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Investigation of the termiticidal and protein composition of Calotropis gigantea (L.) W.T. Aiton) against Cryptotermes tropicalis Gay & Watson (Isoptera: Kalotermitidae)

Pavunraj Manickam[a*] Raja Selvaraju[b], Ramakrishnan Ramasamy[c], Nagarajan Kalimuthu[d], Rajeshkumar Shanmugam[e] and Ponnarasu Selvam[a]

[a*]Post Graduate & Research Department of Zoology, Vivekananda College, Affiliated to Madurai Kamaraj University (MKU), Tiruvedakam West, Madurai District – 625 234, Tamil Nadu, India.
[b]Post Graduate & Research Department of Zoology, Kongunadu Arts and Science College, Coimbatore – 641029, Tamil Nadu, India.
[c]Post Graduate & Research Department of Zoology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi West - 626 124, Tamil Nadu, India.
[d]Department of Animal Science, Manonmaniam Sundaranar University, Tirunelveli – 627 012, Tamil Nadu, India.
[e]Nanobiomedicine Lab, Centre for Global Health Research, Saveetha Medical College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai – 602 105, Tamil Nadu, India.

***Corresponding Author:** Pavunraj Manickam
*Email: mpavunraj@gmail.com (M. Pavunraj).

ABSTRACT
There is an increasing awareness of the use of natural products for termite and pest control because of their environmental safety. There are several indigenous plant materials that have been reported as potential alternatives to synthetic termiticides. In our study, we examined the anti-termite potential of chloroform, methanol, methanol + aqueous (1:1), and aqueous extracts of *Calotropis gigantean* against *Cryptotermes tropicalis*. The anti-termitic activity was performed by no-choice bioassay methods at 31.2, 62.5, 125, 250, and 500 mg/mL. The anti-termite potential of each extract revealed that the chloroform extract of *C. gigantea* exhibited the highest anti-termitic activity (94.0%) at 500 mg/mL against *C. tropicalis*, over a period of 72 hrs. followed by methanol (86.0%), methanol + aqueous (1:1), (59.0%), and aqueous (24.0%) extracts. It was found that the LC_{50} and LC_{90} values of chloroform extract from *C. gigantea* were 7.94 (6.61–9.55) and 23.82 (20.15–26.87), followed by methanol (15.31–13.60–17.24) and 45.93 (42.94–48.02), methanol + aqueous (1:1), 27.49 (21.82–36.51), and 61.63 (47.13–82.19), and aqueous less than 50%, respectively. The SDS-PAGE analysis revealed that the treated termite had a lower protein concentration. The termite groups that were aqueously treated and the control group showed negligible or no changes in protein expression. The preliminary phytochemical analysis of the plant also showed that the extracts contained alkaloids, terpenoids, flavonoids, phenolic compounds, glycosides, and steroids. *C. giganteam* leaves could be enhanced with an environmentally friendly termiticide.

Keywords: Anti-termitic activity; *Calotropis gigantea*; *Cryptotermes tropicalis*; Protein profile; Eco-friendly pesticide.

1. Introduction

Termites, which are arthropods with soft bodies and are a member of the Isoptera order, are a common occurrence worldwide and are recognized for their eusociality and cellulophagy (Engel and Krishna 2004). The ecosystem is greatly aided by termites, which are also known as social engineers and recycle waste materials such as dead wood, feces, and plants. Termites play a significant role in maintaining most of the world's ecosystems. The world has 2800 termite species, but 185 of them are classified as pests (Verma et al., 2009). Termites have the potential to cause serious damage to various crops, such as wheat, sugar cane, pandemonium, cotton, ground nuts, maize, and soybeans, as well as infect wild plant species (Sileshi et al., 2005). Despite being used for a long time, synthetic pesticides have not only failed to control termite infestations, but they have also been linked to toxic chemicals in ecosystems.

The discovery of phytochemicals and plant defense mechanisms has brought about a new era in medicine and pestology (Nerio et al., 2010; War et al., 2012). The use of biodegradable materials from natural sources can replace resistant synthetic pesticides, which can help prevent the harmful environmental effects of the past, as it is well known. Due to the great diversity within the kingdom of plants, a large variety of phytometabolites are produced; some of these can be applied as insecticides or pesticides to decrease environmental impact and pest resistance (Guclu-Ustundag and Mazza, 2007; Sahay et al., 2014).

C. gigantea, locally known as Erukku in Tamil and milk weed in English, belongs to the family Apocynaceae and subfamily Asclepiadoideae and is common to Africa and Asia and widely available throughout India (Saratha and Subramanian, 2012). The whole plant is used for skin diseases such as boils and sores, as a tonic, and as a as a purgative.

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In particular, the stem bark is used as diaphoretic, expectorant, and useful in complaints such as dysentery, spleen enlargement, convulsions, scabies, ringworm, pneumonia, and to induce labour in pregnant women. Fruit pulp is used as an abortive. Powdered root is used for elephantiasis, leprosy, and dysentery (Pattanaik et al., 2006).
The latex is used for stings, toothaches, caries, leprosy, ringworm, syphilis, tumors, rheumatism, antiseptics, vermifuges, and purgatives. The flowers are also used for treating jaundice, inflammation, ulcers, and asthma (Mandal, 2023). The latex showed digitalis-like action on the heart as infanticide and abortive properties (The Wealth of India, 2004). The leaves are crushed, warmed, and applied to burns, headaches, rheumatic pains, and in the form of a tincture for fever. Even the flower infusion is used for treating rheumatism, intestinal worms, and epileptic attacks (Shilpkar et al., 2007). Decoction of flowers is used for cough and asthma; the plant powder mixed with cow's milk can be used for rheumatism, diarrhea, dysentery, syphilis, ulcers, and leprosy (Alagesaboopathi, 2009). The present investigation examined the anti-termite activity of *C. gigantea* leaf extract as a potential bio-based material for termite control. Furthermore, the protein composition of the termites *C. tropicalis* in various solvent-based leaf extracts of *C. gigantea* was examined.

2. Materials and Methods

2.1. Collection and identification of plant material

The study was conducted at Department of Zoology, Vivekananda College, Tiruvedakam West, Madurai. The fresh and healthy leaves of *C. gigantean* **(Fig. 1)** were collected from Sholavandan, Madurai Districts, Tamil Nadu, India. The plant specimen was identified by an authentic plant taxonomist.

Fig. 1. *Calotropis gigantean:* a plant with leaves and flowers
(*Photo credit: Dr. M. Pavunraj*)

2.2. The process for the extraction of crude substances from leaves

The healthy leaves were shade-dried at room temperature for 3–5 days and ground into a fine powder. 10 g of fine powder was macerated for 24 hours with 100 ml of chloroform solvent. Then, the extract was filtered through Whatman No.1 filter paper and collected as filtrate. The collected filtrate was concentrated by a vacuum rotary evaporator. The crude extract obtained was stored at 4 °C until further use. The remaining residue was sequentially extracted with methanol, methanol + aqueous (1:1), and aqueous. The resulting colloidal extract was used in preliminary phytochemical analysis test and anti-termitic activity studies.

2.3. Collection and establishment of test organism

The population of termite’s *C. tropicalis* was collected from a Vivekananda College Campus (a residential institution featuring a self-sustaining agroecosystem) Sholavandan, Tiruvedakam West, Madurai District, Southern Tamil Nadu, India, and was identified by an authentic entomologist. Termite mounds were dug up using shovels, and soil containing termites was kept in polyethylene plastic boxes. Termites were collected from the plastic sheets using a camel hair brush and placed in plastic containers as described by Addisu et al. (2013). Termites were fed dry wood inside the container, and the top of the container was covered with muslin cloth to allow free flow of air and to prevent the termites from escaping.

2.4. Taxonomic classification of C. tropicalis.

Phylum	:	Arthropoda
Subphylum	:	Hexapoda
Class	:	Insecta
Infraclass	:	Neoptera
Subclass	:	Pterygota
Order	:	Isoptera
Family	:	Kalotermitidae
Genus	:	Cryptotermes
Subject	:	*Cryptotermes tropicalis* Gay & Watson

2.5. Preliminary analysis of phytochemical constituents

A preliminary investigation of the phytochemical composition of *C. gigantia* crude extracts was performed as per the methodology of Parekh and Chanda, 2008 to determine the presence of alkaloids, terpenoids, flavonoids, tannins, phenolic compounds, glycosides and steroids.

2.6. Bioassay to determine the toxicity of extracts on termites

The anti-termite activity of *C. gigantean* leaf was assessed using the no-choice bioassay method that was developed by Kang et al. in 1990. About 1 mL of plant extract with various concentrations ranging from 31.2 to 500 mg/L was applied to Whatman No.1 filter papers with a diameter of 09 cm. Filter paper treated with acetone was used as a control. The solvent was removed from the treated filter papers by air-drying at ambient temperature, and batches of 20 *C. tropicalis* worker termites were randomly selected from the stock population and kept into respective Petri dishes (10 cm in diameter, 1.5 cm in height). Treated and control termites were held under laboratory conditions in darkness at 27 ± 2 °C and 60–80% relative humidity. All treatments were replicated three times. The number of dead termites was counted every 24 hours of exposure, and the percentage mortality was calculated. A termite was considered dead when it was lying flat on its back and showing no sign of body movement after being touched with a soft camel brush. Percentage mortality was calculated using the following formula, which is given below:

$$\text{Percentage mortality} = \frac{\text{No of dead termite}}{\text{Total no of termite}} \times \frac{100}{1}$$

2.7. Protein extraction

The total protein content of whole insect samples in each treatment was extracted according to a prior protocol outlined by Choi et al. *(*2017).

2.8. Statistical analysis

The percentage termite mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90}, and other statistics at 95% fiducidal limits of the upper and lower confidence limits, and $\chi 2$ values were calculated using Finney's method (1971). The SPSS software package 16.0 was used. Results with a $P \leq 0.05$ were considered to be statistically significant.

3. RESULTS

3.1 Preliminary phytochemical analysis of C. gigantea leaf extracts

Preliminary phytochemical screening of *C. gigantea* leaves revealed the presence of various bioactive compounds. Chloroform extract showed maximum percentage mortality against *C. tropicalis*, due to the presence of alkaloids, terpenoids, flavonoids, tannins, phenolic compounds, and steroids **(Table 1).** The present findings coincide with the findings of Mann and Kaufman, (2012) who reported that both the botanicals *A. occidentale* and *C. burhia* caused more than fifty per cent mortality in *O. obesus* under controlled conditions which proves they are effective against termites. The present findings on the presence of secondary metabolites in ethanolic leaf extract of *C. gigantea* was well supported by the findings of Rafia, et al. (2023).

3.2. Anti-termitic activity of Calotropis gigantea leaves

The efficacy of the *C. gigantea* leaf extract against the *C. tropicalis* termites at various concentrations with a 72-hour time interval is shown in **Fig. 2 & Table 2**. The result showed that the percentage mortality rate was concentration-dependent; mortality increased with the increase in concentration and the time of exposure. Higher mortality rates were recorded at increasing concentrations. The result revealed that the chloroform extract of *C. gigantea* recoded (94.0%) at a higher concentration of 500 mg/L after 72 hours of exposure, followed by methanol (86.0%), methanol + aqueous (59.0%), and aqueous extract (24.0%), respectively. The results indicate that the treated termite mortality was significantly different from that of the control groups ($p < 0.05$). The probit analysis (LC_{50}, LC_{90}) shows their 95% fiducial limits at 72 h. The result showed the $LC5_0$ and LC_{90} values gradually decreased with the exposure periods. LC_{50} and LC_{90} values of chloroform extract from *C. gigantea* were 7.94 (6.61–9.55) and 24.82 (20.15–26.87), followed by methanol (15.31–13.60–17.24) and 45.93 (42.94–48.02), methanol + aqueous (1:1), 27.49 (21.82–36.51), and 61.63 (47.13–82.19), respectively, at 125 mg/mL. The control experimental set-up for this study showed no mortality.

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3.3. *Qualitative analysis of protein samples using SDS-PAGE*
According to Engelmann (1979), proteins are the fundamental building blocks of all living things and change both quantitatively and qualitatively as they develop. In addition, it plays a crucial role in the synthesis and breakdown of structural materials and it is constantly changing. In the present study, the concentration of protein was reduced in the treated termite. The total protein composition changes in *C. tropicalis* workers in response to different plant extracts **(Fig. 3).** SDS–PAGE results presented here show differences in hemolymph protein expression of *C. tropicalis* in various extracts of *C. gigantean* are presented herewith. In order to more thoroughly investigate changes in total proteins, the protein composition was compared in treated insect groups exposed to different plant extracts. The SDS-PAGE analysis showed that the protein expression was found to be less in the 3rd lane (methanol extract treated) than in other extracts treated. There are two dark bands observed at 66 KDa and 55 KDa in the 2nd lane, which is considered an effective treatment. This treatment also drastically reduced the protein bands. The intensity of the expression of 66 KDa and 55 KDa proteins in the 2ndlane band was found to be lower among the protein samples. The differences in band 4 expression are significant between 72-hour exposures. Protein expression changes are non-existent or slight in aqueous-treated control termites. The variations in protein band might be due to the susceptibility of the larvae to extracts. In the present study, qualitative changes in the haemolymph protein were observed through electrophoretic separation of the haemolymph. Haemolymph volume changes under stress, resulting in fluctuations in the protein pattern. The appearance of protein bands in the haemolymph in the treatment indicated developmental changes due to toxic stress on fractions. This is in accordance with the reports of Shaurub, 2012; Joykishan, et al., 2017, Ayoub Ajaha et al., 2019, and Pavunraj et al., 2024.

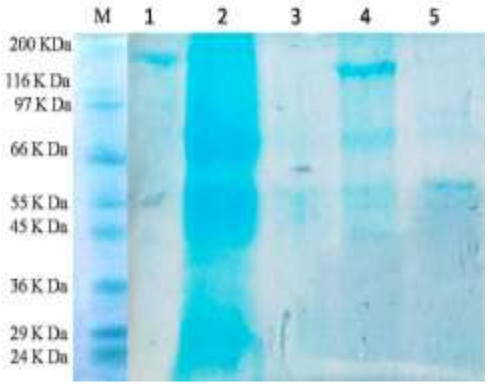

Fig. 3: SDS-PAGE analysis of *C. tropicalis* total proteins by coomassie staining.

SDS-PAGE (10%) Showing the protein expression of termite proteins: Lane-M protein marker; Lane-1 control; Lane-2 chloroform extract treated; Lane 3 methanol extract treated; Lane-4 methanol + aqueous (1:1) extract treated; and Lane-5 aqueous extract treated.

4. DISCUSSION
In present scenario, termite menace is a serious problem in tropical and subtropical regions. Indian wood termite *C. tropicalis* is a terrible insect pest which causes economic damage to commercial wood, fibers, paper sheet, clothes, woolens, and mats and seriously infests agricultural crops and forest products. Considering the resistance of insects to conventional synthetic insecticides and environmental safety issue, the recent trend is to search the plant-based products. There are several well documented studies showing that the plant essential oils and extracts could be used as antifeedant (Pavunraj et al., 2024), larvicidal (Pavunraj et al., 2014), adulticides (Afolabi et al., 2018), repellents (Henagamage et al. 2023), growth regulant (Cespedes et al., 2021), ovicidal (Pavunraj et al., 2020) and oviposition deterrent (Coria et al., 2008; Jeyabalan et al., 2003; Senthilkumar et al., 2009). In this study it was also observed that, leaf extract of *C. gigantea* has showed anti-termic activity against the wood termite *C. tropicalis*. The anti-termitic activities are strongly influenced by the concentration and duration of exposure.

According to various reports, the anti-termic functions of plant extract can be attributed to various secondary compounds viz., alcohols, aldehydes, fatty acid derivatives, phenolics, tannins, alkaloids, saponins, sterols, triterpenes and flavanoids which might be contributing jointly or independently (Boue and Raina, 2003; Cornelius et al., 1997; Ohmura, Doi et al., 2000; Pavunraj et al., 2013). It has been reported that the chemical composition of the crude plant extract and essential oils from several plants could vary significantly depending on species, plant parts, geographical origin, season, and extraction procedure (Nattudurai et al., 2012). The present studies also revealed the presence of steroids, saponins, terpenoids, alkaloids and flavonoids in leaf extract of *P. juliflora* which might be responsible for its insecticidal activity.
In order to understand the insecticidal activities from this plant, the chemical compounds such as: alkoloid, flavonoid, steroid, triterpenoid, saponin, and tannin were extracted and analyzed using phytochemical test, in which those compounds thought to have insecticidal effect (Ge et al., 2015; Ismayati et al., 2017; Meshram et al., 2019; Pavunraj et al., 2024). The

termiticidal effect of saponin has been documented by earlier studies in preliminary evaluation of anti-termite activity of *P. juliflora* extract against Macrotermes sp. (Bezuneh et al., 2019), they reported that the presence of steroids, saponins, terpenoids, alkaloids and flavonoids in leaf extract of *P. juliflora* might be responsible for its insecticidal activity. On the other hand, the presence of flavonoid can also induce termiticidal effect. The common link among flavonoid compounds is the presence of two hydroxyl groups at C-5 and C-7 in A-rings that incite high antifeedant activity. Furthermore, the presence of a carbonyl group at C-4 in the pyran rings of the compounds is needed for the occurrence of high activity (Ismayati et al., 2018). The present studies also revealed the presence of steroids, saponins, terpenoids, alkaloids and flavonoids in leaf extract of *P. juliflora* which might be responsible for its insecticidal activity.
The present study the SDS-PAGE separation of haemolymph protein revealed that the polypeptide of molecular weight 66 KDa showed variations among the different treatments. Further analysis of the protein banding pattern showed that a polypeptide of nearly 200 KDa molecular weight was found in treatments 2, 3, and 4 but was disintegrated in treatments 5 and 6. The variations in protein band might be due to the susceptibility of the larvae to fractions. This is in accordance with the reports of Shaurub, et al., 2012; Saleh, et al. 2018; Abdou et al., 2023.

5. CONCLUSION

In conclusion, the chloroform leaf extract of *C. gigantean* has been found to be toxic to worker termites of *C. tropicalis*, as demonstrated by the results of this study. The termiticidal activities of the *C. gigantean* leaf extract was attributed to potent secondary metabolites in the plant extract. Therefore, this study suggests that the isolation and characterization of the bioactive chemical constituents in the plant that are responsible for termiticidal activity are necessary. Moreover, it is important to evaluate the application of the extract in the field for proper management of termites.

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Pavunraj Manickam: Conceptualization, Writing – original draft, Writing – review & editing. **Ramakrishnan Ramasamy:** Methodology, Supervision. **Raja Selvaraju:** Resources. **Nagarajan Kalimuthu:** Supervision. **Rajeshkumar Shanmugam:** Investigation. **Ponnarasu Selvam:** Formal analysis, Data curation and Validation.
Declaration of competing interest

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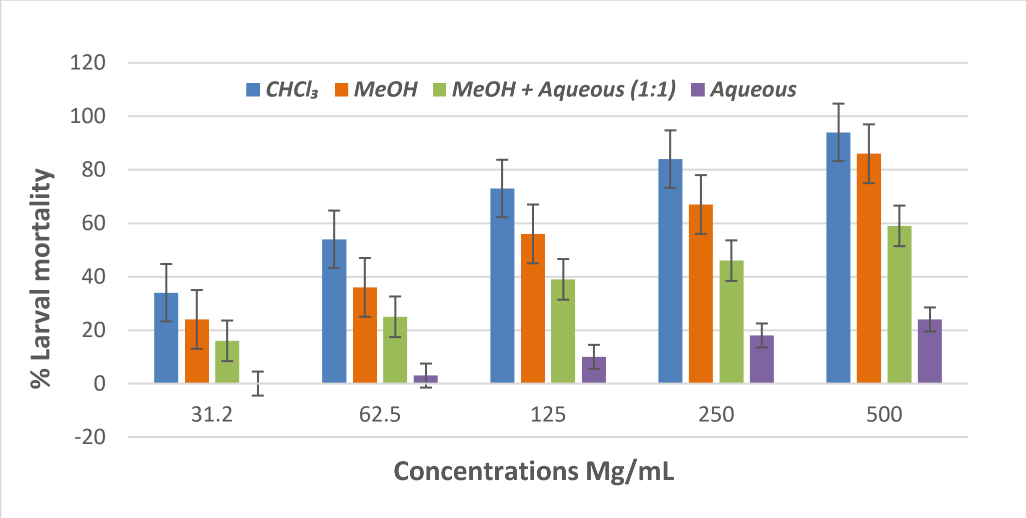
Table 1. Preliminary phytochemical analysis of various solvent leaf extracts of *C. gigantea*

S. No.	**Test**	**Test applied**	**Name of the extracts**			
			Chloroform	**Methanol**	**Methanol + Aqueous (1:1)**	**Aqueous**
1	Alkaloids	Mayer's test	+	-	+	+
2	Terpenoids	Salkowski test	+	-	-	+
3	Flavonoids	Alkaline reagent test	-	+	+	+
4	Tannins	Iron iii trichloride (FeCl3)	-	-	-	-
5	Phenolic compounds	Gelatin Test	+	+	-	-
6	Glycosides	Keller Killiani test	+	-	+	+
7	Steroids	Salkowski test	+	+	-	-

Note: (+) sign indicate the presence and (-) sign indicate the absence of phytochemical.

Fig. 2 A graphical representation depicting the percentage of mortality associated with various crude extracts against *C. tropicalis*.



Values are statistically different from the corresponding control at $p \leq 0.05$ by LSD.
The values are represented as the mean ± SE of three replicates.

Table 2. Termiticidal potential of various solvent extracts of *C. gigantea* leaves against *C. tropicalis*

Name of the Extract	LC_{50} ppm (LCL-UCL)	LC_{90} ppm (LCL-UCL)	Slope	r^2
$CHCl_3$	7.94 (6.61-9.55)	23.82 (20.15-26.87)	54	0.898
MeOH	15.31 (13.60-17.24)	45.93 (42.94-48.02)	36	0.988
MeOH + Aqueous (1:1)	27.49 (21.82-36.51)	61.63 (47.13-82.19)	39	0.856
Aqueous	Less than 50%	Less than 90%	18	0.456

Control—nil mortality, Significant at $p<0.05$ level,

LC_{50} lethal concentration that kills 50% of the exposed larvae,
LC_{90} lethal concentration that kills 90% of the exposed larvae,
UCL upper confidence limit, LCL lower confidence limit,
r^2 regression coefficient