

Full Length Research Paper

Protozoidal activities of *Eucalyptus cammeldulensis*, *Dalbergia sissoo* and *Acacia arabica* woods and their different parts on the entozoic flagellates of *Heterotermes indicola* and *Coptotermes heimi*

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Different parts of three woods of *Eucalyptus cammeldulensis*, *Dalbergia sissoo* and *Acacia arabica* were analyzed for their toxicity potentials against two species of termites (*Heterotermis indicola* and *Coptotermis heimi*). Termite workers were allowed to feed on 2 g complete wood powder of plant species and their parts, including; bark, sapwood and heartwood. Samples of flagellates were collected after each 24 h from the termites' gut and they showed a significant variation in their mortality rate as per the wood species and their parts used in the experiments. After six days, mortality rates in flagellates were 100% with all wood parts of *E. cammeldulensis*, whereas it was 87.2, 47.61 and 100% with bark, sapwood and heartwood of *D. sissoo* respectively. However, in the case of *A. Arabica*, only bark inflicted 44.5% mortality on the flagellates in termites on the 6th day. It is revealed from the results that different woods or their specific parts have some specific toxic compounds that inflicted varying degree of toxicity on enteric flagellates of termites. Considering the toxigenic nature of different woods and their respective parts, the three woods; *E. cammeldulensis*, *D. sissoo* and *A. arabica* and their parts barks, sapwoods and heartwoods were analyzed for the presence of water soluble constituents such as lignin, benzene-ethanol soluble components and alpha cellulose contents. However, it is highly recommended that such protozoicidal compounds should be isolated, purified and biochemically characterized in order to apply them as commercial products for the control of pest like termites, which cause a huge damage to woody plants, and their products.

Key words: Bark, sapwood, heartwood, *Eucalyptus cammeldulensis*, *Dalbergia sissoo*, *Acacia arabica*, termite flagellates.

INTRODUCTION

Natural organics as pesticide highlight pests like termites cause huge damage to wood, wood products and cellulose material that is a major source of instant energy

for the growth and development of living organisms. Though termites are hugely dependent on cellulose products as their food, however, they lack basic catalytic tool, that is, cellulases to digest these polymers into monomers in order to assimilate them into their bodies (Beckwith and Rose, 1929, Pierantoni 1951, Ikeda et al., 2007, Stingl et al., 2004,2005). Comparatively, it has been noticed that flagellates play major role in such

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transformation of cellulose (Mannesmann, 1972,1973) by developing a mutual association with their host termites. It has been observed that defaunated termite's worker do not survive for more than two days without flagellates and subsequently die of starvation (Honigberg, 1970). Termites as being devastating organisms to the wood and related products are controlled by a variety of techniques and in them; the use of artificial pesticides is the most common practice. Artificial pesticides or termiticides are mostly; dieldrin, chlordane, heptachlor, depachlox aposide lindare and sodium arcinite. These chemicals are no doubt quite effective against termites, however, they proved to be recalcitrant in nature and become part of our food chain thereby leading to process of magnification. These insecticides cause serious environmental hazards, if there are any leakages after heavy rain. The pesticides have strong pungent odors (Logan et al., 1990; Martius, 1998; Jamil, 2005). These are a source of many disorders; chlordane cause cancer, monochrotophos, chlorpyriphos, dimethoate, and endosulfan cause genotoxicity (Jamil, 2005). Polychlorinated biphenyls and organochlorine pesticides (α - and γ -chlordane, p, p'- dichlorodiphenyltrichloroethane (DDT), p,p'- dichloro diphenyl dichloro ethylene (DDE), methoxychlor, and pentachlorophenol) remain persistent in indoor carpets and cause leukemia (in children), neuroses and also fatal for reproductive and immune system. One popular termiticide chlordane proved to be causing cancer and has been banned in some countries. Therefore, these are not recommended for indoor applications (Ward et al., 2009).

Currently, scientists are seriously thinking of using less hazardous techniques to control different types of pests. Among them, the use of plants and or their specific parts that are toxic to termites and their enteric micro-fauna have been taught as an effective and environmentally friendly technique. In this reference, certain reports have mentioned the marked variations in termite's survival and associated colonization when feeding on different woods and their respective parts (Carter et al., 1972; Mac Mahan, 1966). Highly resinous woods proved to be termite resistant as being woods with high lignin contents (Wolcott, 1940, 1957). Kofoid and Bowe (1934) suggested that the factors determining wood selection by the termites in nature include; physical hardness (soft or hardwood), moisture contents, the amount and chemical nature of the wood extracts and the extent of pre-existing fungal attack. They suggested that the high death rate of termites in redwood might be due to the lethal effect of certain chemicals present in the extracts on it or on protozoans living inside termite' gut.

A number of studies on the effect of woods (Smythe and Carter 1969, 1970, Beal, 1976, Bultman et al., 1978, 1979, Bultman and Perrish, 1979, Carter et al., 1972, Carter, 1976, Carter and Smythe, 1974) and wood extracts (Chuhan,1987), on termites have been carried out however, these studies did not specifically reveal the effect of diverse woods and their different parts on the

flagellate's population of their associated host termites.

Considering the hazardous effects linked with the artificial pesticide, scientists are working for the development of organic pesticides or plant based pesticides. Such chemicals will be biodegradable and inflict less hazardous effects on the environment (Alesso et al., 2003; Azmi et al., 2004; Pathak et al., 2000; Bläske and Hertel, 2001; Siddiqui et al., 2009; Verma et al., 2006). In this context, the present research was planned to look at the relationship of whether or not the termites' resistance quality of timbers and their part have a supporting or damaging effects on the protozoan population residing in the hind gut of termites. If certain natural resistant woods or their specific parts such as bark, sapwood or heartwood are toxic to the entozoic flagellates of termites, then the isolation of these chemicals can be a good substitute for other synthetic ones used as termiticides. Further, it will be helpful in choosing termite resistant woods for furniture and other purposes.

MATERIALS AND METHODS

Preparation of wood samples

The woods, *E. cammeldulensis*, *D. sissoo* and *A. arabica* and the portions of their bark, sapwood and heartwood were used in the study. 200 g of each part were cut into fragments and pulverized separately. 40 meshed and 60 retained of the pulverized material was taken and kept in sterilized plastic jars at 30°C in an oven for experimental use.

Insect procurement and maintenance

Termite workers of species *Coptotermes heimi* (Wasman) and *Heterotermes indicola* (*syn. Leucotermes indicola*) (Wasman) isoptera, insecta were collected from old trees present in the gardens of Government College University Lahore.

Termite workers were kept, before using them for the present work, at 30°C in five (1000 termite workers in each) sterilized plastic jars of 10 cm height and 4 cm in diameter containing Wattmann filter paper (No.1) of 6.2 cm radius. The jars were covered with narrow net and filter paper was moistened with 2 ml distilled water after every 24 h. Termite workers remained in the plastic jars for at least one week until their intestines cleared of debris and other wood particles following the method of Ramos and Rojas (2005) with some modifications.

Chemical analysis of woods

The different parts, that is, bark, sapwood and heartwood of three woods used in the experiment were analyzed for the presences of alpha cellulose, lignin, water solubility and benzene-ethanol solubility using TAPPI's methods

Extraction of cellulose (TAPPI's methods, No. T203)

1.5 g powdered samples were taken in a 250 ml beaker, 75 ml of 17.5% NaOH was added to the sample and the mixture was kept for ten min, 25 ml (17.5%NaOH) was added to the above mixture and was allowed to rest for 30 min. 100 ml of water was added and

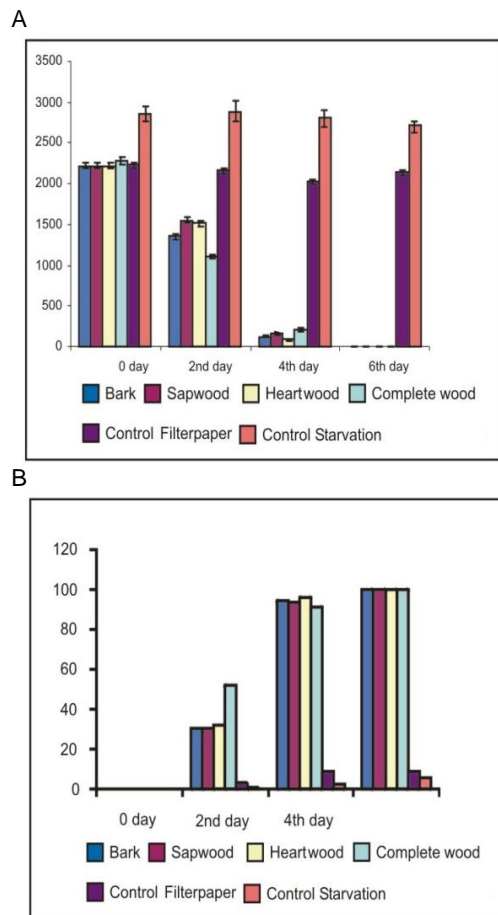


Figure 1: Comparison of the average flagellate (Figure 1a) and percentage mortality (Figure 1b) in the workers of *C. heimi* fed on wood *E. commeldulensis* and its different parts compared with that of control

allowed to rest 30 min again. The residue was filtered and washed with 8.3% acetic acid to neutralize the caustic. The retained material (alpha cellulose) on filter paper was weighed and percentage was calculated.

Extraction of lignin (TAPPI's methods, No. T222)

1 g wood sample was taken in 1000 ml flask, 15 ml of 72% H_2SO_4 was added and kept for 2 h with continuous vigorous stirring and then was diluted to 3% by adding 575 ml of distilled water, the mixture was left for 4 h and filtered. The retained substance which was lignin was dried and percentage was calculated.

Solvent extracts

The extraction of water soluble and benzene-ethanol solvent were carried out using Soxhlet apparatus (TAPPI's methods, No. T204). 20 g of wood sample were taken in a soxhlet extractor (PILZ, Heraus-Witmann, and Heidelberg, Germany). The flask was filled with 300 ml of solvent, connected with the extraction apparatus and the water started flowing to the condenser section. Heating was adjusted to boiling temperature, and extraction cycle of the solvent

was set at least 6 times per hour.

Extraction was done for a period of 4 to 5 h, then the flask was removed from the apparatus and allowed to cool. The extracts were kept in dark brown sealed bottles at 5°C. The same procedure was repeated for both water soluble and benzene-ethanol (2:1) (Civelek and Weintraub, 2004). In order to calculate percentage, 10 ml from each extract was taken in pre-weighed Petri dish dried in the air and then weighed again to find the weight of extracts which was then divided by total weight of sample taken and multiplied by 100.

Experimental set up

Pulverized wood (2 g) was taken from every sample in separate sterilized Petri dish (12 cm diameters) and hundred termite workers were kept in each one at 30°C. The wood samples were moistened with 2 ml distilled water after every 24 h. In control experiments Wattmann filter paper (No.1) of 6.2 cm radius was put in each Petri dish instead of wood samples and in the starvation control, no filter paper even was present in the jars. Five termite workers were selected randomly from each set after every 24 h and the gut of each one to study flagellate's population was dissected.

Quantitative analysis of flagellate, population

A drop of 0.2% saline solution was dropped on a cover slip. A decapitated termite worker was placed near the drop of saline solution, and its entire gut was pulled out with the help of a sterilized needle. The gut was opened in saline solution and all of its contents were squeezed out. The empty gut was removed from the cover slip after making sure that no material of gut content remained sticking to it. The drop containing the gut content was spread evenly by rotating the cover slip in all directions. The cover slip was inverted on haemocytometer and the flagellate's population counted under 10 x 10 magnifications in four random squares (1 mm²) of the haemocytometer. The average flagellates population was calculated in each square. The diameter of spread smear was measured and radius was found by dividing diameter by two. The area of the smear according to the formula of πr^2 and total flagellate's population in a smear was found using the following formula:

Total number of flagellates = Average number of flagellates in 1W or 1mm² x total area of the smear in mm².

The data were analyzed using two-way analysis of variance (ANOVA), and separated by means of Tukey's HSD quantile function, using SPSS 10.0. All tests were conducted at $\alpha = 0.05$ levels.

RESULTS

Population studies of entozoic flagellates

The population studies on entozoic flagellates in the *C. heimi* workers showed a significant mortality rate when they were fed on *E. commeldulensis* (complete wood). The mortality rates were; 51.88, 91.22 and 100% on 2nd, 4th and the 6th day of the experiment (Figure 1 (a and b)). However, it was comparatively lesser, that is, 34.9, 61.58 and 76.55% on 2nd, 3rd and the 6th days respectively in flagellates when the associated termites fed on wood of *D. sissoo* (Figure 2 (a and b)). Contrarily, wood of *A. arabica* proved to be a favorable food for

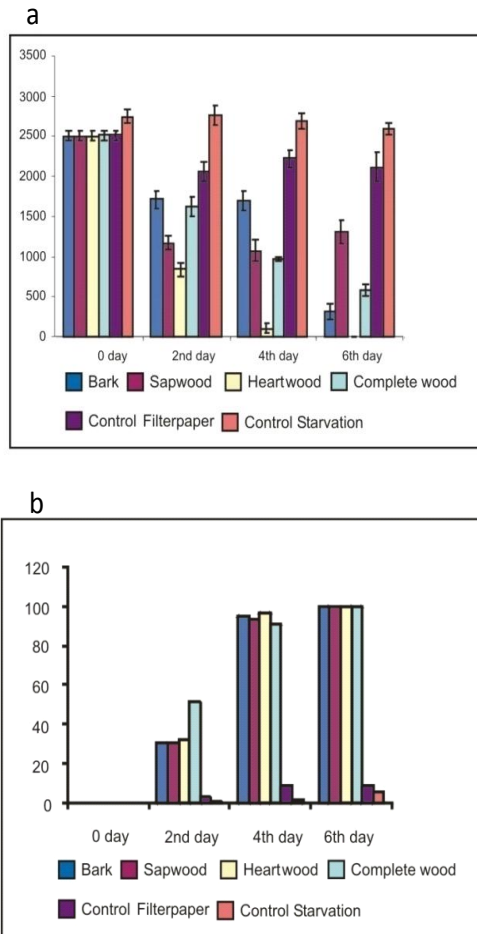


Figure 2: Comparison of the average flagellate population (Figure 2a) and percentage mortality (Figure 2b) in workers of *C. heimi* feeding on the complete wood of *D. sissoo* and their parts separately on corresponding days.

termites as flagellate's population hardly showed any mortality {Figure 3 (a and b)}. The comparative studies of different parts of the three woods revealed that with the bark of *E. cammeldulensis*, the flagellate's population reduced by 30.34, 94.70 and 100% on the 2nd, 4th, and 6th day of the experiment. Almost similar findings were made when the termites were grown either on sapwood or on heartwood ($P < 0.05$). However, the flagellates' population was hardly affected in the control and in the starvation control during the period of experimentation {Figure 1 (a and b)}. The data on flagellate populations in the termites which fed on the various portions of the *D. sissoo* showed that the heartwood was the most toxic, followed by the bark and then sapwood. The percentage age mortality of flagellates in the termites feeding on the bark, the sapwood and the heartwood was 87.2, 47.61 and 100% on the 6th day respectively {Figure 2 (a and b)}. In the case of termites feeding on bark of *A. arabica*,

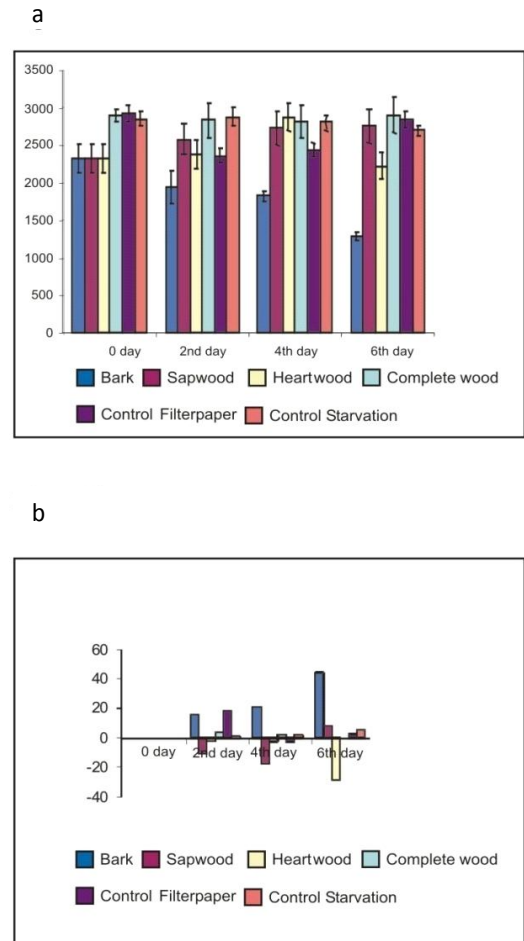


Figure 3: Comparison of the average flagellate population (Figure 3a) and percentage mortality (Figure 3b) in workers of *C. heimi* feeding on the complete wood of *A. arabica* and their parts separately on corresponding days.

the flagellates population reduced by 21.3 and 44.5% on the 4th and the 6th day of the experiment. Contrarily, the flagellates population of termites feeding on the sapwood and the heartwood of *A. arabica* registered a considerable increase. The flagellate's population of termites in the control (feeding filter paper and starvation) showed an insignificant decrease during the period of experimentation [$P < 0.05$, {Figure 3 (a and b)}]. Flagellates population studied on another group of termites, *H. indicola* indicates almost the same mortality patterns as were observed in the case of *C. heimi*. The complete wood of *E. cammeldulensis* inflicted 37.39, 68.11 and 100% mortality in flagellate's population on day 2, 4 and 6 of the experiment, respectively. The wood *D. sissoo*, however, showed less toxicity for flagellates population and the mortality rate were 20.37% on the 4 and 78.11% on the day 6 of the experiment. *A. arabica* proved to be quite less toxic for the entozoic flagellates

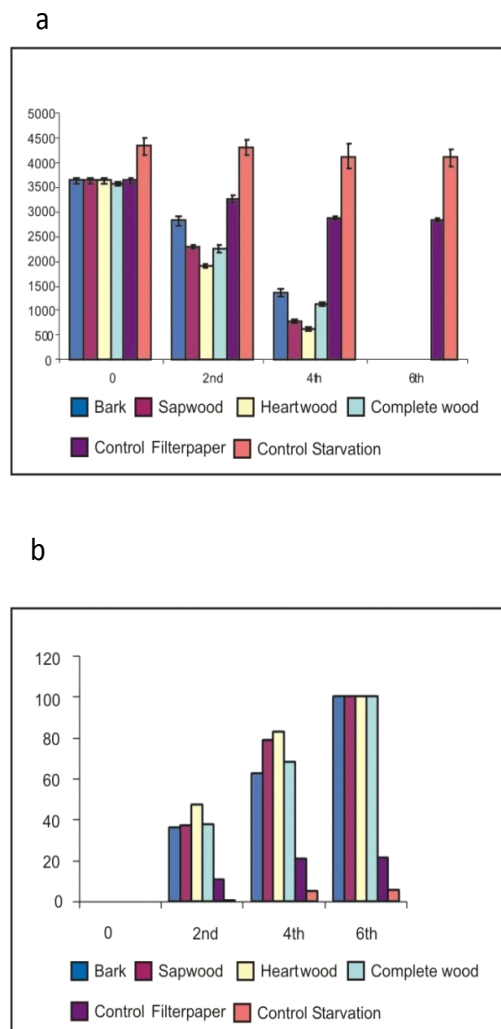


Figure 4: Comparison of the average flagellate population (Figure 4a) and percentage mortality (Figure 4b) in *H. indicola* feeding on the complete wood of *E. cammeldulensis* and their parts separately on corresponding days.

as the mortality were just 12.58% even on the 6th day of the experiment and was comparatively higher than the one obtained in the control experiments 25.55%). The bark, sapwood and heartwood of the three woods behaved differently in force-feeding by *H. indicola* workers but in a same manner as was observed in *C. heimi*. There was a decrease observed in the population of entozoic flagellates of termites with increasing time of the feeding of their respective host with all the parts of *E. cammeldulensis* and almost 100% mortality in flagellate's population at day 6 of the experiment ($P < 0.05$, {Figure 4 (a and b)}). Among different parts of *D. sissoo*, the least toxic was proved to be sapwood followed by a bark and then heartwood. The mortality rate of flagellates in *H. indicola* fed on the bark of *D. sissoo* was 69% on day 4 and 84.66% on day 6 of the experiment. Sapwood even

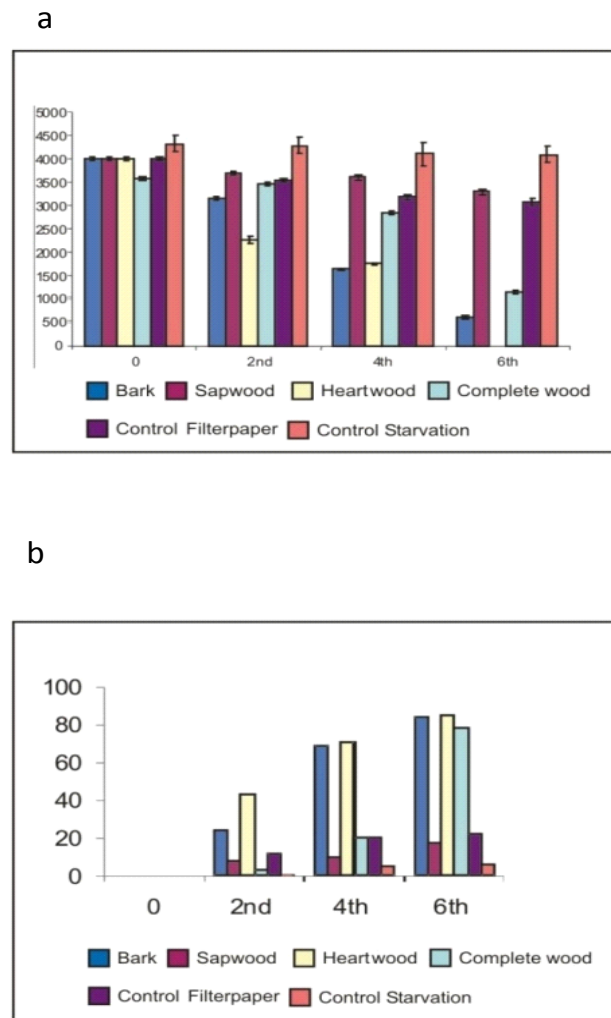


Figure 5: Comparison of the flagellate population (Figure 5a) and percentage mortality (Figure 5b) of *H. indicola* feeding on the complete wood of *D. sissoo* and their parts separately on corresponding days.

on the 6th day of the experiment inflicted only 17.1% mortality. The heartwood of *D. sissoo* also showed toxicity for flagellates which was 56% on the 4th day and 100% on the 6th day of the experiment and it was significantly greater compared to the one observed with sapwood ($P < 0.05$, {Figure 5 (a and b)}). The three parts of *A. arabica* was found to be the least toxic compared to the three parts of the other two woods experimented woods. On the 6th day of the experiment, the percentage mortality of the flagellate's population of the termites fed on the bark, reduced by 70% whereas, on the sapwood and the heartwood, there was very little reduction in flagellate population and it was only 13.4 and 10.7% respectively, on day 6 ($P < 0.05$, {Figure 6 (a and b)}). Comparison of the water soluble constituents, lignin, benzene-ethanol soluble components and alpha cellulose contents in barks, sapwoods and heartwoods of *E.*

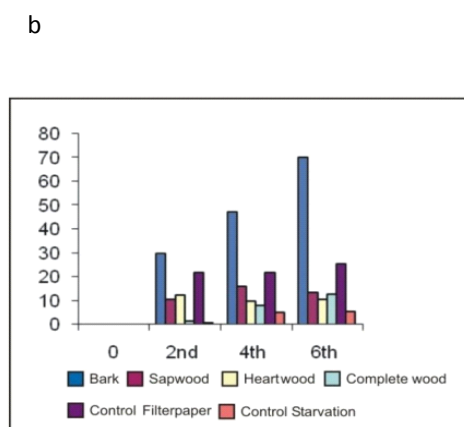
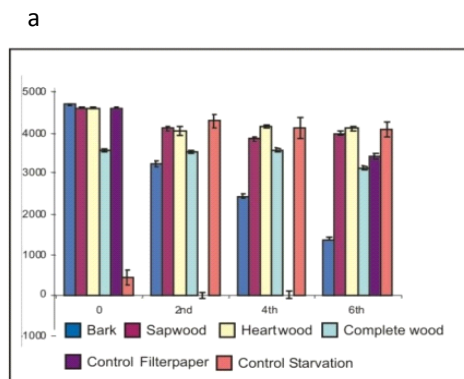


Figure 6: Comparison of the flagellate population (Figure 6a) and percentage mortality (Figure 6b) of *H. indicola* feeding on the complete wood of *A. arabica* and their parts separately on corresponding days.

cammeldulensis, *D. sissou* and *A. Arabica* is represented in Tables 1 to 4.

DISCUSSION

Woods and their parts of the three different plants inflicted a variable mortality rate on entozoic flagellates harboring two different species of termites. Amongst three woods used in the experiments, the decreasing order of toxicity to flagellates was *E. cammeldulensis* > *D. sissou* > *A. arabica*. Furthermore, the different parts of the same wood showed varying levels of toxicity in terms of death in flagellates. Akhtar and Jabeen (1981) reported that *D. sissou* in block forms were highly resistant to the attack of *C. heimi*. In the present studies, the death of the termites owes to the mortality of their protozoan population during the period of experimentation which appears to be due to the toxic effect of corresponding wood and not because of non-feeding of the woods. This could be inferred from the observation that in the starvation control, there was hardly any

mortality of termites and their flagellate's population.

It was detected that the flagellate populations were significantly reduced by feeding the termites on complete wood of *E. cammeldulensis*, and on the 6th day of the experiment, it was completely eliminated. On the 4th day of the experiment when 24% of the termites were still surviving, the number of flagellates in them reduced by 91.22% {Figure 1 (a and b)}. On the 2nd day of the feeding on *E. cammeldulensis*, 51.88% of flagellate population had died, whereas the mortality in termites was only 30%. This result clearly suggests that reduction in flagellate's population had a significant effect on mortality of termites. The mortality of the termites might have mainly resulted from the direct action of the toxicant on protozoans because on 4th day of the experiment, the workers had only 199.9 ± 21.05 protozoans, which were only 8.78% of the original population. The *D. sissou* also produced similar results except that it was less toxic than *E. cammeldulensis* {Figure 2 (a and b)}.

The wood of *A. arabica* had been found to be well supportive for the survival of termites and their flagellates, as no termite had died on feeding on the powdered wood of *A. arabica* up to the 6th day of the experiment while flagellates showed only 0.15% mortality {Figure 3 (a and b)}. This might be due to the different chemical nature of the woods and its associated different parts. Like in the case of *E. camaldulensis* stem, the overall biomass contained 45% of cellulose, 19.4% of hemicelluloses and other polysaccharides 31.3% of lignin and 2.8% alcohol-benzene extracts (Sjöström and Alén et al., 1999). However, *D. sissou* stem had 53% holocellulose, 25% lignin, 19% pentasans. 1% alcohol-benzene extract solubility in 1% NaOH was 22% and in hot water, it was 6% (Schmelzer and Fakim, 2008). Similar findings were made in the case of *A. arabica*, however, the benzene- alcohol extracts was considerably high, that is, 8.58% personal calculations using TAPPI methods. The quantity of cellulose did not prove limiting for the survival of protozoans or a termite as in starvation control, there was no significant mortality in them during the six day's experiments. The percentage of lignin also was not significantly different among the three woods but the mortality in flagellates in the hind gut of *C. heimi* was significantly greater in *E. camaldulensis* and *D. sissou* than in *A. arabica*. It is inferred from the above mentioned reports that the toxicity of wood for flagellates might be related to the nature of benzene -alcohol extracts in them and also not on quantity as in *A. arabica* which proved to be a most favorable wood for flagellates and termites; has higher quantities of benzene- alcohol extracts. So many other elements which are secondary metabolites of higher plants such as phenols, flavonoids, quinones, tannins, essential oils, alkaloids, saponins and sterols (Dubey, 2008) might be responsible for the toxicity of plants for termites and their flagellates. The chemical nature of extract also has termiticidal activity as quinine (an anti-malarial) in the extract of *Diospyros sylvatica*

Table 1. Comparison of percentage composition of water extract in bark, sapwoods and heartwoods of the three woods.

Wood part	<i>E. cammeldulensis</i>	<i>D. sissoo</i>	<i>A. arabica</i>
Barks	13.7	14.35	19.02
Sapwoods	9.34	5.53	4.77
Heartwoods	7.89	6.35	6.09

Table 2. Comparison of percentage composition of lignin in bark, sapwoods and heartwoods of the three woods.

Wood part	<i>E. cammeldulensis</i>	<i>D. sissoo</i>	<i>A. arabica</i>
Barks	19.2	17.49	37.85
Sapwoods	9.01	12.05	29.39
Heartwoods	11.7	5.08	25.03

Table 3. Comparison of percentage composition of alpha cellulose in bark, sapwoods and heartwoods of the three woods.

Wood part	<i>E. cammeldulensis</i>	<i>D. sissoo</i>	<i>A. arabica</i>
Barks	65.58	64.42	40.6
Sapwoods	81.23	79.05	69.18
Heartwoods	78.9	72.07	68.06

Table 4. Comparison of percentage composition of organic solvent extract in bark, sapwoods and heartwoods of the three woods.

Wood part	<i>E. cammeldulensis</i>	<i>D. sissoo</i>	<i>A. arabica</i>
Barks	1.65	3.31	4.65
Sapwoods	0.85	3.42	1.48
Heartwoods	1.65	15.5	2.45

roots, showed termiticidal activity on exposure to *Odontotermes obesus* (Ganapaty et al., 2004). Similarly, the extract of the Tung tree and pine resin has been reported to have anti-termite properties (Bläske and Hertel, 2001; Nunes, 2004). It is reported that the extracts of many plant species have antibacterial activities such as *Justicia adhatoda*, *Glycyrrhiza glabra* and *Hyssopus officinalis* extracts showed activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Staphylococcus aureus* (Shinwari et al., 2009). As the termites depend mainly upon protozoa and bacteria residing in their gut for the nutrient (Ikdea et al., 2008), so the plants having anti-microbial activities can be tested for the toxicity of termites and there is thus a need to search for natural anti-termite chemicals.

As far as the different portions of (bark, sapwood and heartwood) the three woods are concerned, the sapwood proved to be less toxic than heartwood and the bark of the same plant. The reason might be that the

sapwood consists of living cells, meant for conduction not for storage besides they are continuously being replaced each year. The deposited materials usually present in the bark and heartwood (Sjöström and Alén, 1999) might be the sites containing the toxicant, like anti-termite or anti-protozoan substances. In the case of *E. cammeldulensis*, all the parts that is bark sapwood and heart wood showed toxicity for flagellates which had no significant difference indicating that these parts may possess even distribution of toxicants among all parts of the wood. While *D. sissoo* bark and heartwood showed toxicity, sapwood did not show any type of toxicity towards flagellates. The bark of *A. arabica* proved to be toxic to protozoan fauna of termites (present investigations) while sapwood and heartwood did not show any toxicity for flagellates {Figure 3 (a and b)}. This might be due to low concentrations or absence of toxicants in them. The mortality of the termites might be related to the death of their entozoic protozoa as the dissected termites were found to be defaunated or they had very few, if any flagellates was

alive. A few dead termites, however were found to contain living specimens of flagellates but their number was exceedingly low. This small number of protozoans was probably not the required amount of digested food associated to the termites in order to sustain their life. Present data showed that feeding of the *H. indicola* on the three woods had similar effects on the mortality of their entozoic protozoan flagellates as seen in the case in *C. heimi*. The result could be explained on the basis of similar food preferences of the two termite species. These two termite species belongs to two separate genera but they are both subterranean termites and anchorage almost similar fauna. The *H. indicola* harbor nine species of flagellates belonging to three genera which are *Holomastigotes*, (*H. campanula*, *H. metchnikowi*, *H. annandalei*), *Holomastigotoides* Grassi and Foa (*H. metchnikowie*, *H. koidzumi*, *H. hemigynum*, *H. hartmanni*, *H. kempi*) and *Pseudotriconympha* (*P. grassi*) (de Mello, 1927). In the present observations, the same species of flagellates were diagnosed from the gut contents of *H. indicola*. The investigations on the gut contents of the *C. heimi* revealed that it harbor nine species of flagellates which were same as in the gut contents of *H. indicola*. The entire average flagellate's population in *H. indicola* was found to be more as compared to *C. heimi*. It was 39.95% in *H. indicola* while it was 22.28% in *C. heimi*.

It was reported that feeding by *Coptotermes formosanus* on red spruce might cause the total loss of flagellates and proved that different termites fed on different types of wood, and showed different food preferences as evidenced by the concentration of protozoan symbionts in their hind gut (Manesmann, 1972, 1973). Since in the present work the two termite species had almost similar entozoic fauna, similar food preferences of the two termites was not a matter of surprise. This is in agreement with the reports made here that various parts of *E. cammeldulensis* and *D. sissoo* cause mortality of protozoan fauna. The reason why the wood of *E. cammeldulensis* was more toxic to the termite gut flagellates as compared to other two plants studied is far from clear. It might be opined that some toxic material/component present in the *E. cammeldulensis* were either absent or in lower concentration in *D. sissoo* and *A. arabica*. Furthermore, these toxic components seemed to be other than volatile essential oils because the pulverized wood used in the present study was not fresh though it was not an oven-dried wood. The volatile acid might have evaporated out by the time the powdered wood was used in the experiments.

In further studies, the protozoacidal components present in the *E. cammeldulensis* and *D. sissoo* can be extracted and isolated for commercial use as termiticides.

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