ISSN impresa 0717-3644
ISSN online 0718-221X
Maderas. Ciencia y tecnología 21(2): 153 - 162, 2019
DOI: 10.4067/S0718-221X2019005000203

TOXICITY POTENTIAL OF HEARTWOOD EXTRACTIVES FROM TWO MULBERRY SPECIES AGAINST *Heterotermes Indicola*

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ABSTRACT

Choice and no-choice tests were run to evaluate natural resistance of the woods of two *Morus* species (*Morus alba* and *Morus nigra*) against the subterranean, *Heterotermes indicola,* under field conditions. Toxicity, antifeedant and repellency potential of the heartwood extractives was also investigated under laboratory conditions. Heartwood extractives were removed from wood shavings by using methanol or an ethanol: toluene (2:1) mixture. Results of choice and no-choice tests with sap and heartwood blocks exposed to termites, showed that both mulberry species were resistant to termites but in comparison, *Morus alba* wood was more resistant than *Morus nigra* to termite feeding as it showed <5 % weight loss after 90 days. Termites exhibited a concentration dependent mortality after exposure to either mulberry species' heartwood extractives. The highest termite mortality occurred after termites were exposed to filter paper treated with *Morus alba* extractives at a concentration of 5 %. At this concentration, antifeedancy and repellency were calculated to be 91,67 and 84 % respectively. . Our results also showed that extractives from either mulberry species imparted resistance to vacuum-pressure treated non-durable *Populus deltoides* wood. Termite mortality was greater than 75 % after feeding on *Populus deltoides* wood treated with extractives from *Morus alba*. Solvent only (methanol) treated *Populus deltoides* controls, showed a minimum weight loss of 2,69 % after 28 days. These results suggest that *Morus alba* extractives have antitermitic properties and may be potentially useful in the development of environment friendly termiticides.

Keywords: Field tests, subterranean termite, toxicity, transferable durability, wood protection.

INTRODUCTION

Heterotermes spp. are structure-infesting termites that account for a significant amount of the damage attributed to subterranean termites in southern Asia, particularly areas of Pakistan. *Heterotermes indicola* previously limited to the Indian subcontinent, and the Arabian Peninsula , has now spread to other areas and is commonly encountered in Punjab, as well as in the Khyber Pakhtunkhwa provinces in Pakistan (Misbah-ul-Haq *et al.* 2015).

Comparative susceptibility/resistance tests of woods against *H. indicola* have indicated that *Dalbergia sissoo*, *Azadirachta indica*, *Syzygium cumini* and *Pinus roxburghii*, were resistant while *Populus euramericana*, *Bauhinia variegate, Mangifera indica* and *Populus deltoides* were ranked as susceptible to termite attack (Dugal and Latif 2015, Afzal *et al.* 2017). The nature of resistance of some heartwoods to termite attack is mostly attributed to toxic compounds sequestered by the living tree within the heartwood (Kirker *et al.* 2013, Hassan *et al.* 2017). Heartwood compounds extracted from *D. sissoo*, *P. roxburghii*, *Tectona grandis*, *Cedrus deodara*, *Eucalyptus camaldulensis*, *Acacia arabica*, *Betula utilis*, *S. cumini* and several other durable woods

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Received: 06.09.2017 Accepted: 29.08.2018

have been found to have toxic, antioxidant, repellent and antifeedant effects on *H. indicola* workers as well as toxicity to the termite gut microbiota (Peralta *et al.* 2004, Ragon *et al.* 2008, Hassan *et al.* 2016a, Hassan *et al.* 2016b, Qureshi *at al*. 2016, Hassan *et al.* 2017, Hassan *et al.* 2018a, Hassan *et al*. 2018b). Studies reported that these characteristics were attributed to phenolic compounds such as terpenoids, flavonoids, stilbenes, tannins and alkaloids (Ohmura *et al.* 2000, Ganapaty *et al.* 2004, Watanabe *et al.* 2005, Little *et al.* 2010). In the living tree, these compounds play a role in protecting the wood from decay and insects as they have been shown to have fungicidal, bactericidal and insecticidal properties (Schultz and Nicolas 2000, Taylor *et al.* 2002). Recently, certain synthetic termite control chemicals have been withdrawn from the commercial markets in the past years because of toxicological and environmental concerns (Little *et al*. 2010). This has prompted an increased interest in the study and use of less toxic alternatives. One approach has been the examination of botanical biocides, which occur naturally in the heartwood of durable wood species as wood protectant (Kirker *et al*. 2013). In this way, phenolic compounds extracted from these naturally durable species can be used to protect less durable wood species.

Morus nigra and *Morus alba* (black and white mulberry respectively) are widespread in northern India, Pakistan and Iran. These occur as shrubs or perennial trees which grow up to 10-20 m tall. The fruits and leaves of both species have also been used in traditional medicines (Datta 2000, Chen and Li 2007).

The woods of both *M. nigra* and *M. alba* are considered durable due to presence of a combination of stillbenes, phenols, sterols and flavonoids (Rowe and Conner 1979, Sadeghifar *et al.* 2011), but the toxicity of these two heartwoods has not been fully examined against termites, particularly against *H. indicola*. In the studies here, we investigated the efficacy of solvent extracted compounds from the heartwood of *M. nigra* and *M. alba* against *H. indicola* and discussed their potential application for termite control as an alternative to synthetic insecticides.

MATERIALS AND METHODS

Wood source and sample preparation

Logs of *Morus alba* (L.), *Morus nigra* (L.) and *Populus deltoides* (poplar) were purchased from a timber market located at Jhang Road, Faisalabad, Pakistan. Wood specimens measuring 130L x 50T x 20R mm (LTR = longitudinal, tangential and radial) were prepared from heartwood and sapwood of each mulberry species. The poplar, *P. deltoides*, control blocks were only from sap wood. Blocks of size 19 x 19 x 19 mm from sapwood portion of *P. deltoides* were also prepared by using an electric saw.

Preparation of extractives

Air dried heartwood from each mulberry species was converted into wood shavings using a planer. Two solvent systems were used to remove extractives from the heartwood. Methanol and an ethanol: toluene (2:1) mixture (Sigma-Aldrich) were sourced from a local market and used for the preparation of extractives. A total of 450 g of shavings of each species were added in one liter of each solvent system and flasks were regularly shaken by hand for a period of 20 days after each 4 hours. After filtration of extractives, the resulting aliquot was placed in a tared round-bottom flask and vaporized using a rotary evaporator. After the flask had cooled to room temperature, it was reweighed and the resulting residue was rehydrated with the extraction solvent to produce a stock solution of 10 mg/ml, based on the dry weight of the extractive residue. The extractives were stored at 4°C in small vials after preparation of three different concentrations from stock solution.

Choice and no-choice tests to determine natural resistance of mulberry woods

The 130 x 50 x 20 mm (LTR) samples were exposed to *Heterotermes indicola* (Wasmann) under choice and no-choice field tests at Post Agriculture Research Station, Jhang Road Faisalabad. The selected site had very good termite activity. In the choice test, one wooden stake (pre-weighed and conditioned) from *M. nigra* and one from *M. alba* were tied together with the help of a cable tie. These packed wooden stakes were buried partially underground (3/4 part of wood was below ground) for 90 days and then re-weighed after conditioning to determine the weight loss. In the no-choice test, wooden stakes from both mulberry species were offered to termites individually in the same way as described above. Each test consisted of five replicates of each tested heartwood as well as five stakes of the *P. deltoides* to serve as control. Method described by Ahmed *et al.* (2014) was used to calculate weight loss of stakes after exposure to termites.

Bioassay of extractive treated filter paper

Oven dried (60°C for 12h) and weighed Whatman No. 1 filter papers (42,5 mm diameter) were treated with three different concentrations (1, 3 and 5 % w/v) of extractives. Concentrations were prepared using both solvents separately. Using a pipette, 200µl of each solution was applied to the center of a single filter paper in a petri dish. Each treatments was replicated three times along with control treatments consisting of a solvent only control using each of the solvent systems and a water only control. A total of 50 termite workers of *H. indicola* were released into plastic jars (Diameter: 72,4 mm; capacity 250 ml) containing 20 grams of sand 3,6 ml water and treated filter papers. Filters papers were placed on small foil instead of direct contact with sand to avoid leaching of the extractives. These jars were maintained in an incubator at 27°C and 75 % R.H for fifteen days. At the end of the test, termite mortality was calculated by counting the number of live termites. Filter papers were cleaned, oven dried at 60°C for 12 hours, and weight loss was calculated as described by Hassan *et al*. 2017. A vacuum desiccator was used to equilibrate the weight of filter paper after drying.

Repellency and antifeedancy tests

The method described by Kadir *et al*. (2014) was followed to test repellent activities of the heartwood extractives. Filter paper (90 mm diameter) was divided equally into two halves. One half was treated with extractives (conc. 1, 3 or 5 % w/v) of each wood separately while other half was treated with their respective solvent only. After air drying (12 h) , both halves were rejoined, placed in a petri dish (91 mm) and a total of 50 termites were released on the filter paper. The number of termites present on the solvent and extractive treated halves were counted after 1, 2, 3, 4, 5, 6, and 12 hours. The percent repellency was then calculated. Antifeedant indices were calculated using the method outlined by Dungani *et al*. (2012).

Termite bioassay with cottonwood pressure treated with extractives

Weighed and conditioned (33°C, 62 ± 3 % R.H.) *P. deltoides* sapwood blocks (19×19×19 mm) were treated by pressure application method with different concentrations (1, 3 and 5 %) of heartwood extractives from either mulberry species. Treated blocks were placed in glass screw cap jars (80 mm diameter and 100 mm height) along with 150g and, 30 ml of distilled water. The water was added to the sand in each jar 2 hours prior to placing samples in the jars. After this, 400 termites (396 workers+ 4 soldiers) were released in each jar. Jars were placed in a growth chamber at 28±1 ^{o}C and 65 % RH for 28 days. Three replications for each treatment and a control treated with solvent were used in this test. At the end of test period the blocks were removed from the jars, conditioned and weighed to determine weight loss. Surviving termites were counted to calculate mortality.

Statistical analysis

Field experiments were conducted by using Randomized Complete Block Design (RCBD), whereas the laboratory study was analyzed using a Completely Randomized Design (CRD). Data was analyzed by using Minitab 16 statistical software in ONE -WAY analysis of variance. Means were separated at the 5 % level of significance using Tukey's HSD test.

RESULTS AND DISCUSSION

Mean weight loss of sapwood and heartwood from *M. nigra* and *M. alba* tested against *H. indicola* in choice and no-choice tests compared with weight losses in poplar controls is shown in Figure 1. Weight loss in sapwood blocks was significantly higher compared to that of heartwood blocks for both species of mulberry tested in choice and no-choice tests. Maximum weight loss (> 45 %) was observed for *P. deltoides* control (Figure 1). In choice tests, termites fed less on *M. alba* stakes compared to *M. nigra* for both the sapwood and heartwood. In the no-choice test, weight losses for *M. alba* sapwood were higher compared to the weight loss in this species in the choice test. Overall heartwood of both mulberry species were consumed less than sapwood by termites in the two types of tests.

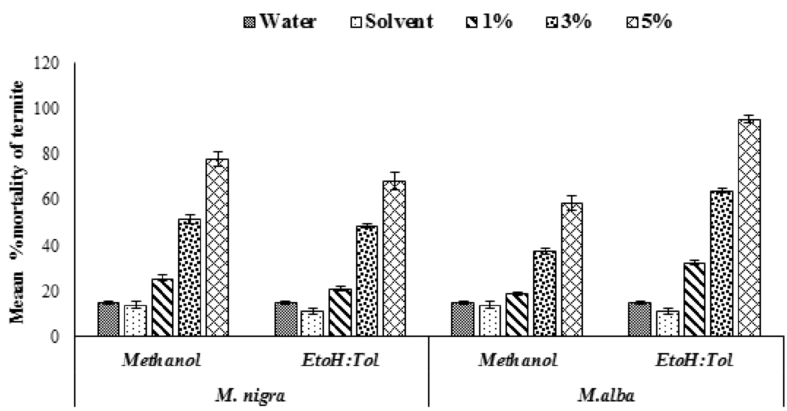

Figure 1: Mean % weight loss of *M. nigra* and *M. alba* sapwood and heartwood in choice and no-choice tests for 90 days.

Toxicity of extractives as termite mortality showed increasing toxicity with increasing extractive concentration (Figure 2) All mortalities incurred by the three concentrations of extractive treatments were found to be significantly different from each other ($p<0,05$). A maximum mortality of 95,5 % was observed for termites that were fed on filter paper with the maximum (5 %) concentration of *M. alba* extractives in ethanol: toluene, followed 77 % mortality of *M. nigra* in methanol. Highest concentration of heartwood extractives from *M. alba* caused significantly high mortality compared to extractives of *M. nigra*. However, methanol extracted *M. nigra* specimens were more toxic to *H. indicola* compared to *M. nigra* extractives in ethanol: toluene (Figure 2).

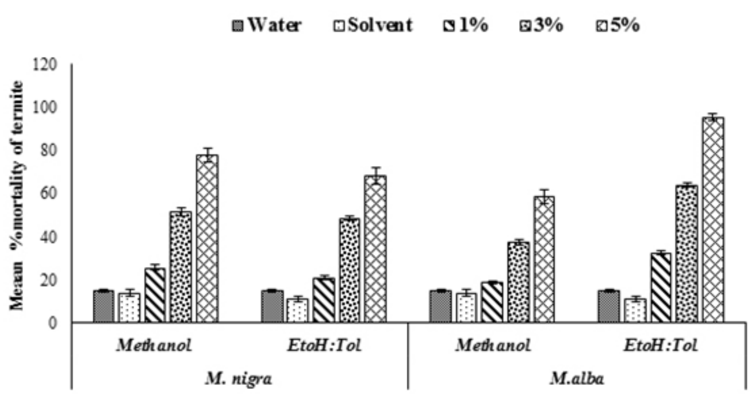

Figure 2: Effect of different concentrations of extractives from heartwood of *M. nigra* and *M. alba* on mortality of *H. indicola* in laboratory tests.

Repellent and antifeedant activities of both mulberry species at different concentrations are shown in Table 1 and Table 2. A significantly higher number of termites were observed on the solvent treated filter paper half compared to extractives treated half. Our ANOVA of the termite repellency data revealed that there was a significant interaction of heartwood type and solvents, heartwood type and concentration, while heartwood type concentration and solvents were non-significantly different ($p > 0,05$). There was a significant difference between the performances of both heartwoods. There was non-significant difference in number of termites present on filter paper treated with solvent only and untreated. A maximum repellent activity of 84 % was found in *M. alba* extracted with ethanol: toluene treated filter paper at the 5 % concentration.. However, antifeedant activity did not differ significantly between the two *Morus* species.

Table 1: Repellent activities (± SE) of heartwoods extractives from *M. nigra* and *M. alba* against *H. indicola.*

Wood type	Solvents	Concentrations of heartwood extractives			Main effect
		1%	3%	5%	
M. nigra	Methanol	22,67 ±3,53 ^{ef}	44,00±2,31 $^{c-e}$	64,00± 2,31^{a-c}	43,56±6,13 ^{B}
	EtoH: Tol	21,33 ±3,53 ^{ef}	37,33±5,81 $^{d-f}$	73,33± 2,67 ^{ab}	44,00±7,97^{B}
M. alba	Methanol	26,67± 5,81 ^{ef}	57,33±3,53 $^{b-d}$	78,67±5,81^{ab}	54,22± 7,98^{A}
	EtoH: Tol	33,33 ± 5,81 ^{ef}	58,67±3,53 $^{b-d}$	84,00± 4,62^{a}	58,67±7,69^{A}

Means sharing the same letters are not significantly different from each other at $p>0,05$.

Table 2: Antifeedant activities (± SE) of heartwoods extractives from *M. nigra* and *M. alba* against *H. indicola.*

Wood type	Solvents	Concentrations of heartwood extractives			Main effect
		1%	3%	5%	
M. nigra	Methanol	26,50±0,42^{c}	41,41±1,04^{c}	85,19±1,41^{a}	44,76±1,05^{A}
	EtoH: Tol	20,15±0,93^{c}	37,37±1,04^{c}	71,85±1,93^{ab}	52,36±1,11^{A}
M. alba	Methanol	20,00±0,00^{c}	33,33±0,00^{c}	80,95±9,52^{a}	51,02±1,58^{A}
	EtoH: Tol	20,20±3,54^{c}	45,19±5,19^{bc}	91,67±8,33^{a}	43,11±1,98^{A}

Means sharing the same letters are not significantly different from each other at $p>0,05$.

The mean percent weight loss of treated and non -treated *P. deltoides* exposed to *H. indicola* is shown in Figure 3. Solvent only treated *P. deltoides* controls showed 39 to 40 % weight loss. Conversely, *P. deltoides* treated with both types of mulberry extractives showed significantly less weight loss (2,69-6,23 %) at the highest 5,0 % concentration. Whereas, the lower concentrations did not incur as high a rate of damage. The weight loss was found to be inversely related to extractive concentration. Extractives of either *Morus* species at every concentration tested was toxic compared to the solvent only treated controls (Figure 4). Mortality of *H. indicola* was greater than 875 % in the highest concentration tested for all type of extractives except ethanol: toluene extractive of *M. nigra*.

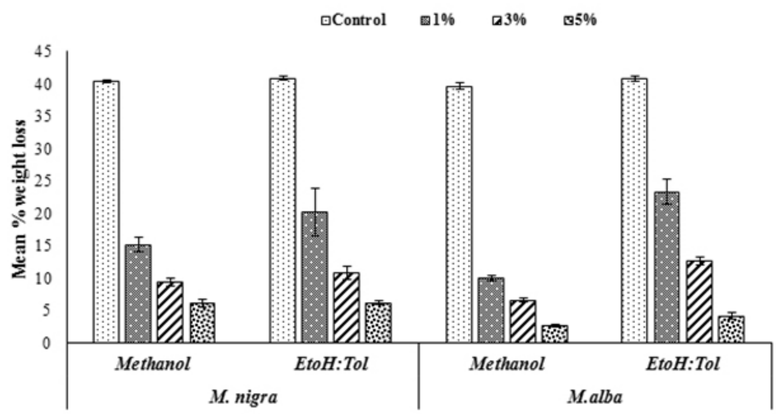

Figure 3: Mean weight loss (%) of *P. deltoides* treated with different concentrations of extractives from heartwood of *M. nigra* and *M. alba* after feeding of *H. indicola* in laboratory tests.

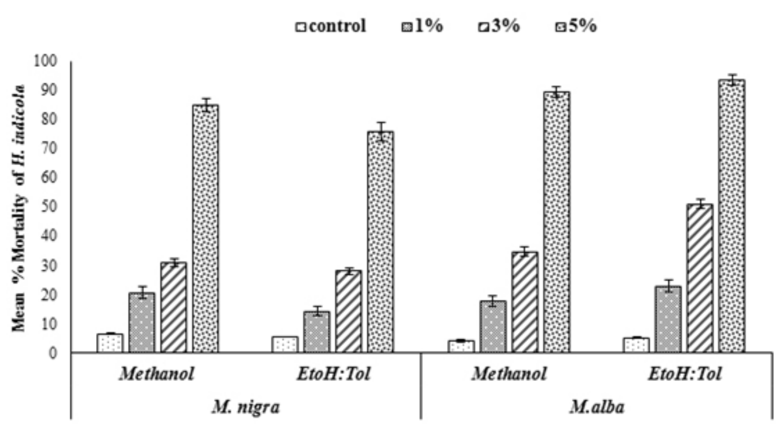

Figure 4: Mean % mortality of *H. indicola* after feeding on pressure-impregnated *P. deltoides* blocks.

In previous studies, choice and no- choice tests were used to examine the preference of wood by termites and for determination of resistance (Morales-Ramos and Rojas 2001). In our study, heartwood of both mulberry species tested showed higher resistance to termites compared to sapwood of the same species. This is not surprising, in that sapwood is generally softer and has lower amounts of toxic compounds compared to heartwoods. Sapwood also has higher level of starch and sugars that may be attractive to termites (Kasseney *et al.* 2011, Rasib and Ashraf 2014). In our study, termites in the choice test avoided the heartwood of *M. alba* compared to *M. nigra*. This is in agreement with Rasib, (2008) who showed that *M. alba* was not palatable to *Microcerotermes championi* under choice and no-choice tests. In our no-choice tests the mass loss of *M. alba* was significantly less compared to *M. nigra*. Sapwood of the positive control, *P. deltoides* was the most preferred wood species as *H. indicola* workers consumed much higher amounts of this wood.

The results of this study indicate that heartwood extractives from both *Morus* species have a negative impact on termite activity and feeding. Extractives were found to be significantly repellent to termites and showed strong antifeedant properties when termites were exposed to *P. deltoides* wood pressure impregnated with extractives removed from either *Morus* species. Between the two *Morus* species tested, ethanol: toluene extractions of *M. alba* heartwood were more toxic, repellent and better wood protectants compared to *M. nigra* extractives. Toxic and repellent activity of *M. alba* has also been observed by Rasib and Aihetasham (2016). These authors y found 70 % mortality of *Coptotermes heimi* after termites were fed on filter paper treated with methanol extractives of *M. alba* and their repellent activities at 30 % extractive concentration were 100 %. Mankowski *et al*. (2016) found 100 % mortality of *R. flavipes* after feeding on southern pine and popular wood impregnated with heartwood extractives of *M. alba*. They also identified Resorcinol from this wood in high quantities. Extractives from *M. alba* were also found to be very toxic against gut protozoan of this termite (Hasan *et al.* 2018b). Leaves and fruits of *M. nigra* have been recorded to have medicinal value and show antimicrobial and anti-oxidants activity as well (Pasheva *et al.* 2015). Heartwood extracts from *M. nigra* have been characterized to show high polyphenol content and antioxidant activity (Pasheva *et al.* 2013). To our knowledge, no work has been done previously to identify the toxic compounds from heartwood and their insecticidal/ antitermitic activities.

Previous studies have shown that termite resistance and toxicity of *M. alba* is due to the presence of certain chemical compounds in the heartwood (Mankowski *et al.* 2016, Hassan *et al.* 2018b). Sadeghifar *et al*. (2011) analyzed the heartwood extractives of *M. alba* and found that it contained 90 % resorcinol, a hydrophilic phenolic compound. Se-Golpayegani *et al.* 2010, Se-Golpayegani *et al.* 2014) observed higher termite feeding rates on solvent extracted shavings of white mulberry compared to un-extracted. They stated that durability of white mulberry might be due to resorcinol solely or synergy with other compounds. In the early 20th century synthetic resorcinol was used as antitermitic compound for the protection of wood (Lyons 1936). Resorcinol has been shown to have antifungal properties (Adikaram *et al.* 2010, Salem *et al.* 2013, Mansour *et al.* 2015) and insecticidal, anti-termitic properties against *Coptotermes formosanus.* Resorcinolated mimosa tannins were shown to kill 100 % of *Coptotermes formosanus* in a feeding test (Yamaguchi *et al.* 2002).

CONCLUSIONS

Some heartwood extractives can potentially be used for the protection of susceptible, non-durable wood species. Our laboratory experiments showed that transferring durability from white mulberry to a susceptible wood species can improve the resistance of the non-durable wood against *H. indicola*. Solvent extraction can remove enough extractives from mulberry heartwood of mulberry to enhance the resistance of susceptible woods. Future studies should include characterization of extractives, and chemical identification of the compounds responsible for antitermitic activity along with field tests examining the durability of non-durable wood species treated with transferred heartwood components from the durable mulberry species.

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