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Comparative protozoacidal activities of different chemical extracts from various parts of three wood species against entozoic flagellates of *Heterotermes indicola* and *Coptotermes heimi*

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Abstract

Lower termites (Insecta: Isoptera: Rhinotermitidae) are one of the most dangerous pests causing serious damages to buildings, crops or plantation forests. Currently insecticidal use for termite control is reported hazardous for other life forms. However the lower termite harbor protozoa in their gut which aid digestion of cellulose by releasing cellulases. The present study is based on the use of plant extracts against gut protozoa (flagellates) of *Heterotermes indicola* and *Coptotermes heimi* to kill termites indirectly. The extracts of bark, sapwood and heartwood of three different plants *Eucalyptus camaldulensis, Dalbergia sissoo* and *Acacia Arabica* were obtained in different solvents (water, benzene-ethanol (2:1) and chloroform) by using Soxhlet apparatus and evaluated against gut flagellates of selected termite species in no choice feeding bioessay. It was found that water extracts of all the woods was growth promoter whereas organic solvent extracts proved to be toxic for termite gut symbionts and demonstrated variable mortality in their population in different parts of the same wood and among the three different plants also. Maximum mortality was shown by heart wood of *D. sissoo* ($P<0.05$) in benzene-ethanol extractive which is 15.5% significantly greater than its bark and sapwood. On the sixth day of the experiment, it was 964.6±59.4, 461.6±207.6 and 328.3±33.2 compared with day zero i.e. 6832.1±187.2 fed on filter papers impregnated with 1, 5 and 10% extracts respectively and no significant mortality was observed in control. It is suggested that the plant parts showing toxicity can be characterized and the affective component can be isolated by GC-MS for preparing environment friendly termiticides.

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Introduction

H. indicola and *C. heimi* are lower termites (family: Rhinotermitidae) which comprises sixteen genera and 305 species (Abe *et al.*, 2000). They solely survive upon the lingocellulosic material and possess a variety of symbiotic microorganisms in their hindgut including bacteria, Archaea and Eukarya (Konig, 2006; Ikeda-Ohtsubo *et al.*, 2007) that live in a 1-mL environment (Brune, 2007). Bacteria also contribute to the digestion of cellulose in the lower termites, but their rate of cellulase production is very low as compared to protozoa (Honigberg, 1970). About 600,000 flagellates can be found in a single hindgut of termite by weight, it is approximately 33% of the total fresh weight of a termite (Hungate, 1955). A diverse fauna of protozoa (flagellates) are found in hindgut on different termite species and about 434 flagellates species have been identified which belong to one of three orders: Trichomonadida, Hypermastigida, and Oxymonadida (Inoue, 2005) of phylum Protozoa.

In lower termites symbiotic flagellates are mainly responsible for the digestion of woods and wood materials (Mannesmann, 1972). Therefore, it seems logical to consider that any harm to the entozoic flagellates would produce a fatal effect on their host termite. *E. camaldulensis* is considered of having allelochemicals and a volatile compound in its all parts (Ghafar, 2000), cosmopolitan in nature and native to Australia (Ozel, 2008).

Allelochemicals released either from the leaves, stem, bark of living or dead trees or plants can be classified into terpenes, glucocides, coumanines, aldehydes and phenolic compounds. The leaves of *Eucalyptus* are a main releasing source of toxic compounds (Alam and Islam, 2002). *D. sissoo*, another economically important plant found in most areas of the world and is used in furniture making and other woodwork materials is attacked by a number of diseases like powdery mildew, leaf rust, leaf blight, collar rot, wilt, die-back and *Ganoderma* root fungal diseases, but there is no report of bacterial or viral disease available (Khan *et al.*, 2004) owing to this quality in the present studies, it is selected as to find its antiprotozoal activities.

A. arabica commonly known as Kikar or Babul, is found in arid and semi-arid areas. It has an antimalarial, anti-fertility activity and used for the treatment of biliousness, colds, bronchitis, diarrhoea, bleeding piles, Leucoderma, and dysentery (Chopra, 1999; Jain, 2005; Kubmarawa, 2007; Rajvaidhya, 2012). Ethyl acetate extract of *A. arabica* has antiplasmodial activity against *Plasmodium falciparum* (El-Tahir, 1999).

Owing to advantageous properties of *E. camaldulensis, D. sissoo* and *A. arabica,* present research work is designed to investigate toxic nature of chemical extracts in different solvents of above mentioned plants against entozoic flagellates of termites.

Materials and methods

Plants Selected

Woods of *E. camaldulensis* (Myrtaceae), *D. sissoo* (Fabaceae) and *A. arabica* (Rubiaceae) were selected and collected from Lahore, Pakistan and identified using key by Nasir and Ali (1970).

Extracts preparation

The stem (10cm in length and 12cm in diameter) of each selected wood was divided into three portions radially i.e. bark, sapwood and heartwood. Each wood part was ground with a pulverizer separately. The twenty grams of 40 meshed and 60 retained wood powder of each sample was extracted in three different solvents i.e. Benzene-ethanol (2:1), chloroform and water using Soxhlet apparatus at temperature 60-100^{o}C for 8 hours (2 cycles per hour). The volume of each extract was made up to 300ml for experimental use.

Samples collection and identification

Termite species selected were *Heterotermes indicola* and *Coptotermes heimi*, identified using key by Akhtar (1983) and collected from mulberry and populous trees respectively from Government

College, University Lahore, Pakistan.

Filter paper Bioessay

The filter paper (Whatmann No. 1, 2.5×2.5 inches) was impregnated with 10ml of each extract in separate Petri dishes. The solvent was evaporated. Placeded hundred termite workers in each petridish containing filterpaper impregnated with extract along with a water soaked cotton plug for humidity and kept at 25°C for no choice feeding bioassay (Kang *et al.,* 1990).Three replicates of each extract were prepared. Five termite specimens were dissected after each 48 hours from each set and observed protozoan population using haemocytometer (Mannesmann, 1972). A control experimen was run simultaneously to see whether the effect produced was due to solvent or substance having been extracted from wood material where filter paper was dipped in 10ml of solvent only without extract, dried and used as food for termites.

The heartwood extract in benzene-ethanol solvent was tested for three different concentrations i.e. 1ml, 5ml, 10ml to study the variations on survival of flagellates population as among all woods and their parts used in the experiment, the heartwood of *D. sissoo* contained highest concentration (15.1%) of organic solvent extracted (Qureshi, 2012).

Statistical analysis

Mortality ratio percentage of protozoans was determined and analysed by using one way analysis of varianvce (ANOVA) at significance level of 0.05 by SPSS software version 19.

Results

Effect of water extracts

Water extracts of all three woods appeared to be supportive for flagellates life of both termite species experimented i.e. *C. heimi* and *H. indicola* to varying extent. Feeding of *C. heimi* workers for six days on water extracts of bark, sapwood and heart wood of *E. camaldulensis,* led to an increase in the flagellate populations by 55% (8857±187.26), 40% (5929±92.67), 18.9% (5377.5±156), *D. sissoo* by 47% (8626±139.0), 13% (4798.7±46.33), 16.9% (5273.8±70.77) and *A. arabica* by 43.4 % (8399±141.55), 10% (4650.8±328.7), 19.05% (7108±212.3) respectively of the populations at day zero of the experiment ($P<0.05$). Whereas in control no extract was applied and only water was used for feeding, the flagellates population was actually reduced slightly and insignificantly by 8.8% (4897.35±26.6) on day sixth of experiment as compared to the populations of flagellates on day zero as shown in Fig 1.

Symbiotic flagellate's population in gut of *H. indicola* feeding on water extracts of bark, sapwood and heartwood of all three woods studied demonstrates similar results and was found to be supportive of life of flagellates as shown by *C. heimi* symbionts.

Table 1. Effect of different concentrations of benzene-ethanol extracts of *D. sissoo* (heartwood) on flagellates population of *C. heimi*.

Days	% Mortality at different Concentrations			LC_{50}
	1ml	5 ml	10ml	
0 days	0	0	0	0
2nd days	0.68	1.90	11.80	18.33
4th days	34.96	52.23	60.62	5.70
6th days	85.88	93.24	95.19	-14.35

After sixth day of experiment, water extracts of bark, sapwood and heartwood of *E. camaldulensis* led to an increase in flagellates population by 68.16% (6331.52±141.33), 31.91% (4452.92±241), 31.09% (4317.12±79), *D. sissoo* by 67.9% (6723.98±71.76), 16.33% (4658.25±180.19), 15.08% (4255.98±141.5) and *A. arabica* by 57.9% (6446.86±149), 19.18% (4819.4±233.2), 28% (5230.55±123) respectively as compared to the growth at day zero of the experiment. In control where no extract was applied

only water was used for feeding, flagellates population was actually reduced slightly and insignificantly by 10.8% (4074.31±7 2) on day six of the experiment as compared to day zero as shown in Fig. 2.

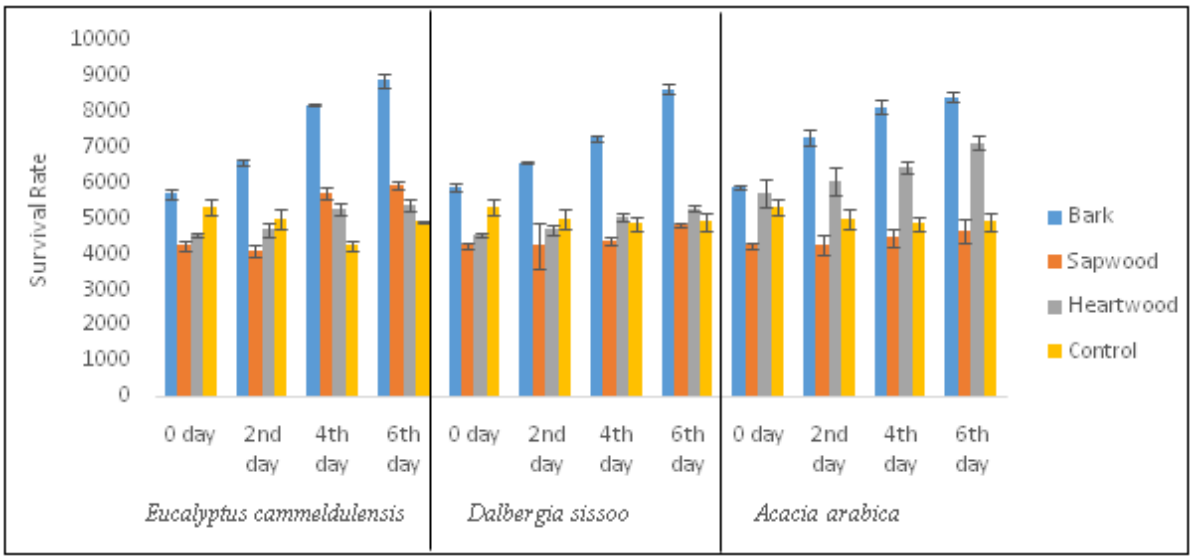

Fig. 1. Survival rate of flagellates' population of *C. heimi* on different concentration of water extracts of *E. camaldulensis* (Bark), *D. sissoo* (Sapwood) and *A. arabica* (heartwood) for 6 days in laboratory conditions.

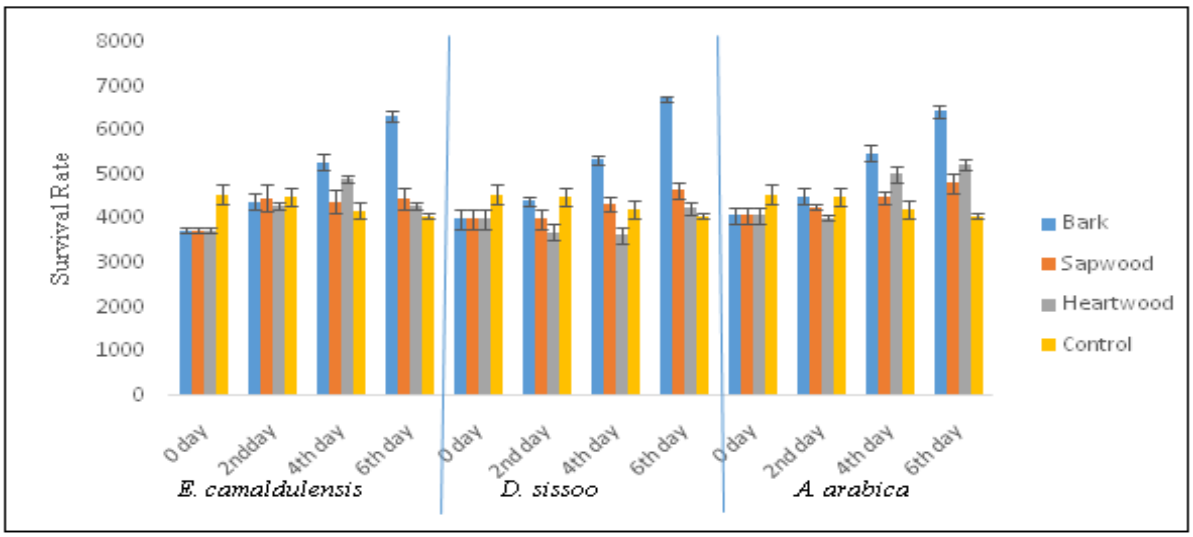

Fig. 2. Survival rate of flagellates' population of *H. indicola* on different concentration of water extracts of *E. camaldulensis* (Bark), *D. sissoo* (Sapwood) and *A. arabica* (heartwood) for 6 days in laboratory conditions.

Effect of chloroform extracts

Chloroform extracts of bark sapwood and heartwood of all three plants appeared to be toxic to flagellates as their population in both termite species was decreased when they were fed on filter papers impregnated with extracts. Flagellate populations of the *C. heimi* workers fed on the bark, sapwoods and heartwoods of *E. camaldulensis* were reduced by 81.5% (908±135.3), 95.3% (226±71.50), 91.3% (408.6±43.66), on *D. sissoo* by 74.4% (1253.5±41.5), 100% (0.0), 100% (0.0) and on *A. arabica* by 46.5% (2270±141.5), 100% (0.0), 46.5% (2270±141) respectively, on 6th day as compared to the flagellates population on day one of experiments whereas in control no significant mortality was observed Fig 3.

Flagellates population of *H. indicola* workers showed similar results as demonstrated by *C. heimi* when

nourished for six days on the chloroform extracts of barks, sapwoods and heartwoods. *E. camaldulensis* extracts significantly (P<0.05) decrease growth by 83.58% (908±570), 95.65% (226.7±71.50), 92.62% (408.6±43.66), *D. sissoo* 95.50% (249.7±103), 95.50% (1681±164), 100% (0.0) and *A. arabica* by 96.01% (204.3±113.04), 96.01% (908±34.33), 93.93% (310.74±36.26) respectively on day zero while in controls where no extract was applied and filter paper was impregnated only in pure solvent (chloroform), was used for feeding. Flagellate population was actually reduced slightly and insignificantly by 6.93% (4024.81±219.03) on the six day as compared to day zero (Fig 4).

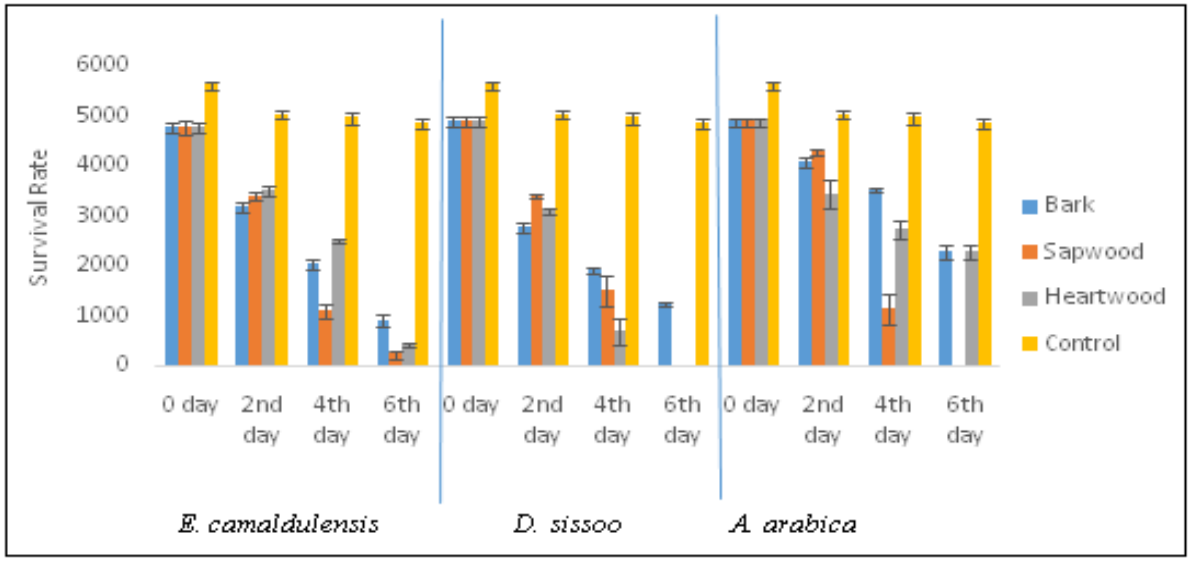

Fig. 3. Survival rate of flagellates' population of *C. heimi* on different concentration of chloroform extracts of *E. camaldulensis* (bark), *D. sissoo* (sapwood) and *A. arabica* (heartwood) for 6 days in laboratory conditions.

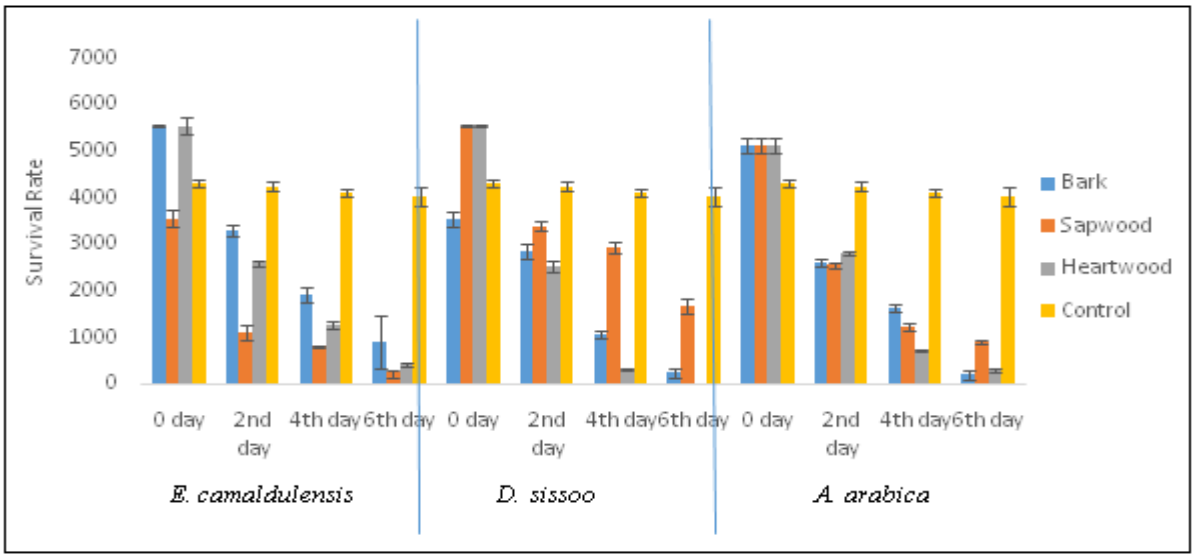

Fig. 4. Survival rate of flagellates' population of *H. indicola* on different concentration of chloroform extracts of *E. camaldulensis* (Bark), *D. sissoo* (Sapwood) and *A. arabica* (heartwood) for 6 days in laboratory conditions.

Effect of benzene-ethanol extracts

Benzene-ethanol extract of bark, sapwood and heartwood also appeared to be toxic to flagellates in both termite species as their population decreased significantly (P<0.05). The flagellate populations in *C. heimi* on the bark, sapwood and heartwood of *E. Camaldulensis* was reduced by 86. 66% (908 ± 82.7), 90.6% (681±214), 100% (0.0), *D. sissoo* by 95%

(381±70.77), 93.1% (489±71), 100% (0) and *A. arabica* by 93.3 % (454±70.69), 91.68% (568±89), 29.9% (2040±98) respectively on the 6th day of the experiment as compared to the day zero. While in control flagellates population was decreased by 18.58% (4029.25±129.4) on day 6th as compared to day zero which is non-significant (Fig 5).

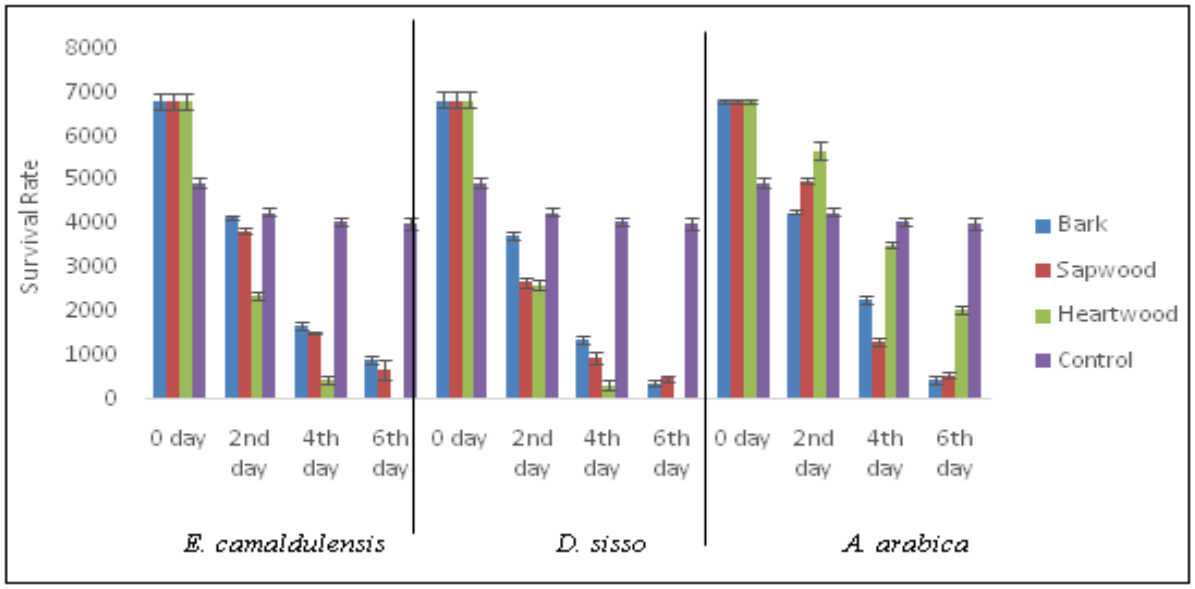

Fig. 5. Survival rate of flagellates' population of *C. heimi* on different concentration of Benzene-ethanol extracts of *E. camaldulensis* (Bark), *D. sissoo* (Sapwood) and *A. arabica* (heartwood) for 6 days in laboratory conditions.

Feeding of *H. indicola* workers for six days on benzene-ethanol extracts of barks of *E. camaldulensis* led to decrease in flagellate's populations by 90.5% (469.88±14.38), 81.55% (880.76±48.96), 78.5% (1062.30±44.66), *D. sissoo* 88.54% (567.5±44.6), 81.22% (930.346±34.33), 100% (0.0) and *A. arabica* 94.95% (249.7±103.52), 88.81% (553.45±79.24), 81.23% (928.37±106.6) respectively as compared to a zero day of the experiment. Whereas in the control termites were forced to fed on filter paper, dipped only in 10ml of benzene-ethanol solution and then dried to evaporate chemicals. Reduction in flagellates population was insignificant i.e. Only 14.07% (4460.45±117.44) on the 6th day of the experiment as compared to the day zero of the experiment (Fig 6).

Significant decrease in flagellate's population was observed when concentrations of benzene-ethanol extracts was increased. On sixth day of the experiment the flagellates population in the termite gut feeding on 1% extract of *D. sissoo* was found to be 328.3±33.2 as compared to day zero, i.e. 6832.7±187.2 whereas about 0.5% and 0.1% the flagellates population was found to be 461.6±207.6 and 964.6±59.4 respectively (Table 1).

Discussion

Termite infestation globally causes billions of dollars in economic loss each year (Tsunoda, 2003). Among these species of genera *Coptotermes* and *Heterotermes* are world wide distributed and their workers harbors divers and rich protozoan fauna as symbionts in their hind guts, which enhances the digestion process of cellulosic material (Yamin, 1979). The present investigations was based upon to study the effect of extracts obtained in different solvents i.e. Water, chloroform and benzene-ethanol of barks, sapwoods and heartwoods of three plants *E. camaldulensis, D. sissoo* and *A. arabica* on the flagellates population of *C. heimi and H. indicola.*

The outcome of present research indicated that water extracts of all parts i.e. Barks, sapwoods and heartwoods of all plants supported the growth of flagellate's population in both termite species i.e. *C. heimi* and *H. indicola*. The water soluble substances are usually carbohydrates and its derived compounds.

It is reported that the wood of *E. camaldulensis* contain 31.3% Lignin, 45% cellulose, hemicelluloses and 19. 4% of other polysaccharides (Sjostrom and Alen, 1999). However, the concentration of lignin increases with age due to the deposition of dead parenchyma tissues (Kasmani *et al.*, 2011). All the three parts (bark, sapwood and heartwood) of *E. camaldulensis* have varying concentrations of lignin contents, i.e. 19.2%, 9.01 and 11.7, cellulose 65.58%, 81.23%, 78.9%, respectively, and the percentage of water extracts in the three parts of wood is 13.7, 9.34 and 7.89 respectively (Qureshi, 2012). Lignin is a polymer, high in concentration in the barks of the three woods and it corresponds to the water extracts, is hydrophobic and aromatic but it degrades during extraction into haphazard subunits (Boerjan *et al.*, 2003) and is mainly consists of methylated three monomers coniyferyl alcohol, p-coumarly alcohol and sinapyl alcohol of polysaccharides (Freudenberg, 1968). Although lignin itself is not soluble in water but its fractions being soluble may have increased its nutritive values.

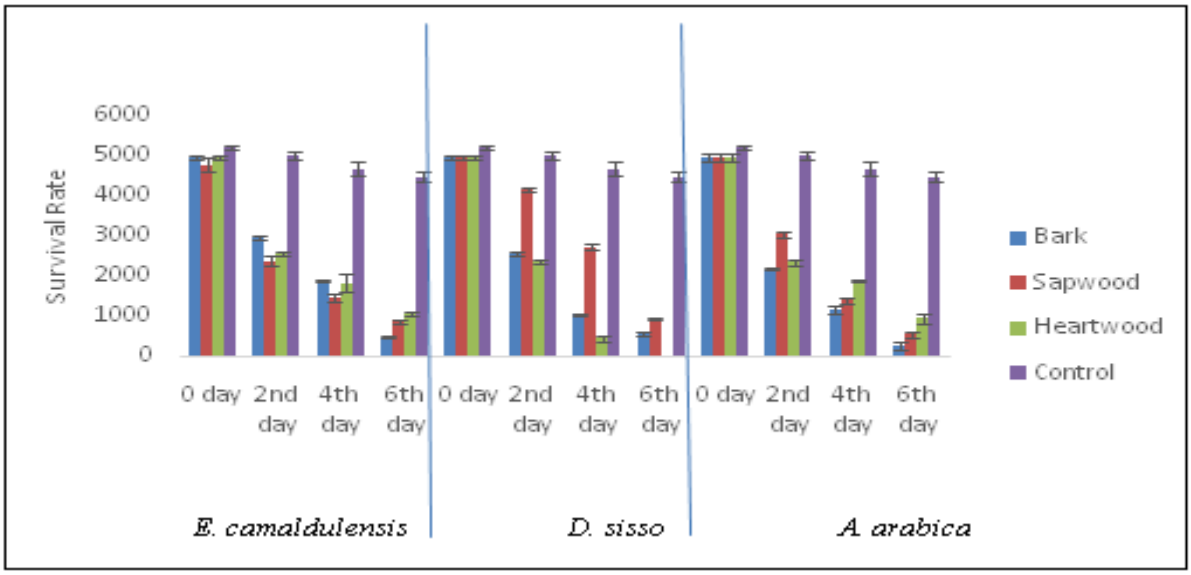

Fig. 6. Survival rate of flagellates' population of *H. indicola* on different concentration of Benzene-ethanol extracts of *E. camaldulensis* (Bark), *D. sissoo* (Sapwood) and *A. arabica* (heartwood) for 6 days in laboratory conditions.

The chloroform and benzene-ethanol extracts of barks, sapwood and heartwoods of all the three woods were found to be toxic for flagellate's population in both the termite species experimented. This toxicity may have been the result of some toxic substances, soluble in organic solvents. When the chloroform extracts of the barks, sapwoods and heartwoods of each of the plants were compared between themselves, it was found that both chloroform and benzene-ethanol extracts of the sapwoods were found to be more non supportive as compared to their own barks and heartwoods in the case of *E. camaldulensis* whereas in *D. sissoo* extracts of the heartwoods and sapwoods were found to be more non supportive as compared to their own barks. However in *A. arabica* the chloroform extracts of the sapwood is more toxic to the flagellate's life than the chloroform extracts of its own barks and heartwoods. The variation in the toxicity of both chloroform and benzene-ethanol extracts of the various parts of wood indicates that bark sapwood and heartwood have different concntration of the harmful substances in them.

Higher the amount of these toxic substances greater may be the toxicity; greater might be the toxic effects on the flagellate's populations and which results in the death of the termites also on the 6th day of the experiment (personal observations). This may be due to both the death of their flagellate's population or the direct toxic effect of these harmful chemicals on the

termites themselves. Smyth and Mauldin (Smythe and Mauldin, 1972) have reported that eight day starvation period is necessary for elimination of some of the flagellate's species from termite. These studies therefore point out that the mortality of termite in the present case may be at least partially due to the direct toxic effect of these harmful substances.

It was observed that among all the parts of all the three woods experimented the heartwood benzene-ethanol extract of *D. sissoo* was found to be most toxic as it showed maximum percentage of flagellate's death. There is gradual decrease in flagellate's population with increase in concentrations of benzene-ethanol extracts. It is reported that among the three parts of *D. sissoo* wood, the heartwood has high concentration of benzene-ethanol extract which is 15.5% significantly greater compared to its bark and sapwood which is 3.31 and 3.42%, it is also larger compared to bark, sapwood and heartwood of *E. camaldulensis* i.e. 1.65, 0.65 and 1.65% and that of *A. arabica* is 4.65, 1.48 and 2.45% respectively (Qureshi *et al.*, 2012). Considering the high concentration of organic solvent extracts in heartwood of *D. sissoo* and its toxicity for flagellates' population, this portion of wood may be used for isolation and characterization of their bioactive compounds for further analysis via HPLC or GC-MS. Each compound in these plant can be identified and evaluated by *invitro* and *invivo* methods for their phormocological effects in future and can be an effective and efficient drug source if they will not prove to be toxic for vertebrates and found to be environment friendly. These bioactive components can be strong antitermitic agents of agriculutural importance.

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