Leach and Mold Resistance of Essential Oil Metabolites

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ABSTRACT

Purified primary metabolites from essential oils were previously shown to be bioactive inhibitors of mold fungi on unleached Southern pine sapwood, either alone or in synergy with a second metabolite. This study evaluated the leachability of these compounds in Southern pine that was either dip- or vacuum-treated. Following laboratory leach tests, specimens were evaluated for inhibition of mold growth for 12 weeks and analyzed quantitatively by GC-MS for residual metabolite(s). With the exception of geraniol, vacuum treating the specimens generally provided longer protection than dip-treating. Citronellol and carvone protected vacuum-treated specimens from mold growth for the 4-week duration of the ASTM D4445-10 test, but efficacy diminished after extending incubation beyond 4 weeks. Thymol completely inhibited mold growth for 12 weeks in both dip-treated and vacuum impregnated specimens even though GC-MS analysis showed that 49% thymol leached from the vacuum-treated specimens.

Key words: leachability, essential oil, metabolite, mold fungi

INTRODUCTION

Essential oils are actively being evaluated as fungitoxic and insecticidal wood protectants. Select herbaceous plant essential oils have been reported to possess inhibitory properties against mold and decay fungi (Yang and Clausen 2006; 2007; 2008; Kartal et al. 2006; Klaric et al. 2006; Li et al. 2007), and subterranean termites (Chang and Cheng 2002; Zhu et al. 2003; Park and Shin 2005; Raina et al. 2007; Clausen and Yang 2008). There are concerns that the bioactivity of essential oil can be quite variable. There are no regulations for essential oils in the US; however, AFNOR (Association French Normalization Organization Regulation) and ISO (International Standards Organization) certification standardizes the chemical profile and principal constituents that differentiate therapeutic grade from lower grade (referred to as Grade A) essential oils. Analysis of the principal constituents is reported in certified oils to ensure that a minimal level of activity is present based on those constituents. Essential oils are complex mixtures comprised of a large number of constituents in variable ratios (van Zyl et al. 2006). However, certain plant species, such as thyme, oregano, basil, rosemary and mint are consistently bioactive due to one or more of the constituents of the oil (Isman and Machial 2006). Presumably, if the purified metabolites that are responsible for bioactivity can be identified their use in purified form for wood protection would alleviate concerns about variability.

This study is a follow-up to a report that evaluated the bioactivity in unleached Southern pine sapwood of purified principal metabolites from four essential oils (Clausen et al. 2010). The objectives of this study were 1) to evaluate the leachability of metabolites from Southern pine treated by dipping or vacuum impregnation and 2) to compare the efficacy of metabolites in unleached and leached Southern pine against mold fungi.

MATERIALS AND METHODS

Test Chemicals

Test chemicals obtained from Sigma-Aldrich (St. Louis, MO) are listed in Table 1. Concentrations evaluated were those that inhibited mold growth in unleached specimens (Clausen et al. 2010). Previous tests suggested that thymol and cymene might be bioactively synergistic so they were also evaluated together. Ninety-five percent ethanol was used as the diluent.

Table 1. Chemicals evaluated for resistance to leaching.

Test Chemical	Concentration (%)
Carvone	5
Citronellol	2.5
Geraniol	1.25
Thymol	10
Thymol:Cymene	10:5

Treatment

Southern pine sapwood specimens (7 x 20 mm cross section by 7 cm long) cut from kiln-dried lumber conditioned at 27ºC and 70% relative humidity (RH) were pre-weighed. For the dip-treatment, five random replicate specimens were dipped for ~30s in individual test solutions (Table 1). Dip-treated specimens were held in a covered container overnight according to ASTM standard test method D4445-10 (ASTM 2010) to allow chemical to penetrate. For vacuum impregnation, 5 random replicate specimens were vacuum-treated for 40 min at -172 kPa submerged in individual test solutions. All specimens were re-weighed to determine gross absorption and air-dried for 1 week prior to conditioning at 27ºC and 70% RH for 21 days.

Chemical leaching

Leaching was performed using a modification of AWPA E11-06 standard method for determining the leachability of wood preservatives (AWPA 2010). After conditioning to equilibrium moisture content, 4 specimens from each treatment group were weighed and placed into individual 500-mL beakers and submerged as a group in 285 mL of deionized (DI) water with mild agitation for a total of 14 days. Leach water was replaced with an equal amount of fresh DI water after 6 h, and 1, 2, 4, 6, 8, 10, 12, and 14 days. Following leaching, specimens were dried at 40ºC for 3 days before reconditioning at 27ºC and 70% RH to equilibrium moisture content. They were again weighed to allow estimation of chemical loss from leaching.

Specimen Analysis

Leached and unleached specimens were separately ground to 20-mesh and 2 g of the composite ground sample for each treatment group was extracted in 5 mL hexane. Extracts were analyzed for the test chemical by GC analysis with an Agilent 5973 GC/MSD (Agilent Technologies, Santa Clara, CA), equipped with DB-5HT capillary column (J&W Scientific, CA, 30 m x 0.25 mm i.d., 0.10 µm film thickness). Ultra pure helium at 2mL/min was used as carrier gas. Five replicate 2 µl splitless injections were made using a 7683 Series Autosampler and Injector (Agilent Technologies, CA). The injector was held at 280°C. Mass Spectra of the standard and samples were generated in the electron impact mode using the Mass Selective Detector (MSD). The scan range used was 45-350 amu. Individual metabolites served as control; calibration standards at 150, 100, 50, 20, 10 and 1.0 µg/mL were used to generate a standard curve. Unknown residual metabolite concentrations were calculated using the standard curve.

Test Fungi

Mold fungi, *Aspergillus niger* 2.242, *Penicillium chrysogenum* PH02, and *Trichoderma viride* ATCC 20476 (Forest Products Laboratory, Madison, WI), were grown on 2% malt extract agar (Difco, Detroit, MI) at 27° C, and 70% RH for 2 weeks. A mixed mold spore suspension was prepared by washing the surface of one Petri dish for each test organism with 10 mL of sterile deionized water (DI) according to American Society for Testing and Materials standard D4445-10 (ASTM 2010). Spores were collected, counted and equal numbers of spores for each test organism were transferred to a spray bottle. The spore mixture was diluted with DI water to yield approximately 3×10^7 spores mL^{-1}. The spray bottle was adjusted to deliver 1 mL inoculum per spray and was mixed frequently during inoculation to ensure homogeneous inocula.

Mold resistance Test

Leached specimens were evaluated for resistance to a mixed mold spore suspension consisting of three test fungi and prepared as described above. Leached specimens grouped by treatment were arranged over 4 layers of blotting paper that was saturated with 30 mL DI water and a polyethylene mesh spacer in sterile disposable Petri dishes (150 x 25mm) (B-D Falcon, Los Angeles, CA). Ethanol-treated wood blocks served as a diluent control. After spraying with 1 mL mixed mold-spore inoculum, plates containing test specimens were sealed in polyethylene bags to prevent drying and incubated at 27ºC and 70% RH. Specimens were individually visually rated for mold growth at 4, 8, and 12 weeks on a scale of 0–5 with 0 indicating the specimen was completely free of mold growth and 5 indicating the specimen was completely covered with mold growth.

RESULTS AND DISCUSSION

It was previously reported that primary metabolites from bioactive plant essential oils were effective inhibitors of mold spore germination for 12 weeks in unleached dip-treated Southern pine sapwood (Clausen et al. 2010). The purified bioactive metabolites included carvone, citronellol, geraniol, thymol, borneol and cymene. Cymene was only effective when it was combined with thymol in the same proportion that it occurs in the essential oil. This study evaluated five metabolites for their ability to inhibit mold growth following laboratory leach tests. Borneol was not included in this study due to availability and cost. Results from the Clausen et al. (2010) study suggested that thymol and cymene are synergistic, so the combination of thymol and cymene was tested at the same concentration and ratio that inhibited mold in the previous study.

Treatment Absorption

Gross absorption of the test chemicals based on wet weight after both treatment methods is shown in Figure 1. For all treatments, chemical uptake varied between 7 and 16 kg/m^3 for dip-treated specimens and between 7 and 17 kg/m^3 for vacuum-treated specimens. The greatest absorption difference between treatment methods was in the thymol:cymene group (6.86 kg/m^3). For all other chemicals tested, average absorption differences were $\leq$2 kg/m^3 between dip-treated and vacuum-treated specimens. The relatively small differences noted in absorption between treatment methods may be due to the combination of natural passive absorbance of oils into wood after dip-treatment and resistance of oil penetration into wood during vacuum treatment.

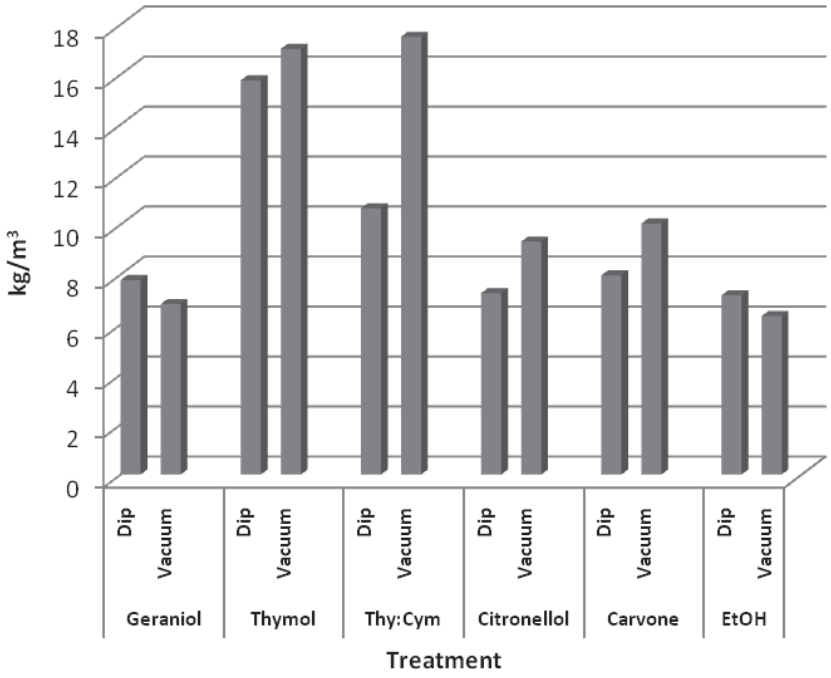

Figure 1. Pre-leach absorption of test chemical for dip-treated and vacuum impregnated Southern pine sapwood.

Mold Test

Mold resistance following leaching is summarized in Figure 2. Average mold ratings are represented for leached specimens that were either dip-treated or vacuum-treated with metabolites. One set of specimens was treated with a combination of thymol and cymene at a concentration previously shown to work synergistically and in proportions equivalent to those found in therapeutic grade thyme oil.

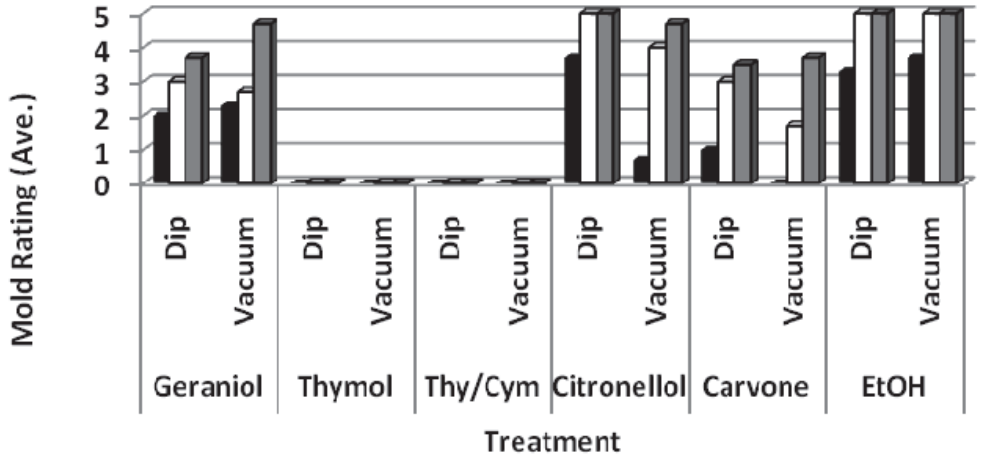

Figure 2. Average mold ratings for leached Southern pine dip- or vacuum-treated with metabolites after 4 weeks (black bars); 8 weeks (white bars); and 12 weeks (gray bars). Rating scale was 0–5 with 0 indicating specimens were completely free of mold growth and 5 indicating specimens were completely covered with mold growth.

Following leaching all thymol-treated specimens maintained complete inhibition of mold growth after 12 weeks in the ASTM D4445-10 test. Vacuum treatment was obviously more effective than dip treatment for citronellol- and carvone-treated specimens. Vacuum-treated carvone specimens continued to provide moderate protection against the mold fungi for 8 weeks.

GC-MS Analysis

Chemical retention was determined using GC-MS analysis of extracted wood and the total amount of chemical remaining after leaching was extrapolated for the whole specimen ($\mu g/cm^3$) (Table 2). The estimated percentage of chemical leached from representative sample treated by both methods is shown in Figure 3. The percentage of treatment chemical leached was based on the initial retention of chemical in treated wood specimens. Natural variations in treatment from specimen to specimen and extrapolation from a homogeneous sample to the whole specimen are possibly responsible for the variability seen between specimens and treatment methods. Results for dip-treated samples frequently suggested that more chemical was retained following leaching than before leaching. However, that was not true for specimens dip-treated with geraniol where GC-MS results showed that 100% geraniol leached out. Forty-nine to 65% of the thymol, geraniol and carvone leached from the vacuum impregnated specimens following leaching, but only 14 percent of the citronellol leached from vacuum impregnated specimens. Leach results did not correlate with mold inhibition or lack of inhibition shown in Figure 2. For example, despite 49% of the thymol leaching from vacuum-treated specimens, complete inhibition of mold test fungi was observed for at least 12 weeks.

Table 2. Estimated residual chemical after leaching of dip- and vacuum-treated Southern pine.

Chemical	Leach test	Treatment method	Residual[a] (µg/cm^3)
Geraniol	Yes	Dip	0.00
	No	"	1.37
	Yes	Vac	2.53
	No	"	5.60
Thymol	Yes	Dip	63.03
	No	"	46.52
	Yes	Vac	96.65
	No	"	190.74
Thy/Cym	Yes	Dip	57.01
	No	"	55.96
	Yes	Vac	88.13
	No	"	201.26
Citronellol	Yes	Dip	2.91
	No	"	2.51
	Yes	Vac	3.93
	No	"	4.57
Carvone	Yes	Dip	2.03
	No	"	1.74
	Yes	Vac	2.10
	No	"	6.00

[a]Residual chemical determined by GC-MS after leaching; results are extrapolated for the entire specimen.

Residual cymene was negligible in the specimens dip-treated with the combination of thymol and cymene and not detected in the vacuum-treated specimens. It was previously surmised that thymol and cymene worked synergistically to inhibit mold fungi, but results from this study showing that efficacy is retained in dip-treated specimens after cymene and thymol are leached did not support or disprove that theory (Clausen et al. 2010). Only 14% citronellol leached from the vacuum-treated specimens; citronellol provided good protection from mold growth for 4 weeks. Sixty-five percent of the carvone leached from vacuum-treated specimens yet vacuum-treated specimens controlled mold growth only slightly better than dip-treated specimens (100% leached) for 8 weeks.

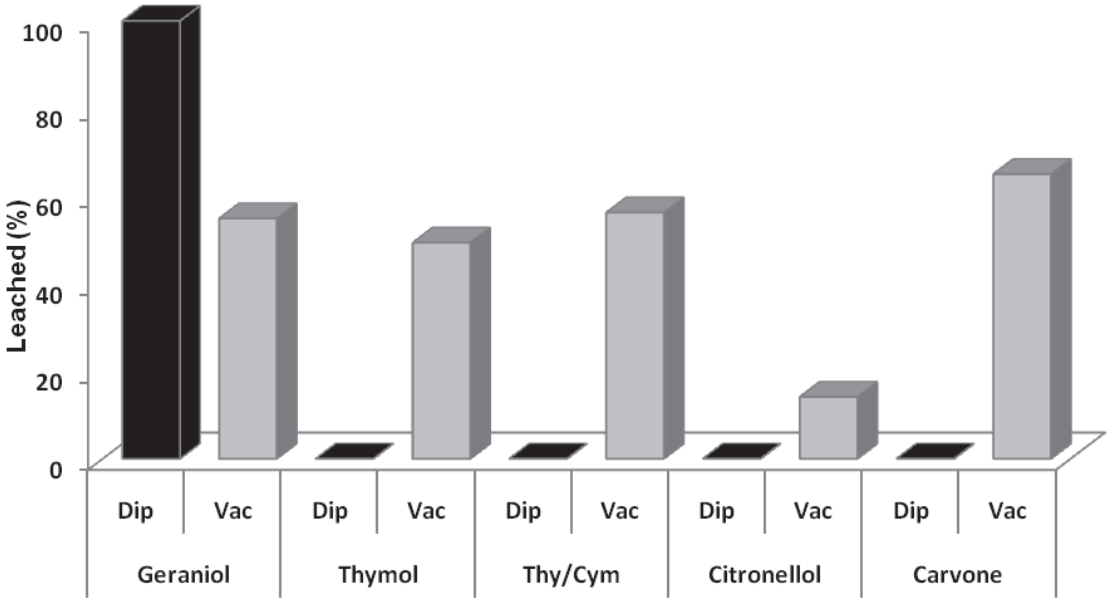

Figure 3. Percentage chemical leached from dip- and vacuum-treated specimens based on analysis by GC-MS of residual chemical following leaching.

CONCLUSIONS

Purified metabolites of bioactive essential oils were evaluated for leach resistance in Southern pine sapwood. Despite analysis by GC-MS that revealed 49% of the thymol leached out of vacuum-treated specimens, the leached specimens provided complete protection against mold fungi used in this study for at least 12 weeks. Likewise, citronellol and carvone provided adequate protection in vacuum-treated specimens for 4 weeks with diminishing efficacy after continued incubation. Chemical leaching was quite variable due to inherent variability in treatment methods and extrapolating to calculate residual chemical for an entire specimen. Leach resistance did not correlate with mold resistance.

Purified bioactive metabolites like those evaluated in this study would be more economical, readily available, and subject to even higher quality control than therapeutic essential oils. Utilizing purified forms of select bioactive metabolites from essential oils could have broad applications for wood protection, particularly if they retain long-term bioactivity even under conditions conducive to leaching.

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