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Effects of heartwood extractives on symbiotic protozoan communities and mortality in two termite species

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ABSTRACT

Lower termites (Isoptera: Rhinotermitidae) are considered severe pests of wood in service, crops and plantation forests. Termites mechanically remove and digest lignocellulosic material as a food source. The ability to digest lignocellulose not only depends on their digestive tract physiology, but also on the symbiotic relationship between termites and their intestinal microbiota. The current study was designed to test the possible effects of four heartwood extractives (*Tectona grandis*, *Dalbergia sissoo*, *Cedrus deodara* and *Pinus roxburghii*) on the mortality, feeding rate and protozoan population in two lower termites, *Reticulitermes flavipes* and *Heterotermes indicola*. All wood extractives tested rapidly lowered protozoan numbers in the hindgut of termite workers, which was closely correlated with worker mortality. The average population of protozoans in both termite species was diminished in a dose-dependent manner after fifteen days feeding on treated filter paper. Mortality of termites increased when fed on filter paper treated with *T. grandis* or *D. sissoo* heartwood extractives with minimum feeding rate at the maximum concentration (10 mg ml^{-1}). Protozoan number and termite survival was also compared with starved termites and results showed that protozoan populations ware reduced up to 99.60 and 65.71% in *R. flavipes* and *H. indicola*, respectively as compared to untreated filter paper controls with ≅ 80% survival for both termite species. Characterizations of heartwood extractives were performed using Gas Chromatography-Mass spectrometry (GC-MS) and chemical profiles were obtained for extractives from each wood species. The largest chemical components, based on percentage of sample, identified from *T. grandis* were Squalene, 2-methyl-9, 10-Anthracenedione and 1-Methyl-3,4-dihydroisoquinoline. Tris-methoxyresveratrol, 1, 3-Diamino-8-n-butyl-5, 6 dihydrobenzoquinazoline and 6, 8-dimethyl-Benzanthracene were the largest components from *D. sissoo*. The largest percentage components from *C. deodara* were (E) − Atlantone, Di-epi-alpha-cedrene and alpha-Cuprenene. The main components of *P. roxburghii* were identified as 1, 2, 3, 4-tetrahydro-5, 8-dimethyl-Acridin-9-amine, 2, 3-dihydro-5, 7-dihydroxy-2phenyl-4H-1-Benzopyran-4-one and 5-hydroxy-7-methoxy-2-phenyl4H-1-Benzopyran-4-one. These are discussed in terms of their termiticidal and protozoicidal properties.

1. Introduction

Reticulitermes flavipes (Kollar) and *Heterotermes indicola* (Wasmann) (Isoptera: Rhinotermitidae) are major pests of wood and wood products. *H. indicola* is a common wood destroying termite, predominantly in areas north of 20°N latitude which includes Pakistan, India, and Afghanistan (Maiti, 2006). Besides causing general damage to wooden structures, this species has also been observed to destroy paper, clothing and other stored products with cellulosic components. *R. flavipes* is found in the eastern North America as far north as Ontario, Canada, and south to Key Largo, Florida and it is considered to be one of the most destructive termite pest species in this region (Su et al., 2001; Hassan et al., 2016). To prevent termite damage, non-durable wood is often treated with chemical preservatives, some of which have been shown to be environmentally toxic to both humans and animals. Hence, some chemical treatments are not approved for indoor applications (Gautam and Henderson, 2008; Ward et al., 2009; Tascioglu et al., 2013). Due to their less toxic nature, other

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alternative methods have been developed such as use plant/wood oils, tannins and other naturally occurring plant extracts (Fatima and Morrell, 2015; González-Laredo et al., 2015) to make the cellulosic material inaccessible or repellent to termites as a food source.

Naturally durable woods with antitermitic properties, such as Alaskan yellow cedar (*Chamaecyparis nootkatensis*) (*D. Don*), Western red cedar (*Thuja plicata*) D. Don, Tung tree (*Aleurites fordii*), Red wood (*Sequoia sempervirens*) (D.Don), Western juniper (*Juniperus occidentalis*) Hook, and Teak (*Tectona grandis*) L. f, are considered very resistant to termites and are good alternatives to commonly used non-durable commercial timbers like southern yellow pine (*Pinus* spp.) and cotton wood (*Populus* spp.) (Beal et al., 1974; Bultman et al., 1979; Carter and Huffman, 1982; Carter et al., 1983; Grace and Yamamoto, 1994; Hutchins, 1997; Maistrello et al., 2003). In addition, they represent a valuable source of natural products (wood extractives) that could potentially be applied to more susceptible woods to control termite infestation (Adedeji et al., 2017). Wood extractives are substances present in the wood that, unlike lignin and cellulose, do not contribute to the structural integrity of wood. They are typically concentrated in the heartwood and are important in conferring resistance to attack by xylophagous organisms (Verma et al., 2009; Kirker et al., 2013). These compounds generally act as toxins, repellants or antifeedants to termites. For example, chamaecynone, an acetylenic sesquiterpenoid from *Chamaecyparis pisifera* (Siebold & Zucc.) Endl (Saeki et al., 1973), 7-methyljuglone, the naphthoquinone from *Diospyros virginiana* L (Carter et al., 1978), loganin, the iridoid glycoside from *Guettarda speciosa* L (Yaga and Kinjo, 1985) and the sesquiterpenoid alcohols, cedrol and widdol, from *Juniperus* spp. (Adams et al., 1988). have been reported to be toxic to the termites. Patchouli alcohol from *Pogostemon cablin* and nootkatone and its derivatives from *Vetiveria zizanioides* were very toxic and repellent to *Coptotermes formosanus* Shiraki (Maistrello et al., 2001a,b; Zhu et al., 2001a,b; Zhu et al., 2003; Ibrahim et al., 2004). Similarly, 2-furfuraldeyde from *Pinus sylvestris* L and other compounds were found to be repellent to termites (Becker et al., 1971). Compounds such as carboxylic acid from *Pinus lambertiana* Douglas (Scheffrahn and Rust, 1983), limonoids from *Phellodendron amurense* (Hayata & Kaneh.) C.E. Chang (Kawaguchi et al., 1989), and *Azadirachta indica* A. Jussieu (Ishida et al., 1992) and the diterpenes, ferruginol, manool and nezukol from *Taxodium distichum* (L.) Rich were feeding deterrents to termites (Scheffrahn et al., 1988). Solvent and aqueous extract of woods such as *Diospyros sylvatica* Roxb, *Eucalyptus camaldulensis* Dehnh, *Dalbergia sissoo* Roxb, *Morus alba* L, *Cedrus deodara* (Lamb.) G. Don, *Pinus roxburghii* Sarg and *Azadirachta indica* Adr. Juss, have also showed termiticidal activities (Hassan et al., 2016; Mankowski et al., 2016).

R. flavipes and *H. indicola* are lower termites (Family: Rhinotermitidae) that possess a variety of symbiotic microorganisms, including, protists, bacteria and archaea (Ikeda-Ohtsubo et al., 2007; Duarte et al., 2016), which are harbored in the distended portion of the hindgut known as the paunch. In this symbiotic system, termites contribute endogenous cellulases and mechanical maceration of wood fibers, while flagellate protists phagocytose the wood particles and digest them (Brune, 2014). Prokaryotes in the hindgut play a significant role in maintaining the physicochemical equilibrium within the termite hindgut (Brune and Ohkuma, 2010; Xie et al., 2012).

Increasing interest in the ligno-cellulolytic degradation process performed by termites and their symbiotic fauna, has led to a number of metagenomic and metatranscriptomic studies examining the effects of different diets on these holobionts (Scharf, 2015; Duarte et al., 2016). Current research suggests that changes in protozoan species composition and community structure in the termite gut appear to be influenced by wood extractives of different woods as well as by high or low weight carbon sources of diet (Mannesmann, 1972; Cook and Gold, 2000; Tanaka et al., 2006). Chemical defensive compounds present in wood (i.e. extractives) are introduced in the gut system by feeding, which has the potential to negatively affect hindgut protozoa. This influences the feeding rate of the host on different nutritive sources and can lead to a complete loss of symbionts, ultimately causing termite death.

It is apparent that the toxicity of various woods and their extractives to lower termites is related to their deleterious effect on hindgut protozoa (Mannesmann, 1972; Mauldin et al., 1981; Breznak, 1982). Limiting termite development through the destruction of the gut microbes is a control method that has shown some success (Adams, 2004; Doolittle et al., 2007). In this perspective, the current investigation was intended to examine the effects of extractives from the heartwood of durable wood species on protozoan populations within the termite hindgut. Extractives from heartwoods with protozoicidal properties may be potential substitutes for current synthetic chemicals used in termite treatment. Thus, these natural extracts are a unique approach for developing new wood preservatives (Salem et al., 2016).

2. Material and methods

2.1. Wood species and preparation of extractives

Four economically important timber species used in Pakistan were selected for this experiment. Raw lumber of *Dalbergia sissoo* Roxb, *Cedrus deodara* (Lamb.) G. Don and *Pinus roxburghii* Sarg were purchased from a timber market located on Jhang Road in Faisalabad (Pakistan). While marine grade *Tectona grandis* L. f was acquired from a supplier in the United States (McIlvain, Pittsburg, PA). These woods were shipped to the Forest Products Laboratory in Starkville, Mississippi (USA) where they were air dried and made into wood shavings using a planer. Air-dried wood shavings (12 g) were Soxhlet extracted using 300 ml of ethanol: toluene (2:1) according to ASTM D1105-96 (ASTM, 2014). Shavings were added to Soxhlets with a small amount of cotton placed below and above to contain the shavings and extracted for a total of six hours. The solvent containing extractive solutions were placed in a pre-weighed and tared round bottom flask and then evaporated to dryness at reduced pressure by using a rotary evaporator (BUCHI, Rotavapor R-114). Extraction yield was calculated per gram of wood shavings (Ordonez et al., 2006) for all wood species extracted, and the resulting dried extractive residue was re-weighed to identify the amount of dried extractives. The dried residue was re-dissolved with solvent (ethanol: toluene) for a final concentration of 100 mg ml^{-1} based on the final weight of the dried residue from the tared flask. This was used as a stock solution for production of a series of concentrations ranging from 10 mg ml^{-1} to 1.25 mg ml^{-1}. The stock solutions were stored in 1 L screw cap jars sealed with parafilm and kept in darkness at 4 °C.

2.2. Termite procurement and maintenance

Workers and soldiers of *R. flavipes* were collected from fallen logs and dead trees at Sam D. Hamilton Noxubee National Wildlife Refuge (Mississippi) and maintained in the laboratory at 25 °C in the dark. For the collection of *H. indicola*, foraging points were identified at Peshawar, Pakistan by installing untreated poplar (*Populus* sp.) stakes (4 cm wide x 2.5 cm thick x 28 cm high), which were examined every two weeks for the presence of termites. The infested stakes were exchanged with underground monitoring stations by digging a hole in the soil so that the upper edge of the station just touched the soil surface. The monitoring station was

comprised of five wooden poplar slices (15 cm high x 8 cm wide x 1 cm thick) wrapped in a blotting paper, and held together by a rubber band, which was surrounded by a 2 mm thick plastic collar (17 cm diameter x 22 cm high). The space between the poplar and the plastic collar was filled with soil, and the exposed end of the PVC pipe was enclosed with a plastic bag to prevent water from entering the trap. These traps were inspected every two weeks and infested bundles exchanged with new ones. Infested bundles were carried to the laboratory for testing. The collected termites were kept in glass Petri dishes (14 cm dia.) containing two pieces of moist blotting paper (14 cm dia.) in incubators at 27 ± 2 °C and 75% R. H.

2.3. Termite toxicity assay

Feeding trials with *R. flavipes* were conducted at FPL (Starkville, MS, USA), while tests against *H. indicola* were conducted at NIFA (Nuclear Institute for Food and Agriculture, Peshawar, Pakistan). Whatman No. 1 filter paper (42.5 mm diameter) was oven dried (60 °C) and weighed before treatment with different concentration of extractives. For treatment, five concentrations, 1.25, 2.5, 5.0, 7.5, and 10.0 mg ml^{-1}, diluted from the 100 mg ml^{-1} stock solution of each extractive were applied on the filter paper using micro pipette. Concentrations were prepared from stock solution using ethanol: toluene as a solvent and 200 μl of this solution was applied to each filter paper. Treatments were performed in triplicate along with a control treatment with only ethanol: toluene. After treatment, all filter paper was oven dried at 60 °C for 12 h and weighed again after drying. A total of 50 termites (*R. flavipes*/*H. indicola*) were released into jars containing 20 g sand, 3.6 ml water and treated filter papers, and maintained in an incubator at 27 °C and 75± 2% R.H. for fifteen days. At the end of the test, termite mortality was calculated by counting the number of live termites. Tested filter paper was brush cleaned, oven dried at 60 °C for 12 h, and the weight loss was calculated. Filter papers were stored in a vacuum desiccator for cooling prior to weighing. Image J software (Developed by Wayne Rasband, Bethesda, Maryland) (Schneider et al., 2012) was used to calculate the area of filter paper consumed by the termites.

2.4. Gut protozoa counts

Total gut protozoan population was calculated using methods described by Lewis and Forschler (2004). Termites were fed on filter paper treated in the same manner as described for the filter paper bioassay. Sets of termites were also given no filter paper to determine the effect of starvation on survival and gut protozoans in both termite species. These starvation controls were set up similarly to the filter paper feeding tests with the absence of paper (food) in the replicate. After 15 days, termite hindguts were removed using a fine needle and forceps by gently removing the last two abdominal segments. The contents of five guts per treatment were pooled to form a single sample for each concentration. This sample was homogenized using a disposable pestle in a 1.5 ml micro centrifuge tube and 250 μl of Trager U solution for each sample (Trager, 1934; Lewis and Forschler, 2004). Ten microliters of the resulting solution was loaded onto a hemocytometer to count protozoa. The total population of all protozoan species in each termite was calculated by using following formula:

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

Percentage reduction of protozoans was calculated by comparing this value to the population in the control treatment (fresh termites out of colony and filter paper fed termites).

2.5. GC-MS analysis and characterization of extractives

Analyses were performed with an Agilent 7890B GC/MS fitted with an Agilent 19091S-433UI HP-5ms Ultra Inert column (30 m × 250 mm x 0.25 μm) and a split less injector maintained at 270 °C. Solvent delay was set at 3–6 min. For all extracts the sample size injected was 1 μL.

For extracts of *T. grandis*, the starting temperature was 75 °C ramped to 230 °C at 5 °C/min and held for 80 min (Xie et al., 2011). For *D. sissoo* a dual ramp up was used where the starting temperature was 45 °C ramped to 165 °C at 4 °C/min then ramped to 280 °C at 4.5 °C/min and held for 35 min (Aly et al., 2013). *Cedrus deodara* extracts also used a dual ramp up with a starting temperature of 70 °C ramped to 200 °C at 10 °C/min, held for 5 min then ramped to 300 °C at 10 °C/min and held for 10 min (Chaudhary et al., 2011). For *P. roxburghii* a dual ramp up was used where the starting temperature of 45 °C ramped to 165 °C at 4 °C/min then ramped to 280 °C at 15 °C/min and held for 9 min (Shah et al., 2014; Hassan and Amjid, 2009). Helium was used as the carrier gas at a constant flow rate of 1 ml/min.

The temperature of ion source in the mass 180 spectrometer was held at 230 °C and the quad temperature was 150 °C. All mass spectra were recorded in the electron impact ionization (EI) at 70 electron volts. The mass spectrometer scanned from m/z 3–700 at a rate of 2 scans per second. An integrator automatically calculated peak area. The top three compounds were identified on a percentage of sample basis using the NIST14 library.

2.6. Statistical analysis

Probit analysis was performed to calculate median lethal concentration (LC_{50}) of each extractives and each species treatment. Mortality recorded at the end of experiment, filter paper weight loss, area loss and gut protozoan numbers were subjected to one way ANOVA followed by mean separation through Tukey's HSD quantile function. Pearson correlation was calculated to determine any relationship of termite mortality and reduction in gut protozoans. All tests were run at $\alpha = 0.05$ levels using the MINITAB 17 program. All the experiments were set up in Completely Randomized Design (CRD).

3. Results

3.1. Feeding rates and termite mortality

Heartwood extractives of the four tree species showed natural biological variability in the amount of extractable phenolics and flavonoids, which resulted in different feeding and mortality rates on both species of termites (Figs. 1–2). Termite mortality and weight loss of the filter paper also varied significantly among the different extractives.

T. grandis heartwood extractives showed concentration dependent mortality on *R. flavipes* and *H. indicola* (Fig. 1) with LC_{50} at 4.43

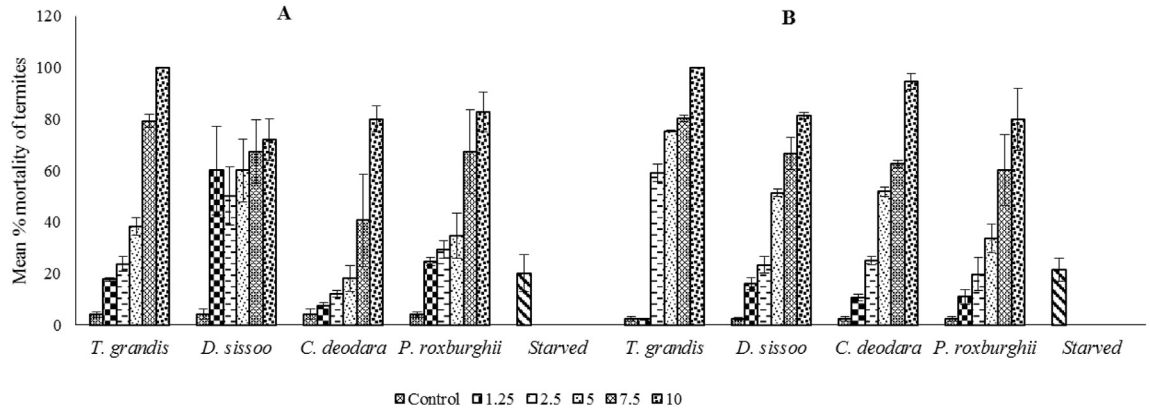

Fig. 1. Mean % mortality of *R. flavipes* (A) and *H. indicola* (B) after toxicity tests of filter paper treated with different concentrations (mg ml^{-1}) of heartwood extractives.

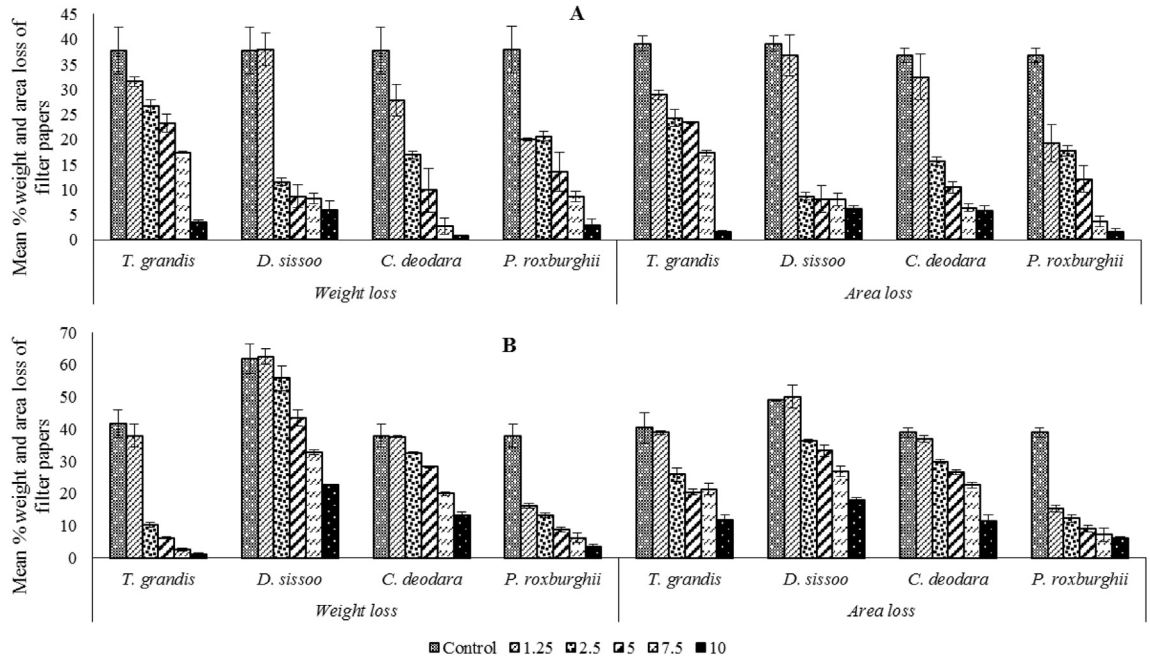

Fig. 2. Mean % weight loss and area loss of filter papers treated with different concentrations (mg ml^{-1}) of heartwood extractives after feeding of *R. flavipes* (A) and *H. indicola* (B).

(n = 50; R^2 = 0.67) and 3.21 mg ml^{-1} (n = 50; R^2 = 0.67), respectively (Table 1). *T. grandis* extractives appeared to be more toxic to *H. indicola* as compared to *R. flavipes*. Both termite species consumed significantly more filter paper treated with solvent only compared to filter paper treated with *T. grandis* heartwood extractives (Fig. 2 ab). Both termite species fed on solvent treated filter paper alone exhibited lower mortality (4%, 2.3% respectively) than those fed the extractives treated. Weight loss and area of consumption of filter paper (Fig. 2) were significantly reduced with increasing extractive concentration. At the highest concentration (10 mg ml^{-1}), weight loss and area consumed by *R. flavipes* was 3.5 ± 0.35 and 1.6± 0.33% respectively, with 100% mortality after 15 days of exposure. Similarly, *H. indicola* caused a weight loss of 1.0± 0.57%, with 100% mortality.

Table 1
Median lethal concentrations (LC_{50}) of filter paper treated with four type of wood extractives and the correlation between number of gut protozoans and termite mortality.

Termite spp.	Wood extractives	LC_{50} (mg ml^{-1})	r^2	R^2	Regression equation (Y = ax + b)
R. flavipes	*Tectona grandis*	4.43	−0.87	0.67	−221.66x + 63,489
	Dalbergia sissoo	3.89	−0.77	0.60	−238.32x + 56,079
	Cedrus deodara	7.47	−0.61	0.37	−74.618x + 56,876
	Pinus roxburghii	4.60	−0.83	0.69	−202.18x + 58,573
H. indicola	*Tectona grandis*	3.21	−0.89	0.79	−24.489x + 3582.5
	Dalbergia sissoo	5.5	−0.97	0.91	−35.408x + 3756.2
	Cedrus deodara	4.80	−0.96	0.93	−16.067x + 3521.5
	Pinus roxburghii	5.66	−0.92	0.85	−21.471x + 3602.6

Feeding rates and mortality of *R. flavipes* and *H. indicola* after exposure to *D. sissoo* heartwood extractives are shown in Fig. 1. *D. sissoo* heartwood extractives showed concentration dependent mortality against *H. indicola,* with all treatments significantly ($P < 0.05$) different from each other. All *D. sissoo* extractive treatments showed similar levels of mortality in *R. flavipes*, despite differences in concentration. In the case of *R. flavipes*, *D. sissoo* extractives incurred LC_{50} at 3.89 mg ml^{-1} ($n = 50$; $R^2 = 0.60$) after termites fed for 15 days. While extractives showed antitermitic activity with an LC_{50} at 5.5 mg ml^{-1} ($n = 50$; $R^2 = 0.91$) against *H. indicola* after 15 days of exposure (Table 1). Maximum mortality was 81.3% at the highest extractive concentration (10 mg ml^{-1}), while 16% mortality was found at the lowest extractive concentration (1.25 mg ml^{-1}). The percentage of filter paper consumed was found to be significantly lower in treated filter paper groups compared to controls ($p < 0.05$).

C. deodara heartwood extractives showed antitermitic activity with an LC_{50} at 7.47 mg ml^{-1} ($n = 50$; $R^2 = 0.37$) after *R. flavipes* fed for 15 days. *C. deodara* extractives were more toxic to *H. indicola,* with a LC_{50} at 4.80 mg ml^{-1} ($n = 50$; $R^2 = 0.93$) (Table 1). Maximum mortality of *R. flavipes* was 80% at the highest extractive concentration (10 mg ml^{-1}), while 7.3% mortality was found at the lowest extractive concentration (1.25 mg ml^{-1}). Mortality of *H. indicola* was 94.6% at 10 mg ml^{-1} while 10.6% at 1.25 mg ml^{-1} extractive concentration (Fig. 1). In both termite species, the percentage of filter paper consumed was found to be lower in treated groups than in the controls. The least filter paper was consumed at the highest concentration (10 mg ml^{-1}) where there was also maximum termite mortality (Fig. 2).

Mortality in both *R. flavipes* and *H. indicola* exposed to *P. roxburghii* heartwood extractives was shown to increase with increasing extractive concentration (Fig. 1). *P. roxburghii* heartwood extractives showed antitermitic activity with an LC_{50} at 4.60 mg ml^{-1} ($n = 50$; $R^2 = 0.69$) after 15 days of exposure against *R. flavipes*. Maximum mortality of *R. flavipes* was found (80%) at the highest extractive concentration level (10 mg ml^{-1}), while 10.8% mortality was found at the lowest extractive concentration (1.25 mg ml^{-1}). The percentage of filter paper consumed was again found to be lower in treated filter paper groups compared to the controls. Against *H. indicola*, antitermitic activity was shown with a LC_{50} at 5.66 mg ml^{-1} ($n = 50$; $R^2 = 0.85$) after 15 days of exposure. At highest concentration, mortality was 80% with minimal consumption of filter paper (Figs. 1–2).

In order to rule out the effects of worker starvation as an overriding cause of termite mortality when exposed to extractive treated specimens, starvation controls were performed. The results of the starvation controls are shown in Fig. 1. The mean mortality rate for *R. flavipes* was 20% and mean mortality rate for *H. indicola* was 21.33%.

3.2. Effect of wood extractives on gut protozoa

Although exposure to extractive treated filter paper had a detrimental effect on gut protozoa in both termite species, complete protozoan defaunation was not observed, even at the highest concentrations of extractives. Protozoan densities in both termite species were, however, dose dependent (Fig. 3). In the control treatments, a single termite gut contained an average of 60,042 ± 1877 and 3530 ± 62.4 total protozoa for *R. flavipes* and *H. indicola*, respectively. Termites fed on untreated filter paper had similar numbers of protozoa compared to termites that fed on solid *Pinus* and poplar wood. Protozoa numbers in *R. flavipes* were significantly reduced after feeding on the various concentrations of the four types of heartwood extractives. In both termite species, results showed a maximum reduction in protozoa at the highest extractive concentration (10 mg ml^{-1}), which was not correlated with maximum termite mortality except for *C. deodara* and *P. roxburghii* in *R. flavipes* and *H. indicola*, respectively (Table 1). Starvation of termites had a significant negative effect on total protozoan population. Protozoa numbers were reduced up to 99.6% in *R. flavipes* as compared to control (fresh collected termites). In contrast, protozoa numbers in *H. indicola* were reduced up to 65.71% after termite starvation (Fig. 3).

The percent reduction of protozoa in *R. flavipes* fed on filter paper treated with the highest concentration of extractives (10 mg ml^{-1}) of each wood species was 39.2, 45.7, 15.0 and 36.4% for *T. grandis*, *D. sissoo*, *C. deodara* and *P. roxburghii*, respectively. The correlation between mortality of *R. flavipes* and depletion of gut protozoa is shown in Table 1. The results suggest that extractives of *D. sissoo*, *T. grandis*, and *P. roxburghii*, at a concentration of 10 mg ml^{-1}, had protozoicidal effects in *R. flavipes*. This was not highly correlated with overall termite mortality as these have r^2 close to zero despite causing 72–80% termite mortality. The effect of *C. deodara* extractives on gut protozoa, however, was more correlated with the depletion of gut protozoans ($r^2 = -0.61$) (Fig. 3).

Reduction of protozoan symbionts in *H. indicola* demonstrated a similar dose response as *R. flavipes*, with all four extractives at the highest concentration (10 mg ml^{-1}) causing higher reduction in protozoan numbers compared to the controls. But the correlation between destruction of protozoa and mortality of termites was very low except for *P. roxburghii* extractives ($r^2 = -0.85$). The percentage reduction of protozoa in *H. indicola* fed filter paper treated with 10 mg ml^{-1} of each wood species were 82.3, 81.5, 45.3 and 52.9% after feeding on the filter paper treated with the extractives of

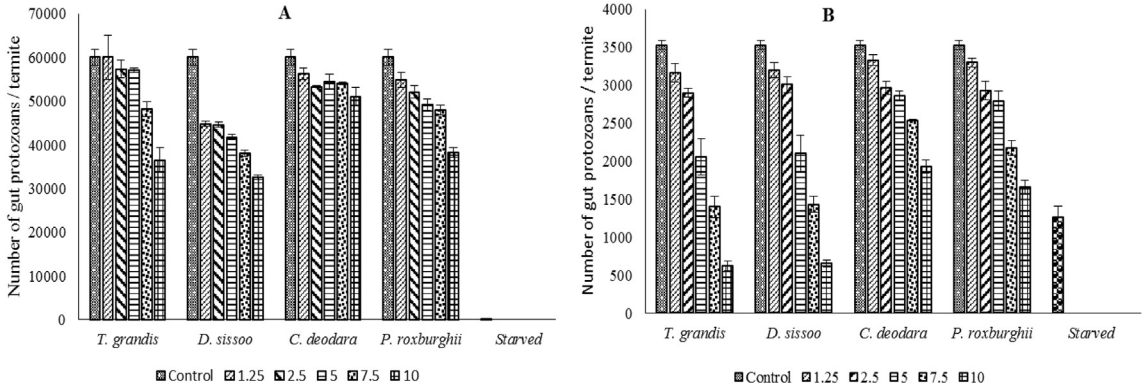

Fig. 3. Mean number of protozoa per gut in *R. flavipes* (A) and *H. indicola* (B) after toxicity tests of filter paper treated with different concentrations (mg ml^{-1}) of heartwood extractives.

T. grandis, *D. sissoo*, *C. deodara* and *P. roxburghii*, respectively.

3.3. GC-MS analysis and characterization of extractives

Table 2 shows the retention times, molecular weights, quality of identification (based on NIST14 library used by the GCMS software), and percent of sample for the three largest chemical components identified from each of the tested wood extractives in our experiment. GC-MS analysis identified a total of 96 chemical compounds from *T. grandis*, 52 from *D. sissoo*, and 147 from *C. deodara* and 12 from *P. roxburghii*. Chromatograms from GC-MS analysis of solvent extracted woods are shown in Fig. 4.

4. Discussion

Lower termites (Rhinotermitidae) are globally distributed and rely on a diversity of protozoan symbionts in their guts to augment digestion of cellulosic material (Yamin, 1979). Symbiotic gut protozoa are considered crucial, with similar protozoan species existing among the various castes and within the colony as a whole. Termites repopulate their gut fauna through horizontal transmission following ecdysis at each instar (Noda et al., 2007; Duarte et al., 2016). The average population of protozoa in both termite species tested diminished in response to increased concentration of heartwood extractives and similarly termite mortality increased with increasing extractive dosage. The effect of the extractives on termites and their protozoan fauna was most likely due to chemical constituents in the heart wood (Mauldin et al., 1981; Breznak, 1982). In starved *R. flavipes*, protozoa were drastically reduced (99.6% reduction), but survival of the termites was higher (80% survival) than termites exposed to extractives at 10 mg ml^{-1} concentrations indicating some toxicity of the extractive components.

Some species of protozoa are crucial for termite survival, e.g. *Trichonympha agilis* in *Reticulitermes* spp. (Hu et al., 2011). Previous studies showed that *R. flavipes* died soon after loss of protozoa as they are critical in termite physiology and digestion of cellulose (Mauldin et al., 1981; Hu et al., 2011). In this study, although protozoa were almost completely removed, starved termites survived possibly due to feeding on each other and the lack of any exposure to wood extractive toxins in the treated filter paper replicates. Similarly, survival of starved *H. indicola* (78.67%) was comparable to

Table 2
Three largest components from GC-MS analysis of solvent extracted heartwood of tested species.

Wood sp.	Compound Name	Retention Time [min]	Molecular Weight	Quality	% of Sample
T. grandis	Squalene	68.82	410.39	99.00	28.24
	2-methyl-9,10-Anthracenedione	42.18	222.07	97.00	24.03
	1-Methyl-3,4-dihydroisoquinoline	56.67	264.12	45.00	5.22
D. sissoo	Trismethoxyresveratrol	44.49	270.12	64.00	40.60
	1,3-Diamino-8-n-butyl-5,6-dihydrobenzoquinazoline	60.03	268.14	59.00	17.09
	6,8-dimethyl-Benzanthracene	45.44	256.12	64.00	11.41
C. deodara	(E) - Atlantone	28.89	218.16	99.00	17.92
	Di-epi-alpha-cedrene	24.19	204.18	90.00	8.14
	alpha-Cuprenene	18.78	204.18	99.00	3.84
P. roxburghii	1,2,3,4-tetrahydro-5,8-dimethyl-Acridin-9-amine	37.61	226.14	70.00	27.46
	2,3-dihydro-5,7-dihydroxy-2-phenyl-4H-1-Benzopyran-4-one	38.69	256.07	99.00	24.42
	5-hydroxy-7-methoxy-2-phenyl-4H-1-Benzopyran-4-one	39.42	268.07	99	17.41

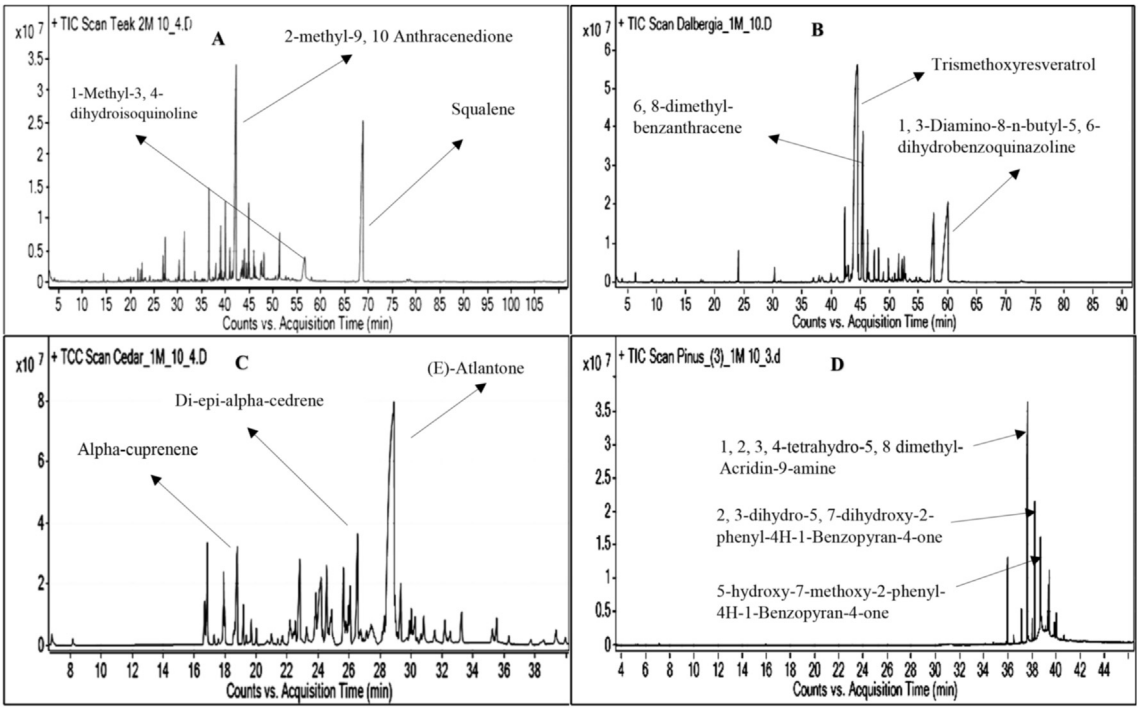

Fig. 4. Chromatograms from GC-MS analysis of solvent extracted *T. grandis* (A), *D. sissoo* (B), *C. deodara* (C) and *P. roxburghii* (D).

R. flavipes, but reduction of protozoa was significantly smaller (65.71%). Future experiments need to address the effects of extractives and their individual components on protozoa species in both termites. Haverty and Howard (1979) reported that starved *R. flavipes* lost protozoa after 13 days which is a timeframe similar to that observed in this study, Hu et al., 2011, however, observed a much longer time of 40 days for similar protozoan elimination.

Different concentrations of extractives can affect protozoa prior to the death of their host subsequently leading to termite mortality. Qureshi et al. (2014) found that the death of protozoa due to toxicity of *Mentha arviensis* L. caused mortality of *Coptotermes heimi* (Wasmann) and *H. indicola*. In our control treatments, a single *R. flavipes* worker contained 60,042 ± 1877 total protozoa on average, which was similar to the number of protozoa reported by Lewis and Forschler (2004) (58,369 ± 16,021). In the present study, the total number of protozoa were greater than those typically described from the workers of *R. flavipes* in previous studies, which range from 40,083 ± 3643 (Mannesmann, 1969) to 32,320 (Mauldin et al., 1981), 31,120 ± 8405 (Howard, 1984), and 14,642 ± 3395 (Cook and Gold, 1999). The difference between number of symbiont protozoa in the control treatment (filter paper treated with ethanol: toluene) and of laboratory-reared termites was not statistically significant, unlike to the results of Jones et al. (1983) who found 31,520, 31,920, and 48,400 protozoans in control termites fed on treated paper with hexane, acetone and methanol, respectively. Thus, an individual *R. flavipes* contained 17 times more protozoa than a single *H. indicola* in the non-treated replicates. Lewis and Forschler (2004) morphologically identified a total of eleven species of protozoans from the hindgut of *R. flavipes*. There were seven different species of protozoa in the hindgut of *H. indicola* unlike Qureshi et al. (2012), who identified nine species in the guts of this termite. The correlation between termite mortality and reduction in total number of protozoa suggests that protozoan death was the primary cause of host mortality. However a potential toxic effect of components in the heartwood extractives on the termite host cannot be ruled out (Qureshi et al., 2016). An eight-day starvation period is generally required for eradication of some of the flagellate protozoan species from termites. These studies point out that the mortality observed in this case may be due to the direct toxic effect of the heartwood extractives tested (Smythe and Mauldin, 1972). It was observed among the wood extractives tested, that *D. sissoo* heartwood extractives were the most toxic to *R. flavipes* and exposure resulted in reduced protist numbers as well. Tests with *H. indicola*, showed that *T. grandis* heartwood extractives were more toxic to the protozoan population as compared to the other three extractives. Qureshi et al., 2012 found the complete elimination of gut protozoans in *H. indicola* after feeding on heartwood of *D. sissoo* and Eucalyptus. This suggests that heartwood extracts of *D. sissoo* have potential as protozoicidal compounds. Qureshi et al., 2016 found that benzene-ethanol extractives of *D. sissoo* were found very toxic to gut protozoa of *H. indicola* and *C. formosanus*. Our findings were different from Mannesmann (1972) who observed total elimination of gut protozoa after feeding of *R. virginicus* on red spruce, although it is likely that different wood extractives influence symbionts differently (Mannesmann, 1972). Weight loss and percent consumption of filter paper was reduced when there was a high reduction in protozoa compared to the control treatment (Jones et al., 1983). Protozoan mortality cannot be generalized for all termites exposed to various wood species because different species of termites can react differently to different wood species. Mauldin et al. (1981) found a 15% reduction of symbionts per termite after feeding on white mulberry blocks for one week, but the population was drastically reduced after three weeks. In our study, we observed >50%–82% reduction in protozoa after 15 days of feeding on extractive treated filter paper treated. This may be due to feeding rates and the solvent used for the extraction of antitermitic compounds.

The GC-MS analysis showed several identified compounds have been reported to have biological activity. Of the largest percentage components identified from *T. grandis*, anthracenedione has been shown to have biocidal activity against termites (Lukmandaru and Ogiyama, 2005; Gori et al., 2009, Lukmandaru et al., 2009; Bhat et al., 2010; Xie et al., 2011). Squalene has also been shown to have biological activity against decay e.g. *T. versicolor* (Kartal et al., 2006). Reyes-Chilpa et al. (1995), found that benzofurans had antitermitic properties. Dihydroisoquinoline identified from this wood extractive has also shown insecticidal activities against *Spodoptera littoralis* (El Sayed et al., 1997). Anthracenedione (anthraquinone) and squalene made up the majority of the sample at 24.03 and 28.24%, respectively.

The most abundant compound in *D. sissoo* was trimethoxyresveratrol (40.6% of total). Trimethoxyresveratrol is a stilbene with published medicinal and free radical scavenging activities (Shang et al., 2009; Dias et al., 2013). It also exhibits potent antimicrobial and antibacterial properties (Taylor et al., 2014; Hwang and Lim, 2015). Stilbenes are regarded as potential inducers of heartwood durability (Schultz and Nicholas, 2000) and are present in many other resistant woods (Hart and Shrimpton, 1979). Resveratrol has been found to be biologically active and toxic to insects in previous studies (Hart and Shrimpton, 1979; Harmatha and Dinan, 2003; Aly et al., 2013; Nascimento et al., 2013). Dihydrobenzoquinazoline and anthracene have been reported to have antibacterial, Antiprotozoal, antiviral and anticancer activities (Agarwal et al., 2009; Kim et al., 2009; Asif, 2014).

The sesquiterpene, (E)-Atlantone) identified from *C. deodara* has been shown to have biological activity (Chaudhary et al., 2011; Bacci et al., 2015). There are numerous instances in the literature where sesquiterpenes have been found to illicit behavioral response in subterranean termites. Nootkatone isolated from *Vetiveria zizanioides* and *Callitropsis nootkatensis* has been characterized as a repellent, antifeedent, wood protectant and protozoicidal in *C. formosanus* (Zhu et al., 2001a,b; Zhu et al., 2003, 2010; Nix et al., 2003). Cuprenene and cedrene have been found associated with frontal gland secretions of soldier termites and are secreted as repellents to others nest mates at the time of the dispersal flight (Maistrello et al., 2001a; Piskorski et al., 2009).

Forms of benzopyran identified from *P. roxburghii* extractives compromised 51% of the sample. Duchowicz et al. (2009) found benzopyran was toxic and antifeedant to *Spodoptera litura*. Acridin-9-amine was not found in the literature to be toxic or biologically active and we are not certain of its function.

Heartwood chemicals targeting gut symbionts have potential as useful termiticides. Although mechanisms responsible for the natural durability of wood are not fully understood, previous studies have shown that heartwood extractives possess distinct anti-oxidant properties. It might not exclusively be toxicity and repellency of extractives in durable wood that gives it termite resistance, but may be the extractives' dual toxicity and antioxidant properties. The two (or more) factors may act together synergistically. It has been suggested that the presence of antioxidants in the heartwood may interfere with the numerous single electron redox reactions that take place in the termite gut as cellulose in converted to acetate via the shikimate acid pathway (Ragon et al., 2008). The majority of the active compounds identified in heartwood extractives of the species in this study are classified as polyphenols, which contain many powerful antioxidants. These phenols can also act as enzyme and metabolism inhibitors i.e. these molecules can bind to proteins, acting as nutritional protein precipitating agents, thus reducing their digestibility (Torres et al., 2003).

Additional studies investigating the free radical scavenging

abilities of heartwood compounds are currently underway. Based on the results of these experiments it would appear that extractive compounds with markedly different chemical profiles have similar mortality responses in response to termite feeding. Wood extractives from resistant wood species, such as *D. sissoo* and *T. grandis* have potential as protozoacidal compounds. In addition, these chemicals are slow acting and preferred because foraging termites can feed on them, return to the colony and potentially transfer them via trophallaxis to other nestmates (Mauldin et al., 1981). This may cause a reduction in vigor of the termite colony ultimately decreasing the ability of the colony to effectively attack wood (Yoshimura et al., 1992, 1993). Our study showed that feeding by *R. flavipes* and *H. indicola* on the treated filter paper had dissimilar effects on the mortality of their gut protozoa. The results could be explained on the basis of dissimilar food preferences of the two termite species. The two termite species tested belong to dissimilar genera with dissimilar gut fauna. This study suggests that the toxicity of wood extractives to protozoa might be related to the nature of the compounds removed from the various heartwoods via the ethanol: toluene solvent system used. Future studies of the physiological requirements of this sensitive symbiotic system as well as an intensive study of extractive components will elucidate this matter. A study of the extractive influences on hindgut protozoa, kept as separated species in vitro would be valuable, since it eliminates the interaction factor between the fauna members and between host and symbionts.

5. Conclusion

All heartwood extractives examined, except *C. deodara*, rapidly depleted protozoa from the hindgut in workers of both *R. flavipes* and *H. indicola* termite species tested. This appears to have caused up to 80% worker mortality within fifteen days of exposure. The results elucidate the potential for controlling these species with heart wood extractives, although the long-term effects of these volatile compounds remains unclear.

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