	$CHCl_3$
1	25.42	$C_{15}H_{24}$	α-Cedrene	19.53	9.89
2	25.65	$C_{15}H_{24}$	β-Cedrene	5.48	3.09
3	26.18	$C_{15}H_{24}$	(*Z*)-Thujopsene	11.11	5.73
4	27.82	$C_{15}H_{24}$	β-Chamigrene	2.04	1.21
5	28.77	$C_{15}H_{24}$	(*E*)-Thujopsene-(I2)	5.38	2.99
6	28.97	$C_{15}H_{24}$	α-Chamigrene	1.53	—
7	29.10	$C_{15}H_{22}$	α-Cuparene	1.92	1.25
8	29.31	$C_9H_{12}O$	2-Ethyl-methyl phenol	1.63	—
9	30.14	$C_{15}H_{24}$	Cedrene isomer	2.21	1.36
10	33.11–34.85	$C_{15}H_{26}O$	Cedrol (may contain small amounts of widdrol)	38.00	31.08
11	35.24	$C_{15}H_{24}O$	Cedr-3-en-15-ol	Det.	1.21
12	35.65	—	Thujopsene alcohols?	1.76	1.31
13	36.06	—	Unknown	1.43	1.38
14	36.25	—	Cedrene alcohols?	2.14	1.66
15	39.87–42.78	—	Sesquiterpene alcohols	3.85	16.84
16	43.04	$C_{15}H_{26}O_2$	Clovan diol	—	2.48
17	45.69–53.17	—	Unknown	1.97	10.63
18	62.88	$C_{21}H_{30}O_3$	Hinokione (diterpenoids)	—	1.27
19	76.20	$C_{20}H_{22}O_6$	Matairesinol?	—	2.76
20	79.01	—	Triterpenoids	—	3.86
Total				100.0	100.0

[a] RT = retention time.
[b] Total ion chromatogram (TIC) peak area of more than 1 percent is presented. Det. = detected but less than 1 percent.



1 2 3 4 5

Figure 4.—Major sesquiterpenes and sesquiterpene alcohol found in hexane and chloroform soluble fractions of methanol extract from Juniperus virginiana *heartwood. 1 = (E)-thujopsene; 2 = (Z)-thujopsene; 3 = α-cedrene; 4 = β-cedrene; 5 = cedrol.*

Table 2.—Inhibition of selected terpenes and their concentrations against wood decay fungi, Gloeophyllum trabeum *and* Trametes versicolor.

Analytes	Concentration (mg/mL)	Inhibition (%)[a]	
		G. trabeum	*T. versicolor*
Thujopsene	2.5	47.1 ± 0.9	43.7 ± 2.8
Cedrol	1.0	80.6 ± 2.8	25.8 ± 7.3
	0.5	61.3 ± 4.8	15.2 ± 2.3
	0.125	61.3 ± 2.8	1.5 ± 2.8
Cedrene	2.5	31.5 ± 17.1	13.5 ± 5.5
	0.5	3.6 ± 1.6	6.0 ± 1.3
	0.125	9.2 ± 2.1	10.4 ± 2.0

[a] Values are means ± standard deviations.

Although we could not test the antifungal activity of cedrol at the highest concentration of 2.5 mg/mL used in this study for the other solvent extracts of *J. virginiana* heartwood, it is clear that cedrol plays a very important role in the growth inhibition of *G. trabeum*. Thujopsene showed the highest (44%) inhibition effect of compounds screened against *T. versicolor* at 2.5 mg/mL while cedrene showed only 31 and 13 percent inhibition against *G. trabeum* and *T. versicolor,* respectively. These results indicate that other components in addition to the three main components in the chloroform and hexane extracts of *J. virginiana* heartwood may also contribute to the antifungal activity against *G. trabeum* and *T. versicolor,* but further detailed studies will be needed to verify this assumption.

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