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Research Article

Effects of White Mulberry (*Morus alba*) Heartwood Extract Against *Reticulitermes flavipes* (Blattodea: Rhinotermitidae)

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Abstract

Heartwood extract from white mulberry (*Morus alba* L.) (Rosales: Moraceae) were investigated for antitermitic activity against *Reticulitermes flavipes* (Kollar) (Blattodea: Rhinotermitidae) in laboratory experiments. An ethanol:toluene (2:1) solvent system was used to remove extract from heartwood shavings. A concentration-dependent feeding response and mortality were observed for termites exposed to a concentration series range of 1.25 to 10 mg/ml of extract based on their dry weight. Results showed that maximum termite mortality occurred at 10 mg/ml. Based on the concentration series data, LC_{50} was calculated at 1.71 mg/ml. In filter paper feeding and repellency assays, extract significantly decreased the total number of gut protozoa compared with untreated and solvent controls. After feeding on filter paper treated at 10 mg/ml for 2 wk, protozoan populations were reduced by >55%. In choice and no-choice tests with mulberry heartwood, greater wood loss from termite feeding was found on solvent extracted blocks compared with nonextracted. Complete (100%) mortality was observed after feeding on nonextracted blocks compared with extracted blocks. Heartwood extract from white mulberry imparted resistance to vacuum pressure treated, nondurable southern pine and cottonwood. At every concentration tested, 100% mortality was observed after feeding on extract-treated southern pine or cottonwood. GC-MS analysis of extract showed high levels of the phenol compound, resorcinol. Results indicated that heartwood extract from white mulberry have antitermitic properties and might be potentially valuable in the development of environmentally benign termiticides.

Key words: white mulberry extract, *Reticulitermes*, antitermitic, gut protozoa, resorcinol

White mulberry (*Morus alba*), a perennial tree, that grows up to 10–20 m tall is widely distributed in Asia, Europe, Africa, and North America. In southern Europe and the United States, it is used for landscaping because of its drought tolerance and suitability for urban conditions (Tipton 1994). Mulberry leaves, fruits, and stems are used for their medicinal properties (Datta 2002). Its wood has been used for manufacturing sporting goods (tennis rackets and hockey sticks) in south Asia and other handicrafts, cabinetry, and musical instruments (Singh and Makkar 2000, Se Golpayegani et al. 2014). It is considered an invasive species in North America where it crossbreeds with indigenous red mulberry (*Morus rubra* L.) (Rosales: Moraceae) and appears to out compete this native species in areas where the two plants co-occur (Hoffman and Kearns 1997).

Past studies indicated that chemical compounds present in *M. alba* heartwood are a combination of stillbenes, phenols, sterols, and flavinoids, but their toxic potential has not been fully investigated (Rowe and Conner 1979, Se Golpayegani 2007, Sadeghifar et al. 2011). Chemicals present in the heartwood of many trees exhibit toxic and repellent activities against termites (Scheffrahn 1991, Nakayama et al. 2000, Peralta et al. 2004, Ragon et al. 2008). Patchouli alcohol from *Pogostemon cablin* (Blanco) Benth (Lamiales: Lamiaceae), nootkatone, and its derivatives from *Vetiveria zizanioides* (L.) Nash (Poales: Poaceae) were toxic and repellent to *Coptotermes formosanus* Shiraki (Blattodea: Rhinotermitidae) (Maistrello et al. 2001a,b; Zhu et al. 2001a,b; Zhu et al. 2003; Ibrahim et al. 2004). Similarly cedrol and widdol from *Juniperus* spp. have been reported to be toxic to termites (Adams et al. 1988). Other researchers reported that these features are due to phenolic compounds such as stilbenes, terpenoids, flavonoids, tannins, and alkaloids (Ohmura et al. 2000, Morisawa et al. 2002, Ganapaty et al. 2004, Watanabe et al. 2005, Coelho et al. 2006, Morimoto et al. 2006a,b; Little et al. 2010). These compounds increase the resistance of wood to decay fungi and insects, and have fungicidal, bactericidal, and insecticidal properties (Tsunoda 1990, Schultz and Nicholas 2000, Taylor et al. 2002). It is often difficult to assess the efficacy of a single component of heartwood extract because toxicity cannot be assigned to a single compound, and there may be synergy between the compounds

present (Arango et al. 2006, El Hanbali et al. 2007, Hwang et al. 2007, Ragon et al. 2008).

Many studies have been published on bark and leaf extract of white mulberry (Chen and Li 2007, Yatsunami et al. 2008, Nakamura et al. 2009, Piao et al. 2009, Zheng et al. 2010). Although the wood of the white mulberry is considered durable, its potential toxicity has not been fully examined against termites (Venkataraman 1972; Rowe and Conner 1979; Se Golpayegani 2007; Se Golpayegani et al. 2010, 2012; Sadeghifar et al. 2011).

Environmental and human concerns regarding conventional wood preservatives, such as creosote and chromated copper arsenate, have highlighted an examination of less toxic substitutes. Extract of naturally resistant woods are easy to detoxify and dispose of without impairing the quality of the environment (Chen et al. 2004). Use of heartwood extract from durable species as wood preservatives for less durable wood species is one strategy to reduce environmental and health hazards.

In this study, we wanted to test the effects of extract from the heartwood of *M. alba* on *Reticulitermes flavipes* via tests exposing termites to heartwood compounds. We assessed the effects of exposure on consumption rates, mortality, hindgut protozoa populations, repellency, and antioxidant properties, as well as the effects of transferring mulberry extract to nondurable wood species. Mulberry heartwood extract were also analyzed via gas chromatography-mass spectrometry (GC-MS) to identify common and potentially toxic components to further understand their efficacy against termites and suggest their potential application for termite control as an alternative to synthetic insecticides.

Materials and Methods

Termite Source

A single colony of *R. flavipes* was collected from a log at Sam D. Hamilton Noxubee National Wildlife Refuge, Mississippi. The infested log was cut into smaller pieces in the field and placed in 50-liter metal trash cans with lids. The cans containing the termites were kept at 25°C until the termites were needed for testing. Damp cardboard was placed on the cut log sections in the cans to supply moisture for the collected termites.

Wood Material and Extract Preparation

White mulberry wood was purchased from a timber market in Faisalabad, Pakistan, shipped to Starkville, MS, cut into boards measuring 457 × 127 × 19 mm and air dried at 25°C and 35% relative humidity. Boards were weighed weekly until an equalized weight was reached (approximately 4 wk). Blocks measuring 1,919 × 19 mm were cut from the boards. Extract for testing were prepared by converting some of the conditioned boards to shavings using a planer. Shavings were air dried as above and placed in 12-g batches in each of several Soxhlet extractors and processed according to ASTM D1105-96 using 300 ml of ethanol:toluene (2:1) as solvent (ASTM International 2014). Wood shavings were contained in the Soxhlet extractors by placing a small amount of cotton underneath and above the shavings and then these were extracted for 6 h. A rotary evaporator (BUCHI, R-114) was used to vaporize the resultant aliquot to dryness at reduced pressure in a tared round bottom flask. Calculation of extraction yield per gram was done according to methods suggested by Ordonez et al. (2006). A stock solution of 10 mg/ml of extract was prepared by re-weighing the dried extract in a pretared flask and re-solubilizing it with solvent (ethanol:toluene) based on the weight of the dry extract. Thus, the 10 mg/ml stock solution was based on a known quantity of dried extract. The resultant stock solutions of extract were individually kept in 1-liter jars in darkness at 4°C.

Preparation of Solvent Extracted Wood

Extracted blocks of white mulberry heartwood were prepared according to ASTM D1105-96. Blocks were conditioned at 25°C and 35 ± 3% RH and then numbered and weighed before placing them into Soxhlet extractors. Blocks were refluxed for 6 h with 300 ml of mixed ethanol:toluene (2:1) solvent. To remove excess solvent, extracted blocks were washed with alcohol and exposed in the Soxhlet extractors a second time using only ethanol (95%) for 6 h. Ethanol-extracted blocks were air dried overnight and then boiled for 6 h in 1 liter of distilled water with 1-liter water changes every hour.

Filter Paper Bioassay

We used a method described by Hassan et al. (2017). Filter paper (Whatman No.1) was oven dried at 60°C and weighed before treatment. Filter papers were individually treated with one of five concentrations, 1.25, 2.5, 5.0, 7.5, or 10.0 mg/ml, of heartwood extract (0.058, 0.11, 0.23, 0.35, and 0.47 mg/cm^2 of filter paper). These concentrations were prepared from a 10.0-mg/ml stock solution using ethanol:toluene solvent. Each individual filter paper was treated with 200 µl of solution. Treatments were replicated three times along with a control treatment that was treated with ethanol:toluene only. Weight gain after treatment was calculated by oven drying (60°C for 12 h) and weighing. Two-inch round plastic containers (Pioneer Plastics #002C) were each filled with 20 g of sand and 3.6 ml of water added. Treated filter papers were placed on an aluminum foil square on top of the moist sand to prevent leaching of compounds from the treated filter paper into the sand. Fifty worker termites (*R. flavipes*) were released into each container, and the containers were placed in an incubator at 25°C and 75% RH for 15 d. Termite mortality was calculated by counting the number of live termites at the end of the test. Filter papers were cleaned, oven dried at 60°C for 12 h, and weight loss calculated and recorded. A vacuum desiccator was used to equilibrate the filter paper after drying. Image J software developed by Wayne Rasband was used to estimate the area of filter paper eaten by the termites.

Gut Protozoa Counts

The method described by Lewis and Forschler (2004) was used to count total gut protozoa number per termite. Field collected termites were fed on filter paper treated with extract as described for the filter paper bioassay. Sets of termites (50) were also given no filter paper to determine the effect of starvation on gut protozoa according to Hassan et al. (2017). Starvation controls were set up similarly to the filter paper feeding tests minus paper (food) in three replicates. After 15 d, termite hindguts were removed using a fine needle and forceps. Contents of five guts per treatment were combined to make a single sample for each extract concentration exposure. Five samples per treatment were homogenized using a disposable pestle in a 1.5-ml microcentrifuge tube and 250 µl of Trager U solution for each sample (Trager 1934). Ten microliters of the resulting solution were loaded onto a hemocytometer, and numbers of protozoans were counted from 0.4 µl. The total combined number of all protozoa species in each termite was calculated by using the following formula (Lewis and Forschler 2004):

$$\frac{(\text{Number of cells counted} \times \text{Volume of saline solution in original sample})}{(\text{Volume of hemocytometer} \times \text{Number of termites per original sample})}$$

Percentage reduction of protozoans was calculated by comparing this value to the protozoa numbers in the control treatments and the freshly collected field termites remaining in the holding cans.

Repellency and Antifeedant Tests

We used the method described by Kadir et al. (2014) to test the extract repellent activities. Filter paper (9 cm diameter) was sliced into two halves. One half was treated with 1.0 ml of each concentration of mulberry extract, and the second half was treated with solvent (ethanol:toluene) only. After drying under a fume hood for 12 h, these were rejoined using adhesive tape attached on the bottom side of filter paper and placed in Petri dish (9.1 cm diameter). Fifty termite workers were released in the petri dish, and the number of termites present on each filter paper half was counted after 1, 2, 3, 4, and 12 h. The following formula as described by Kadir et al. (2014) was used to calculate percent repellency.

$$\text{Repellency}(\%) = 100 \times \frac{(\text{Nc} - \text{Nt})}{(\text{Nc} + \text{Nt})}$$

where Nc and Nt are the number of termites present on control and treated half of filter paper, respectively. Antifeedant indices were calculated on the basis of filter paper weight loss as described by Dungani et al. (2012). The following formula was used to calculate indices of the activity of the extract.

$$\text{Absolute coefficient of antifeedancy}\,(A) = 100 \times \frac{(\text{KK} - \text{EE})}{(\text{KK} + \text{EE})}$$

where KK and EE are the weight losses of the control and treated filter papers. All extract were classified into four groups according to their *A* values (Table 1).

DPPH Radical Scavenging Assay

Extract from heartwood can be a rich source of antioxidants or radical scavengers, which may act synergistically with other compounds to affect termite mortality. To determine DPPH (1,1-Diphenyl-2-picryl-hydrazyl) scavenging activity of the heartwood extract, the method described by Lu et al. (2014) was followed. Extract were dissolved in methanol to make a series of concentrations (25 to 800 µg/ml). One hundred microliters of methanolic DPPH solution (2.5 µM) was added to 100 µl of each extract concentration. Two hundred microliters of solution was then added to each well of a 96-well microtiter plate. Methanol was used as a control. The plate was shaken for 2 min and incubated for 20 min at 37°C in darkness. Color change results were measured spectrophotometrically at 517 nm using a BioTek's PowerWave HT microplate spectrophotometer linked to a computer (Gen5 software). Percentage of radical scavenging activity was calculated using the formula:

$$\text{DPPH radical scavenging activity}\,(\%) = \frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control})} \times 100$$

where Abs control is the absorbance of DPPH + methanol and Abs sample is absorbance by DPPH radical + each extract concentration.

Butylated hydroxytoluene (BHT) was used as the positive control antioxidant compound, and IC_{50} values were calculated by using Graph pad Prism 6 software.

Choice and No-Choice Test on Solvent Extracted or Nonextracted Wood

Choice and no-choice feeding tests on solvent extracted and nonextracted wood blocks were run according to AWPA E1-17 standard test (AWPA 2017). Screw-top jars were filled with 150 g of sand that was moistened with 27 ml of distilled water. Jars were held for 2 h to equilibrate after moistening. For the no-choice test, an extracted or nonextracted block, conditioned at 33°C and 62 ± 3% RH, was positioned on top of the moist sand, with one block in each jar. For the choice test, one extracted and one nonextracted block were placed in each jar together. In total, 400 termites consisting of 396 workers and 4 soldiers were released into each jar (AWPA 2017, Haverty and Howard 1981). Jars were placed in an incubator for 28 d at 27°C and 75 ± 2% RH. Five replicates were used in both tests. After 28 d, the number of live termites was counted to calculate percentage mortality. Blocks were brushed to remove sand, conditioned for 1 wk (33°C and 62 ± 3% RH), and re-weighed to calculate the weight loss. All blocks were visually rated using the 0–10 scale described in the AWPA E1-17 standard.

Termite Bioassay on Southern Pine and Cottonwood Pressure Treated With Extract

The AWPA E1-17 standard was followed for the termite bioassay (AWPA 2017). Blocks of southern pine and cottonwood (19 × 19 × 19 mm) conditioned at 33°C and 62 ± 3% RH and weighed. Blocks were vacuum pressure treated with three different concentrations (2.5, 5.0, and 10.0 mg/ml) of mulberry heartwood extract. A subset of pine or cottonwood blocks was treated with ethanol:toluene or water as control treatments. For vacuum pressure treatment, five blocks were placed in a beaker (300 ml) containing 250 ml of the treatment solution and placed in a vacuum pressure chamber. A vacuum pressure of 91.4 KPa was held for 30 min, and then pressure was applied at 275.8 KPa for 60 min. Treated blocks of both woods were blotted dry using paper towels, weighed, reconditioned at 33°C and 62 ± 3% RH, and re-weighed.

GC-MS Analysis

Analyses and characterization of heartwood extract were performed using an Agilent 7890B gas chromatograph mass spectrometer. This system used a 19091S-433UI HP-5ms Ultra Inert column (30 × 250 × 0.25 µm). The temperature of the column ranged from 50 to 270°C, with a solvent delay of 3–6 min (Mankowski et al. 2016). For the mass spectrometer, the temperature of the ion source was held at 230°C with a quad temperature of 150°C. The system used an electron capture detector and a splitless injector (270°C; Mankowski et al. 2016). For extract analysis, the initial temperature

Table 1. Antifeedant and repellent activity (±SE) of white mulberry heartwood extract against *Reticulitermes flavipes*

Concentration of extract	Absolute coefficient of antifeedancy %	Activity level[a]	% Repellent activity
Control (solvent only)	—	Minimal activity	3.39 ± 0.021
1.25 mg/ml	10 ± 0.57	Minimal activity	13.33 ± 0.069
2.50 mg/ml	37 ± 1.15	Moderate activity	34.67 ± 0.188
5.00 mg/ml	46 ± 0.99	Moderate activity	50.40 ± 0.306
7.50 mg/ml	61 ± 0.11	Strong activity	52.80 ± 0.110
10.0 mg/ml	62 ± 0.57	Strong activity	70.40 ± 0.176

[a]Mini = 0 ≤ A < 25; Moderate = 25 ≤ A < 50; Strong = 50 ≤ A < 75; Very strong = 75 ≤ A < 100.

was 50°C that ramped to 265°C at 5.5°C/min for 45 min (Se Golpayegani et al. 2014). Helium was used as the inert carrier at a flow rate of 1.0 ml/min, and sample (1 µl) was injected in the splitless mode. All mass spectra were recorded at 70 eV in the electron impact ionization. The mass spectrometer scanned from *m/z* 3 to 700 at a rate of two scans per second. Peak area was calculated automatically by an integrator. The NIST14 library was used to identify the top five compounds present in heartwood extract (Mankowski et al. 2016).

Statistical Analysis

Probit analysis was used to calculate lethal concentration (LC_{50}) for extract by using Polo-PC software (Finney 1971). A one-way ANOVA was used for the analysis to determine any significant variation between treatments in all tests using MINITAB 16. Means were separated using Tukey's HSD test ($P = 0.05$). Graph pad Prism 6 was used to calculate IC50 values in the DPPH radical scavenging test.

Results

Filter Paper Bioassay

Mulberry heartwood extract exhibited a concentration-dependent effect on mortality in *R. flavipes* (Fig. 1A). All treatments were significantly different from the control treatment ($F = 7.63$; $P < 0.005$; df = 4). After a 15-d exposure, white mulberry extract showed antitermitic activity, with an LC_{50} of 1.71 mg/ml ($n = 50$; $\chi^2 = 21.24$; Slope ± SE = 2.53 ± 0.17; FL 95% = 0.93–3.39). Mortality was higher (93.3%) at the maximum extract concentration (10 mg/ml), while mortality was significantly lower (17%) at the lowest concentration (1.25 mg/ml) compared with all other treatments except the control. The percentage area of filter paper consumed by termites was lower in treated filter paper groups compared with controls. Results showed a positive correlation ($r = 0.900$; $P < 0.005$) between the amount of filter paper consumed and termite mortality. All treatments were significantly different from the control treatments ($F = 10.24$; $P < 0.005$; df = 5, 12). A parallel trend was observed between termite mortality and filter paper weight loss (%). Reduced feeding (weight loss 3.1%) was found at the maximum concentration of extract where mortality was highest (Fig. 1 B). Termite mortality and filter paper weight loss (%) were correlated ($r = 0.906$; $P < 0.005$), and all treatments were significantly different from the control treatment ($F = 15.17$; $P < 0.005$; df = 5, 12).

Effects on Gut Protozoa

Feeding on the heartwood extract resulted in a dose-dependent reduction in gut protozoa numbers in *R. flavipes* (Fig. 1A). Termite mortality and decreasing gut protozoa number were correlated ($r = 0.700$; $P < 0.005$), suggesting that the reduction of the gut protozoa was directly associated with termite mortality. The highest percentage reduction in protozoa (55.2%) was observed at the maximum extract concentration (10 mg/ml) where mortality was 93%. Elimination of even a single species of protozoan has been shown to cause high termite mortality (Mauldin et al. 1972). Protozoa numbers at each concentration were significantly different from each other ($F = 40.34$; $P < 0.005$). In starved termites, protozoa were reduced by 99.6%, but termite mortality was lower (20%) than in treatments exposed to white mulberry extract (Fig. 1A).

Repellency and Antifeedant Tests

Repellent and antifeedant activity of the white mulberry extract concentrations are shown in Table 1. Termite repellency was shown to be concentration dependent, ranging from minimal to strongly repellent. More termites were observed on paper treated with solvent only (control) compared with extract-treated paper (13.3 vs 70.4%) indicating minimal repellent activity in the control. All treatments were significantly different from one another except 5.0 and 7.50 mg/ml ($F = 3.19$, $P < 0.05$). The antifeedant activity of extract acquired from mulberry ranged from 10 to 62% for the minimum to maximum concentrations of extract. Antifeedant activities were not significantly different at 7.50 and 10 mg/ml, but all treatments were significantly different compared with 1.25 mg/ml ($F = 68.89$, $P < 0.05$).

DPPH Radical Scavenging Assay

The method used to assess DPPH radical scavenging is based on a decrease of methanolic DPPH in the presence of hydrogen-donating antioxidant. Our results indicated that white mulberry extract has free radical scavenging activity even at low extract concentrations (Fig. 2). The concentration required to inhibit 50% free radical of DPPH is the IC_{50}. In our tests, the IC_{50} for white mulberry extract was 26.6 µg/ml which was lower than the IC_{50} for the positive control synthetic antioxidant BHT at 42.63 µg/ml. Antioxidant activity increased with increasing concentration of extract. At the lowest and highest concentrations, scavenging activity was 48 and 94%, respectively. At the first three concentrations (25–100 µg/ml) of extract and BHT, there was a significant difference in percent inhibition.

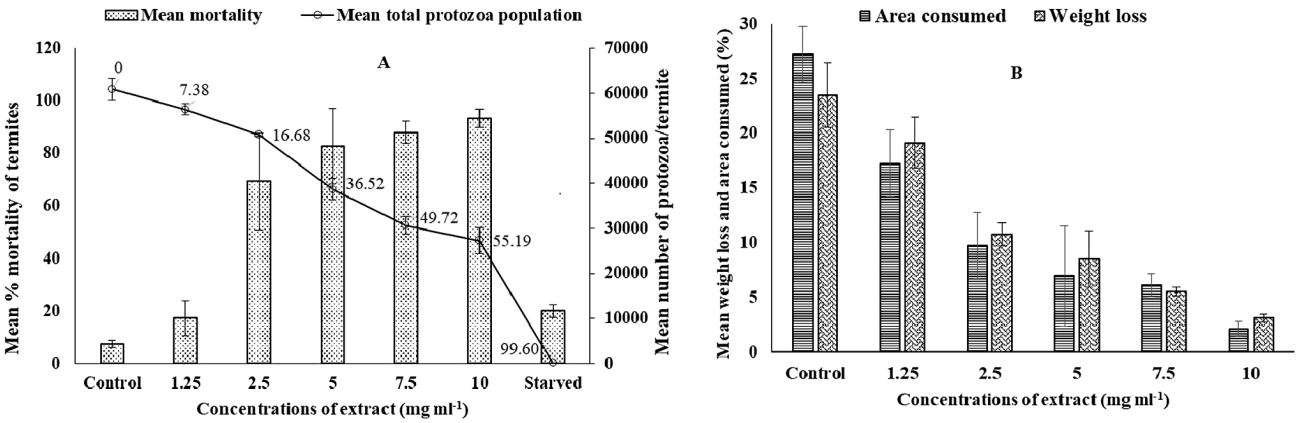

Fig. 1. Mortality and protozoan number per termite (A); filter paper area consumption and weight loss (%) (B); after feeding *Reticulitermes flavipes* with treated or nontreated filter paper for 15 d.

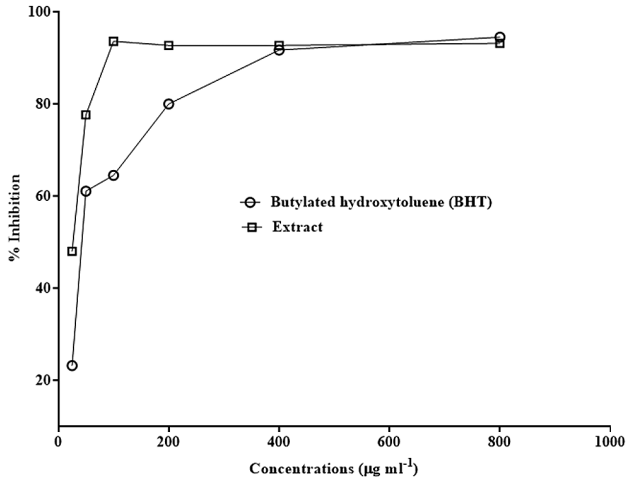

Fig. 2. Effect of different concentrations of white mulberry heartwood extract on DPPH free radical scavenging activity. BHT = butylated hydroxytoluene.

However, at the higher extract concentrations, the scavenging activity was not significantly different.

Choice and No-Choice Test on Solvent Extracted or Nonextracted Wood

Results for the choice and no-choice bioassays with solvent extracted and nonextracted (normal) white mulberry wood are shown in (Table 2). In both the no-choice and choice tests, termites avoided the nonextracted, normal mulberry blocks and significantly consumed more of the extracted wood (>8%; $F = 32.92$; $P \leq 0.05$). Consumption was much lower in the nonextracted, normal wood in both tests, indicating mulberry wood with its natural chemical components intact was not a palatable food source. Total (100%) termite mortality was observed in the no-choice test when termites were exposed to nonextracted wood. Mortality in either test involving solvent-extracted blocks was lower (77.4, 75.6%; $F = 11.10$; $P < 0.05$), indicating the wood was slightly more palatable with some of its chemical components removed. However, a mortality of 77% is relatively high, indicating the food source contained toxic components after the extraction process.

Termite Bioassay on Wood Pressure Treated With Extract

Mean weight loss (%) of treated and nontreated southern pine or cottonwood exposed to *R. flavipes* is shown in Table 3. Water or solvent (ethanol:toluene) treated southern pine controls lost 26.46 or 25.11%, respectively. Cottonwood incurred weight losses of 37.53 and 36.45 for water- and solvent-treated blocks, respectively. Southern pine and cottonwood treated with mulberry heartwood extract at the highest concentration had the lowest weight losses at 3.71 and 5.14%, respectively. Weight loss was inversely related to extract concentration. Weight loss for southern pine control treatments was significantly greater compared with all other treatments ($F = 53.08$; df = 4, 20; $P < 0.005$). A similar trend was observed in cottonwood ($F = 142.03$; df = 4, 20; $P < 0.005$). Ratings according to the AWPA E1-17 Standard indicated less feeding on southern pine and cottonwood treated at 10 mg/ml extract, with mean ratings of 8.8 and 7.8 for pine and cottonwood, respectively ($F = 72.6$; $P < 0.05$ and $F = 81.74$; $P < 0.05$). In contrast, water-treated or solvent-treated control blocks failed, with average ratings of zero. Comparison between extract-treated southern pine and cottonwood showed that mulberry extract were more effective at protecting southern pine than cottonwood. Vacuum pressure treatment of susceptible woods with extract was effective against termites in both tests.

GC-MS Analysis

Fifty-five compounds were identified from white mulberry heartwood extract. Table 4 shows the top five compounds identified (their molecular weights, identification quality, retention time, and percent compound in the sample. The compounds were resorcinol, 2, 4-dihydroxy-benzaldehyde, gamma-sitosterol, 4,14-dimethyl-9,19-cycloergost-24(28)-en-3-ol, and 24-methylene-9, 19-cyclolanostan-3-ol. These were approximately 58% of the total chemical makeup of the solvent fraction analyzed. The chromatogram for this analysis is shown in Fig. 3. Other authors have noted similar compounds from analyses of *M. alba* (Hosseinihashemi and Kanani 2012, Mankowski et al. 2016).

Discussion

M. alba extract displayed antitermitic activity. Our results were comparable to Raya-González et al. (2013) who observed that mortality and feeding rates were concentration dependent when *Incisitermes marginipennis* Latreille (Blattodea: Kalotermitdae) fed on filter paper treated with extracts of *Enterolobium cyclocarpum* Jacq. Griseb (Fabales: Mimosaceae). In our study, a sixfold decrease in feeding on treated filter paper was observed at the highest concentration of extract compared with control treatments. Additionally, Se Golpayegani et al. (2014) observed >95% termite mortality after exposure to acetone and methanol white mulberry extract-treated filter paper. However, these authors also showed that termite mortality was >80% when fed filter paper treated with water removed white mulberry extract. All concentrations of mulberry extract tested showed antitermitic activity in our test (Fig. 1A). Termites feeding on treated filter paper became sluggish and developed shrinking abdomens within a few days of exposure.

Protozoa numbers in the control treatment averaged 60,917 ± 2,434. This is comparable to Lewis and Forschler (2004) for *R. flavipes* (58,369 ± 16,021). Our protozoa population estimate was greater than reported in earlier studies for *R. flavipes* that showed ranges from 40,083 ± 3,643 (Mannesmann 1969) to 32,320 (Mauldin et al. 1981), to 31,120 ± 8,405 (Howard, 1984), and 14,642 ± 3,395 (Cook and Gold 1999).The number of protozoa in the control treatment (filter paper treated with ethanol:toluene) and in our field source collected termites were similar to each other, but higher than the aforementioned studies (60,917 ± 2,434). Our results also differed from Jones et al. (1983) who found 31,520, 31,920, 29,440, and 48,400 protozoans in hexane, acetone, a mixture of acetone-hexane-water, and methanol treatments, respectively. Our results were different from Mannesmann (1972) who found complete reduction of gut protozoa after feeding *Reticulitermes virginicus* (Banks) (Blattodea: Rhinotermitidae) red spruce, although it is likely that different wood extract influence symbionts differently (Mannesmann 1972). Weight loss and filter paper percent area reduction decreased when protozoa decreased compared with the control treatment (Jones et al. 1983). Mortality of termite gut fauna may be affected by the solvent used to extract the antitermitic compounds. Jones et al.(1983) observed greater numbers of protozoa in *R. flavipes* occurred after feeding paper treated with methanolic extract of *Platymiscium ulei* Harms (Fabales: Fabaceae) while the lower numbers occurred after exposure to hexane extract. Protozoan mortality cannot be generalized for all termites and wood species because

Table 2. Mortality of *Reticulitermes flavipes* and wood consumption in choice and no-choice bioassays on solvent extracted and nonextracted (normal) mulberry wood

	Mean mortality (%)		Mean weight loss (%)	
Type of test	Extracted	Nonextracted	Extracted	Nonextracted
No-choice test	77.40 ± 0.90	100 ± 0.00	8.67 ± 0.27	0.2 ± 0.15
Choice test	75.60 ± 2.00		8.93 ± 0.44	0.51 ± 0.13
Southern pine	32.5 ± 3.08		26.46 ± 2.00	
Cottonwood	32.0 ± 4.64		37.53 ± 3.2	

Table 3. Mean termite mortality (%) and wood weight loss (%; ±SE) for vacuum-impregnated nondurable wood blocks and visual damage rating averages of white mulberry extract-treated southern pine and cottonwood exposed to *Reticulitermes flavipes* (0 = failure; 10 = sound)

	Vacuum-impregnated nondurable wood blocks					
	Southern Pine (SP)			Cottonwood (CW)		
Concentration of extract	Mortality	Weight loss %	Rating	Mortality	Weight loss %	Rating[a]
Water	32.5 ± 3.08	26.46 ± 2.00	0	32.0 ± 4.64	37.53 ± 3.2	0
Solvent	29.0 ± 2.29	25.11 ± 1.05	0	36.8 ± 2.21	36.45 ± 0.46	0
2.5 mg/ml	100 ± 0.00	8.86 ± 1.29	7.6	100 ± 0.00	14.85 ± 0.38	5.2
5.0 mg/ml	100 ± 0.00	5.60 ± 0.67	8.2	100 ± 0.00	10.17 ± 0.81	6.4
10 mg/ml	100 ± 0.00	3.71 ± 0.92	8.8	100 ± 0.00	5.14 ± 0.24	7.8

[a]0 = failure; 4 = very sever attack; 6 = sever attack; 7 = moderate or severe attack; 8 = moderate attack; 9 = slight attack; 9.5 = trace; 10 = sound.

Table 4. Five largest components from GC-MS analysis of solvent extracted white mulberry

Compound name	Retention time (min)	Molecular weight	Quality	% of Sample
Resorcinol	7.78	110.037	94	40.54
2,4-Dihydroxy-benzaldehyde	9.71	138.03	95	6.97
Gamma-sitosterol	26.81	414.39	99	4.31
4,14-Dimethyl-9,19-cycloergost-24(28)-en-3-ol	27.27	426.39	78	3.68
2,4-Methylene-9,19-cyclolanostan-3-ol	28.62	440.4	93	2.85

different species of termites react differently to different wood species. Mauldin et al. (1981) found a reduction of 5,000 protozoa per termite after feeding on white mulberry blocks for 1 wk, a 15% reduction from the source population (32,320). In our study, we observed a 15 ± 1% reduction in protozoan after feeding for 2 wk on filter paper treated with 2.5 mg/ml extract and a 55.2% reduction after feeding on paper treated with 10.0 mg/ml extract. This may be due to feeding rates and the solvent used for the extraction. Our results also differ from Lewis and Forschler (2010) who found 30% reduction in gut protozoa after treatment with chitin synthesis inhibitors. Results of this study agree with Hassan et al. (2017) who found 45.7, 39.2, 36.4, and 15% reduction in protozoa in *R. flavipes* after feeding on filter paper treated with heartwood extract of *Dalbergia sissoo* (Roxb) (Fabales: Fabaceae), *Tectona grandis* Linn (Lamiales: Lamiaceae), *Pinus roxburghii* Sarg (Pinales: Pinaceae), or *Cedrus deodara* (Roxb.) G. Don (Pinales: Pinaceae), respectively. Reduction of gut protozoa may not be the only cause of termite mortality, as several other physiological mechanisms can be involved. (Raje et al. 2015). Protozoa numbers in starved termites were reduced, but termite survivorship was 80%, suggesting another mode of action of the extract caused toxicity (Fig. 1A). Raje et al. (2015) showed that tumerone from turmeric extracts was toxic to termites by acting on the termite nervous system causing respiratory disruption. With no exposure to extract and no food, survival may be due to cannibalism (Hu et al. 2011, Hassan et al. 2017). Lower termites cannot digest cellulose without the aid of their symbiotic protozoa (Cleveland 1925). Chemicals that eliminate symbionts have potential as natural termiticides. In this study, antiprotozoan compounds present in white mulberry heartwood extract appear to be slow acting. This could be a benefit for termite control with these compounds. Foraging termites feed and return to the colony with these chemicals and transfer them to other members via trophallaxis (Mauldin et al. 1981, Hassan et al. 2017). This may cause a reduction in vigor, weakening of the colony, and ultimately decrease the ability of termites to attack wood (Yoshimura 1992, Yoshimura et al. 1993).

Simple laboratory experiments can be useful to screen and explore insect repellent properties of natural extracts (Smith 1979, Sharma et al. 1994). White mulberry heartwood extract showed repellent and antifeedant activities. Concentrations tested showed both repellent and lethal effects, with termites appearing sluggish, possibly due to neurotoxic effects of extract (Price and Berry 2006, Raje et al. 2015). Antifeedant activity was similar to Dungani et al. (2012) who showed that teak extract concentration was a vital factor in termite mortality and antifeedancy. This may be due to the presence of phenolic compounds that can be strong antioxidants, antifeedants, and act as natural protectants for the living tree (Gupta et al. 1972, Morimoto et al. 2006a,b; Ateş et al. 2015).

The mechanism by which wood extract kill or repel termites remains unclear (Ragon et al. 2008). Previous studies indicate that extract chemical compounds present in heartwood such as flavones,

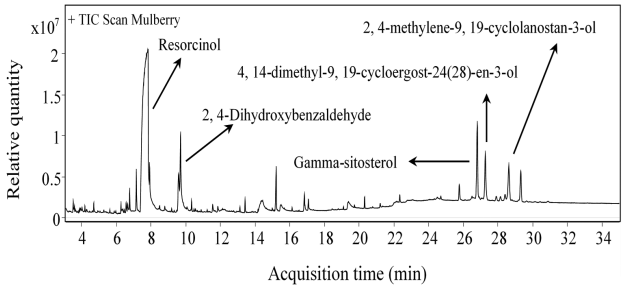

Fig. 3. Chromatogram of 10 mg/ml white mulberry heartwood extract.

tannins, stilbenes, and flavonoids possess both termiticidal and antioxidant properties (Doi et al. 2001, Torres et al. 2003, Fava et al. 2006, Morimoto et al. 2006a,b; Ragon et al. 2008, Little et al. 2010, Hassan et al. 2018). Free radical scavengers or antioxidants serve as synergists by interfering with the Glutathione S-transferase (GST)-mediated detoxification mechanism and act as alternative substrate for the antioxidant enzymes. They can also reduce the activities of antioxidant enzyme systems by acting as competitive and noncompetitive inhibitors toward GST and esterase substrates (Das et al. 1984, Hassan et al. 2018). Appel and Schultz (1992) reported that these compounds are toxic to insects causing strand breaks in DNA. Stilbenes and flavonoids have also been characterized from mulberry (Venkataraman 1972, Rowe and Conner 1979). Our GC-MS analysis showed white mulberry contains large amounts of the phenol, resorcinol (40.54%). Previous studies found resorcinol at high levels in white mulberry and showed biological activity against several organisms (Sadeghifar et al. 2011, Salem et al. 2013, Se Golpayegani et al. 2014). Resorcinol has been shown to have antifungal properties (Adikaram et al. 2009, Salem et al. 2013, Mansour et al. 2015) and insecticidal antitermitic properties against *C. formosanus* (Salem et al. 2013, Mansour et al. 2015). Many biologically active compounds such as stilbenes, flavonoids, morusimic acid, oleanolic acid, phytosterols, saponins, anthocyanins, triterpenes, benzofuran derivatives, tannins, anthroquinones, 2-arylbenzofurans, and sitosterol have been identified from extract of white mulberry (Chen et al. 2005, Yogisha and Raveesha 2009). Sadeghifar et al. (2011) extracted the heartwood of white mulberry and found that it contains 90% resorcinol, which is a hydrophilic phenolic compound. Se Golpayegani et al. (2014) stated that durability of white mulberry might be due to resorcinol solely or by synergy with other compounds. Heartwood phenolics such as resorcinol can act synergistically with other compounds to affect insect digestion and metabolism (Duffey and Stout 1996). Resorcinol is hydrophilic and can form several bonds including hydrogen, covalent, or ionic with dietary proteins and digestive enzymes in the insect gut affecting digestion nutrient assimilation (Appel and Schultz 1992). Oxidation of phenols (auto-oxidation or enzymatic) due to the alkaline termite midgut can form quinones that reduce protein digestibility (Bhonwong et al. 2009). The antioxidant and pro-oxidant properties of resorcinol have been observed in previous studies (López et al. 2011, Veliká and Kron 2013). Earlier work showed that heartwood extract from *T. grandis*, *D. sissoo*, *C. deodara*, and *P. roxburghii* reduced the antioxidant enzyme activity in *Heterotermes indicola* (Wasmann) (Blattodea: Rhinotermitidae) (Hassan et al. 2018). Similarly, antioxidants as wood preservatives (BHA) caused maximum mortality and minimum loss of treated wood (Schultz and Nicholas 2000, Little et al. 2010).

In our tests, the highest concentration of extract caused maximum termite mortality while in bioassays using solid southern pine and cottonwood blocks, 100% mortality was observed at all concentrations with minimum feeding. Mortality may be due to the presence of flavonoids in *M. alba* heartwood, which have dual toxicity and antioxidant properties (Ragon et al. 2008). Antioxidants may interfere with lignocellulosic digestion by the termites and their symbiotic microbes (Abe et al. 2000). Our results indicated this because at the maximum concentration of extract there was the greatest reduction in protozoa (Fig. 1A).

Results of choice and no-choice tests with solid wood indicated that white mulberry is naturally durable due to the presence of antitermitic compounds. These results agreed with Se Golpayegani et al. (2010, 2014), who observed 100% mortality and lower wood consumption after termite feeding on un-leached samples compared with leached white mulberry wood. Se Golpayegani et al. (2014) found 70% termite survival after feeding on white mulberry wood powder extracted with methanol, but on acetone-extracted powder, survival was 30%. In our tests, termite survival was less than 25%, which may be due to the use of different solvents and differences in extracting whole wood blocks versus wood powder. White mulberry extract was toxic to termites even at the lowest concentration and showed 100% mortality at every concentration after termites fed on treated southern pine or cottonwood (Table 2). Results suggest that white mulberry heartwood extract can increase the resistance of less durable wood species such as southern pine and cottonwood. Comparable results were observed in several previous studies using heartwood extract (Yamaguchi et al. 2002; Syofuna et al. 2012; Tascioglu et al. 2012; Kirker et al. 2013, 2015; Hassan et al. 2016).

Results of this study indicate that *M. alba* heartwood extract had a significant negative impact on termite activity. White mulberry heartwood extract was found to be repellent, termiticidal, and reduced numbers of hindgut protozoa in force feeding tests. These are foundational studies to establish biological relevance of white mulberry heartwood extract on our target system. Future studies will provide targeted investigation of extract components to isolate bioactive properties and assign stoichiometric ratios that illicit physiological responses in termites.

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