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RESEARCH ARTICLE
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TERMITICIDAL ACTIVITY OF ETHANOLIC LEAF EXTRACTS OF MEDICINAL PLANTS *CALOTROPIS GIGANTEA* AND *MORUS ALBA* AGAINST *HETEROTERMES INDICOLA* (WASMANN)

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Abstract
The present study was designed to determine the chemical constituents of leaf extracts from two medicinal plant species *Morus alba* and *Calotropis gigantea* against *Heterotermes indicola* under laboratory conditions. A no-choice bioassay technique using impregnated filter paper was applied. The extracts of leaves of selected plants were obtained in ethanol solvent by using a soxhlet apparatus. A laboratory bioassay was used to investigate the biological activity of leaf extracts against *H. indicola*. Gas chromatography-mass spectrometry (GC-MS) was used to characterize leaf extracts, and chemical profiles for extracts from each plant species were obtained. In GC-MS analysis of *M. alba*, twelve compounds were identified in which Phytol, Squalene, Vitamin E, and Ergost-8-en-3-ol, 14-methyl-, (3β,5α) were the main compounds. γ-Sitosterol, Octacosanol, and Nonadcane were the main chemical components obtained from *C. gigantea*. Results showed that extracts of *C. gigantea* exhibited antitermitic activity in a dose-dependent manner and showed significant activity. The LC50 values of *M. alba* and *C. gigantea* were 18.48% and 11.30%, respectively, and LC90 values of these extracts against *H. indicola* were 34.85% and 23.13%, respectively. Gas Chromatography components identified by Mass Chromatography (GC MS) may be further investigated for their antitermitic activity against different termite pest species in Pakistan.

Key words: *Calotropis gigantea*, *Morus alba*, *Heterotermes indicola*, Gas chromatography-mass spectrometry

INTRODUCTION
Termites are notoriously destructive to wooden structures- in the tropics (Ajayi et al. 2012). An estimated 10-25% of nations' crop may lose their whole output due to termites, resulting in an enormous annual financial loss of several hundred million rupees (Ranjith et al. 2021). Chemical pesticides are typically used to tackle the enormous task of termite control. Using plant-based products as insecticide alternatives in pest management programs have gained popularity recently because

many commercial termiticides have the potential to be long-lasting environmental pollutants (Rana et al., 2021).

Heterotermes spp. is a type of structure invading termite that cause a lot of damage everywhere it turns up and can only live in certain climate zones due to the moisture and temperature constraints of the soil (Emerson 1971; Saljoqi et al. 2012). As termite infestations continue to rise, so does the need for effective, environmentally friendly methods of controlling them (Meepagala et al. 2006). There have been several attempts both in the field and in the lab to harness the activity of plants to combat termites. In the past, researchers have tested the effectiveness of many plant species against termites (Adams et al., 1988). The study of plant-based toxins has recently gained attention (Chang et al. 2001; Elango et al. 2012). Plants produce secondary metabolites for defense, such as alkaloids, coumarins, terpenoids (particularly monoterpenoids), and chromenes. The effectiveness of these secondary metabolites in preventing or reducing household pests has been assessed. In the 1990s, the oils from the Zoological Society of Pakistan were revitalized with more and more evidence of their insect-killing fumigant and contact properties being made public (Isman, 2000; Koul et al., 2008). Insects show their distaste for phytochemicals in a wide variety of ways, including a lack of calling activity (Khan and Saxena 1986; Ahmad et al. 2011), a slowing of growth (Breuer and Schmidt 1995; Ahmad et al. 2011), toxicity (Hiremath et al. 1997; Ahmad et al. 2011), a refusal to lay eggs (Zhao et al. 1998; Muthukrishnan and pushpalatha, 2001; Ahmad et al. 2011).

In Southern Asia, especially in parts of Pakistan, *Heterotermes spp. are structure-infesting* termites responsible for a sizable portion of the damage attributed to subterranean termites. The distribution of *Heterotermes indicola* has expanded beyond its original range of the Indian Subcontinent and the Arabian Peninsula; it is now frequently seen in the Pakistani provinces of Punjab and Khyber Pakhtunkhwa (Misbah-ul-Haq et al. 2015).

Calotropis procera, also known as Ak, is a plant in the Asclepiadaceae family. It is used in many Ayurvedic medicines, such as Arkelavan. Traditional forms of medicines have known for a long time that *Calotropis procera* can be used to treat health problems, and its leaves are often used. Modern pharmaceutical compounds have always been built on the use of plants, plant extracts, and pure compounds taken from natural sources (Evans, 2005). *C. procera* is a well-known plant that has been used traditionally to treat stomatitis, sinus fistula, diarrhea, and skin diseases (Ranghubir et al. 1999). The leaf part is used to treat jaundice. There are a lot of reports about where the plants grow, where they live, and what they look like. However, work has yet to be done on the leaves of these plants which could be useful as traditional medicines (Murti et al. 2010).

One of the most common medicinal plants is the mulberry (Morus spp., Moraceae). Most people associate the genus *Morus* with the *white mulberry* (*Morus alba*), *The black mulberry (Morus nigra)*, and the *red mulberry (Morus rubra)* (Yigit et al. 2010). Numerous mulberry species can be found in both warm and cold climates. The majority of plants are distributed in Asia, including Korea, Japan, China, and India (Sánchez, 2000). It's not just for eating. There are many other uses for mulberry. Mulberry has been recognized as a functional food due to its high nutritional and phytochemical content (Srivastava et al. 2006). Padwal et al. 2023 evaluated the efficacy of seven different plant extracts against termites and found that extracts of neem and congress grass cause the highest mortality. Ileke et al. 2022 found out the efficacy of cowpea pod powder against termites. According to their results, this powder caused 100% mortality in workers of termites at 12g after exposure of 24 hours.

The current study was done to find out the toxical potential of the leaf extracts of *Morus alba* and *Calotropis gigantea* against *Heterotermes indicola*. It includes the structural characterization of compounds in ethanolic leaf extracts of all selected plant species through GC-MS analysis, Ethanolic extraction of selected plant leaves using a soxhlet extractor, and termite (*H. indicola*) collection to determine the feeding bioactivity in extracts under laboratory conditions.

MATERIALS AND METHODS

Termite collection

Heterotermes indicola (workers) were collected from different areas of Lahore. Termites with their substrates were placed in Petri dishes lined with moistened filter paper and maintained in a controlled laboratory at 26 ± 2 °C and 75-80% humidity.

Collection of plant leaves

Leaves of selected plants were collected from trees in the University of the Punjab's Botanical Garden. They were dried for three days and kept in polythene bags for experimental purposes.

Soxhlet extraction procedure

Soxhlet equipment was used to get plant extracts. For use, the leaves of selected plants were ground mechanically into fine powder with an electric blender (Daigger Scientific, USA). 200ml of solvent was used to get powder from 20g of each plant leaf. Ethanol was used as a solvent. Powdered plant materials were put in the thimble; it was then attached to a soxhlet apparatus. The solvent was put in a round bottom flask. Attached to a machine that makes distilled water flow continuously is a condenser. The solvent was heated in an isomantle that had a round flask with a flat bottom. The temperature was kept at the same level as when ethanol and methanol boiled. When the solvent was heated, it started to evaporate and move through the condenser. When the solvent reached the level of the siphon and was pumped back to the round bottom flask, one cycle was finished. For each extract to be finished, 6-8 cycles had to be done. After the extraction process was done, the flask was taken off the isomantle and allowed to cool.

Gas chromatography-mass spectrometry (GC-MS)

For GC-MS analysis, extracts from the soxhlet extractor were passed through hydro distillation to find out what kind of parts they contained. Analysis was performed with an Agilent 7890A GC-MS fitted with an Agilent 5975C. Samples were distilled at a temperature below 200°C and filtered through filter paper with pores of 0.20m. The Gas Chromatography temperature had gone from 50 to 250°C at a rate of 4°C/min, with a 5-minute wait for the solvent. The injector will be heated to 250°C. Helium is an inert gas and has flown at a rate of 1.0 ml/min. In the splitless mode, two µl of samples will be injected. The percentages of the sample parts were worked out. Each compound's mass spectrum was compared to the mass spectrum from the spectra library NIST 98 (USA National Institute of Science and Technology software).

Anti-termite assay

No-choice bioassay was used to evaluate the efficacy of plant extracts against *H. indicola* by following the (Elango et al. 2012; Ashraf et al. 2020) protocol. The petri dishes were cleaned, washed, and dried in the oven for a full day. After cutting out circles from filter paper, we soaked them in 1ml of our extract solutions of choice and then dried them. Termites were divided into experimental and control groups. Experimental groups were treated with concentrations of extract solutions, and the control group was impregnated with distilled water. One hundred termite workers were released into each Petri plate. Humidity was kept constant by placing a cotton plug soaked in water on each Petri plate. All experimental petri plates were stored or kept in the dark at 26, and the humidity level was maintained between 75-80%. All the experiments were conducted in replicates, and the control was treated with distilled water only. Mortality percentage was recorded after each 24 hours for ten days by applying the formula.

Mortality rate= ODP ÷ TP × 100

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

Repellency assay

Filter papers 9cm in diameter were cut into two halves for the repellency test. Half of each filter paper was exposed to extracts of 30%, 20%, 10%, and 5% concentrations. At the same time, the other half was treated with distilled water (untreated). With the cut space in the middle, the separated pieces of filter paper were placed into petri plates. Ten termites were dropped into the central area. The experiment was conducted for two hours, and the reading of repellency was noted every 15 minutes by counting the number of termites on untreated and treated filter paper. Each concentration of the plant extracts was made with three replicates. At the end of two hours, the number of termites on treated and untreated filter paper discs was counted to determine the level of repellency. When 21 (average of three replicates) of 30 termites were found on untreated filter paper for five consecutive readings, the treatment was deemed repellant.

Statistical analysis

The percentage mortality of termites was calculated and analyzed by using two-way ANOVA at P=0.05, which was considered statistically significant ($P<0.05$). LC50 and LC90 values were calculated by "Probit analysis" Minitab version 21.

RESULTS

Anti-termitic assay

This leaf extract of *C. gigantea* at 30% concentration gives 91.66% mortality, which is quite significant, and at 5% concentration, it gives 19% mortality. In this, a sharp decrease in mortality has occurred, depicting variable results of toxicity of this extract. When workers of *H. indicola* were exposed to different concentrations of *M. alba the*, 74%, 61.33%, 37.33%, and 12% were observed at 30%, 20%, 10%, and 5% concentrations, respectively, as shown in **Figure 1.** Both plants showed concentration-dependent mortality on *H. indicola* with LC50 value 11.12% (n=100, R square= 0.90) and 18.50% (n=100, R square=0.99) respectively, for *C. gigantea* and *M. alba* and gave LC90 value of 23.13% and 34.85% for each plant respectively as shown in **Table 1** and ANOVA results shown in **Table 2**.

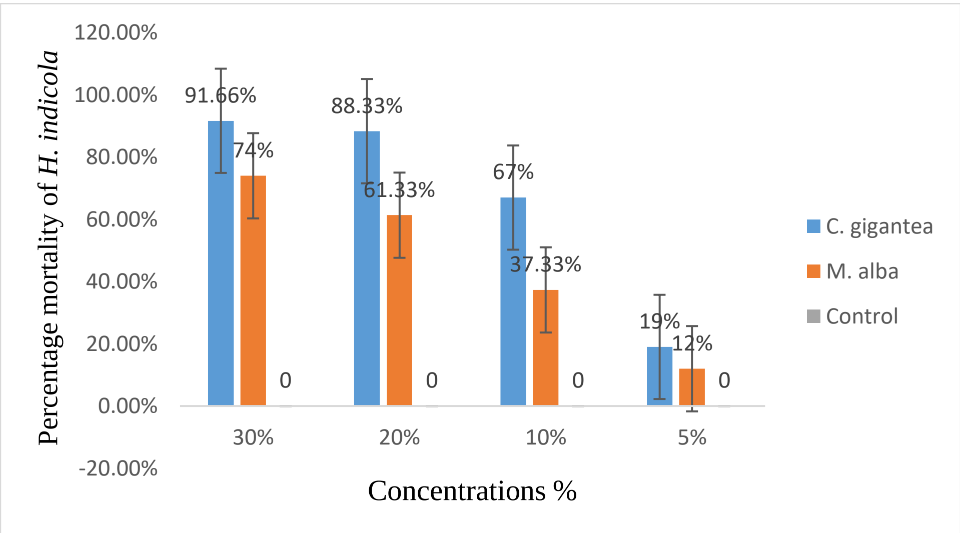


Figure 1. Percentage mortality of *H. indicola* at different leaf extract concentrations

Table 1. Median lethal concentration (LC50) and (LC90) of filter paper treated with *C. gigantea* and *M. alba* leaf extracts

Sr No.	Plants name	LC50	LC90	R	R squared	Significance	95% confidence interval
1	*Morus alba*	18.50	34.85	-0.99	0.99	Significant	-0.9999-0.2409
2	*Calotropis gigantea*	11.12	23.13	-0.95	0.90	Significant	-0.9990-0.0992

Table 2. Two-way ANOVA for mortality of termites

ANOVA Table	SS (Type III)	Df	MS	F (DFn, DFd)	P value	P value summary	Significance
Plants type	826.8	1	826.8	F (1, 3) = 15.67	P=0.0288	*	Yes
Concentrations	5458	3	1819	F (3, 3) = 34.48	P=0.0080	**	Yes
Residual	158.3	3	52.76				

Filter paper consumption

In the case of *C. gigantea*, the maximum consumption of 0.41g was observed at an extract concentration of 5%, and the minimum consumption was 0.14 at an extract concentration of 30%. In the case of *M. alba*, 0.43g, 0.27g, 0.21g, and 0.18g filter paper consumption was observed at 30%, 20%, 10%, and 5% concentrations, respectively, as shown in **Figure 2.**

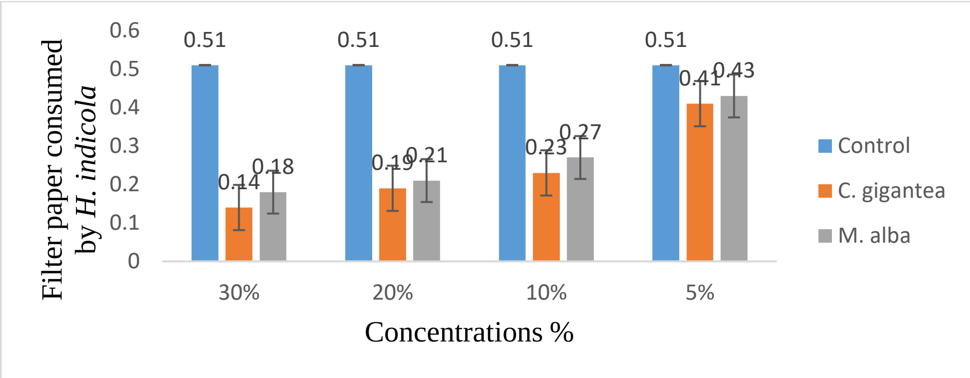


Figure 2. Filter paper consumption by *H. indicola* at different extract concentrations

Repellency test

Almost all concentrations of leaf extracts of *C. gigantea* and *M. alba* were repellent, as fewer termites were present on treated filter paper. This indicates repellency, as most of the termites were present on untreated filter paper except at a 5% concentration, as shown in **Figure 3.**

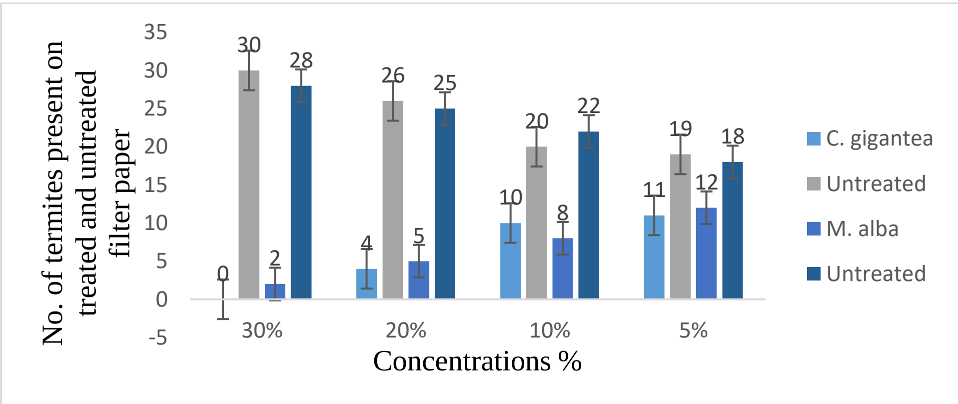


Figure 3. Repellency test of *C. gigantea* and *M. alba* against *H. indicola*

GC-MS analysis and characterization of extracts

Table 3 and 4 show the retention time, molecular formula, structural formula (based on the NIST14 library used by the GCMS software**),** and percent composition of the sample for chemical components identified from each of the tested leaf extracts in our experiment. GC-MS analysis identified 12 chemical compounds from *C. gigantea* and 12 from *M. alba*. Chromatograms from GC-MS analysis of solvent-extracted leaves are shown in **Figures 4 and 5.**

Table 3. Phytocompounds identified in ethanolic extracts of *C. gigantea*

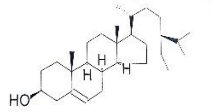
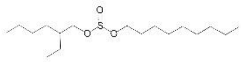
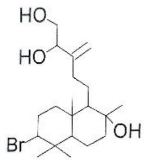
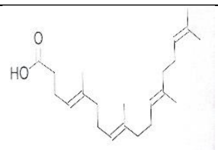
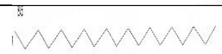
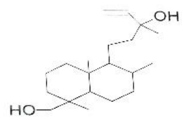
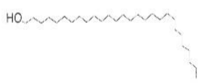
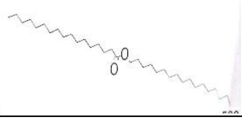
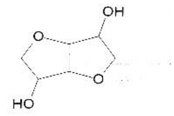
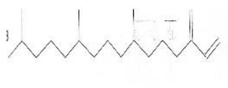
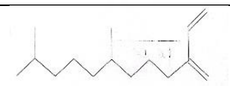
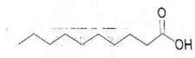
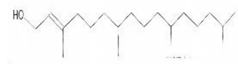
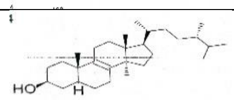
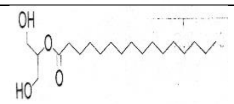
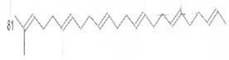
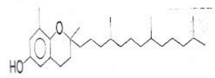
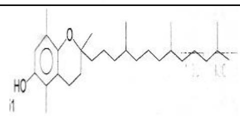
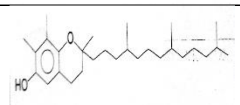
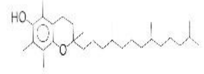
Sr No.	Retention time minutes	Phytocompounds	Relative percentage composition	Structural formula
1	23.782	Neophytadiene	1.729%	
2	26.702	Phytol	3.647%	
3	28.730	Nonacos-1-ene	1.602%	
4	30.042	γ-Sitosterol	26.737%	
5	31.505	Sulfurous acid, 2-ethylhexyl nonyl ester	2.442%	
6	32.024	1,2-Pentanediol, 5-(6-bromodecahydro—hydroxy-2,5,5a,8a-tetramethyl-1-naphthalenyl)-3-methylene-	5.470%	
7	32.490	5,9,13,17-Tetramethyl 4,8,12,16-octadecatetraenoic acid	4.796%	
8	32.915	Nonadcane	8.705%	
9	33.720	1-Naphthalenepropanol	1.734%	
10	34.442	Carbonic acid, eicosyl vinyl ester	6.145%	
11	34.512	Octacosanol	34.104%	
12	35.416	Heptadecanoic acid, heptadecyl ester	2.889%	

Table 4. Phytocompounds identified in ethanolic extracts of *M. alba*

Sr No.	**Retention time minutes**	**Phytocompounds**	**Relative percentage composition**	**Structural formula**
1	19.883	Hexahydrofuro[3,2-b]furan-3,6-diol	5.851%	
2	23.788	Neophytadiene	4.429%	
3	24.283	3-Methylene-7,11-dimethyl-1-dodecene	1.159%	
4	25.227	n-decanoic acid	2.190%	
5	26.714	Phytol	24.795%	
6	30.019	Ergost-8-en-3-ol, 14-methyl-, (3β,5α)	9.778%	
7	30.158	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	2.439%	
8	32.490	Squalene	17.809%	
9	33.399	δ –Tocopherol	2.143%	
10	34.104	β –Tocopherol	3.200%	
11	34.233	γ –Tocopherol	2.963%	
12	34.961	Vitamin E	23.243%	

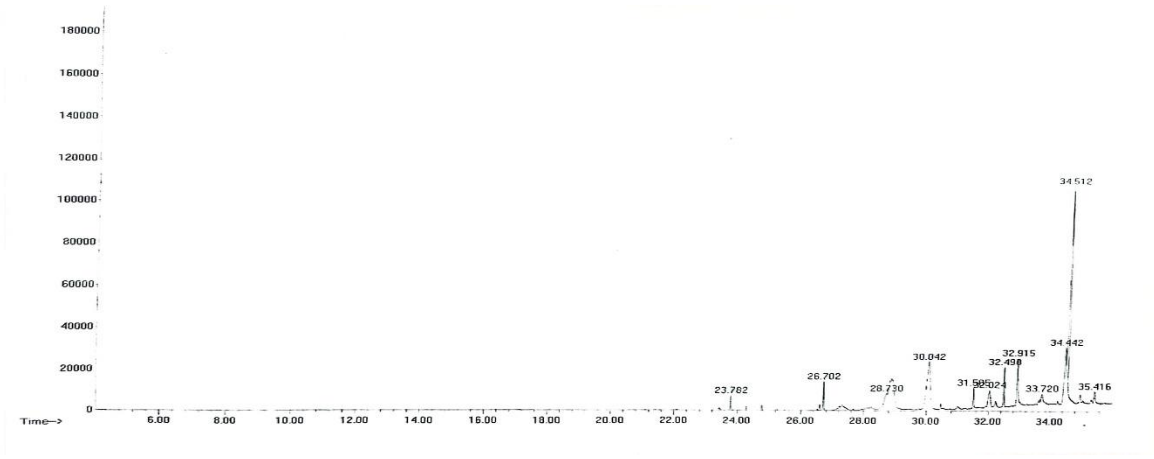


Figure 4. GC-MS chromatogram of leaf extract obtained from *Calotropis gigantea*

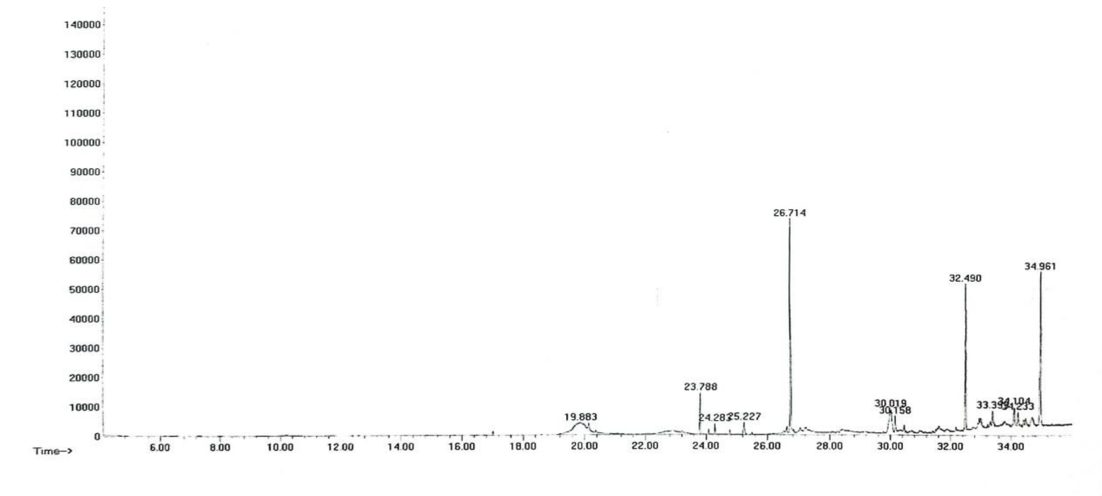


Figure 5. GC-MS chromatogram of leaf extract obtained from *M. alba*

DISCUSSION

The present research, of significant importance, is conducted to evaluate the response of *H. indicola* against ethanolic leaf extracts of *Morus alba and Calotropis gigantea*. To evaluate the termiticidal properties of extracts with distilled water control setup was established using filter papers with distilled water. Controls had no effect on termite life as they were active and lived for long duration. To observe mortality and repellency various quantities of leaf extracts were made and applied. As a result, plant extracts of *C. gigantea* and *M. alba* showed maximum mortality and repellency against *H. indicola*. This finding holds great promise for the field of entomology and plant biology.

GCMS analysis, a reliable method, revealed the presence of phytochemicals in leaf extracts of all selected plants which are effective against insects. Researchers have also reported the presence of effective phytochemicals which are responsible for its biological especially its insecticidal activity in various parts of *C. gigantea* especially in leaves (kumar et al. 2013). Parwin et al. (2014) found the therapeutic potential and insecticidal effects of leaves of *C. gigantea* against pathogenic bacteria, insects and fungi. Sumathe et al. 2017 evaluated the efficacy of flower extracts of *C. gigantea* against *Papaya mealy bug* and observed 90-95% insecticidal activity at 2000 ppm within 24hours of treatment. Jacob and Sheila 1994 evaluated the antifeedant activity of aqueous leaf extracts of *C. gigantea* on *Pericallia racini* (hairy caterpillar) at 5% concentration. Similar effect of antifeedant activity of *C. procera* against *teak skeletonizer*. was reported by Meshram in 1995. Saika and Paremeswaran 2000 also reported similar results with *Allium cepa*, *neem*, and *C. gigantea* plant extract against *Cnaphalocrocis medinalis* (rice leaf roller). So, the results of our findings confirmed the repellency and efficacy of *C. gigantea* leaf extract. Maximum repellency was observed on filter papers, which were treated with *Calotropis gigantea* and *Morus alba* extractives in all concentrations against the termite species except the lowest concentration, further validating our research.

Our findings were similar to those of Raya- Gonzalez et al (2013), who found that percentage of mortality and feeding rate was concentration dependent when *Incisitermes marginipennis* Latreille (Blattodea:Kalotermitdae) fed on filter paper treated with *E. cyclocarpum* Jacq. Griseb (Fabales:Mimosaceae) extracts. At highest concentration of extract, our study showed a highest decrease in feeding on treated filter paper as compared to control treatments. Furthermore, after exposing termites to filter paper treated with aceton and methanol white mulberry extracts Se Golpayegani et al. (2014) reported >95% termite mortality. After feeding on white mulberry wood powder extracted with methanol, Se Golpayegani et al. (2014) observed that termite survival was 70%, however, termite survival was only 30% on powder extracted with acetone. Termite survival was less than 25% in our testing which might be because we used different solvent and use leaf extracts instead of wood powder. Both plant extracts at all tested concentrations cause mortality so both plants exhibited antitermitic efficacy. Within a few days of exposure, termites feeding on treated filter paper became lethargic and their abdomens shrank.

Different chemicals were found in all selected plants by GCMS analysis. According to the GCMS analysis of *M. alba*, the primary constituents include Vitamin E, Squalene, Ergost-8-en-3-ol,14-methyl-, (3β,5α), phytol, Hexahydrofuro[3,2-b] furan-3,6-diol. Based on the percentage of samples that were identified, Octacosanol, Nanodcane, and γ-Sitosterol were the primary constituents of *Calotropis gigantea*.

Squalene was different in chemical nature, and the active portion on the basis of percentage was not shared among extractives. According to Lukmandaru and Ogiyama (2005), Kartal et al., (2006), Gori et al., (2009), Lukmandaru et al., (2009), Bhat et al., (2010) and Xie et al., (2011), extracts containing Squalene have been shown to have termiticide activity. The majority of the sample consisted of Squalene. In 2018, Mary and Giri demonstrated the antibacterial, anti-inflammatory, and anti-cancer properties of squalene and phytol.

CONCLUSION

It is concluded that the botanical leaf extract of *C. gigantea* in an ethanol solution demonstrated maximum repellency and was more lethal than *M. alba* against the subterranean termite *Heterotermes indicola*.

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CONFLICT OF INTEREST

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

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