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Critical fluid extraction of *Juniperus virginiana* L. and bioactivity of extracts against subterranean termites and wood-rot fungi☆

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ABSTRACT

Eastern red cedar (*Juniperus virginiana* L.) is an abundant renewable resource and represents a vast potential source of valuable natural products that may serve as natural biocides. Both the wood and needles from *J. virginiana* were extracted using liquid carbon dioxide (L-CO_2) as well as ethanol (EtOH) and the yields determined. Wood blocks were vacuum impregnated with the extracts and subsequently tested for resistance against eastern subterranean termites (*Reticulitermes flavipes*) and two species of brown-rot fungi (*Gloeophyllum trabeum* and *Postia placenta*). Cedarwood oil (CWO) yields (fresh weight) were 2.3% and 5.9% using L-CO_2 (CWO-CO_2) and EtOH (CWO-EtOH), respectively. The yield (i.e., fresh weight) of juniper leaf oil using EtOH was 6.6% of the unground needles. Laboratory termite testing indicated that the ethanol extract of needles was lethal to the termites and resulted in no damage to the test blocks. The CWO-CO_2 and the CWO-EtOH treatments were statistically equivalent and both exhibited significant resistance to termite damage compared to untreated controls. The CWO extracts were significantly more effective against *G. trabeum* than *P. placenta*. For *G. trabeum*, the CO_2-derived CWO was statistically equivalent to the uninoculated control. On the other hand, for *P. placenta*, only the EtOH-derived CWO conferred any significant inhibition. The ethanol extract of the needles did not inhibit either test fungus. These extracts from *J. virginianna* may provide a renewable source of safe natural wood preservatives.

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1. Introduction

Eastern red cedar (ERC) (*Juniperus virginiana* L.) (Cupressaceae) is the most widely distributed North American conifer east of the Mississippi River (Folwells, 1965) and the area covered by junipers has been expanding recently (Schmidt and Leatherberry, 1995). Junipers have encroached on 10.2 million acres in Texas and Oklahoma (Weidemann and Cross, 1996) and there has been a concerted effort to control this species (Bidwell et al., 1991). Because of its invasive character and its encroachment onto rangeland with the subsequent loss of forage production, it is considered a pest species in some areas (Alemayehu et al., 1998). These junipers, however, represent a vast underutilized resource with potential economic opportunities.

Eastern red cedar wood is known for its aromatic smell, toxicity and repellency to several species of insects including clothes moths (Huddle and Mills, 1952), flour beetles (Sighamony et al., 1984), cockroaches (Appel and Mack, 1989) and ants (Thorvilson and Rudd, 2001; Meissner and Silverman, 2001). Zhu et al. (2001) found that cedarwood oil (CWO) repelled termites. Juniper has been shown to be resistant to both Formosan (Morales-Ramos and Rojas, 2001) and eastern subterranean (Carter and Smythe, 1974; Arango et al., 2006) termite attack and has long been used for fence posts (Hemmerly, 1970; Adams, 2004). Particleboard-chip panels made from ERC are moderately resistant to termite damage (Kard et al., 2007).

Antitermitic compounds can be removed from ERC heartwood by organic solvent (i.e., acetone, pentane, hexane or methanol) extraction and these extracts are toxic to eastern subterranean termites (Carter and Smythe, 1974; Carter, 1976; Adams et al., 1988; McDaniel et al., 1989). An extract (acetone/hexane/water mix) of ERC significantly reduced termite attack when applied to southern pine by vacuum impregnation (McDaniel and Dunn, 1994).

The needles of juniper also represent a potential resource. Adams (1987b) reported that methanol extracts of juniper needles gave very high yields and stated "the foliage is extremely rich

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name USDA implies no approval of the product to the exclusion of others that may also be suitable.

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in phytochemicals". "However, their chemical composition needs to be fully investigated to determine the economic feasibility for utilization". Hexane and methanol extracts of ERC needles both have termiticidal activities using a filter paper assay (Adams et al., 1988). Monoterpenes have demonstrated toxicity towards termites (Cornelius et al., 1997) and cedar leaf oil (CLO) from *J. virginiana* is known to contain many monoterpenes (Comer et al., 1982). It is likely that CLO monoterpenes may impart resistance against termites and/or wood-rot fungi.

Hexane and methanol extracts of *J. virginiana* heartwood and needles have antibacterial and antifungal activities, however, wood-rot fungi were not included in the study (Clark et al., 1990). Resistant wood species other than juniper (i.e., black locust, osage orange and redwood) can be extracted using methanol and aspen blocks treated with these extracts have significant resistance towards a brown-rot fungus (Kamden, 1994). Although CWO has been tested against termites, CWO has not been studied previously against wood-rot fungi.

CWO is generally obtained by steam distillation, however, because components of CWO can be degraded under the high temperature employed during steam distillation, solvent extraction is preferable to avoid such changes (Semen and Hiziroglu, 2005). Solvents such as acetone, pentane, hexane and methanol have been used to obtain active extracts (i.e., CWO and CLO) from *J. virginiana*, however, these solvents are hazardous due to their toxicity and flammability. Carbon dioxide, on the other hand, is both non-toxic and non-flammable and leaves no solvent residue in the extract and has been demonstrated to effectively extract CWO (Eller and King, 2000; Eller and Taylor, 2004).

A typical steam-derived CWO contains ca. 15.8% cedrol and 27.2% α-cedrene (Adams, 1987a) and the ratio of cedrol to α-cedrene is generally quite low (i.e., 0.11, 0.58, and 1.05) (Runeberg, 1960; Adams, 1987a, 1991a,b). However, when a solvent such as hexane, SC-CO_2 (40 °C; 1500 psi) or L-CO_2 (25 °C; 1500 psi) is used, the ratio of cedrol to α-cedrene is much higher (i.e., 10.1, 2.6 and 13.3) (McDaniel et al., 1989; Eller and King, 2000; Eller and Taylor, 2004). The degradation (i.e., dehydration) of the sesquiterpene alcohol cedrol to the sesquiterpene hydrocarbon cedrene is promoted by the acidic conditions found during extractions of wood at elevated temperatures with water (e.g., steam distillation) (Eller and Taylor, 2004).

Avoiding the degradation of cedrol to α-cedrene is critical because cedrol and widdrol were identified from CWO extracts as having the greatest termiticidal activity, while the sesquiterpene hydrocarbons were essentially innocuous (McDaniel et al., 1989; McDaniel and Dunn, 1994). Sesquiterpene alcohols also confer resistance to other wood species. Cataponol (catalpa) has antitermitic activity (McDaniel, 1992), muurolol (port orford cedar) has antifungal activity (Gao et al., 2008), and cadinol (incense cedar) has both antitermitic and antifungal activity (Cheng et al., 2004).

Although organic solvent extracts of ERC have been tested against termites, the bioactivity of CO_2-derived or EtOH-derived extracts of ERC has not been previously investigated. In addition, CWO has not been previously tested against wood-rot fungi. The objective of this research was to test the hypothesis that L-CO_2 and ethanol can effectively extract natural biocides from ERC heartwood and needles and these extracts can be impregnated into wood to impart resistance against termites and wood-rot fungi.

2. Materials and methods

2.1. Eastern red cedar extraction

Cedarwood sawdust (from both heartwood and sapwood), obtained from a commercial sawmill, was extracted using L-CO_2 (CWO-CO_2) (25 °C; 1500 psi) and the CWO composition was determined by gas chromatography (relative peak areas) as described previously (Eller and Taylor, 2004). Cedar leaf (i.e., needle) samples, including both old and new foliage, were collected in Woodford County, IL, from several individual male trees in March 2006, transported to the laboratory, separated from the stems and extracted as quickly as possible. A L-CO_2 extraction (25 °C; 1500 psi) of the needles was performed but resulted in essentially no collected CLO. A Dionex Accelerated Solvent Extractor (ASE) 300 (Dionex Corp., Sunnyvale, CA) was used to extract both the cedarwood sawdust as well as the unground needles using absolute ethanol (CWO-EtOH and CLO-EtOH, respectively). The extraction conditions were the same for the sawdust and needles and were as follows: 100 °C, 1500 psi, 7 min static, 3 cycles, 60 s purge. Extracts were dried under a gentle stream of nitrogen to a constant weight. All yield data were based on the fresh weight of the sawdust or needles. There were five replications of each extraction treatment.

2.2. Wood block preparation, conditioning and impregnation

Southern pine blocks were prepared from a board milled to 2.54 cm × 2.54 cm × 0.64 cm blocks for termite resistance testing (AWPAS, 2007a) and 1-cm^3 blocks for fungal decay testing (AWPAS, 2007b). The milled wood blocks were conditioned to a constant mass at 50% relative humidity (RH) and 25 °C prior to impregnation. Extracts were impregnated using a vacuum impregnation apparatus as previously described (AWPAS, 2003). Solutions of CWO in EtOH and CLO in EtOH were prepared to give target incorporation rates of ca. 5% of the wood block mass. The extracts tested were: EtOH solvent only control (Control); CWO derived by L-CO_2 extraction (CWO-CO_2); CWO derived by EtOH extraction (CWO-EtOH); and CLO derived by EtOH extraction (CLO-EtOH). After vacuum impregnation, the EtOH was allowed to evaporate and the blocks re-conditioned to a constant mass at 50% RH and 25 °C. The actual mean (±SEM) incorporation rates for the 2.54 cm × 2.54 cm × 0.64 cm blocks for the three extracts were determined to be: CWO-CO_2 5.0% (±0.9); CWO-EtOH 5.7% (±0.9); and CLO-EtOH 3.8% (±0.9). The mean (±SEM) incorporation rates for the 1-cm^3 blocks were determined to be: CWO-CO_2 5.7% (±0.6); CWO-EtOH 5.6% (±0.6); and CLO-EtOH 6.3% (±0.6).

2.3. Termite resistance testing

Using a no-choice test (i.e., only one treatment per container), impregnated wood blocks were compared for resistance to eastern subterranean termites, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) using Standard Method for Laboratory Evaluation to Determine Resistance to Subterranean Termites E1-06 (AWPAS, 2007a). The five treatments tested were: EtOH Control; CWO-CO_2; CWO-EtOH; CLO-EtOH; and cedarwood heartwood blocks (Cedarwood) milled from a kiln-dried ERC board purchased locally. Weight loss was determined after a 4-week exposure to the termites. There were six replications of each treatment.

2.4. Fungal decay resistance testing

Impregnated wood blocks were tested for resistance to wood-rot fungi using Standard Method of Testing Wood Preservatives by Laboratory Soil-Block Cultures E10-06 (AWPAS, 2007b). Two species of brown-rot fungi (basidiomycete) were tested: *Gloeophyllum trabeum* MAD-617 (Persoon: Fries) Murrill and *Postia placenta* MAD-698 (Fries Larsen et Lombard). The four treatments tested were: EtOH Control; CWO-CO_2; CWO-EtOH; and CLO-EtOH. Weight loss was determined after an 8-week exposure to the fungi at 27 °C and 70% RH. There were four replications of each treatment per test fungus, including EtOH solvent controls and two replications of an uninoculated treated control.

2.5. Statistical analyses

Analyses of variance (ANOVA) were conducted on the mean percentage weight loss data using Statistix 7 software (Analytical Software, Tallahassee, FL, USA) and means were compared using least significant difference (LSD) at $P = 0.05$.

3. Results and discussion

3.1. Extraction yields and compositional analysis

The mean (±SEM) CWO extraction yields using L-CO_2 and EtOH were 2.3% (±0.1) and 5.9% (±0.06), respectively based on the fresh weight of the sawdust. These L-CO_2 and EtOH yields are close to the 3.0% and 4.0% yields previously reported for hexane-derived and methanol-derived CWOs, respectively (Adams, 1987b). The CWO-CO_2 was light amber in color while the CWO-EtOH was much darker than the CWO-CO_2 and burgundy in color. GC analyses indicated that the two CWOs were essentially identical in composition to each other and similar to a previous report using SC-CO_2 (Eller and King, 2000). The composition means (±SEM) of the four most abundant components were: α-cedrene 12.8% (±1.4), thujopsene 17.3% (±1.4), cedrol 52.4% (±3.5), and widdrol 4.9% (±0.4). The ratio of cedrol to α-cedrene was 4.1 and similar to previously described solvent-derived CWOs (i.e., 10.1, 2.6 and 13.3) (McDaniel et al., 1989; Eller and King, 2000; Eller and Taylor, 2004). The mean (±SEM) CLO yield using EtOH was 6.6% (±0.2) based on the fresh weight of needles. Adams (1987b) reported yields of 1.9% and 39.9% for unground needles of *J. virginiana* using hexane and methanol, respectively.

3.2. Termite resistance

The mean weight loss percentages for the wood blocks exposed to the subterranean termites are shown in Fig. 1. The EtOH solvent control lost 58.0% of its mass and this weight loss was statistically higher than all other treatments. Conversely, the wood treated with the CLO-EtOH extract had almost no loss in mass (i.e., 0.4%). The CLO-EtOH extract is apparently very toxic to the termites and caused 100% mortality of the termites exposed to that treatment. The wood blocks of cedarwood *per se*, also had very little weight loss (i.e., 5.2%) and this treatment was statistically equivalent to the CLO-EtOH treated blocks with only 0.4% weight loss. The blocks treated with the CWO-CO_2 and CWO-EtOH had weight losses of 28.6% and 23.3%, respectively and these two treatments were statistically equivalent to one another. These two treatments were less heavily damaged than the control but more heavily damaged than either the CLO-EtOH or cedarwood *per se*.

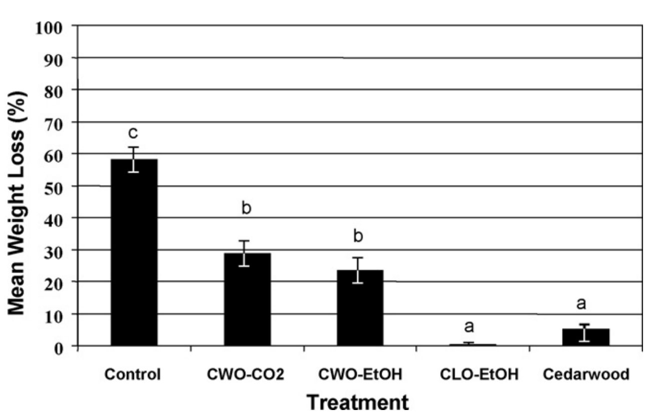

Fig. 1. Mean (±SEM) percentage weight loss for vacuum-impregnated wood blocks exposed to subterranean termites, *Reticulitermes flavipes*. Treatment descriptions: ethanol solvent control (control); CWO derived by L-CO_2 extraction (CWO-CO_2); CWO derived by EtOH extraction (CWO-EtOH); CLO derived by EtOH extraction (CLO-EtOH) and cedarwood heartwood blocks (Cedarwood). Means without letters in common differ significantly using LSD ($P < 0.05$).

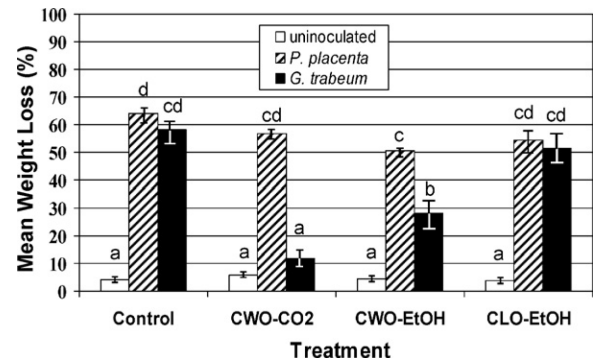

Fig. 2. Mean (±SEM) percentage weight loss for vacuum-impregnated wood blocks exposed to brown-rot fungi, *Gloeophyllum trabeum* and *Postia placenta*. Treatment descriptions: ethanol solvent control (control); CWO derived by L-CO_2 extraction (CWO-CO_2); CWO derived by EtOH extraction (CWO-EtOH); and CLO derived by EtOH extraction (CLO-EtOH). Means without letters in common differ significantly using LSD ($P < 0.05$).

3.3. Fungal decay resistance

The mean weight loss percentages for the wood blocks exposed to the decay fungi are shown in Fig. 2. The weight losses for the wood blocks in the uninoculated jars showed very little weight loss, ranging from 3.9% to 6.0% and there were no statistically significant differences in weight losses between the treatments from the uninoculated jars. In general, the weight losses were higher for the wood blocks exposed to *P. placenta* than for the wood blocks exposed to *G. trabeum*. For *P. placenta*, the mean weight losses for the blocks treated with CWO-CO_2 (i.e., 56.9% loss) or CLO-EtOH (i.e., 54.5% loss) were statistically equivalent to that for the solvent control blocks (i.e., 64.1% loss). The blocks treated with the CWO-EtOH exhibited the most resistance, significantly less than the untreated controls, however, the weight loss was still quite high at 50.5%.

For *G. trabeum*, the CLO-EtOH treated blocks exhibited 51.4% weight loss and this was statistically equivalent to the solvent control blocks (i.e., 58.1% loss). Conversely, both heartwood and leaf oils from Japanese cedar (*Cryptomeria japonica*) showed high activity against *G. trabeum* (Cheng et al., 2005). Interestingly, the leaf oils from *C. japonica* contain high amounts of sesquiterpenes and diterpenes and relatively low amounts of terpenes (Cheng et al., 2005) while the leaf oil from *J. virginiana* contains mostly terpenes. The heartwood oils from both *J. virginiana* and *C. japonica* contain mostly sesquiterpenes and are both active against *G. trabeum* suggesting the role of sesquiterpenes in this resistance.

The CWO-EtOH treated blocks had a 28.1% weight loss for *G. trabeum* which was statistically less than the solvent control (i.e., 58.1% loss) and those treated with CLO-EtOH (51.4% loss). An average weight loss of 25–44% constitutes moderate resistance in a natural decay resistance test (ASTM, 1998). The blocks treated with CWO-CO_2 exhibited the highest resistance towards *G. trabeum* with only 11.7% weight loss, which was statistically equivalent to the uninoculated control (i.e., 6.0% loss). Average weight loss of 11–24% in a natural decay resistance decay test is considered resistant to decay (ASTM, 1998).

The data demonstrate that extracts from *J. virginiana*, especially CWO, can impart significant resistance to both termites as well as wood-rot fungi. Cedarwood oil is Generally Regarded As Safe (i.e., GRAS) by the U.S. Food and Drug Administration, and because CWO poses little or no risk to public health or the environment, the U.S.

Environmental Protection Agency has exempted CWO from federal pesticide regulation. Therefore, CWO could become a safe natural alternative to other wood preservatives as well as being a renewable product from cedars and cedarwood wastes (i.e., sawdust) not currently utilized (Adams et al., 1988).

The overall results suggest that the CWO-EtOH may offer slightly more resistance than the CWO-CO_2. In addition, because the CWO-EtOH was a dark burgundy in color, the impregnated wood also took on some of that color as well, thus making it appear more like actual cedar heartwood. Although the CWO treated wood did confer significant protection against both termites and wood-rot fungi, an even higher level of resistance would be preferable. This is especially true for *P. placenta*, which was only slightly inhibited by the CWO-EtOH treatment. It may be possible to increase the effectiveness of the extracts by using a higher concentration of CWO or using a modified CWO formulation. Additionally, it may be possible to increase the effectiveness of the CWO as a wood preservative by the addition of materials such as free radical scavengers/antioxidants or metal chelators as discussed by Schultz and Nicholas (2001). This may increase the effectiveness against *P. placenta* which was affected only slightly by any of the extracts.

The needles of ERC represent a very large biomass and potentially rich source of biologically active phytochemicals (Adams, 1987b) in the CLO. Small trees have relatively low amounts of CWO because small trees have high percentages of sapwood which contains very low amounts of CWO compared to the heartwood (Payne et al., 1999; Dunford et al., 2007). Consequently, small trees have relatively large amounts of CLO compared to CWO. However, because CLO contains significant amounts of saffrole (Adams, 2004, 1991a,b; Comer et al., 1982; Vinutha and von Rudloff, 1968; Setzer et al., 1992) and it is a suspected carcinogen (Merck Index, 1989), its presence in the leaf oil may limit its potential uses or require its removal prior to its subsequent use.

4. Conclusion

Wood preservation is a huge industry, and there is an immense potential for using natural products as wood preservatives. It had been estimated that if CWO captured a mere 1% of the wood preservation market in 1993, it would constitute a $50 million market for CWO (Mater Engineering Report, 1993). We found that both L-CO_2 and ethanol can effectively extract natural biocides from ERC heartwood as well as needles and these extracts can be impregnated into wood to impart resistance against termites and wood-rot fungi. Cedars are an abundant underutilized resource that could provide a renewable source of natural wood preservative materials from the sawdust co-product stream from cedar sawmills as well as meet consumer demand for safe products.

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