

Original Research

Termiticidal and Protozocidal Potentials of Eight Tropical Plant Extracts Evaluated Against *Odontotermes obesus* Rambur (Blattodea; Termitidae) and *Heterotermes indicola* Wasmann (Blattodea; Rhinotermitidae)

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Abstract

The subterranean termites *Odontotermes obesus* and *Heterotermes indicola* are highly infesting wood pests that cause economic losses that cost billions of dollars throughout the world – especially in tropical and subtropical regions. It is highly significant to formulate insecticides from plant-based chemicals rather than synthetic ones. The eight plant species resistant to termites were selected for screening as anti-termite phytochemicals. Extracts from different parts of the plants were collected by using ethanol solvents in different ratios. These extracts were evaluated for their toxicity against termites and their gut flagellates at subsequent concentrations in the range of 100-2000 ppm to find LC_{50} . Although the plants tested were found to be effective, extract from *Grevillea robusta* showed maximum mortality and reduced termite survival rate to 94% ($LC_{50} = 1391.01$) and 90% ($LC_{50} = 1607.95$) of *O. obesus* and *H. indicola* respectively. This extract also reduced the survival rate of hindgut flagellates of *H. indicola* up to 95% with an LC_{50} value of 502.64 ppm. The extract of *Grevillea robusta* was characterized by UV-Vis spectroscopy, FTIR and GC-MS analysis. Two absorption peaks were recorded by UV-Vis spectroscopy at 208.94 and 282.97nm having absorption of 2.33 and 0.811 respectively. FTIR analysis confirmed the presence of amines, alcohols, alkanes, nitriles, aldehydes, ether and halo compounds. GC-MS analysis revealed the presence of 15 phytochemicals with a high percentage of Coumarin (30.64%), 7-hydroxy, Methyl hexofuranoside (27.63%) and 9,12,15-Octadecatrienoic acid

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(Z,Z,Z) (8.97%). The natural phytochemicals from *G. robusta* having termiticidal and protozoicidal potential being eco-friendly can replace synthetic pesticides for termite control.

Keywords: endosymbiont, termiticidal, *Grevillea robusta*, phytochemicals, spectroscopy, GC-MS

Introduction

Termites are “silent killers” as they ruin houses and wooden materials without showing external damage at initial stages. Globally, 90% of economic loss and 70% of construction damage is caused by the subterranean termites *Coptotermes formosanus* Shiraki and *Coptotermes gestroi* Wasmann [1-3]. Approximately \$1 billion is spent annually on the loss caused by termites in the United States [4]. In Southeast Asia, the cost of termite damage is approximately \$400 million per year [5]. Currently, a number of insecticides are available in the market, like carbamates, organochlorine, synthetic pyrethroids, chlordane, bifenthrin, fipronil and Termidor. Exposure to these synthetic insecticides causes serious health issues to humans, e.g., hyperglycemia, oxidative stress, and metabolic and nervous system disorders [6]. However, at the present time due to the lack of any effective alternative, these chemical insecticides are allowed for crop and soil treatments [7]. The urge for using naturally occurring botanical compounds as wood preservatives has increased from the last few decades [8-10]. Therefore, there is a need to explore natural products for developing pesticides or termiticides being biodegradable and environmentally friendly. Bioactive compounds from different plants that are cheaper, target-specific, biodegradable and non-toxic to humans can be used to replace synthetic insecticides [11]. Plant-derived biocides are naturally occurring compounds in roots, leaves, heartwood and bark, where they act as a defensive agent against xylophagous insects [12]. Termiticidal compounds like 2-methyl-9,10-anthracenedione, 1-methyl-3,4-dihydroisoquinoline and squalene have been reported to be present in the heartwood of *Tectona grandis* [8]. Rasib et al. [13] reported that carboxylic acid isolated from *Magnolia grandifolia* and coniferyl alcohol from *Saraca asoca* were found to be a feeding deterrent against *Odontotermes obesus*. Similarly, a phytochemical “resorcinol” derived from *Morus alba* proved to be toxic against *Reticulitermes flavipes* [14].

Carica papaya (Caricaceae) is native to Mexico and northern South America. It is a medicinally important plant and is widely used as a whole plant as well as its seeds, leaves and fruits being used for pest control. *C. papaya* leaf extract exhibits antifungal, antibacterial, wound healing and antitumor potential [15]. The genus *Eucalyptus* (Myrtaceae) has more than 700 species native to Australia and is widely used as an antiseptic, repellent for insects, flavoring and fragrance agent, as well as in pharmaceuticals [16]. Antitermitic activity of *Eucalyptus citriodora* oil has been reported against higher termites and *Heterotermes indicola* [17] and

antibacterial activity [18]. Antitermitic activities of *E. camaldulensis* leaf oil extract has been reported against *Coptotermes formosanus* in Thailand by both contact and fumigation methods [19]. The current study is based on the use of eight tropical plant leaf extracts for evaluating their termiticidal properties as they are locally available. The chemical characterization of the most effective plant was carried out by VU-Vis Spectroscopy, FTIR and GC-MS analysis. So that the natural plant products could become a good substitute for synthetic insecticides to prevent health hazards.

Materials and Methods

Termite Collection

Termite workers were collected from dead wooden logs and mud mounds using a collection of trap units prepared by plastic bottles containing wet toilet tissue rolls. Molasses was used as a phagostimulant to attract the termites. The traps were buried half underground at termite collection sites and monitored regularly. After 15 days, traps were brought to the laboratory, where active and healthy workers were separated. Live specimens were kept in Petri dishes with a soaked cotton plug in the laboratory at temperature $27 \pm 1^\circ\text{C}$ and relative humidity $80 \pm 5\%$. Termites were fed on No. 2 Whatman filter paper for one week, before the trial, to remove debris and other wood particles.

The plants leaves (Table 1 and Fig. 1) collected from the same vicinity were washed, shade dried at room temperature ($27-37^\circ\text{C}$) and powdered mechanically with an electric blender (Daigiger Scientific, USA). Thirty grams of plant powder were subjected to obtain extract in 250 ml of ethanol solvent by using a Soxhlet apparatus at $40-60^\circ\text{C}$ (two cycles per hour) for 8 hours. The extract was concentrated by evaporating the solvent with a rotary vacuum evaporator (R-300, Rotavapor, Germany). The stock solution was prepared by dissolving 1g of crude extract in 100ml of distilled water and different concentrations of 100, 500, 1000, 1500 and 2000 ppm were prepared by applying formula $C_1V_1 = C_2V_2$.

$$V_1 = \frac{\text{Required ppm} \times \text{Required Volume}}{\text{Stock Solution}}$$

Experimental Design

No-choice bioassay [20] was designed to evaluate the toxic potential of plant extract against two species of termites: *O. obesus* and *H. indicola*. Whatman

Table 1. Plants selected for antitermitic and antiprotozoan bioassays.

Sr. No.	Botanical name	English name	Common name	Family
1	<i>Carica papaya</i>	Papaya	Papita	Caricaceae
2	<i>Eucalyptus camaldulensis</i>	River red gum	Safaïda	Myrtaceae
3	<i>Ocimum basilicum</i>	Sweet basil	Niazbo	Lamiaceae
4	<i>Grevillea robusta</i>	Silky oak	Bahekar	Proteaceae
5	<i>Eucalyptus globules</i>	Blue gum	Safaïda	Myrtaceae
6	<i>Pongamia pinnata</i>	Indian beech tree	Sukchain	Leguminosae
7	<i>Mentha longifolia</i>	Horse mint	Podina	Lamiaceae
8	<i>Melia azedarach</i>	Chinaberry tree	Dhraik	Meliaceae

No. 1 filter paper (2.5"×2.5") was pre-weighed and treated with 1.5 ml of different concentrations of (100, 500, 1000, 1500 and 2000ppm) for each plant extract. Papers were then air-dried at room temperature and kept in Petri dishes (3"×3"). Fifty termite workers of each species were placed in each petri dish containing plant extract-treated filter paper. The Petri dishes were kept in a dark environment at 27±1°C and 80±5 RH, and a few drops of distilled water were periodically added to each petri dish to keep up moisture content. All the experiments were conducted in replicates, and control was treated with distilled water only. Percentage mortality was calculated against each plant extract at every dose treatment after each 24 h for 16 days by applying the formula:

$$\% \text{ Mortality} = ODP \div TP \times 100$$

...where ODP is observed dead population of workers and TP is total population.

Counting Protozoa

Protozoa count was made by using the method described by Hassan et al. [8] for evaluating the toxic potential of each plant extract against the hindgut flagellate population. Every 24 hours, two termite workers were removed and their hindgut contents were opened in 250 µl of 0.2% saline solution by using sterilized forceps and needle. The gut contents of two termites were homogenized in sterilized micro-pestle and used as a single sample for each treatment. Protozoa number was calculated by loading 25 µl of resulting gut content onto Neubauer's chamber (Bright-Line Sigma, USA) under a digital trinocular microscope

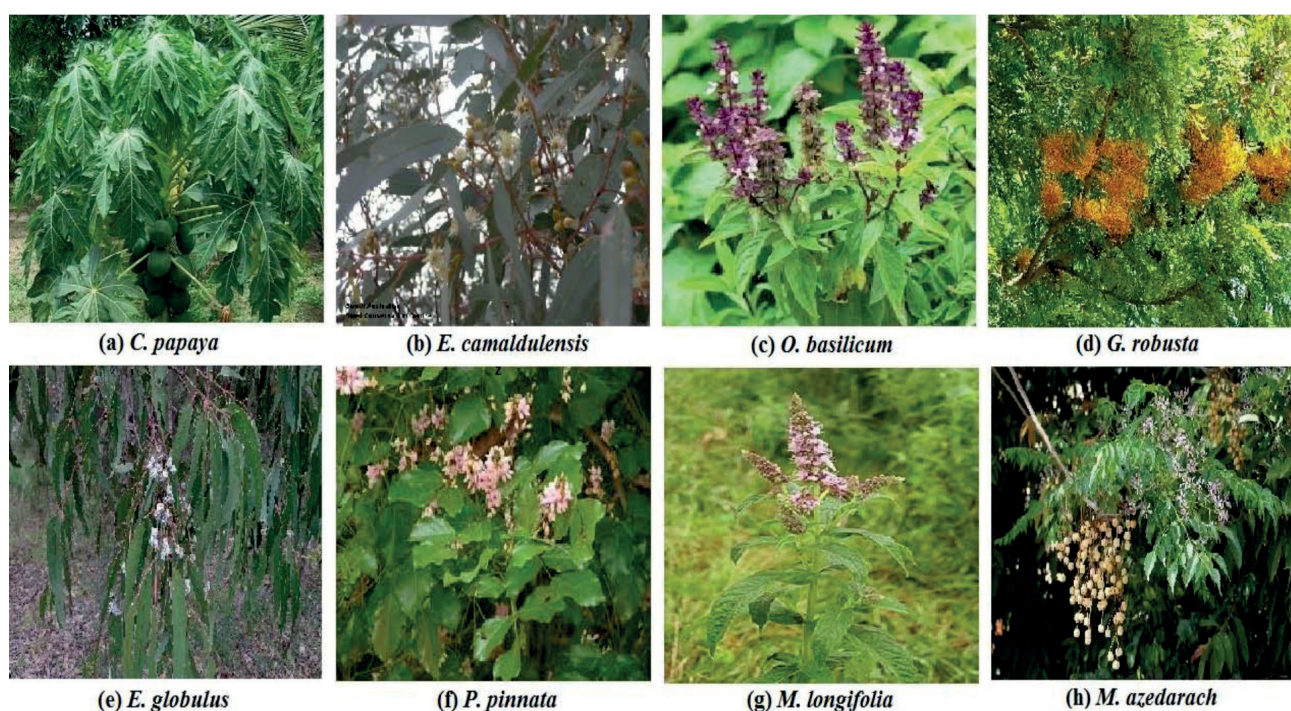


Fig. 1. Plants selected for antitermitic and antiprotozoan bioassays.

(Optika B-500, Italia). Counts were made by calculating the mean number of protozoa in four squares of each chamber according to Lewis and Forschler [21], and results were compared with control for determining a decrease in the protozoa population.

$$X = \frac{\left(\text{No. of cell counted} \times \text{Volume of saline} \frac{\text{solution}}{\text{sample}} \right)}{\left(\text{Volume of Neubauer's chamber} \times \text{No.} \frac{\text{termites}}{\text{sample}} \right)}$$

Characterization of the Most Effective Plant Extract

UV-Vis Spectroscopy

The leaf extract was examined under UV-Vis spectroscopic analysis for its spectral profile. The extract was centrifuged for 10 minutes at 3000 rpm and filtered using Whatman No. 1 filter paper. The sample was then diluted 10 times with ethanol. The spectra were obtained by scanning the extract from 200 to 800 nm wavelength using a spectrophotometer (UV-1601, Shimadzu), and characteristic spectral peaks were recorded [22].

Fourier Transform Infrared Spectroscopy (FTIR)

Functional groups were detected by FTIR analysis and the characteristic peak values were recorded in the infrared region from 400-4000 cm^{-1} [23]. The sample was prepared by mixing 2 mg of leaf extract

in 100 mg of KBr (FTIR grade). The mixture was then compressed to prepare 3 mm (diameter) salt discs which were loaded in FTIR spectroscopy (Shimadzu ETIR spectrophotometer) and scanned to record infrared spectra at different wavelengths (400-4000 cm^{-1}).

Gas Chromatograph-Coupled Mass Spectroscopy (GC-MS)

Leaf extract was further analyzed by using gas chromatography coupled with a mass spectrophotometer (GC-2010, Shimadzu). The chromatographic system was installed with an AOC-20i auto-injector, AOC20s autosampler and QP2010 mass selective detector. The fused silica capillary column (DB-5Ms, 0.25 mm \times 30 m \times 0.25 μm) having a maximum temperature capacity of 325°C was used for screening. The column's initial temperature was 100°C and maintained at this temperature for 0.5 minutes. The temperature of the oven was then increased to 280°C (24°C/min) and maintained for 3 minutes. Injector temperature was 250°C with a flow rate of Helium as 1.0 ml/min. The chromatograph was operated in split injection mode with an injection volume of 1.0 μL and split ratio of 10:1. The ionization voltage was 70 eV and the mass spectral scan range was 50-350 m/z.

Identification of Phytoconstituents

The National Institute of Standards and Technology (NIST) database having more than 62000 patterns was used for interpreting the mass spectrum. The

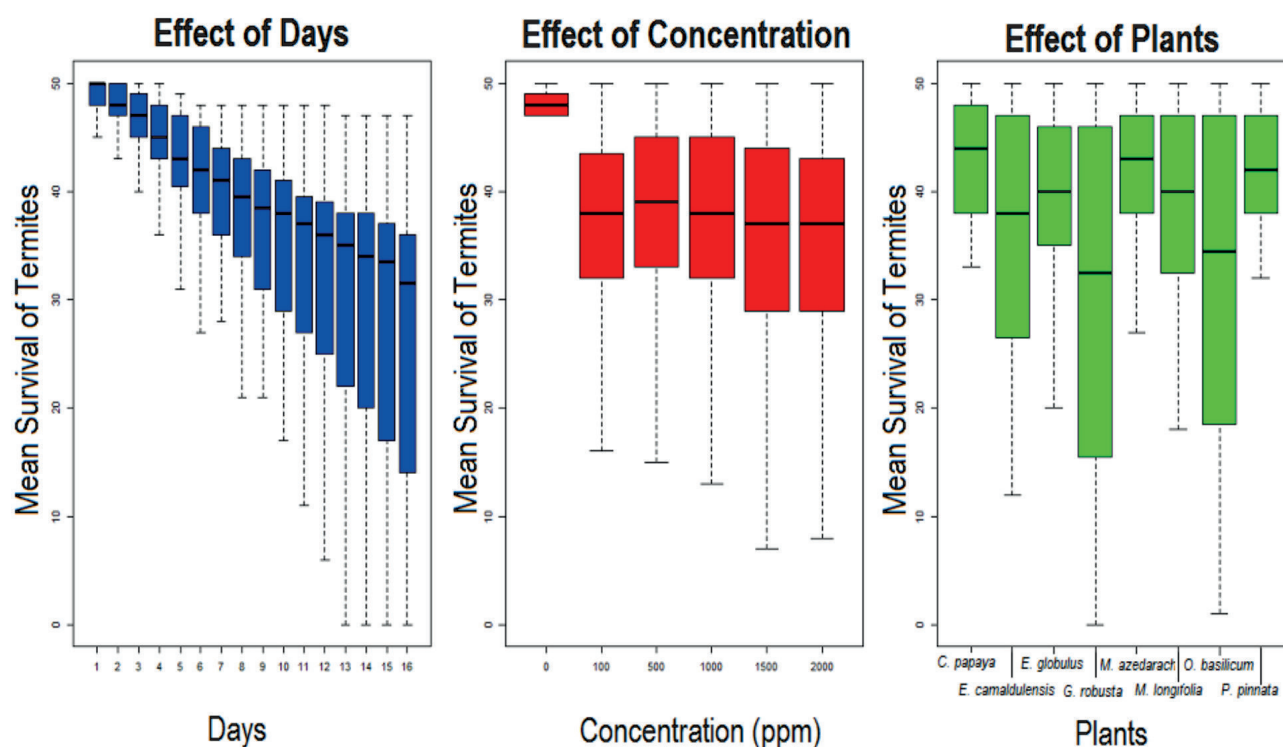


Fig. 2. Box plot representing the effect of days, concentration and plants on the mean survival of *O. obesus*.

comparison between spectra of known and unknown compounds, from the NIST library, helps in interpreting the mass spectrum and determining name, chemical formula, chemical structure and molecular weight of identified compounds [24].

Statistical Analysis

Mean mortality of termites and protozoa for each plant extract and concentration was compared, and statistically significant differences were determined using one-way ANOVA with Tukey's multiple comparison tests using "R language". Results with P value less than 0.05 were considered to be significant and less than 0.005 considered strongly significant. Stars (*, **, ***) denote statistically significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. LC_{50} value of each plant extract against termites and their gut protozoa was calculated by applying "probit analysis" using SPSS software version 19.

Results and Discussion

Antitermitic Activity of Plant Extract Against *O. obesus* and *H. indicola*

Antitermitic activity of ethanolic leaf extract of eight plants against two termite species (*O. obesus* and *H. indicola*) was evaluated by using a no-choice bioassay. Five different concentrations (100, 500, 1000, 1500 and 2000) were used in a 16-day experiment, and the mean survival of termites was calculated. All concentrations were found to have a significant effect

on termite survival against *O. obesus* as compared to control ($F (df) = 289.0 (5)$; $P < 0.05$) in a time-dependent manner, which also differed significantly from each other ($F (df) = 216.0 (16)$; $P < 0.05$). Similarly, all plants showed different antitermitic activity in dose- and time-dependent manners ($F (df) = 209.6 (8)$; $P < 0.05$). The effects of days, concentrations and plants on mean survival of termites is represented in Fig. 2. Among the tested plants, *G. robusta* was found to be most effective, with a significant ($P < 0.05$) reduction in survival of termites (94%) at the 16th day of the experiment. Similarly, *O. basilicum*, *E. camaldulensis* and *E. globulus* also significantly ($P < 0.05$) reduced termite populations with 34, 40 and 60% survival rate, respectively. However, *M. azedarach*, *C. papaya*, *M. longifolia* and *P. pinnata* have a non-significant effect on termite survival with less than 30% reduction in the termite population (Fig. 3).

An almost similar response was observed against the mean survival of *H. indicola* in *O. obesus*. All concentrations were found to have a significant effect on termite survival as compared to control ($F (df) = 343.0 (5)$; $P < 0.05$) in a time-dependent manner, that also differed significantly from each other ($F (df) = 261.6 (16)$; $P < 0.05$). Similarly, all plants showed differential antitermitic activity in dose- and time-dependent manners ($F (df) = 234.8 (8)$; $P < 0.05$). The effect of days, concentrations and plants on mean survival of termites is represented in Fig. 4. Among all plants used, *G. robusta* showed a significant ($p < 0.05$) decrease in the termite population with 50% termite death after the 10th day of the experiment. About 90% of termite mortality was recorded on the 16th day of the experiment. Similarly, *E. camaldulensis*, *O. basilicum*,

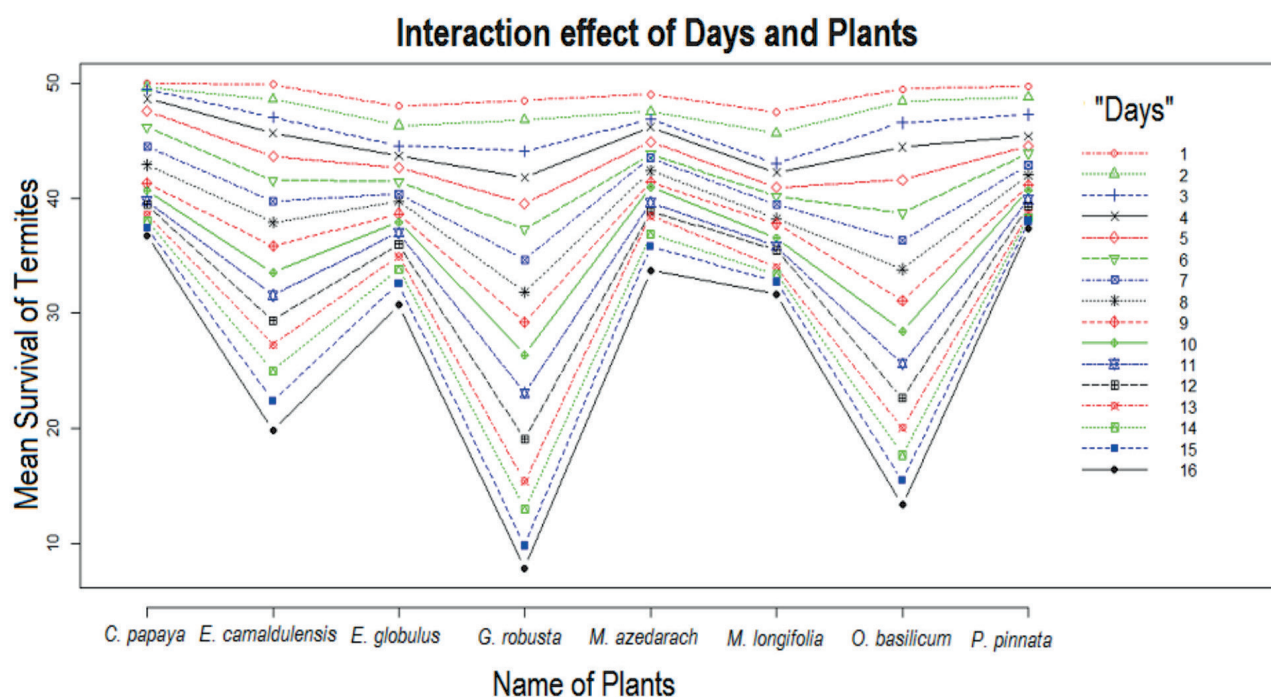


Fig. 3. Interaction plot representing the interaction effect of plant extract with days on mean survival of *O. obesus*.

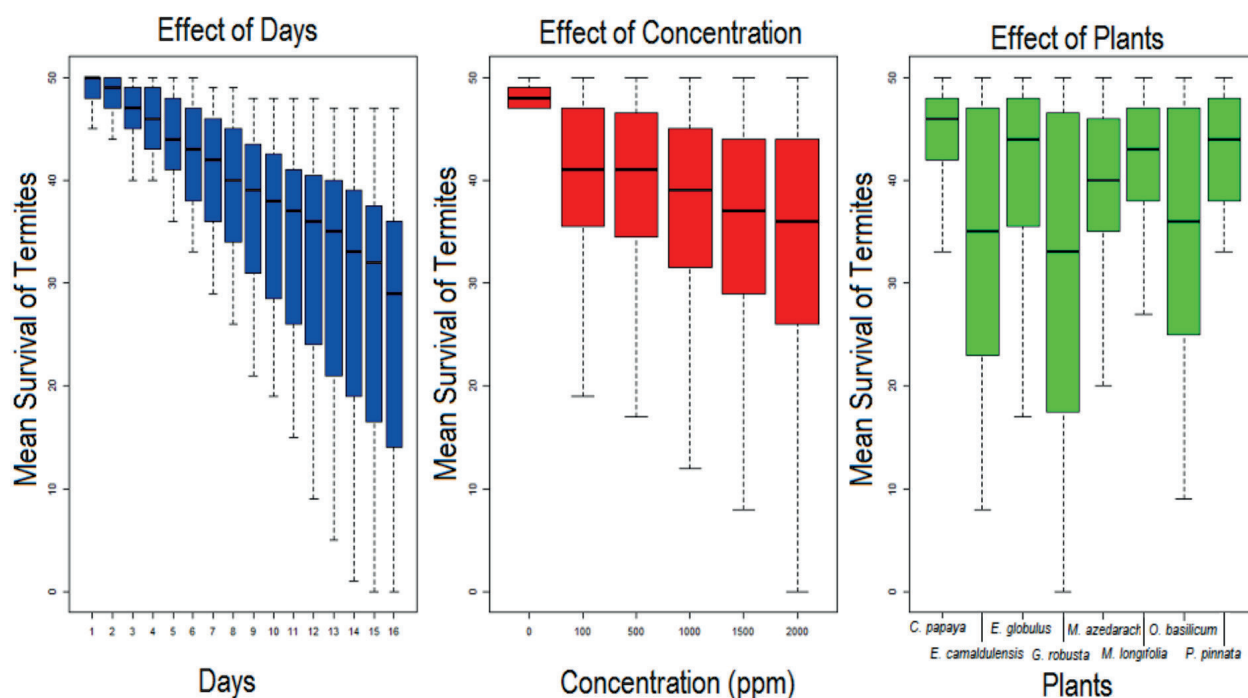


Fig. 4. Box plot representing the effect of days, concentration and plants on the mean survival of *H. indicola*.

E. globulus and *M. azedarach* were also found to have a significant ($p < 0.05$) effect on termite survival and reduced their populations by up to 70, 65, 59 and 54%, respectively. However, *C. papaya*, *M. longifolia* and *P. pinnata* have a non-significant effect on termite survival with less than 30% reduction in the termite population (Fig. 5). Calculated LC_{50} values of all

plants are represented in Table 4. The level of significance of all the plants and their comparative analysis between each other is represented in Tables 2 and 3.

For the control of termites, synthetic insecticides have been used from the last few decades that have resulted in insecticidal resistance, human health disorders, and

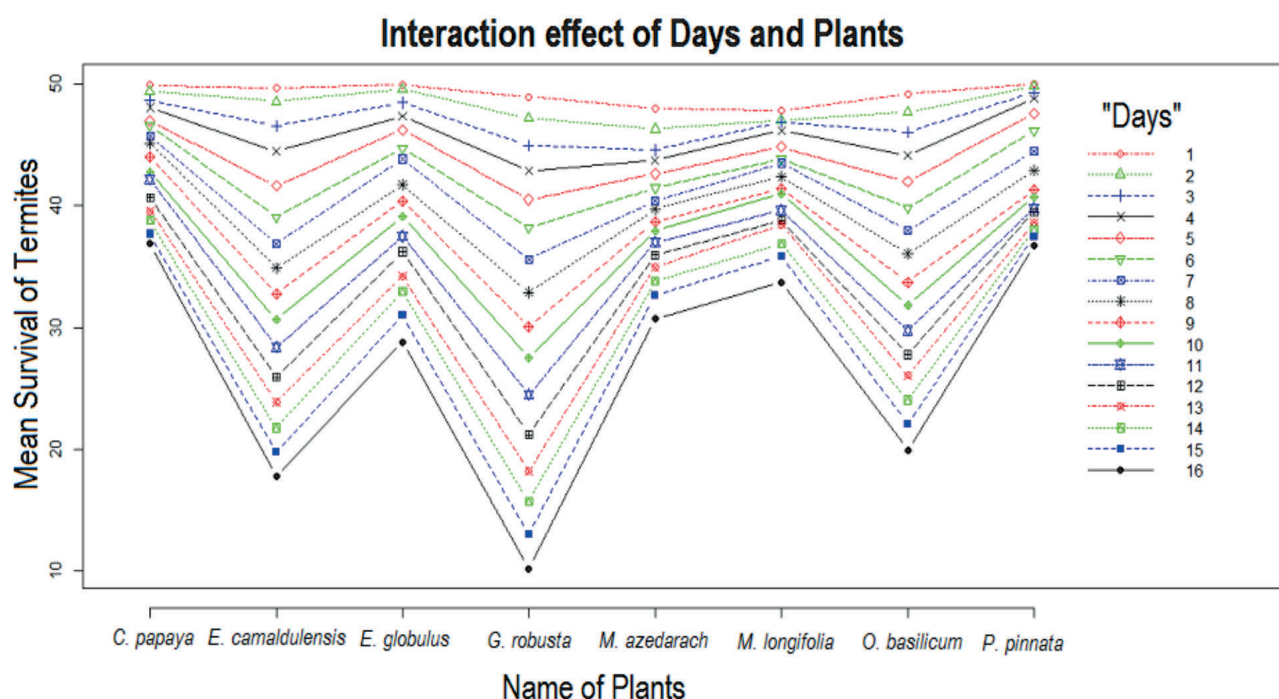


Fig. 5. Interaction plot representing the interaction effect of plants extract with days on mean survival of *H. indicola*.

Table 2. Comparative significance analysis of plants used for antitermitic activity against *O. obesus*, *H. indicola* and its hindgut Protozoa.

Plants Name	Level of Significance		
	<i>O. obesus</i>	<i>H. indicola</i>	Protozocidal
<i>C. papaya</i>	Non-Significant	Non-Significant	Significant***
<i>E. camaldulensis</i>	Significant**	Significant**	Significant***
<i>E. globulus</i>	Significant*	Significant*	Significant**
<i>G. robusta</i>	Significant***	Significant***	Significant***
<i>M. azedarach</i>	Non-Significant	Significant*	Non-Significant
<i>M. longifolia</i>	Non-Significant	Non-Significant	Significant*
<i>O. basilicum</i>	Significant***	Significant**	Significant***
<i>P. pinnata</i>	Non-Significant	Non-Significant	Non-Significant

Table 3. Comparison of significance level ($P < 0.05$) between plants used for antitermitic activity against *O. obesus*, *H. indicola* and its hindgut Protozoa.

Plant Name	<i>O. obesus</i>		<i>H. indicola</i>		Protozocidal	
	Level of Significance	P value	Level of Significance	P value	Level of Significance	P value
<i>C. papaya</i> - <i>E. camaldulensis</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>C. papaya</i> - <i>E. globulus</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>C. papaya</i> - <i>G. robusta</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>C. papaya</i> - <i>M. azedarach</i>	Non-Significant	0.129	Significant	0.000	Significant	0.000
<i>C. papaya</i> - <i>M. longifolia</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>C. papaya</i> - <i>O. basilicum</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>C. papaya</i> - <i>P. pinnata</i>	Non-Significant	0.698	Non-Significant	0.720	Significant	0.000
<i>E. camaldulensis</i> - <i>E. globulus</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>E. camaldulensis</i> - <i>G. robusta</i>	Significant	0.000	Significant	0.000	Non-Significant	0.204
<i>E. camaldulensis</i> - <i>M. azedarach</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>E. camaldulensis</i> - <i>M. longifolia</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>E. camaldulensis</i> - <i>O. basilicum</i>	Significant	0.000	Non-Significant	0.378	Non-Significant	0.996
<i>E. camaldulensis</i> - <i>P. pinnata</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>E. globulus</i> - <i>G. robusta</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>E. globulus</i> - <i>M. azedarach</i>	Significant	0.000	Non-Significant	0.020	Significant	0.000
<i>E. globulus</i> - <i>M. longifolia</i>	Non-Significant	0.641	Non-Significant	0.317	Significant	0.000
<i>E. globulus</i> - <i>O. basilicum</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>E. globulus</i> - <i>P. pinnata</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>G. robusta</i> - <i>M. azedarach</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>G. robusta</i> - <i>M. longifolia</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>G. robusta</i> - <i>O. basilicum</i>	Significant	0.000	Significant	0.000	Non-Significant	0.030
<i>G. robusta</i> - <i>P. pinnata</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>M. azedarach</i> - <i>M. longifolia</i>	Significant	0.000	Significant	0.000	Non-Significant	0.246
<i>M. azedarach</i> - <i>O. basilicum</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>M. azedarach</i> - <i>P. pinnata</i>	Non-Significant	0.974	Significant	0.000	Non-Significant	0.197
<i>M. longifolia</i> - <i>O. basilicum</i>	Significant	0.000	Significant	0.000	Significant	0.000
<i>M. longifolia</i> - <i>P. pinnata</i>	Significant	0.000	Non-Significant	0.027	Significant	0.000

Table 4. LC₅₀ values of plants used for antitermitic activity against *O. obesus*, *H. indicola* and its hindgut Protozoa.

Plants Name	LC ₅₀ (ppm)		
	Upper confidence limit - Lower confidence limit		
	<i>O. obesus</i>	<i>H. indicola</i>	Protozoa
<i>C. papaya</i>	6329.70 (7478.71-5519.87)	3546.81 (3776.76-3350.46)	1046.24 (1052.65-1039.86)
<i>E. camaldulensis</i>	2973.69 (3231.45-2762.92)	2214 (2351.38-2097.04)	594.70 (601.77-588.29)
<i>E. globulus</i>	3148 (3375.31-2957.11)	2754.81 (2889.69-2635.82)	1373.28 (1380.49-1366.13)
<i>G. robusta</i>	1391.01 (1455.08-1331.88)	1607 (1686.03-1537.49)	502.64 (506.97-497.10)
<i>M. azedarach</i>	5308.70 (6093-78-4726.97)	3154.99 (3383.57-2962.57)	2475.97 (2498.83-2453.83)
<i>M. longifolia</i>	9408.11 (14283.4-7124.08)	5395.08 (6220.86-4788.18)	3093.47 (3129.35-3058.63)
<i>O. basilicum</i>	1926 (2039.53-1827.68)	2515.19 (6220.86-4788.18)	683.60 (688.63-678.56)
<i>P. pinnata</i>	5468.71 (6293.14-4860.36)	6240.51 (7348.15-5455.17)	2080.22 (2096.91-2063.87)

environmental issues. So the replacement of synthetic insecticides with bio-pesticides is strongly required as they are a rich source of bioactive compounds and less toxic [25]. In the past, many studies have been conducted to evaluate the toxic effects of plants on termites and their hindgut endosymbionts. Abbas et al. [26] reported the antitermitic activity of 15 medicinal plants, including *Ricinus communis*, *Croton tiglium*, *Ocimum basilicum*, *Psoralea corylifolia*, *Nigella sativum*, *Mentha* sp., *Cichorium intybus*, *Peganum harmala*, *Foeniculum vulgare*, *Allium sativum*, *Plantago ovate*, *Moringa oleifera*, *Capsicum frutescens*, *Melia azedarach* and *Azadirachta indica* against *O. obesus*, and found that *O. basilicum*, *P. harmala* and *F. vulgare* have potential to be used as antitermitic agents. These plants have many bioactive constituents that can be used against termites. In previous studies, the leaf and seed extracts of *Jatropha curcas*, bark extract of *Melia azedarach*, rhizome extract of *Curcuma longa*, leaf extract of *Nerium indicum* and crude seed extract of *Annona* were evaluated for behavioral, feeding and tunneling activities of subterranean termites [27]. Similarly the effect of crude extracts of *Azadirachta indica*, *Pyllanthus niruri*, *Andrographis paniculata* and *Leucaena leucocephala* on behavior and feeding activities was studied against *Coptotermes gestroi* and *Globitermes sulphurues* [28].

In the present study among the tested eight plant species, *G. robusta* was found to have promising activity and cause significant ($P < 0.05$) reduction in termite survival. About 50% termite death was recorded on the 10th day of the experiment, with a survival rate of 6 and 10% (94 and 90% mortality) on the 16th day of the experiment in both termite species, i.e., *O. obesus* and *H. indicola*.

E. camaldulensis and *O. basilicum* were also reported to have a significant ($P < 0.05$) effect on termite survival and reduce up to 70 and 65% *H. indicola* population, but they do not differ significantly from each other ($P = 0.378$). However, in the case of *O. obesus*, *O. basilicum* was found to be more effective than *E.*

camaldulensis and caused 76 and 60% reduction in the termite population. Similarly, *E. globulus* and *M. azedarach* were also active significantly ($P < 0.05$) against termites, but have non-significant ($P = 0.20$) differences among each other. The results of the present study are comparable with a recent study conducted by Hassan et al. [14] showing that the population of *Reticulitermes flavipes* was reduced significantly when exposed to the extracts of *Morus alba*, and we found that the percentage mortality of termites was concentration-dependent. The activity profile of all tested plants against *O. obesus* is as follows; *G. robusta* > *O. basilicum* > *E. camaldulensis* > *E. globulus* > *M. longifolia* > *M. azedarach* > *C. papaya* > *P. pinnata*. Similarly, the activity profile of all tested plants against *H. indicola* is as follows: *G. robusta* > *E. camaldulensis* > *O. basilicum* > *E. globulus* > *M. azedarach* > *M. longifolia* > *C. papaya* > *P. pinnata*.

Protozocidal Activity of Plant Extract

Protozocidal activity of the above-mentioned eight plants were also calculated against the mean survival of the protozoa population isolated from the hindgut of *H. indicola*. All concentrations were found to have a significant effect on protozoa survival as compared to control (F (df) = 553.5 (5); $P < 0.05$) in a time-dependent manner, which also differed significantly from each other (F (df) = 177.2 (7); $P < 0.05$). Similarly, all plants showed differential protozocidal activity in dose- and time-dependent manners (F (df) = 141.3 (8); $P < 0.05$). The effect of days, concentration and plants on the mean survival of the protozoa population is represented in Fig. 6. Among all the plants, *G. robusta*, *E. camaldulensis* and *O. basilicum* were found to be most effective and have a significant ($P < 0.05$) effect on survival of protozoa with 95, 78, and 74% reduction in the population on the 16th day of the experiment. Similarly, *C. papaya*, *E. globulus* and *M. longifolia* also significantly ($P < 0.05$) reduced the protozoa population with 66, 56 and 46% mortality respectively. However, *M. azedarach* and *P. pinnata* have a non-significant

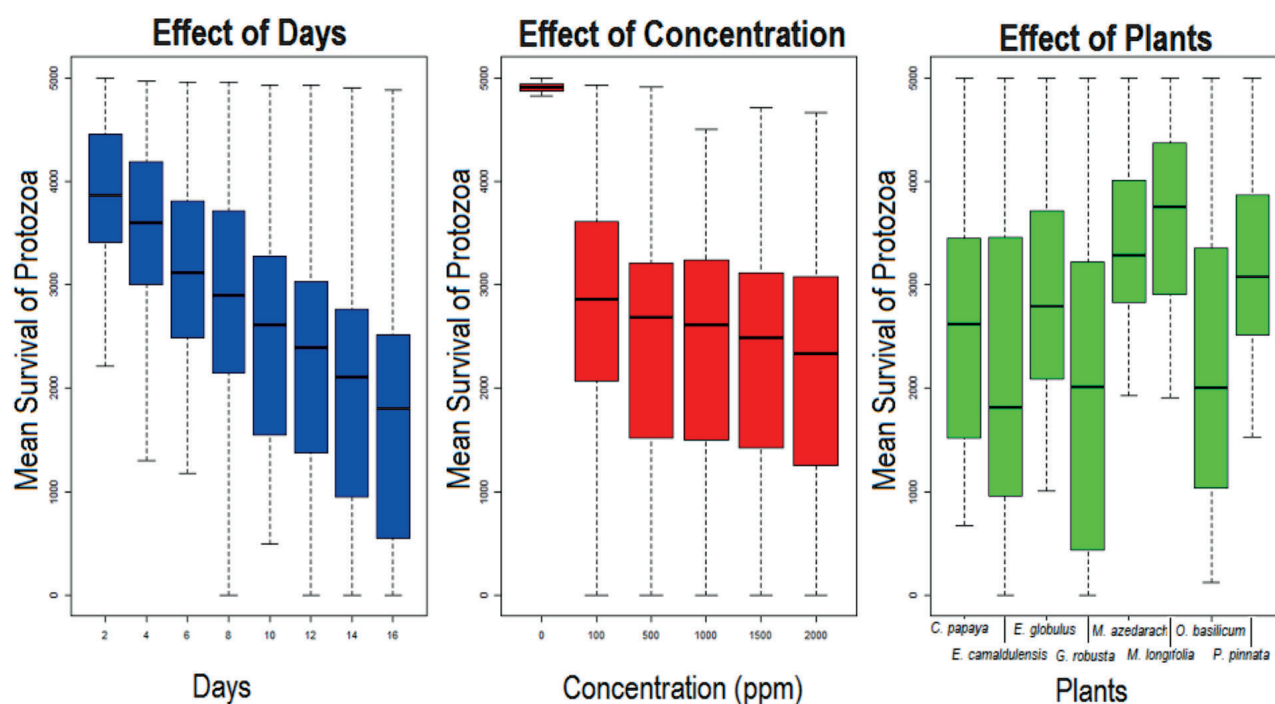


Fig. 6. Box plot representing the effect of days, concentration and plants on the mean survival of protozoa isolated from the hindgut of *H. indicola*.

effect on the protozoa population (Fig. 7). Calculated LC_{50} values of all plants are represented in Table 4. The level of significance of all the plants and their comparative analysis between each other is represented in Tables 2 and 3.

Protozocidal activity by feeding on extract-treated filter papers was also investigated against the endosymbiotic flagellate population of *H. indicola*. In previous studies, the toxic potential of some natural products against hindgut flagellates has been

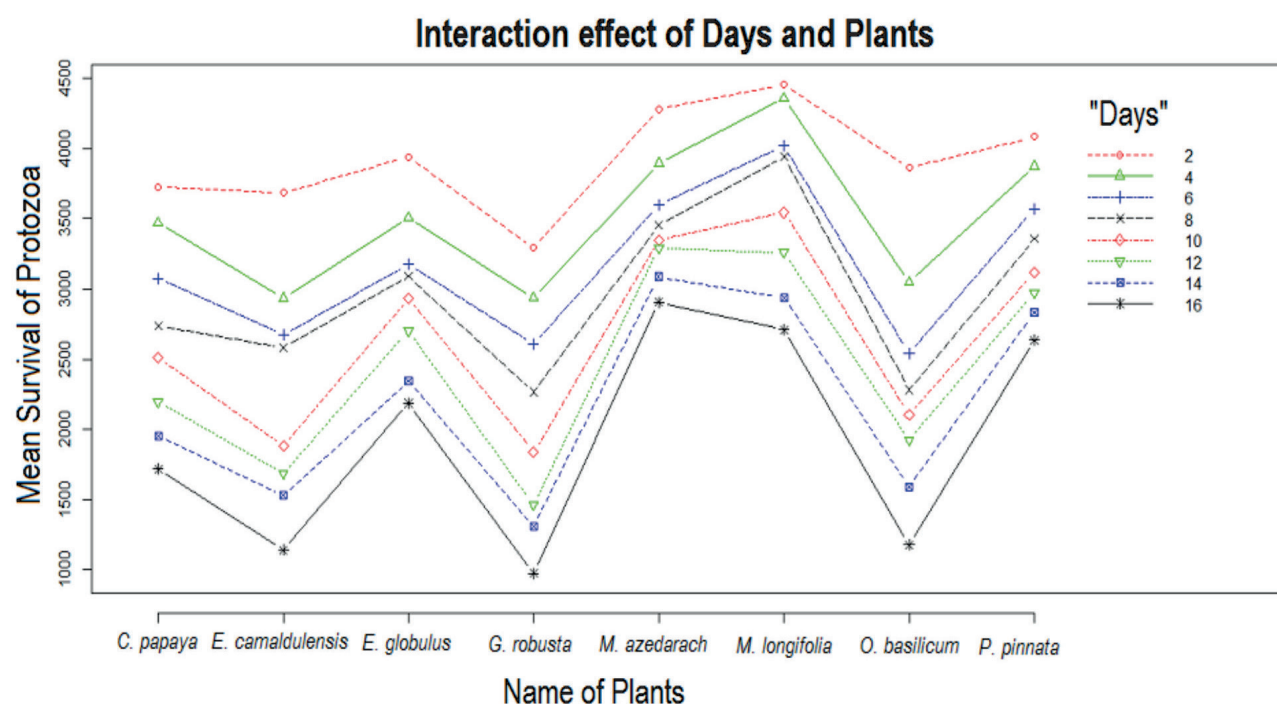


Fig. 7. Interaction plot representing the interaction effect of plant extracts with days on mean survival of protozoa isolated from the hindgut of *H. indicola*.

reported. One study, conducted by Doolittle and his coworkers [29], used bioactive compounds from neem extract, gleditschia from *Gleditschia triacanthas* and capsaicin from cayenne pepper (*Capsicum* sp.) against endosymbiotic fauna of *Formosanus* subterranean termites, and evaluated that neembokil was more significant for decreasing the population of *P. grassii* and *spirochaete* genus. Similarly, the comparative effect of synthetic disodium octaborate tetrahydrate with natural *vetiver* oil and *nootkatone* on endosymbionts to *C. formosanus* was conducted in the laboratory trials for 12 months [30]. In choice and no-choice bioassays it was revealed that *nootkatone* and *vetiver* oil had a more significant ($P \leq 0.05$) effect on termite hindgut flagellates than disodium octaborate tetrahydrate.

In the present study, leaf extract of eight plants was evaluated against protozoan fauna of *H. indicola* and found that all plants have differential protozocidal activity in a dose-dependent manner with an increase in time. *G. robusta*, *O. basilicum* and *E. camaldulensis* showed high activity profile and caused significant ($P < 0.05$) elimination of protozoan fauna from termite hindgut. However, they have non-significant differences among each other, i.e., *G. robusta* and *E. camaldulensis* ($P = 0.204$), *G. robusta* and *O. basilicum* ($P = 0.030$), and *E. camaldulensis* and *O. basilicum* ($P = 0.996$). These results are compatible with a study conducted by Afzal et al. [31], who concluded that a reduction in the protozoa population was directly related to the filter paper area consumption treated with plant extract and found that after two weeks of exposure of termites to treated filter paper in no-choice bioassay at the concentration of 20mg/ml of plant leaf extract reduced the protozoa population by 62.90% as compared to the untreated negative control. Similarly, *C. papaya*, *E. globulus* and *M. longifolia* infected protozoan populations significantly ($P < 0.05$) and caused a decline in their survival of up to 66, 56 and 46% respectively. However, the termite survival rate was high in those experimental units where filter papers were treated with extract of these plants. The present findings are comparable with the study conducted by Hassan et al. [14] on protozoan fauna of *Reticulitermes flavipus*, who reported that heartwood extracts of *Morus alba* at its highest concentration (10mg/ml) reduced the protozoan population by up to 50%, but the survival rate of termites was 7%. So while it is recommended that termite mortality is not only the result of the decline in protozoan population, many other physiological phenomenon may also affect their survival during continuous exposure to plant extract [32]. Similarly, in the present study, a decline in the protozoan population was higher in treated termites, but their survival rate was also high, suggesting that the plant extract might have some other mode of action that causes termite mortality (Figs 5 and 7). The protozoa population was declined totally in termite hindgut when exposed to toxic compounds that eventually led to termite death. The activity profile of all tested plants against protozoa

was as follows: *G. robusta* > *E. camaldulensis* > *O. basilicum* > *C. papaya* > *E. globulus* > *M. longifolia* > *P. pinnata* > *M. azedarach*.

Characterization of Most Effective Plants

UV-Vis Spectroscopy

Qualitative UV-Vis spectroscopic analysis of *G. robusta* leaves extract in ethanol solvent showed a spectrum profile at 200 to 800nm due to the sharpness of peaks and proper baseline. The peak spectra of *G. robusta* showed characteristic peaks at 208.94 and 282.97nm with the absorption of 2.33 and 0.811 respectively (Fig. 8).

Fourier Transform Infrared Spectroscopy (FTIR)

Functional groups of active compounds in ethanolic leaf extract of *G. robusta* were identified using FTIR analysis. Each peak in the spectrum represents the specific peak value corresponding to the specific functional group. The absorbance was noted from 400 to 4000 cm^{-1} wavelength. The results of FTIR analysis confirmed the presence of amines, alcohol, alkanes, nitriles, aldehyde, ether, and halo compounds (Table 5 and Fig. 9).

Gas Chromatograph-Coupled Mass Spectroscopy (GC-MS)

Gas chromatograph-coupled mass spectroscopy (GC-MS) of ethanolic leaf extracts of *G. robusta* indicated 15 peaks, which revealed the presence of 15 phytochemical compounds. The mass spectrum of each compound was characterized by comparison with the NIST library. Compound name, retention time, area percentage, molecular weight and a molecular formula

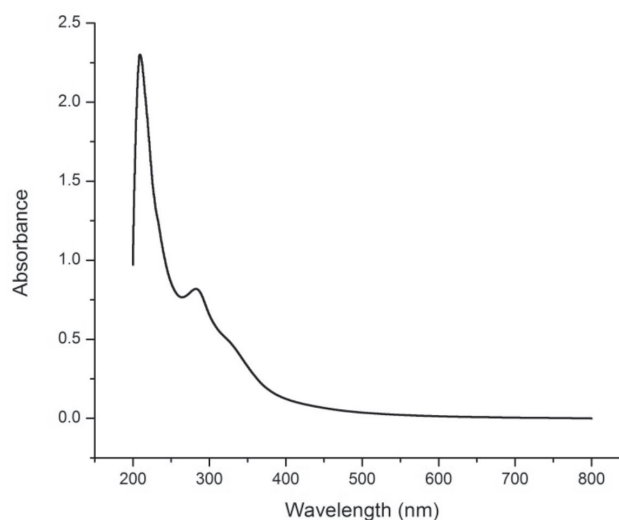


Fig. 8. UV-Vis spectra of ethanolic leaf extract of *G. robusta*.

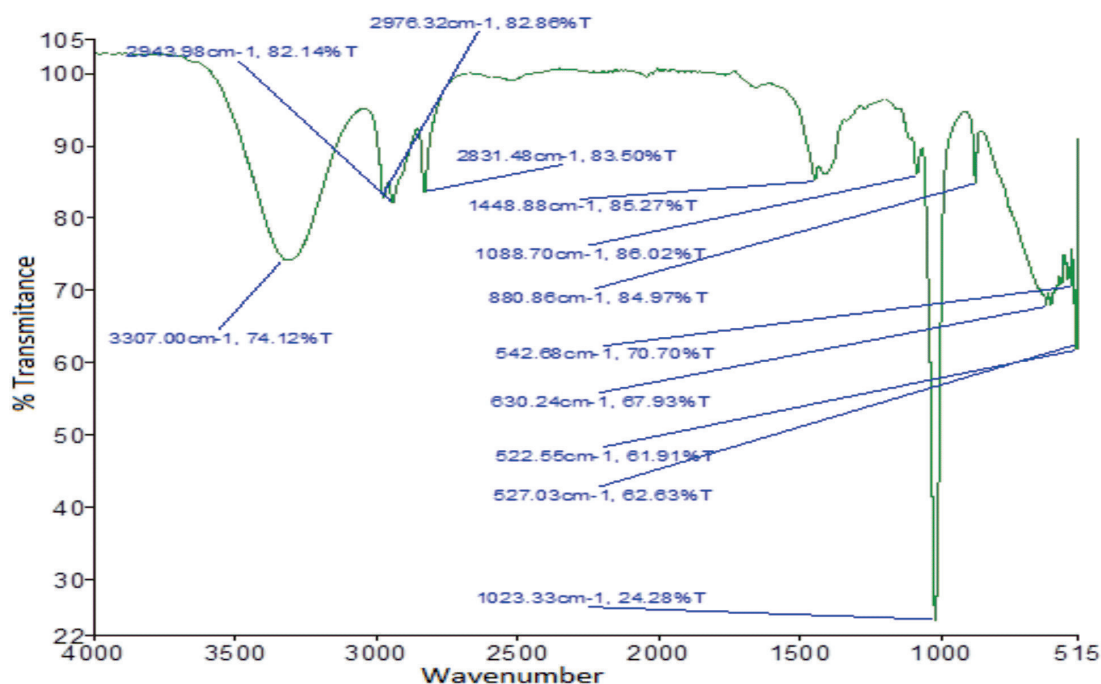
Table 5. FTIR spectral peak values and functional groups obtained from ethanolic leaf extract of *G. robusta*.

Peak #	Peak Value	Type of stretching	Functional group
1	3307.00	N-H stretch/O-H stretch	Amine/Alcohol
2	2976.32	C-H stretch	Alkane
3	2943.98	C \equiv N	Nitriles
4	2831.48	C-H stretch	Aldehyde
5	1488.88	C-H bending	Alkane
6	1088.70	C-O stretch	Ether/Alcohol
7	1023.33	C-N stretch	Amines
8	880.88	C-H bending	1,2,4 trisubstituted
9	630.24	C-X stretch	Halo compound
10	542.66	C-X stretch	Halo compound
11	527.03	C-X stretch	Halo compound
12	522.55	C-X stretch	Halo compound

of identified components are listed in Table 6. The GC-MS chromatograms of identified compounds are presented in Fig. 10.

Extraction and analysis of natural bioactive compounds from the medicinal plants help in quality control of herbal medicines and their use to secure human beings and other animals from an attack of natural toxins [33]. Therefore, in the present study characterization of a most effective plant (*G. robusta*) was carried out using VU-Vis spectroscopy, FTIR and GC-MS analysis. UV-Vis spectroscopic analysis of *G. robusta* leaf extract was performed at a wavelength

of 200 to 800nm, and maximum absorption was observed at 208.94nm (2.33) and 282.97 (0.811). Similar absorption peaks were observed by Rani et al. [34] in the leaf extract of *Meizotropis pellita*. The nearest absorption spectra were also reported by Rajeshkumar and Jayaprakash [35] on selected red seaweed (*Acanthophora specifera*). FTIR analysis of extract was carried out by using Shimadzu ETIR spectrophotometer, and absorption spectra were recorded from 400 to 4000 cm^{-1} . The results of FTIR analysis confirmed the presence of amines, alcohol, alkanes, nitriles, aldehyde, ether, and 1,2,4 trisubstituted and halo compounds at

Fig. 9. FTIR spectrum of ethanolic leaf extract of *G. robusta*.

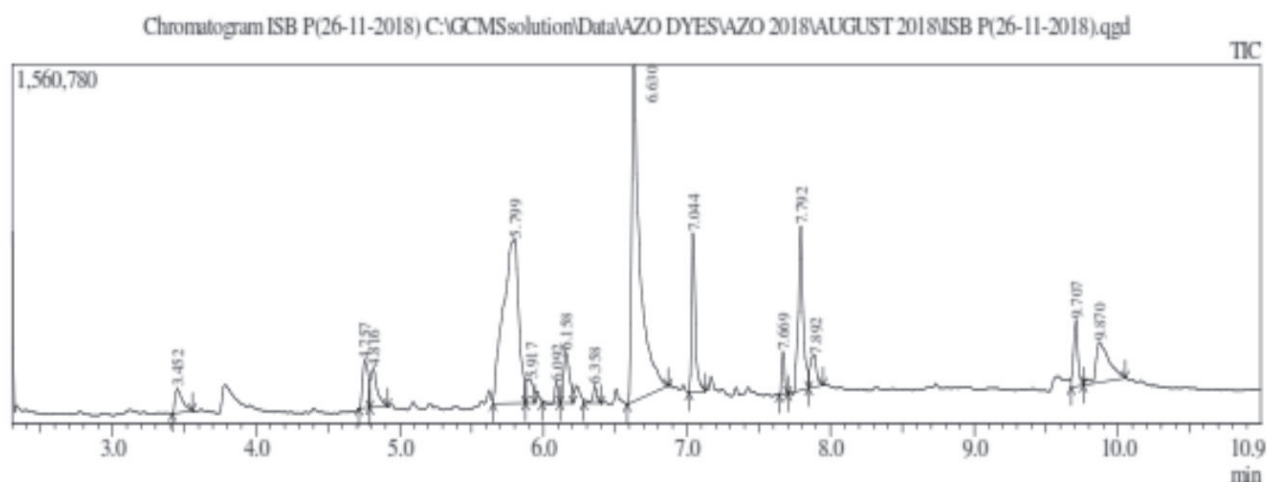
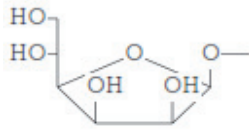
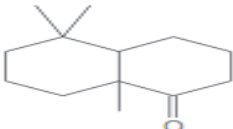
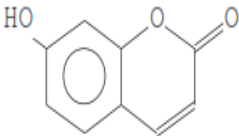
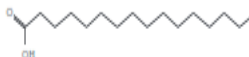
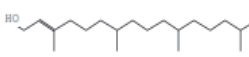


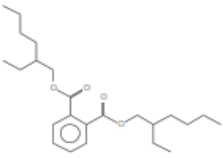
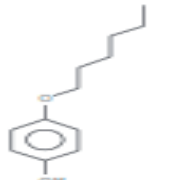


Fig. 10. GC-MS chromatogram of ethanolic leaves extract of *G. robusta*.

Table 6. Phytocomponents identified in ethanolic leaf extract of *G. robusta* by GC-MS peak report.

Peak #	Retention time	Area %age	Molecular Formula	Molecular Weight	Compound name	Structure
1	3.452	1.93	C ₈ H ₈ O	120	2,3-dihydrobenzofuran	
2	4.757	3.07	C ₈ H ₈ O ₂	136	3-Hydroxy-2-methylbenzaldehyde	
3	4.816	2.55	C ₈ H ₆ O ₂	134	2-Coumaranone	
4	5.799	27.63	C ₇ H ₁₄ O ₆	194	Methyl hexofuranoside	
5	5.917	1.77	C ₉ H ₁₄ O ₂	154	1,2,3,4,5-Cyclohexanepentol	
6	6.092	0.69	C ₁₁ H ₁₄ O ₂	162	5-Hydroxy-1-tetralone	

Table 6. Continued.

7	6.158	3.34	$C_7H_{14}O_6$	194	Beta-d-Mannofuranoside, methyl	
8	6.358	0.73	$C_{14}H_{22}O_3$	238	5,5,8a-Trimethyldecalin-1-one	
9	6.630	30.64	$C_9H_6O_3$	162	Coumarin, 7-hydroxy	
10	7.044	6.30	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
11	7.669	1.37	$C_{20}H_{40}O$	296	Phytol	
12	7.792	8.97	$C_{18}H_{30}O_2$	278	9,12,15- Octadecatrienoic acid(Z,Z,Z)	
13	7.892	2.16	$C_{21}H_{36}O_4$	352	9,12,15- Octadecatrienoic acid	
14	9.707	3.08	$C_{16}H_{22}O_4$	278	Bis(2-ethylexyl)phthalate	
15	9.870	5.77	$C_{10}H_{14}O_2$	166	4-Hexyloxyphenol	

the wavelength of 3307.00, 2976.32, 2943.98, 2831.48, 1088.70, 1023.33, 880.88, 630.24, 542.66, 527.03, 522.55 respectively. The presence of these functional groups in plant extracts has been confirmed by many studies previously [36, 37].

The GC-MS analysis of *G. robusta* leaf extracts characterized 15 phytochemicals by comparing their mass spectrum with the NIST library. Among the identified bioactive compounds, n-Hexadecanoic acid has been reported to have antimicrobial, antioxidant, antifibrinolytic, and hemolytic activity [38]. Similarly, n-Hexadecanoic acid, Phytol and 9,12,15- Octadecatrienoic acid(Z,Z,Z) characterized in *G. robusta* leaf extract has also been identified by Sermakkani and Thangapandian [39] in methanolic leaf

extract of *Cassia italica* and reported their biological activities. n-Hexadecanoic acid (Palmitic acid) has an antiandrogenic, antioxidant, hypocholesterolemic, flavoring agent, nematocides, lubricant and pesticidal activities. Phytol belongs to Diterpenes and has anti-cancerous, anti-inflammatory, anti-microbial and anti-nociceptive activities [39, 40]. 9,12,15- Octadecatrienoic acid (Z,Z,Z) is a fatty acid ester compound and has hepatoprotective, anti-inflammatory, insectifuge antihistaminic, 5-alpha-reductase inhibiting, antiandrogenic, pesticidal and cancer preventive activities [39, 41]. 2,3-Dihydrobenzofuran has been reported in *Origanum vulgare* seeds that have antimicrobial and anti-inflammatory activities [40]. In previous studies macrocyclic phenols, cinnamic acid

derivatives and alkylresorcinols have been reported in *G. robusta* [42].

Conclusions

We have concluded from the present study that plant extracts could be an effective alternative for synthetic insecticides for the control of termites by targeting their hindgut endosymbionts. As bioactive insecticides, these plants contain phytochemical compounds that could be used in a refined form to synthesize bioactive preservatives that can be used to prevent infestation and damage caused by termites. Moreover, these plants are easily available and affordable in local areas of Pakistan. The phytochemical analysis of the most effective plant revealed that its leaves contain various valuable bioactive compounds that can be used as an alternative to synthetic insecticides. Further studies are required for isolation, identification and characterization of bioactive compounds from *G. robusta* leaves extract by column chromatography and their characterization by GCMS analysis for their application in industries.

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Conflict of Interest

All the authors have approved the manuscript and declare no conflict of interest.

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