Antifungal Activity of Heartwood Extracts from Three *Juniperus* species

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Heartwood samples from three species of *Juniperus* (i.e., *J. virginiana, J. occidentalis,* and *J. ashei)* were extracted with hexane, ethanol, and methanol. The hexane and ethanol extracts were tested for antifungal activity against four species of wood-rot fungi (i.e, *Gloeophyllum trabeum, Postia placenta, Trametes versicolor,* and *lrpex lacteus).* Ashe juniper (AJ) gave the highest extract yields (6.60 to 11.27%), followed by Eastern red cedar (ERC) (4.78 to 9.56%), and then Western juniper (WJ) (4.26 to 7.32%). WJ contained the highest level of cedrol (over 60%), while AJ contained the highest level of thujopsene (over 30%). Methanol and ethanol gave the highest extract yields as well as slightly higher percentages of cedrol and widdrol. The juniper extracts were more effective against white-rot fungi than brown-rot fungi. The ethanol extracts had higher antifungal activity than the hexane extracts. The AJ extracts had the greatest bioactivity against the wood-rot fungi.

Keywords: Juniperus; Cedarwood oil; Extraction; Wood preservation; Wood-rot fungi; Hexane; Ethanol; Methanol; Impregnation

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INTRODUCTION

Several species of juniper, including Eastern red cedar (ERC) (*Juniperus virginiana* L.), Western juniper (WJ) (*Juniperus occidentalis* Hook.), and Ashe juniper (AJ) (*Juniperus ashei* J. Buchholz) (Cupressaceae) are very abundant conifers in the United States. The area covered by junipers has been expanding (Schmidt and Leatherberry 1995; Ganguli *et al.* 2008). In fact, three species are considered noxious species because of their encroachment onto rangeland and pastures (Adams *et al.* 1988).

Junipers are well known for their pleasant smell as well as their resistance to both termite attack and microbial decay. Because of this resistance, juniper has long been used for fence posts (Hemmerly 1970; Adams 2004). It has been hypothesized that these junipers may serve as a source of safe, natural wood preservatives from an abundant renewable resource. Extracts from these species may be used to impregnate susceptible wood species, making them resistant to subsequent attack by termites and other decay organisms.

Kamden (1994) reported that treating aspen blocks with methanol extracts from resistant wood species, including black locust, osage orange, and redwood, conferred significant resistance to *Gloeophyllum trabeum,* a brown-rot fungus. Cheng *et al.* (2005) reported that steam-distilled heartwood extracts of Japanese cedar *(Cryptomeria japonica* D. Don) had strong antifungal activities against the white-rot fungus *Trametes verisicolor*. Köse and Taylor (2012) investigated the resistance of heartwood and sapwood of *J. virginiana* to mold fungi and termites and reported that the heartwood was more resistant to both than sapwood.

Clark *et al.* (1990) reported that hexane and methanol extracts of *J. virginiana* heartwood and needles had antifungal and antibacterial activity; however, they did not include wood-rot fungi in their study. Using a petri dishlagar bioassay, Du *et al.* (2011) reported only weak antifungal activity of soxhlet hexane and supercritical CO_2-derived heartwood essential oils from *J. virginiana* against *T. versicolor* and *G. trabeum.* However, the yield reported for their hexane extraction (*i.e.*, 0.8%) was very low and may have affected their results. Mun and Prewitt (2011), also using a petri dish/agar bioassay, investigated the antifungal activity of methanol heartwood extracts of *J. virginiana* and several individual components of the essential oil against *T. versicolor* and *G. trabeum.* They reported thujopsene and cedrol as the most active components against *T. versicolor* and *G. trabeum,* respectively.

The purpose of this research was to expand the number of juniper species investigated as well as the number of wood-rot fungi tested and to extend previous antifungal tests from petri dishlagar bioassays to soil block tests. The specific objectives of this study were to determine the extraction yields from heartwood of *J. virginiana, J. occidentalis,* and *J. ashei* using several solvent treatments, compare the chemical compositions of the extracts, and test the bioactivity of the extracts towards four species of wood-rot fungi.

EXPERIMENTAL

Juniper Heartwood Samples

Heartwood samples from Eastern red cedar (ERC) (Woodford Co., Illinois), Western juniper (WJ) (Harney Co., Oregon, USA), and Ashe juniper (AJ) (Edwards Co., Texas, USA) were prepared from freshly cut trees (3 trees per species). Sapwood was removed from the samples using a band saw and heartwood sawdust was prepared using a compound miter saw. Sawdust samples were held in glass containers at room temperature prior to extraction.

Solvent Extraction

Extractions of juniper sawdust were performed with an ASE 200 Accelerated Solvent Extractor (Dionex Corp., Sunnyvale, CA, USA). There were seven solvent treatments: Hexane (Hex), Ethanol (EtOH), Methanol (MeOH), Ethanol after Hexane (EtOHtHex), Methanol after Hexane (MeOHtHex), Hexane after Ethanol (HextEtOH), and Hexane after Methanol (HextMeOH). Extraction conditions were as follows: 1500 psi, 80 °C, 5 min heat, 7 min static, 80% flush, 60 sec purge, and 3 cycles. Sawdust samples of *ca.* 2 g were placed in an 11-mL cell and extracted with *ca.* 25 mL of solvent. Extracts were dried under nitrogen and weighed. The extracted sawdust samples were dried overnight in a vacuum oven at 90 °C, and the percentage

yields were determined based on the dry weight of the wood. There were three replications per tree.

Chemical Analyses of Extracts

The extracts from the three species of juniper were analyzed by gas chromatography (GC) to compare their compositions to their fungal decay resistance. The cedarwood oil compositions of the extracts were determined by GC using a 5890 Series II gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, USA), equipped with an FID and an autosampler/injector. Analyses were conducted on a SP™ 2380 capillary column (60 m x 0.25 mm i.d.; 0.20 µm film thickness) (Supelco, Bellefonte, PA, USA), using helium as the carrier gas at a linear flow velocity of 18 cm/s. The temperature program was 60 °C for 1 min, then 5 °C/min until 250 °C was reached. The injector and detector temperatures were 235 °C and 250 °C, respectively. There were three trees per species and three replications pre tree (*i.e.*, n = 9).

Chemical standards of (-)-α-cedrene (CAS no. 469-6 1-4), (+)-ß-cedrene (CAS no. 546-28-l), (-)-thujopsene (CAS no. 470-40-6), (+)-cuparene (CAS no. 16982-00-6), and (+)-cedrol (CAS no. 77-53-2) were purchased from Fluka (Milwaukee, WI, USA).

Wood Block Conditioning and Impregnation

Spruce/pine/fir (SPF) blocks were used for tests utilizing the brown-rot fungi, and yellow poplar (YP) blocks were used for tests utilizing white-rot fungi. The 1 cm^3 wood blocks were conditioned to a constant mass at 27 °C and 50% relative humidity (RH) and weighed prior to impregnation. Specimens were placed in a beaker with an individual extract and held under vacuum (1 72 kPa) for 20 min twice to ensure complete removal of air from test specimens and penetration with extract solution. Following vacuum treatment, specimens were reweighed, air-dried, and re-conditioned at 27 °C and 50% RH. Treated specimens were gas-sterilized with propylene oxide prior to exposure to test fungi in the soil block test.

Although MeOH gave slightly higher yields than EtOH, the difference was quite small and EtOH is safer to work with than MeOH. In addition, a preliminary study utilizing a petri dish agar block test indicated that the MeOH extracts were no more effective than the EtOH extracts. This preliminary study utilized 13-mm filter paper discs treated with juniper heartwood extracts, the discs were placed on agar in petri dishes with wood-rot fungi and the zone of inhibition measured after a period of time. Therefore, to decrease the number of soil block test treatments, the MeOH extracts were not included in the impregnation experiments.

Extract solutions of Hex or EtOH were prepared to give incorporation rates that matched the original concentrations in the source wood. After vacuum impregnation, the solvent was allowed to evaporate and the blocks were re-conditioned to a constant mass at 27 °C and 50% RH.

After vacuum impregnation, the mean (± SEM) incorporation rates for the 1 cm^3 SPF blocks were determined to be: ERC/Hex 4.6% (±0.09), ERC/EtOH 9.5% (±0.37), WJ/Hex 4.0% (±0.27), WJ/EtOH 6.9% (±0.29), AJ/Hex 7.0% (±0.30, and AJ/EtOH 13.1% (±0.66). The mean (± SEM) incorporation rates for the 1 cm^3 YP blocks were determined to be: ERC/Hex 6.9% (±0.21), ERC/EtOH 15.2% (±0.56), WJ/Hex 6.1% (±0.14), WJ/EtOH 11.8% (±0.19), AJ/Hex 9.8% (±0.32), and AJ/EtOH 20.3% (±0.60).

Fungal Decay Resistance

Wood blocks that were vacuum-impregnated with individual extracts were tested for resistance to wood-rot fungi using Standard Method of Testing Wood Preservatives by Laboratory Soil-Block Cultures E 10-12 (American Wood Protection Association Standards, 2012). Two brown-rot fungi (*Gloeophyllum trabeum* (Pers.: Fr.) Murr (MAD 617) and *Postia placenta* (Fr.) M. Lars., et Lomb (MAD 698)) and two white-rot fungi (*Trametes versicolor* (L. Fr.) Pil. (MAD 697) and *Irpex lacteus* (Fr.: Fr.) Fr. (HHB 7328)) were tested. The nine treatments tested were: untreated control, hexane only, EtOH only, ERC/Hex, ERC/EtOH, WJ/Hex, WJ/EtOH, AJ/Hex, and AJ/EtOH. Weight loss was determined after an 8 week exposure to the fungi at 27 °C and 70% RH. There were six replications of each treatment per test fungus.

Statistical Analyses

Analyses of variance (ANOVA) were conducted on percentage data using Statistix 7 software (Analytical Software, Tallahassee, FL, USA). The main effects were tested using F-tests and means were compared using least significant difference (LSD) ($p = 0.05$). Linear contrasts were used to test for differences between brown-rot and white-rot fungi.

RESULTS AND DISCUSSION

Solvent Extraction

All Hex extracts were nearly colorless and produced light yellow oils after solvent evaporation. Both the polar solvents MeOH and EtOH gave similarly colored extracts. The polar solvent extracts of WJ produced light yellow oils, while the polar extracts of AJ and ERC produced amber and burgundy oils, respectively. The percentage yields for the juniper species and the solvents are shown in Table 1.

Table 1. Effect of Solvent on Mean[a] Percentage Extract Yields from *Juniperus* Heartwood

	Solvent Treatment						
Species	Hex	MeOH	EtOH	MeOH←Hex	EtOHtHex	Hex←MeOH	HextEtOH
ERC	4.78 *gh*	9.56 *c*	7.94 *d*	4.94 *g*	3.14 *j*	0.05 *l*	0.13 *l*
WJ	4.26 *i*	7.32 *e*	6.24 *f*	3.28 *J*	2.34 *k*	0.07 *l*	0.17 *l*
AJ	6.60 *f*	11.27 *a*	10.34 *b*	5.18 *g*	4.29 *h*	0.06 *l*	0.16 *l*

[a] Three trees per species and three replications per tree (*ie.*, $n = 9$ total) ($n = 18$ for hexane), means without letters in common differ significantly (LSD, $p = 0.05$)

The results of ANOVA indicated that both tree species ($F_{2,206} = 100.8$, $p = 0.0000$) and solvent ($F_{7,206} = 394.7$, $p = 0.0000$) had significant effects on extraction yield. AJ gave the highest yields, followed by ERC, and finally WJ. Adams (1987) using soxhlet hexane extraction, reported somewhat lower results for AJ, ERC and WJ (*i.e.*, 7.0%, 1.9%, and 3.0%, respectively). Previously, it was reported that ethanol extraction gave a

yield of 5.9% from ERC (Eller *et al.* 2010). The present results are quite similar to previously reported results.

MeOH gave the highest yields, followed by EtOH, and then Hex. Previously, Mun and Prewitt (2011) reported soxhlet methanol yields of 5.26% from ERC. The somewhat higher results reported in this study are probably a result of the higher extraction temperatures used (*i.e.*, 80 °C). The extraction yields for HextMeOH and extracts of HextEtOH were practically nothing. The sums of the Hex yields plus the subsequent polar solvent yields (*i.e.*, MeOHtHex or EtOHtHex) were equivalent to the MeOH only and EtOH only treatments, respectively. This indicates that both MeOH and EtOH extract everything that Hex extracts (*i.e.*, non-polar compounds) plus additional polar materials not extracted by Hex.

Chemical Analyses of Extracts

The cedarwood oil compositions for the three juniper species are shown in Fig. 1. The ANOVA indicated that the juniper species had a significant effect on the percentage of thujopsene ($F_{2,6}$ = 32.76, p = 0.0006), cedrol ($F_{2,6}$ = 10.07, p = 0.012), and widdrol ($F_{2,6}$ = 37.85, p = 0.0004). The ANOVA also indicated the juniper species had no significant effect on either the percentage of a-cedrene ($F_{2,6}$ = 3.70, p = 0.09) or the percentage ß-cedrene ($F_{2,6}$ = 3.04, p = 0.12). WJ had the highest percentages of cedrol and widdrol (61.7% and 18.0%, respectively), and ERC had a high percentage of cedrol as well (52.4%). AJ had the highest percentage of thujopsene (34.8%). Adams (1987) reported similar results for these three species of junipers using steam distillation, with the exception that cedrol from ERC was only 15.8% and a-cedrene was 27.2%. This may have been a result of the conditions of the steam distillation, causing the dehydration of cedrol to a-cedrene (Eller and Taylor 2004).

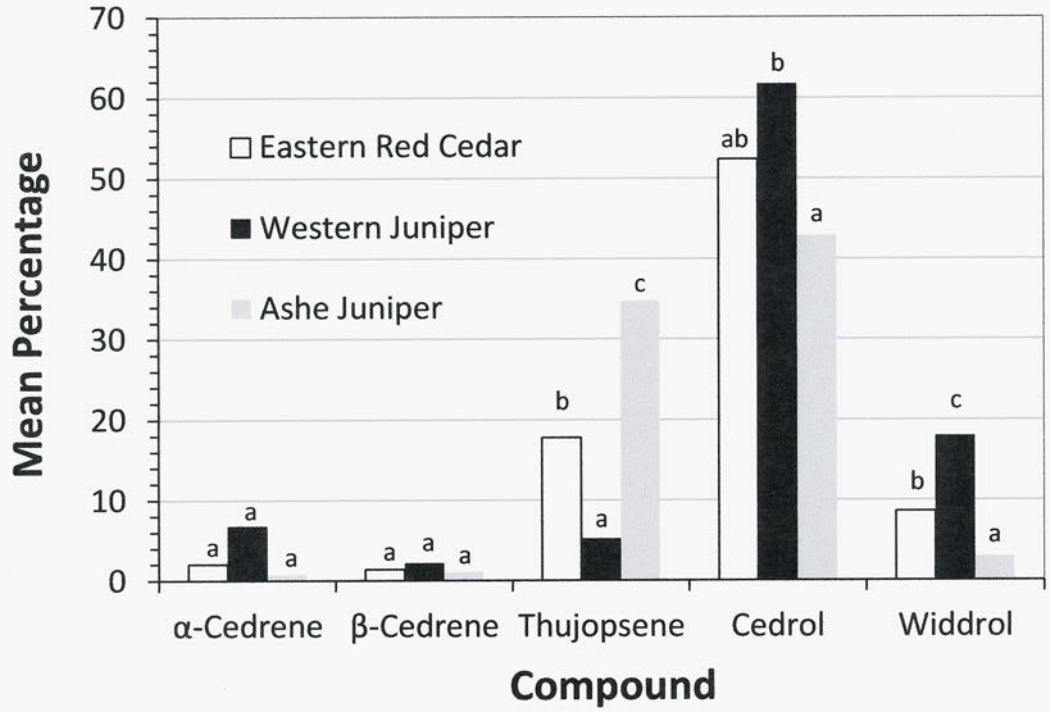

Fig. 1. Mean[a] % of cedarwood oil components in heartwood extracts of *Juniperus* species; [a] Means (n = 9) within a cluster with different letters differ significantly using LSD (p = 0.05)

The effect of solvent on cedarwood oil composition is shown in Fig. 2. The ANOVA indicated that the species of juniper had a significant effect on the percentages of thujopsene ($F_{2,12}$ = 6.72, p = 0.011), cedrol (F2,12 = 5.41, p = 0.021), and widdrol ($F_{2,12}$ = 10.64, p = 0.008). The ANOVA indicated that there were no significant effects of juniper species on either the percentage of a-cedrene ($F_{2,12}$ = 1.03, p = 0.38) or the percentage of ß-cedrene ($F_{2,12}$ = 0.86, p = 0.45). Interestingly, the polar solvents MeOH and EtOH yielded higher percentages of the polar compounds cedrol (over 52%) and widdrol (over 10%), of which both are sesquiterpene alcohols, than did the non-polar solvent, Hex. Conversely, Hex yielded higher percentages of the non-polar thujopsene (over 20%), a sesquiterpene hydrocarbon.

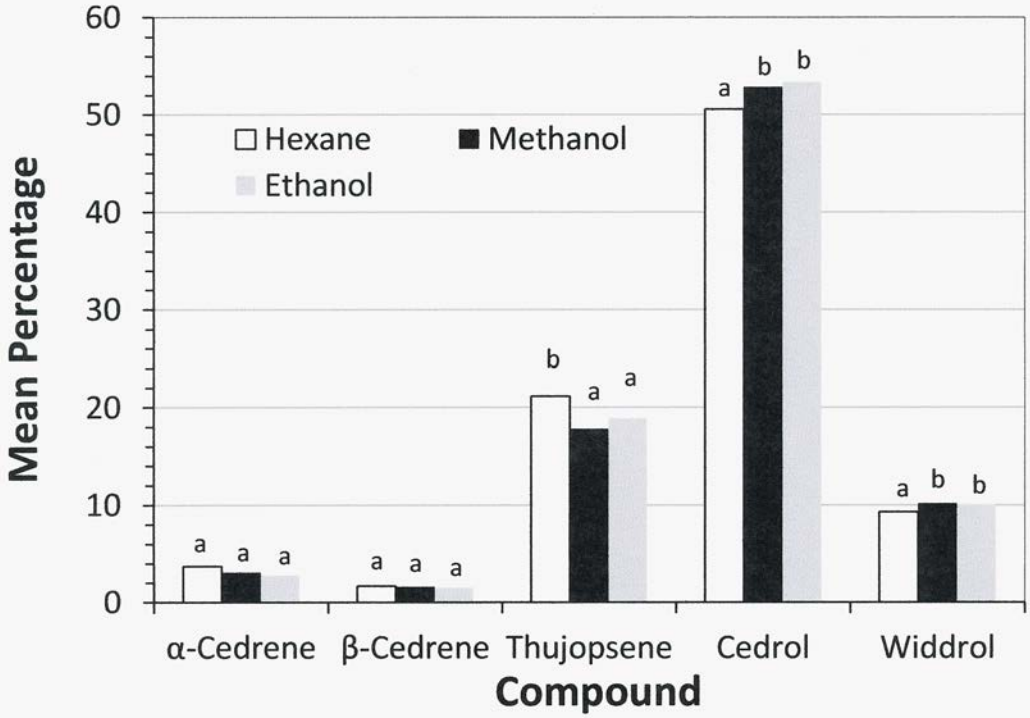

Fig. 2. Effect of solvent on mean[a] percentage cedarwood oil composition
[a] Means (n = **9)** within a cluster with different letters differ significantly using LSD (P = 0.05)

Fungal Decay Resistance

The mean weight loss percentages for the wood blocks exposed to the decay fungi are shown in Table 2. The ANOVA indicated that there were significant differences between fungal species ($F_{3,160}$ = 135.9, p = 0.0000), solvent ($F_{1,160}$ = 16.9, p = 0.0001), and *Juniperus* species ($F_{3,160}$ = 172.7, p = 0.0000) on wood loss percentage. The data indicated that the Juniperus extracts inhibited the white-rot fungi greater than the brown-rot fungi, and the EtOH extracts were slightly more inhibitory than the Hex extracts. In addition, the extracts of AJ showed the greatest inhibitory activity, followed by WJ, and finally ERC. Previously, it was reported that weight losses were higher for extractive-treated wood blocks exposed to *P. placenta* than for treated wood blocks exposed to *G. trabeum* (Eller *et al.* 2010). This was true in this study as well. Wang *et al.* (2011) studied steam-distilled foliage essential oils from several wood species, including *J. occidentalis* against *P. placenta* and *T. versicolor*, and also reported greater inhibition of *T. versicolor*

than *P. placenta*. These results indicate that of the wood-rot fungi studied, the brown-rot *P. placenta* is the most aggressive and the white-rot *I. lacteus* is the least aggressive.

Table 2. Effect of *Juniperus* Species and Solvent on Mean[a] Percentage Weight Loss from Woodblocks Exposed to Decay Fungi

	Decay fungus			
	Brown-rot		White-rot	
Species/solvent	*G. trabeum*	*P. placenta*	*T. versicolor*	*I. lacteus*
Untreated control	56.37 *a*	60.18 *a*	29.10 *a*	51.04 *a*
Hexane only	38.09 *bcd*	61.15 a	30.57 *ab*	50.78 *a*
Ethanol only	48.83 *ab*	60.39 *a*	32.88 *a*	41.2.1 *b*
Eastern red cedar				
Hexane	41.44 *bc* *	49.95 *bc*	24.43 *bc* *	37.52 *b* *
Ethanol	30.23 *cde* *	44.02 *cd* *	12.41 *d* **	9.10 *c* ***
Western juniper				
Hexane	27.67 *de* *	55.35 *ab*	21.26 *c* **	2.44 *c* ***
Ethanol	30.05 *cde* *	38.14 *de* *	7.64 *d* ***	2.20 *c* ***
Ashe juniper				
Hexane	25.43 *e* *	5.24 *f* ***	13.62 *d* **	2.29 *c* ***
Ethanol	18.82 *e* **	32.71 *e* *	7.64 *d* **	3.61 *c* ***

[a] Means (n = 6) within a column without letters in common differ significantly (LSD, P = 0.05); *, **, and *** denote moderately resistant, resistant, and very resistant, respectively American Society for Testing & Materials (1998).

Many of the extracts exhibited at least some resistance to decay fungi, and several were quite resistant, especially the AJ extracts. This may be due in part to the AJ giving the highest yields of any of the junipers tested. Because the AJ gave the highest extract yields and the treatment incorporation rates were chosen to match the concentrations in the source heartwoods, the high antifungal activity of the AJ may have been due in part to the relatively high concentration used. It may be possible to improve the antifungal activity of the other juniper extracts by merely increasing their incorporation rates.

It may also be worth noting that AJ also contained very high levels of thujopsene and cedrol. Mun and Prewitt (2011) reported thujopsene and cedrol as the most active components against *T. versicolor* and *G. trabeum*, respectively. It is possible that these compounds are important components of the essential oil's antifungal activity. Although it is likely some individual components of essential oil extracts will have more antifungal activity than other components, it may not be cost effective to separate them from one another prior to use. It is likely that the other components confer some undiscovered benefit as well and the unrefined extract could be the cheapest and overall most cost effective material to use.

Essential oils are generally perceived as safer alternatives to synthetic pesticides and recently, Duringer *et al.* (2010) reported that heartwood extracts of Port Orford cedar

posed little to no risk to aquatic organisms. These results suggest that essential oils from *Juniperus* species could become an environmentally friendly natural wood preservative from a renewable and currently underutilized resource.

CONCLUSIONS

1. Ashe juniper gave the highest extract yield, followed by Eastern red cedar, and then Western juniper.
2. MeOH and EtOH produced the highest extract yields.
3. The juniper extracts were more active against white-rot fungi than brown-rot fungi.
4. The EtOH extracts had higher antifungal activity than the Hex extracts.
5. Ashe juniper extracts had the greatest bioactivity against the wood-rot fungi.
6, The major components of cedrol and thujopsene are likely largely responsible for the antifungal activity of the extracts, especially against *T. versicolor* and *G. trabeum.*

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