ORIGINAL ARTICLE

Effect of combinatorial essential oil ingredients of *Citrus maxima* on percent wood weight loss and infestation caused by Indian white termite *Odontotermes obesus*

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Abstract

In the present investigation, various field bioassays were performed to evaluate the anti-termite efficacy of *Citrus maxima* essential oil-based combinatorial formulations against Indian white termite. LD_{50} values of various formulations were found in the range of 12.73–702.489 µg/g. In subsequent bioassays, crude essential oils and combinatorial mixtures have shown high toxicity against *Odontotermes obesus*. LD_{50} was obtained in the range of 702.489 µg/g for S-RET-C against *O. obesus.* Active ingredients present *C. maxima* essential oil repel and kill termites in thread binding assays, poison baits, and direct spray in field and garden saplings. Combinatorial mixtures of *Citrus* essential oils have shown synergistic activity against termites. Bait formulation (0.125% w/v) was also used which significantly cut down infestation of crop field termite in various crop fields. Combinatorial mixtures S-RET-B repelled (80%) termites to the opposite arm in Y-shaped tube. Besides this, *C. maxima* extracts have shown significant reduction (89%) in termite infestation in garden saplings. These combinatorial mixtures were also used for wood seasoning of solid wood sticks and hollow wood sticks which gave very good results as test wood sticks have shown significantly 70–80% reductions in termite infestation. *C. maxima* combinatorial mixtures displayed significant protection to garden saplings and resisted against wood invasion in field experiments. These gave an overall sustainable way to termite control in crop field mainly maize and millet crops.

Key words: *Citrus*, *Odontotermes obesus*, Plant essential oils, Poison bait, Termiticides, Wood seasoning

INTRODUCTION

Termites are social insects belonging to the class Insecta (order: *Isoptera*). These are highly destructive polyphagous insect pests of crop plants, forest trees, and buildings and invade stored products, cereal grains, wood fibers, cloths, and papers.[1,2] These also damage green foliages, seedlings, wood, fibers, and other household cellulose-based materials. Termites heavily infest post-harvest stored products, cereal grains, wood fibers, cloths, and papers. Both workers and soldier termites harm non-seasoned commercial wood and its formed materials. In forests, gardens and even in houses termites make tunnels; adjoin them with green biomass, vegetation, or crop fields. Termite mound soil is used as fertilizer, making bricks, geochemical prosperity, pottery, and plastering of houses. There are approximately 2800 termite species have been identified belong to 282 genera worldwide.

Termites colonize in rainy tropical regions of the world; they establish themselves in green forest covers and garden soils. Most of the termite species attack crop plants those results in reduction of crop yield and biomass production significantly. Termites do decomposition of above ground dead wood, mediating the incorporation of suspended and standing dead wood into the soil.[3] Termites help in decomposition of plant litter and play

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an important ecological role in the recycling of wood and other cellulose-based material. These decompose forest and agriculture biomass in ecosystem and assist in numerous ecological processes. Termites forage in forests mainly green leaves and make huge mounds. These invade standing dead trees in tropical forests and degrade the wood and reduce its quality.[4]

For routine control of field termites, various synthetic pesticides such as chlordane,[5] cypermethrin,[6] hydroquinone, and indoxacarb have been used.[7] Although, these synthetic pesticides cause high lethality in termites and are highly toxic as they persist for longer periods in the form of residues. They enter into the food chain and kill non-target organisms. These have been banned and its new alternatives are discovered in the form of natural pesticides. Ruta plants are perennial shrubs belonging to the family Rutaceae, which are traditionally used in folk medicine, since ancient times mostly for the treatment of various ailments of the womb. Different parts of the plants belonging to Ruta genus are used in folk medicine to treat a wide range of different diseases.[8] Few natural products such as flavonoids,[9] sesquiterpenes,[10] and thiophenes[11] isolated from different plants species were found effective against termites.

Plant species synthesize thousands of active biomolecules and so many of them have been extracted and identified for its insecticidal activities.[12] Few of them act at cellular and physiological level.[13] Plant-derived compounds are used as an alternative of synthetic pesticides for termite control. Plant natural products are used in eco-friendly control of termites and its management. *Capparis decidua* and its combinatorial mixtures are used to control the Indian white termite *Odontotermes obesus*[14,15] and stored grain insects[16] showed string termiticidal effects.

For termite control, few bait formulations have been provided wider control against subterranean termite colonies.[17] Noviflumuron is used in poison baits to kill molting worker population of Formosan subterranean termite, *Coptotermes formosanus* Shiraki inside nest.[18] Polyacrylamide/attapulgite composite baits are also applied in agricultural soils. These attract termite population for foraging.[19] Systemic poisons or pesticides could be added to these gels to kill termites. Carbon dioxide release attracts termites. It improves effectiveness of an insecticide to kill termites in nests.[20] Plant essential oils and its components show multiple deleterious effects such as toxic, antifeedant, repellent, growth, and reproductive inhibitory activity in number of insect pest species. Its various components synergists are used in preparation of poison baits where they acted as synergists, these successfully exploit feeding, tunnelling,[21] and reproductive behavior in termites.[22,23] New alternates in the form of various natural plant products such as essential oils, poison baits,[24] deterrents, repellents, fumigants, insecticidal bioorganic chemicals, and genetic and biological control methods should employ to control this white termite.[25] The present study signifies control of termite population in field and garden soil.

MATERIALS AND METHODS

Collection of Termites

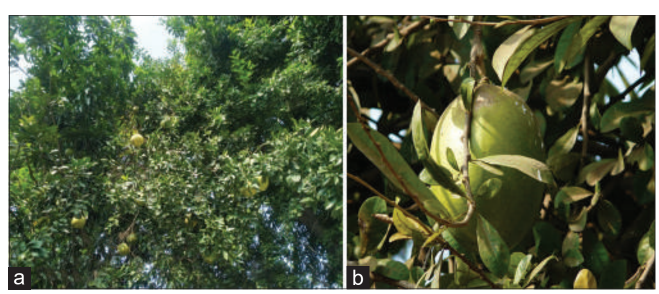
Termites, *O. obesus* (Rambur) both soldier and workers, were collected from the University garden and temporary culture was maintained in the laboratory at 37°C ± 2°C at 80% RH by providing green leaves as food material. Termite culture was protected from light illumination, using black paper sheets wrapped around the glass containers (12 × 9 inch). Termites were provided fresh food material and it was changed regularly after 24 h. The LD_{50} after 24 h of exposure to each was calculated using probit analysis tested using the method of Finney (1971).

Preparation of *Citrus maxima* Extracts for Anti-Termite Effect

C. maxima (Brum.) Chakotra/Pomelo fruit or grapefruit belongs to the family Rutaceae was collected from Deen Dayal Upadhyaya Gorakhpur University garden, living specimen is photographed [Photoplate 1a and b]. It is a natural, non-hybrid, citrus fruit, and native to Southeast Asia. This specimen was authenticated by an expert of botany and help was taken from Taxonomy of Indian Angiosperms. The herbarium specimen is healthy and preserved in Botanical garden of Gorakhpur University for future references. This plant is extensively used for nutritional and therapeutic purposes by local people not only in India but also in Southeast Asia. Peel of fresh fruits was used for the preparation of combinatorial mixture w/v. Fresh peel was weighed and extract was prepared in distilled water in a power mixture and grinder. The extract obtained was dried in rotatory evaporator and kept in refrigerator for further use. All chemicals used in this study were purchased from CDH-laboratory chemicals suppliers India supplied by Eastern Scientific Company, Gorakhpur.

Combinatorial Formulations

Citrus maxima peel oil extract and other ingredients were used in the preparation of combinatorial mixtures. The details of all combinatorial mixtures are mention in table number 1.



Photoplate 1: (a and b) Vegetative parts of *Citrus maxima* plant

FIELD EXPERIMENTS

Determination of LD Values in Extracts and Combinatorial Mixtures

Toxicity bioassay

For the evaluation of dose–response relationship of essential oil extracts, different doses (w/v), that is, 60, 120, 240, 360, 480, 600, and 720 µg of different extracts, were loaded on separate Whatman paper strips (1 × 1 cm^2) and air dried to remove the solvent. These pre-coated solvent free strips were placed in the center of separate Petri dishes (42 mm diameter) as tests and uncoated as control. Twenty worker termites were released in the Petri dish to observe the mortality. After setting the experiment, green leaves were provided as food for both tests and control termites and containers were covered with black paper sheets. Mortality was recorded on the basis of dead and living termites and observations were made in triplicate for each extract and pure compounds up to 24 h. Termites were treated as dead when become immobile and have shown no further activity to the external stimuli. The LD50 after 24 h of exposure to each was calculated using probit analysis tested using the method of Finney.

Repellency bioassay

Repellent responses were observed in a glass Y-tube olfactometer using serial concentrations 20, 40, 60, 80, 100, 120, and 160 µg of different crude extracts/fractions/formulations loaded on separate Whatman paper strips (1 × 1 cm^2) and air dried to remove the solvent. These pre-coated solvent-free strips were placed in the right arm of Y-tube olfactometer (16 mm diameter × 90 cm length) as tests while similar strips uncoated were placed in the left arm as control. Twenty worker termites were released inside the opposite tri-arm to observe the repellent activity. After introduction of termites, tube openings were closed by Teflon tape and number of termites oriented to toward uncoated strips or non-scented area was counted as repelled. Individuals that did not enter at least one of the arms were scored as unresponsive. Tests were conducted for 18 h at 27°C temperature. Same tests were conducted after reversing the arms to test directional bias. A Chi-square test was used to compare the number of termites responding to the olfaction generated by *C. maxima* essential oil components. Number of repelled termites in the presence of each extract was counted after 30 min of treatment with six different concentrations (20, 40, 60, 80, 100, and 120 µg/g) of each *C. maxima* extract which were used. The ED50 values that repelled 50% of termite population were calculated.

Wood Seasoning

For the evaluation of anti-termite potential of combinatorial mixtures, wood seasoning experiments were performed. Both solid and hollow bamboo wood sticks were placed in crop fields, and garden soil in separate plots at an equal length. These wood sticks were treated with various combinatorial mixtures.

Treatment of solid wood sticks

In these experiments, dried solid wood sticks of Sagwan (*Tectona grandis*) (Family: *Lamiaceae*) having 1 feet and ~50 mm average diameter were used. These solid wood sticks were seasoned with different combinatorial mixtures of *C. maxima* for 24 h. Following sets were made –

Set no. I

In set no. 1, solid wood sticks were submerged for 24 h in S-RET-A, S-RET-B, and S-RET-C mixtures in a 30 L plastic tub. Mixture was prepared by adding 3.0 g of sulfur as an inorganic substance [Table 1]. For each mixture, six wood sticks were seasoned by dip method for 24 h for an experiment of 6 months observation (total 18 sticks were planted in this set).

Set no. II

In another set of experiment, solid wood sticks were seasoned with B-RET-A, B-RET-B, and B-RET-C combinatorial mixtures. These were prepared by mixing 3.0 g borate powder while rest of the content were same as above [Table 1] (total 18 sticks were planted in this set).

Set no. III

In the third experiment, wood sticks were seasoned with C-RET-A, C-RET-B, and C-RET-C a mixture which is prepared by mixing 3.0 g of copper sulfate. Other components were similar as above [Table 1] (total 18 sticks were planted in this set).

Set no. IV

In the fourth set of experiment, solid wood sticks were seasoned with CU-RET-A, CU-RET-B, and CU-RET-C mixtures [Table 1] (total 18 sticks were planted in this set).

Set no. V

In the fifth set of experiment, inorganic compounds such as malathion, fipronil, and thiamethoxam were used for the wood seasoning [Table 1] (total 18 sticks were planted in this set).

In above experiments, seasoned wood sticks of 1 feet length were planted inside soil by making separate pits of 0.75 feet depth. Separate pits were used for each stick. Six replicates of each concentration were used for precision. For comparison, unseasoned wood sticks of similar size and diameter were used as control. For observations, one wood stick each from control and test was dug out after 30 days interval and weighed. These wood sticks were tagged with all required information. Experiments were continued up to 6 months [Photoplate 2].

Table 1: *Citrus maxima* and other ingredients used in the preparation of combinatorial mixtures

S. No.	Combinatorial mixtures	Ingredients
1.	S-RET-A	*Citrus maxima* peels (9 g) + Coconut oil (17ml) + Terpene oil (17 ml) + Glycerol (17 ml) + Sulfur (3 g) + Water (5 L)
2.	S-RET-B	*Citrus maxima* peels (12 g) + Coconut oil (17ml) + Terpene oil (17 ml) + Glycerol (17 ml) + Sulfur (3 g) + Water (5 L)
3.	S-RET-C	*Citrus maxima* peels (18 g) + Coconut oil (50ml) + Terpene oil (50 ml)+ Glycerol (50 ml) + Sulfur (3 g) + Water (5 L)
4.	B-RET-A	*Citrus maxima* peels (9 g) + Coconut oil (17ml) + Terpene oil (17 ml)+ Glycerol (17 ml) + Borate (3 g) + Water (5 L)
5.	B-RET-B	*Citrus maxima* peels (12 g) + Coconut oil (17ml) + Terpene oil (17 ml)+ Glycerol (17 ml) + Borate (3 g) + Water (5 L)
6.	B-RET-C	*Citrus maxima* peels (18 g) + Coconut oil (17ml) + Terpene oil (17 ml)+ Glycerol (17 ml) + Borate (3 g) + Water (5 L)
7.	C-RET-A	*Citrus maxima* peels (9 g) + Coconut oil (17ml) + Terpene oil (17 ml)+ Glycerol (17 ml) + Copper (3 g) + Water (5 L)
8.	C-RET-B	*Citrus maxima* peels (12 g) + Coconut oil (17ml) + Terpene oil (17 ml)+ Glycerol (17 ml) + Copper (3 g) + Water (5 L)
9.	C-RET-C	*Citrus maxima* peels (18 g) + Coconut oil (17ml) + Terpene oil (17 ml)+ Glycerol (17 ml) + Copper (3 g) + Water (5 L)
10.	CU-RET-A	*Citrus maxima* peels (9 g) + Photoactivated Cow urine (10 g/L)+ Water (5 L)
11.	CU-RET-B	*Citrus maxima* peels (12 g) + Photoactivated Cow urine (10 g/L)+ Water (5 L)
12.	CU-RET-C	*Citrus maxima* peels (18 g) + Photoactivated Cow urine (10 g/L)+ Water (5 L)
13.	H-RET	*Citrus maxima* peels (40 g) + Hexane (200 ml)
14.	AQ-RET	*Citrus maxima* peels (40 g) + Water (200 ml)
15.	A-RET	*Citrus maxima* peels (40 g) + Acetone (200 ml)
16.	P-RET	*Citrus maxima* peels (40 g) + Petroleum Ether (200 ml)
17.	Malathion	Malathion powder (7.5 g/L) + Water (5 L)
18.	Fipronil	Fipronil powder (7.5 g/L) + Water (5 L)
19.	Thiamethoxam	Thiamethoxam powder (7.5 g/L) + Water (5 L)

Photoplate 2: Experimental set up of solid wood sticks planted in garden soil

Treatment of solid wood sticks

In these experiments, wood seasoning was done by fixed *C. maxima* essential oil extract, fixed oil extract + sulfur, and fixed oil extract +sulfur + cow urine mixtures for overnight. Stick length was 3 feet and 1.25 inches in diameter were planted in soil at 1 m distance from each. In each row, the six stick of *Tectona grandis* were planted. Control was also planted in a row with fixed solid wood stick as control. For observation, one wood stick from each test was dug out 30 days wood weight loss, % of infestation termite population [Photoplate 3]. Besides above experiments, hollow bamboo wood sticks were also used for wood seasoning to observe the tunnelling activity of termites in the presence of various mixtures [Photoplate 3].

Treatment of hollow wood sticks

For the evaluation of toxic and repellent properties of *C. maxima* combinatorial mixtures, bamboo wood sticks (1 feet and ~50 mm diameter) were used for the treatment. For this purpose, bamboo wood sticks were treated inside obstructions present at internodes which were made free by iron rod to fill agar impregnated combinatorial mixture.

Set no. I

In the first experiment, REAT-1, REAT-2, REAT-3, REAT-4, REAT-5, and REAT-6 combinatorial mixture (prepared

Photoplate 3: Experimental set up of solid wood sticks planted in garden soil

from extract [9 g, 10 g, 12 g, 14 g, 16 g, and 18 g, respectively]+Ash [100 g]) [Table 2] [total six sticks were planted in this set].

Set no. II

In the second experiment, hollow bamboo sticks were treated with REAT-CU-1, REAT-CU-2, REAT-CU-3, REAT-CU-4, REAT-CU-5, and REAT-CU-6 mixture (prepared from extract [9 g, 10 g, 12 g, 14 g, 16 g, and18 g, respectively]+Cow Urine [90 ml, 100 ml, 120 ml, 140 ml, 160 ml, and 180 ml, respectively]+Ash [100 g]) [Table 2] (total six sticks were planted in this set).

Set no. III

In the third experiment, hollow bamboo sticks were treated with REAT-CU-CD-1, REAT-CU-CD-2, REAT-CU-CD-3, REAT-CU-CD-4, REAT-CU-CD-5, and REAT-CU-CD-6

Table 2: *Citrus maxima* and other ingredients used in the preparation of combinatorial mixtures.

S. No.	Combinatorial mixtures	Ingredients
1.	REAT-1	*Citrus maxima* Extract (9 g)+Ash (100 g)
2.	REAT-2	*Citrus maxima* Extract (10 g)+Ash (100 g)
3.	REAT-3	*Citrus maxima* Extract (12 g)+Ash (100 g)
4.	REAT-4	*Citrus maxima* Extract (14 g)+Ash (100 g)
5.	REAT-5	*Citrus maxima* Extract (16 g)+Ash (100 g)
6.	REAT-6	*Citrus maxima* Extract (18 g)+Ash (100 g)
7.	REAT-CU-1	*Citrus maxima* Extract (9 g)+Cow Urine (90 ml)+Ash (100 g)
8.	REAT-CU-2	*Citrus maxima* Extract (10 g)+Cow Urine (100 ml)+Ash (100 g)
9.	REAT-CU-3	*Citrus maxima* Extract (12 g)+Cow Urine (120 ml)+Ash (100 g)
10.	REAT-CU-4	*Citrus maxima* Extract (14 g)+Cow Urine (140 ml)+Ash (100 g)
11.	REAT-CU-5	*Citrus maxima* Extract (16 g)+Cow Urine (160 ml)+Ash (100 g)
12.	REAT-CU-6	*Citrus maxima* Extract (18 g)+Cow Urine (180 ml)+Ash (100 g)
13.	REAT-CU-CD-1	*Citrus maxima* Extract (9 g)+ Cow Urine (90 ml)+Cow Dung (90 g)+ Ash (100 g)
14.	REAT-CU-CD-2	*Citrus maxima* Extract (10 g)+ Cow Urine (100 ml)+Cow Dung (100 g)+ Ash (100 g)
15.	REAT-CU-CD-3	*Citrus maxima* Extract (12 g)+ Cow Urine (120 ml)+Cow Dung (120 g)+ Ash (100 g)
16.	REAT-CU-CD-4	*Citrus maxima* Extract (14 g)+ Cow Urine (140 ml)+Cow Dung (140 g)+ Ash (100 g)
17.	REAT-CU-CD-5	*Citrus maxima* Extract (16 g)+ Cow Urine (160 ml)+Cow Dung (160 g)+ Ash (100 g)
18.	REAT-CU-CD-6	*Citrus maxima* Extract (18 g)+ Cow Urine (1800 ml)+Cow Dung (180 g)+ Ash (100 g)
19.	Control-1(PVC)	Cow Urine (90 ml)+Cow Dung (90 g)
20.	Control-2(PVC)	Cow Urine (100 ml)+Cow Dung (100 g)
21.	Control-3(PVC)	Cow Urine (120 ml)+Cow Dung (120 g)
22.	Control-4(PVC)	Cow Urine (140 ml)+Cow Dung (140 g)
23.	Control-5(PVC)	Cow Urine (160 ml)+Cow Dung (160 g)
24.	Control-6(PVC)	Cow Urine (180 ml)+Cow Dung (180 g)
25.	Control-1	Fipronil (9 g) +Cow Dung (90 g)+ Ash (100 g)
26.	Control-2	Fipronil (10 g)+Cow Dung (100 g)+ Ash (100 g)
27.	Control-3	Fipronil (12 g)+ Cow Dung (120 g)+ Ash (100 g)
28.	Control-4	Fipronil (14 g)+ Cow Dung (140 g)+ Ash (100 g)
29.	Control-5	Fipronil (16 g)+Cow Dung (160 g)+ Ash (100 g)
30.	Control-6	Fipronil (18 g) +Cow Dung (180 g)+ Ash (100 g)

mixtures (prepared from extract [9 g, 10 g, 12 g, 14 g, 16 g, and18 g, respectively]+Cow Urine [90 ml, 100 ml, 120 ml, 140 ml, 160 ml, and 180 ml, respectively]+Cow Dung [90 g, 100 g, 120 g, 140 g, 160 g, and 180 g, respectively] +Ash [100 g]) [Table 2] (total six sticks were planted in this set).

Set no. IV

In this experiment, control was prepared with the mixing of cow urine and cow dung (Cow Urine [90 ml, 100 ml, 120 ml, 140 ml, 160 ml, and 180 ml, respectively]+Cow Dung [90 g, 100 g, 120 g, 140 g, 160 g, and 180 g, respectively]+ Ash [100 g]) [Table 2] (total six PVC pipes were planted in this set).

Set no. V

In this experiment, inorganic termiticide fipronil was mixed with different amount of cow dung (Fipronil [9 g, 10 g, 12 g, 14 g, 16 g, and 18 g, respectively] +Cow Dung [90 g, 100 g, 120 g, 140 g, 160 g, and 180 g, respectively]+ Ash [100 g]) [Table 2] (total six sticks were planted in this set).

For above experiment, maize crop (Krishna cultivar) was sowed in middle of March 2021 in the field. For this purposes, loam soil was selected, field was prepared by three consecutive ploughing and watered after 3 days. Field was prepared weed free and soil texture was made fine. The field size was of 8 × 3 m (W × L) in size, both control and test were arranged in the form of six regular replicates in a row and planted at a distance of 1 meter. The gap between the rows was stick to stick distance which was kept 1.5 feet. A control was set using six PVC pipes (1 feet length and 1.25 inch diameter). PVC pipes were planted in the soil to 3 inches (up to humus region of the soil) so that active ingredient could come out and diffuse in the soil and its intake becomes possible to the plants through absorption [Photoplate 4].

Humidity, temperature, and day of sowing were noted down. All climatic regimes average humidity, day temperature, and dew were noted as additional parameters. These were 64–70%, 23–28°C temperature, dew was moderate, day period periodicity was (11:13 h) 6.05 AM–6.45 PM, and sowing day was rainy cloudy day. In the end, % response of ingredient (% control), % infestation, and wood weight loss were enumerated by selecting six random soil samples after 1 month interval. In the end of experiment crop loss, % yield and termite infestation versus termiticidal efficacy of essential oil were calculated.

Treatment of hollow bamboo wood sticks

In these experiment, wood seasoning was done by fixed *C. maxima* essential oil extract, fixed oil extract + sulfur, and fixed oil extract +sulfur + cow urine for overnight. Sticks of 3 feet length and 1.25 inches in diameter were planted in soil at 1 m distance from each. In each row, the six stick of bamboo were planted. Control was also planted in a row with fixed solid wood stick as control. After one month, wood weight loss, % of infestation and termite population were observed in bamboo sticks and these observation was follow up to six month. [Photoplate 5].

Photoplate 4: Experimental set up of hollow wood sticks planted in garden soil

Photoplate 5: Experimental set up of hollow wood sticks planted in garden soil

Poison Bait Experiment

For controlling field termite, homemade baits were prepared using Multani earth and organic ash + loam soil (2:1:2). Disk shape baits were prepared using iron bottle lid of 22 mm diameter. In the cavity of it, fixed oil ingredient was filled, these was air dried inside room condition. In the first set, above soil mixture was used and the active ingredient was fixed oil extract 1.4 g. In the second set, 1.4 g fixed oil extract added with 1.25 g sulfur in bait. In the third set, bait was prepared by 1.4 g fixed oil extract; 1.25 g sulfur; and 10 ml cow urine in poison bait (0.125%w/v). Similar field size was prepared for sowing of maize and millet seeds. Soil condition was same as used in last experiment. Experiments were conducted for 6 months. Termite number was counted from each wood stick just completion of 30 days. Both plastic pipe and hollow wood sticks were used for testing the infestation. Dried wood sticks were weighed for weight loss [Photoplate 6].

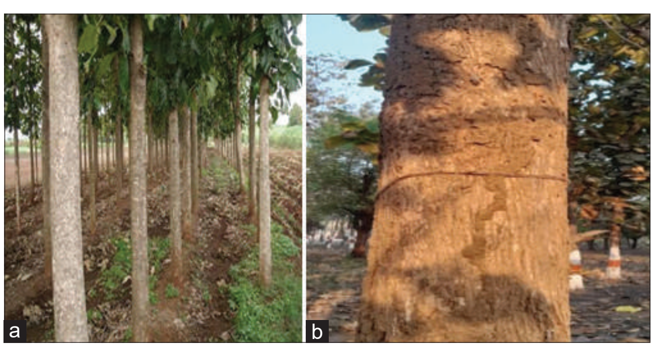
Thread Binding Bioassay

For the evaluation of anti-termite efficacy of *C. maxima* extract, cotton threads were soaked in different combinatorial mixtures for 30 min. After drying, the threads were tagged around the trunks of infested trees at an average height of 5–6 feet above the ground. In control, the same thread was tagged at the same height without coating any active fraction for comparison. Few important observations such as tunnelling and foraging behaviors were significantly noted to evaluate the termite infestation [Photoplate 7a and b].

Statistical Analysis

For the calculation of LD_{50} value, Probit Or LOgit (POLO), a computer program for the analysis of quantal response data such as that obtained from insecticide bioassays by the techniques of POLO analysis. Data were analyzed for the calculation of dose response (LD_{50} and degrees of freedom [df]), heterogeneity, and Chi-squared goodness-of-fit test using POLO computer program. This is specifically developed to analyze data obtained from insecticide bioassays (Russell and Robertson [1979]). Dosage response lines may be compared for parallelism or equality by means of likelihood ratio tests. Statistical features of the program, suggestions for the design of experiments that provide data for analysis, and formats for data input and output are described in detail. Standard deviations Chi-square, t-significance, correlation, and analysis of variance (ANOVA) were calculated from the means using Sokal and Rohlf method (1973). In the experiments, ANOVA was done whenever two means were obtained at a multiple test range and $P < 0.05$.

Photoplate 6: Experimental set up of poison baits planted in garden soil



Photoplate 7: (a and b) Experimental set up of thread binding assay in garden

RESULTS

Determination of Toxicity and LD_{50} Value

All the combinatorial fractions of *C. maxima* have shown very high insecticidal activity. These have shown very low LD_{50} value, that is, 335.677, 526.867, 702.489, 348.091, 446.547, 564.058, 361.552, 584.962, 594.205, 404.801, 498.223, 603.676, 27.825, 22.605, 12.736, and 17.423 µg/g body weight of termites for S-RET-A, S-RET-B, S-RET-C, B-RET-A, B-RET-B, B-RET-C, C-RET-A, C-RET-B, C-RET-C, CU-RET-A, CU-RET-B, CU-RET-C, AQ-RET, A-RET, H-RET, and P-RET, respectively. The lowest LD_{50} was obtained in H-RET mixture, that is, 12.736 µg/g body weight of termite. The upper and lower confidence limits obtained were for each combinatorial fraction and synthetic termiticides [Table 3]. Besides this, LD_{50} value of synthetic pesticides was also determined for comparison. It was obtained 67.026, 27.891, and 50.255 µg/g LD_{50} for malathion, fipronil, and thiamethoxam. Besides this Chi-square, slope function, degree of freedom, and heterogeneity were also calculated to find upper and lower limits of toxicity and its level significance (significant at <0.05). These combinatorial formulations have shown much better toxicity than synthetic pesticides. It was found to be time and dose dependent.

Wood Seasoning

For solid wood sticks

In these experiments, seasoned wood sticks were planted in the soil at an equal distance. Significant anti-termite activity was observed in *C. maxima* extracts and its combinatorial mixture in seasoned wood sticks planted in the soil. Results showed that wood seasoning protected wood weight losses and termite infestation in comparison to unseasoned wood sticks.

Set no. I

Very high anti-termite activity was obtained in S-RET-A, S-RET-B, and S-RET-C seasoned wood sticks in the garden soil. In this set of experiment, both percent weight loss and termite infestation were found to be significantly reduced in comparison to control. However, S-RET-A mixture showed 36–67% wood protection and it effectively prevented termite infestation in comparison to control. Similarly, S-RET-B mixture caused significant decrease both in percent weight

Table 3: LD50 values after the treatment of termites with various combinatorial fractions and pesticides

S. No.	Name of extract/ combinatorial mixture	LD50 μg/g	LD40 μg/g	LD20 μg/g	0.95 confidence limit UCL-LCL	Chi-square	Slope function	Degree of freedom	Heterogeneity
1.	S-RET-A	335.677	134.27	67.13	408.779–282.543	3.976	−0.128442	4	0.994
2.	S-RET-B	526.867	210.74	105.37	741.457–402.078	6.2156	−0.162239	4	1.5539
3.	S-RET-C	702.489	280.99	140.49	988.609–536.104	6.2156	−0.169926	4	1.5539
4.	B-RET-A	348.091	139.23	69.61	518.657–257.072	6.9060	−0.139692	4	1.7265
5.	B-RET-B	446.547	178.61	89.30	528.985–380.638	2.898	−0.140655	4	0.725
6.	B-RET-C	564.058	225.62	112.81	681.271–471.640	2.940	−0.132454	4	0.735
7.	C-RET-A	361.552	144.62	72.31	428.327–311.588	1.320	−0.165391	4	0.330
8.	C-RET-B	584.9	233.98	116.99	1060.174–406.699	8.4758	−0.145411	4	2.1190
9.	C-RET-C	594.2	237.68	188.84	802.549–453.330	5.4707	−0.148692	4	1.3677
10.	CU-RET-A	404.8	161.92	80.96	494.686–343.794	3.047	−0.162205	4	0.762
11.	CU-RET-B	498.22	199.28	99.64	741.966–365.374	6.2522	−0.136723	4	1.5630
12.	CU-RET-C	603.6	241.47	120.73	723.312–510.536	3.367	−0.141853	4	0.842
13.	AQ-RET	27.82	11.13	5.56	49.763–19.092	10.837	−0.696979	4	2.7093
14.	A-RET	22.60	09.04	4.52	50.140–14.676	11.332	−0.651231	4	2.8329
15.	H-RET	12.73	05.09	02.54	39.622–7.583	12.345	−0.454328	4	3.0862
16.	P-RET	17.42	06.96	03.48	27.784–12.453	6.9421	−0.582885	4	1.7355
17.	Malathion*	67.02	26.81	13.40	95.511–52.909	2.083	−0.875498	4	0.521
18.	Fipronil*	27.89	11.15	5.57	58.871–18.100	11.839	−0.715511	4	2.9597
19.	Thiamethoxam*	50.25	20.10	10.05	63.329–41.833	2.844	−0.872107	4	0.711

*Synthetic pesticides

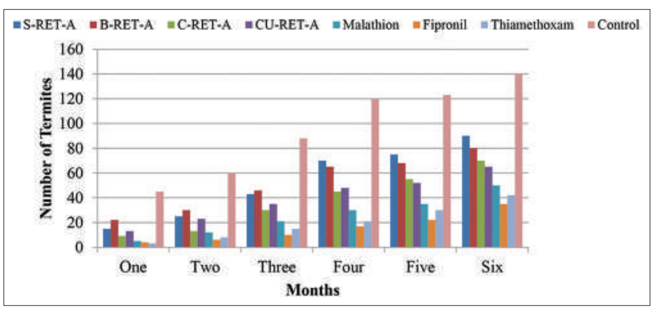


Figure 1: Number of termites in solid wood sticks

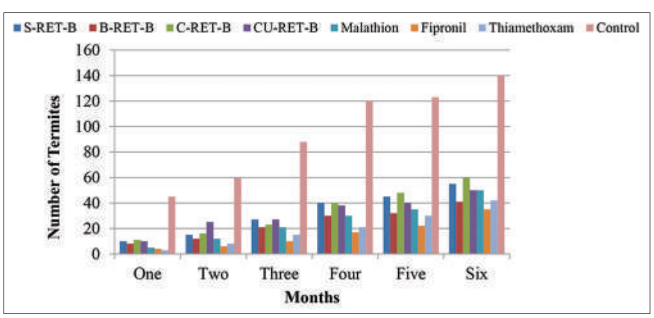


Figure 2: Number of termites in solid wood sticks

loss 67–97%, S-RET-C mixture gave better wood protection approximately 65–83% and a very significant decrease in termites infestation [Figures 1-9].

Set no. II

In another experiment, wood seasoning was done using B-RET-A, B-RET-B, and B-RET-C mixtures out of which B-RET-C mixture was found more effective in comparison to other mixtures. The percent weight loss in wood sticks seasoned with B-RET-A mixture was obtained in a range of 5–53% while termite infestation was obtained in a range of 48–57%. Similarly, both B-RET-B and B-RET-C mixtures have shown very high wood protection, that is, 28 and 89% significant decrease in termite infestation, respectively [Figures 1-9].

Set no. III

In this experiment, the highest wood protection and lowest weight loss were observed in C-RET-B mixture, that is, 2–32% after 180 days. Similar activity was obtained in C-RET-A and C-RET-C mixtures. Above mixture was also successfully controlled the termite infestation in treated wood sticks as percent termite infestation recorded was very low, that is, 13–30% in the presence of C-RET-C mixture, 24–42% in the presence of C-RET-B mixture, and 20–50% in the presence of C-RET-A mixture, respectively [Figures 1-9].

Set no. IV

Photoactivated cow urine showed synergistic activity against termites, it cut down weight loss up to 69–96% up to 6 months [Figure 1-9].

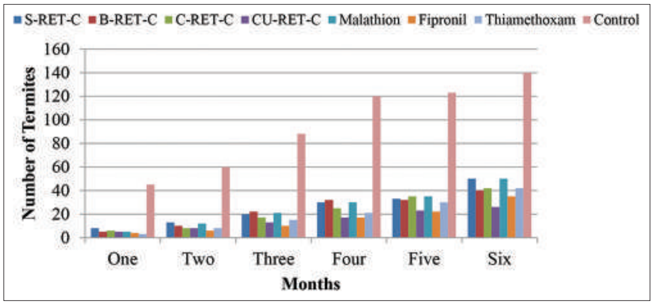


Figure 3: Number of termites in solid wood sticks

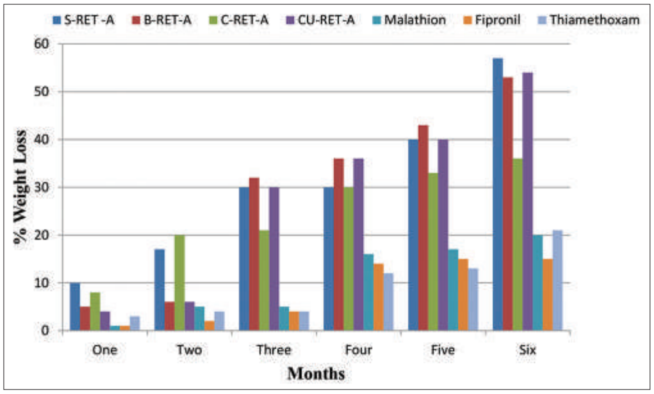


Figure 4: Percent weight loss in solid wood sticks

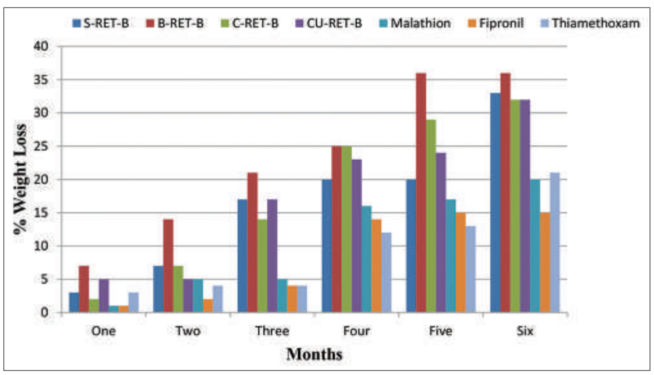


Figure 5: Percent weight loss in solid wood sticks

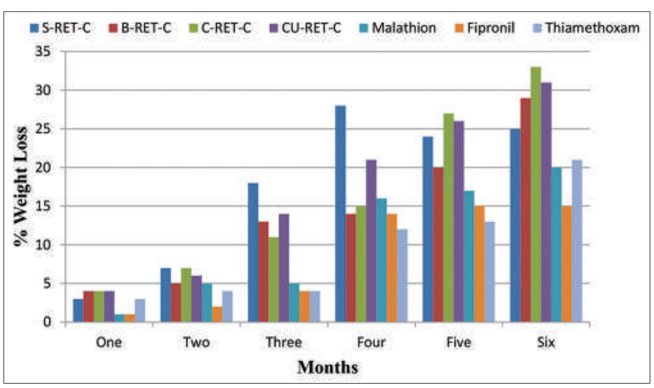


Figure 6: Percent weight loss in solid wood sticks

Set no. V

Percent weight loss in the presence of inorganic pesticides such as malathion, fipronil, and thiamethoxam was also recorded.

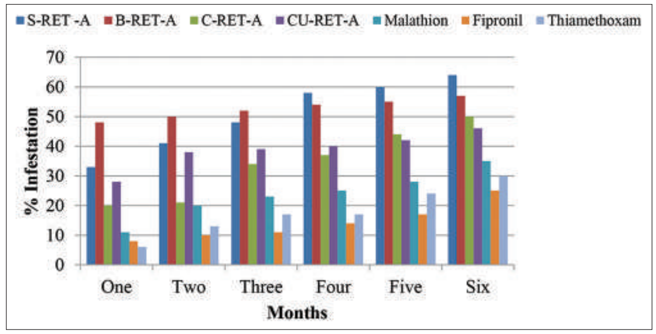


Figure 7: Percent infestation in solid wood sticks

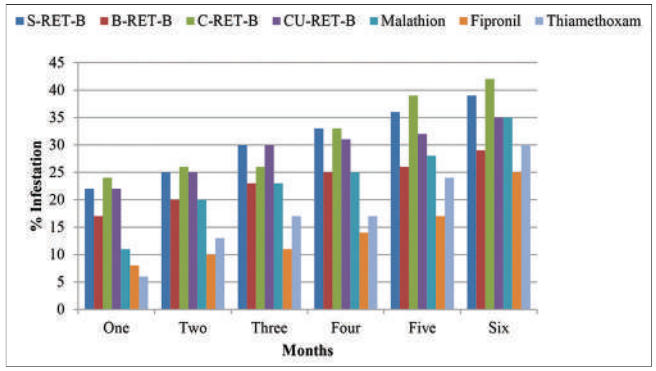


Figure 8: Percent infestation in solid wood sticks

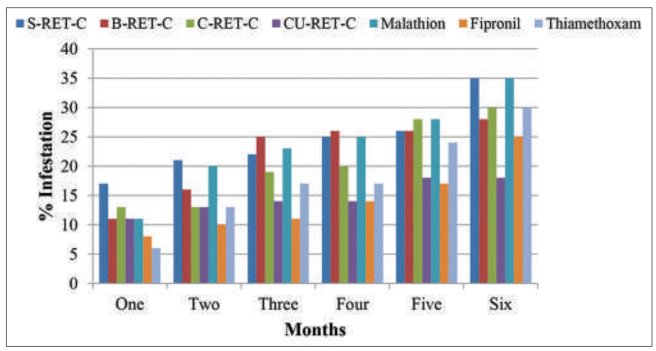


Figure 9: Percent infestation in solid wood sticks

Malathion seasoned wood sticks have shown 1–20% weight loss and 11–35% percent infestation. Fipronil has shown significant reduction in weight loss (99%) and termite infestation (92%). Weight loss of thiamethoxam seasoned wood sticks was noted in a range of 3–21% while percent termite infestation ranges from 6% to 30% [Figures 1-9 and Photoplate 2].

For solid wood sticks

In these experiments, fixed *C. maxima* essential oil extract mixture was shown weight loss 31–41%, fixed oil extract + sulfur is 13–32%, and fixed oil extract +sulfur + cow urine mixtures are 20–46% in 6 months duration. The maximum % of infestation in fixed *C. maxima* essential oil extract mixture was 35%, fixed oil extract + sulfur mixture was 43%, and fixed oil extract + sulfur + cow urine mixture was 43%. Fipronil was also shown 43% termite infestation [Figures 10-12 and Photoplate 3].

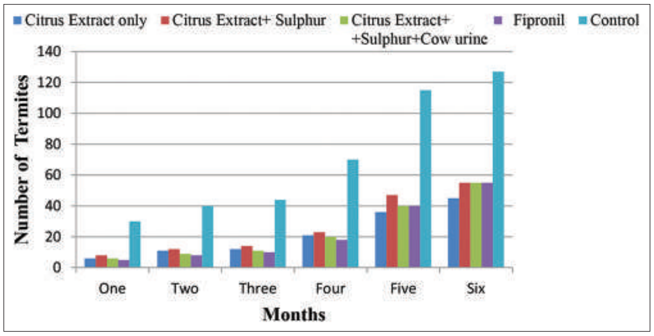


Figure 10: Number of termites in solid wood sticks

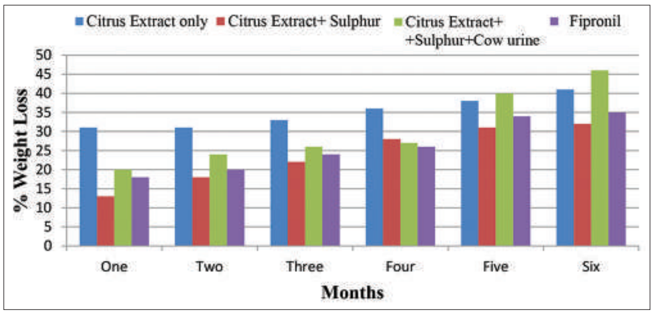


Figure 11: Percent weight loss in solid wood sticks

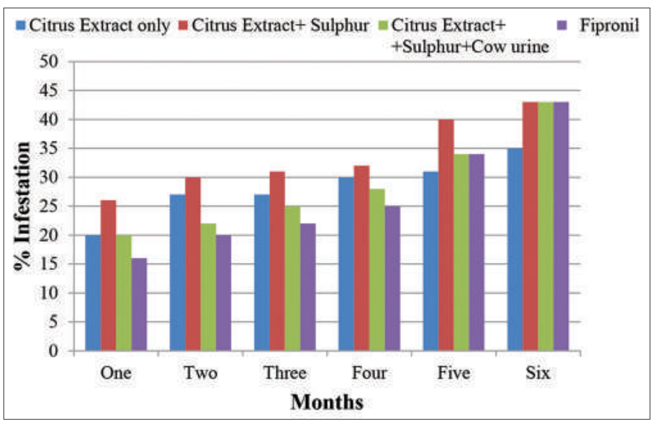


Figure 12: Percent infestation in solid wood sticks

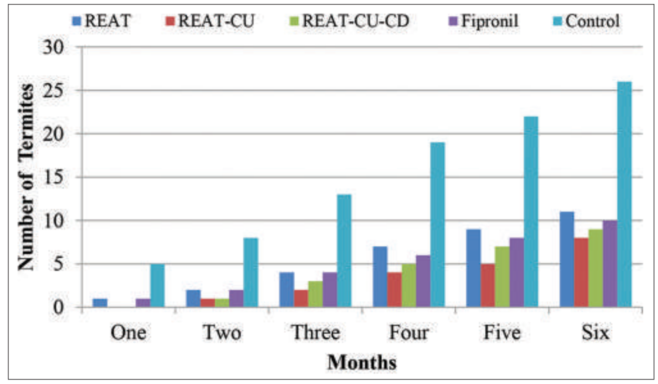


Figure 13: Number of termites in hollow wood sticks

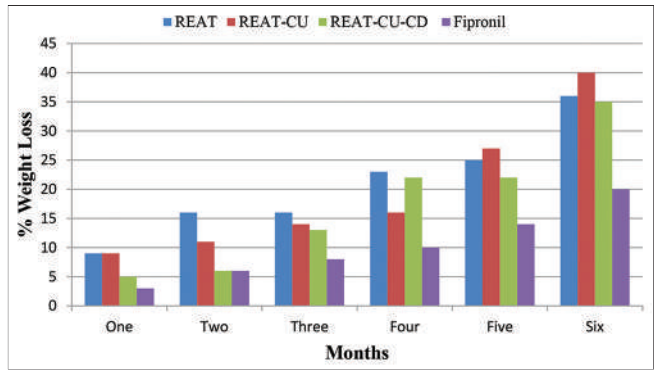


Figure 14: Percent weight loss in hollow wood sticks

For hollow wood sticks

In this set of experiment, bamboo wood sticks were seasoned with the highest concentration of combinatorial mixtures. Therefore, various combinatorial mixtures, that is, REAT-6, REAT-CU-6, and REAT-CU-CD-6 were used for wood treatment. For comparison of termiticidal activity, bamboo wood sticks were also treated with fipronil.

Set no. I

REAT-3 treated bamboo sticks very significantly cut down percent weight loss and recorded 16% in the 3rd month of experiment after which no termite infestation was observed in any bamboo sticks [Figures 13-15].

Set no. II

Similarly, REAT-CU-4 treated bamboo wood sticks have shown a significant decrease in percent weight loss and recorded 16% while percent infestation was significantly decreased up to 21%, respectively [Figures 13-15 and Photoplate 4].

Set no. III

In REAT-CU-CD-5 treated bamboo wood sticks, percent weight loss was decreased and recorded 22% while almost no termite infestation was observed in these wood sticks. In this experiment, wood weight loss and percent termite infestation were found significantly reduced up to 5 months [Figures 13-15].

Set no. IV

In this experiment, control mixture of cow urine and cow dung was shown high termite infestation in comparison to combinatorial mixtures [Figures 13-15].

Set no. V

Percent weight loss was found high in case of fipronil seasoned bamboo wood sticks, that is, 3–20% and high termite infestation was observed in fipronil, that is, 20–38% [Figures 13-15].

For hollow wood sticks

In these bioassays, fixed *C. maxima* essential oil mixture has shown weight loss 07–30%, fixed oil extract + sulfur is 9–33%, and fixed oil extract +sulfur + cow urine mixtures was 05–15% in 3 months duration. The maximum % of

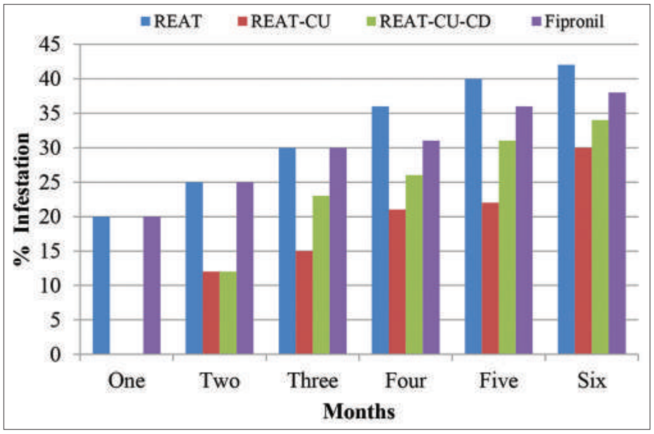


Figure 15: Percent infestation in hollow wood sticks

infestation in fixed *C. maxima* essential oil extract mixture was 25%, fixed oil extract + sulfur mixture was 27%, and fixed oil extract + sulfur + cow urine mixture was 12%. Fipronil significantly cut down 32% termite infestation [Figures 16-18 and Photoplate 5].

Poison Bait

In these bioassay, fixed *C. maxima* essential oil extract mixture was used that has cut down 36% termite infestation during six months, while fixed oil + sulfur showed 52%, and fixed oil extract + sulfur + cow urine mixture cut down infestation up to 38% in 6 months duration. Their maximum termite count was 38, 55, and 40, respectively. Fipronil has shown 38% termite infestation and 40 numbers of termites [Figures 19 and 20, Photoplate 6].

Thread Binding Bioassays

For effective management of termites in garden, pre-soaked cotton threads were tagged around the tree trunks at a height of 5–6 feet above the ground. Termite infestation on these tagged trees was significantly decreased after 6 months of thread binding and mud plastering and tunnels were found shed off from the tree trunk. Lesser number of termites was observed on tested trees in comparison to untreated plants. Further, termite infestation was found to be significantly decreased after 6 months in comparison to control. An overall 96% control was observed in test plants. There was a significant decrease in number of infested plants and termite occurrence after 6 months [Figures 21 and 22, Photoplate 7a and b].

DISCUSSION

In the present investigation, various combinatorial mixtures of *C. maxima* were found highly effective against termites. These were tested in maize and millets crop field, garden soil, and trees.In first set of experiments both hollow and solid wood sticks were seasoned by emerging them overnight

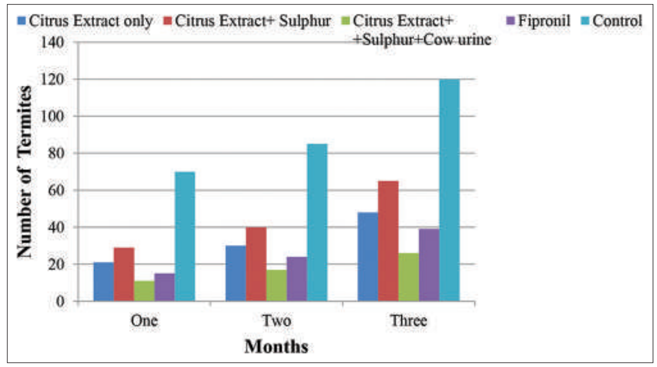


Figure 16: Number of termites in hollow wood sticks

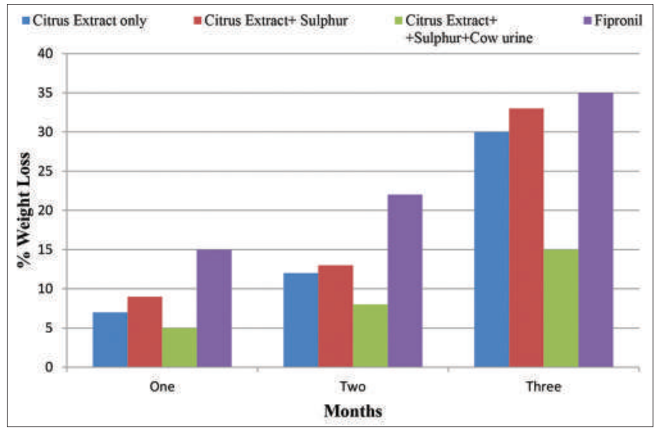


Figure 17: Percent weight loss in hollow wood sticks

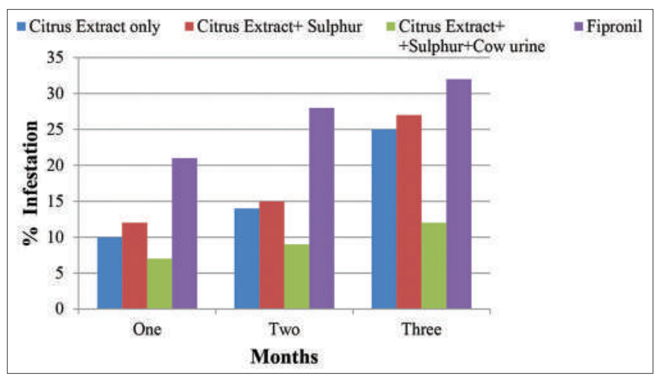


Figure 18: Percent infestation in hollow wood sticks

in various combinatorial mixtures separately. These were planted in garden soil at a fix distance in different rows. In both the test and control wood sticks, termite number was counted and weight loss was measured after digging out wood sticks and dried them for 72 h. From results, it is clear that *C. maxima* and its combinatorial mixtures significantly reduced the wood consumption/infestation by termites that controlled wood weight loss. In test wood sticks, percent termite infestation was significantly reduced due to the action of ingredients. This reduction was found to be concentration and time dependent. This activity of ingredients was remain up to 6 months, though it was little bit reduced later on. There is another reason that due to leaching of pesticides, wood infestation was found to be increased in underground

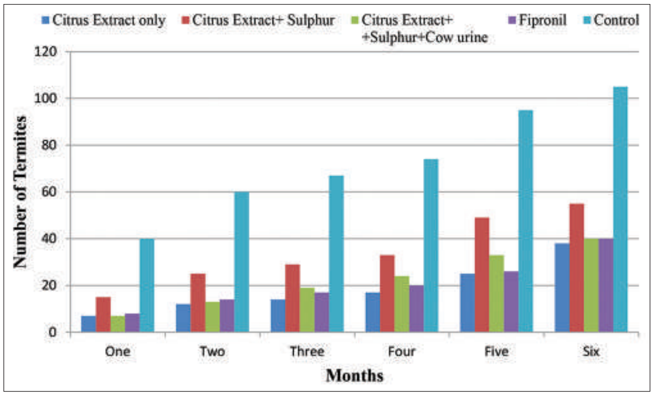


Figure 19: Number of termites in poison bait experiment

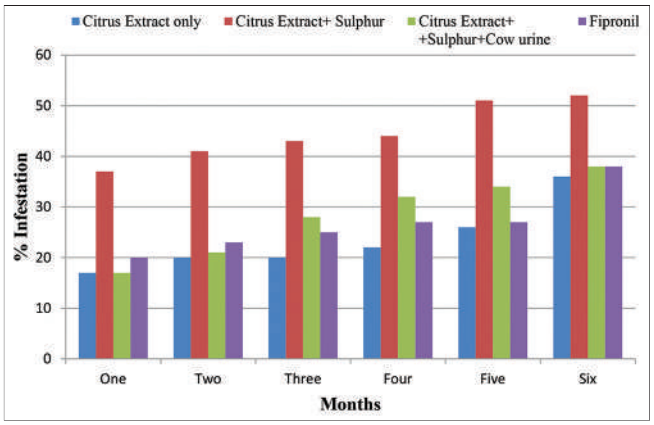


Figure 20: Percent infestation in poison bait experiment

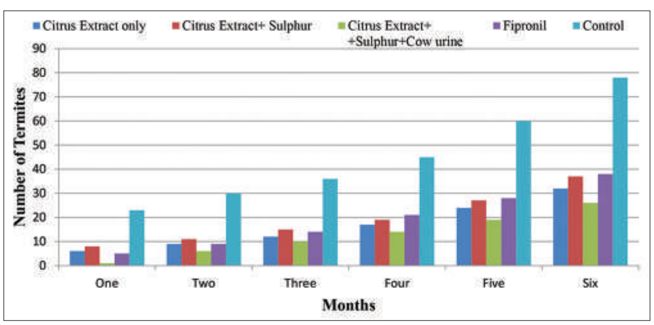


Figure 21: Number of termites in thread binding bioassay

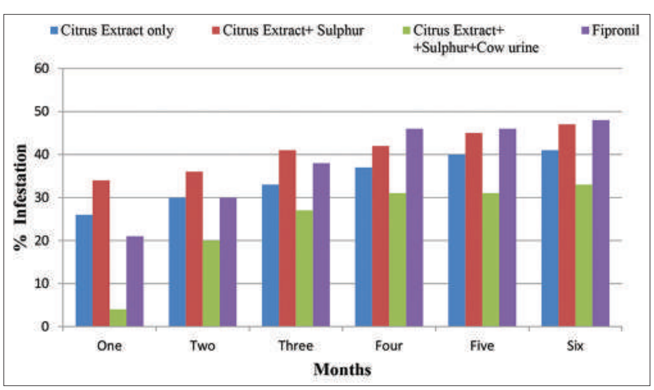


Figure 22: Percent infestation in thread binding bioassay

wood sticks.[26] However, wood consumption is decided on the basis of wood softness due to humidity as termites feed more amounts of soft woods than hard wood.[27,28]

In wood seasoning experiments, 4–07% weight loss was obtained in S-RET-B and S-RET-C treated wood sticks, respectively. Similarly, B-RET-A mixture treated wood sticks showed approximately 5% weight loss up to 30 days of treatment but later on, it was exceeded up to 53% after the 5th month of the treatment [Figures 3 and 4]. Interestingly, termite number was not increased, but bacterial presence in soil degrades the wood that results in wood weight loss. Again in a similar experiment, reduction in wood weight loss was obtained, that is, 4% in photo activated cow urine (CU-RET-C) seasoned wood sticks. Besides this, B-RET-B, C-RET-A, C-RET-B, and CU-RET-B mixtures have also shown similar activity and protected wood sticks from termite infestation very effectively in comparison to other mixtures. Contrary to this, inorganic pesticides were not found able to protect wood as in such cases, wood weight loss was much higher than wood seasoned with *C. maxima* combinatorial mixtures, that is, 1–20% after the 5th month of treatment. It may be due to their fast leaching from wood to soil due to rain water. Results obtained from wood seasoning experiments indicate that *C. maxima* combinatorial mixtures favorably increased resistance in wood sticks against termite up to 6 months. Similar wood protection was obtained after wood seasoning done by applying heat and temperature.[29] Both these ecological factors significantly cut down termite infestation, weight loss, and provide decay resistance to the wood.[30]

Different combinatorial mixtures of *C. maxima* were found highly effective against termites. It was proved by very low percent of infestation values such as S-RET-C, B-RET-C, C-RET-C, and CU-RET-C mixture which were shown 17%, 11%, 13%, and 11%, respectively. Essential oils and various *Citrus* species possess highly effective bio-organic components such as monoterpene, limonene, β-pinene, geranyl acetate and verbenone,[31] citronellal and citronellol,[32] germacrene isomers, pinene, linalool dimmer, bornane, citral, anethole, anisole, safrole, and demitol organic constituents,[33] β-myrcene, and α-pinene.[34] However, major constituents which are commonly found are d-limonene, linalool, geraniol, nerol, monoterpene aldehydes, α- and β-pinene, myrcene, and sesquiterpenes in most of the *Citrus* plant species.[35,36] Sesquiterpene, α-terpinene, and α-pinene have been isolated from *Citrus paradise*[37] and *Citrus latifolia*.[38] *Citrus limon* contains d-limonene, linalool, nerol and monoterpene, etc.[39] Germacrene and hexane isolated from Citrus japonica[40] and sabinene isolated from Citrus *hystrix*.[41] *Aegle marmelos* also contains limonene, α-phellandrene, β-ocimene, α-pinene, (E)-caryophyllene, β-elemene, and germacrene.[42] Only few of them have been evaluated for the presence of anti-termite or insecticidal activity in laboratory and field conditions. Therefore, it can be said that *C. maxima* have few active components which can be used for the development of a new termiticidal formulation, which can be used as an effective wood preservative.[43]

In another experiment, anti-termite activity of all the above mixtures was tested in one foot hollow bamboo wood sticks. For this purpose, each combinatorial mixture was mixed with wood ash and poured inside hollow bamboo sticks and planted underground. REAT, REAT-CU, and REAT-CU-CD treated bamboo sticks have shown minimum weight loss, that is, 5–9% against termite [Figure 14]. While in REAT-CU and REAT-CU-CD treated bamboo sticks, approximately zero percent infestation was found in the 1st month. Contrary to this, fipronil treated wood sticks have shown 20% weight loss up to the 6th month of treatment. In three feet solid and hollow bamboo wood sticks, citrus extract, sulfur, and photoactivated cow urine combinatorial mixture were showed minimum weight loss, that is, 5–46% and termite infestation was 7–43%. Besides this, poison bait was also more significant to control termite infestation in crop field because it reduced the termite count in tested crop field and helpful in better yield of crop. Besides this, thread binding assays on infested saplings of *T. grandis* and poison bait bioassay in maize crop field were also conducted to check the mud plastering, tunneling activity, and up and downward movements of termites. Thread binding bioassay showed positive outcomes for controlling of termite infestation. It was proved by reducing termite count and mud plastering around the plants.

Similarly, plant-derived natural products such as Vulgarone B (*Artemisia douglasiana),* apiol (*Ligusticum hultenii),* and cinicin (*Centaurea maculosa)* significantly exhibited higher mortality than synthetic pesticides.[44] Besides this, flavonoids also showed antifeedant activity against *C. formosanus* Shiraki.[45] Besides this, few low persistence toxic pesticides such as organophosphates, carbamates, and pyrethroids were also used for termite control in soil. But nowadays, many of these synthetic pesticides have been phased out because of their negative impact on human and environmental. Although these pesticides work as repellents,[46] termites have developed the ability to detect them in soil when used as barrier.

It was mostly found that on average *C. maxima* and its combinatorial mixtures have shown better toxicity than synthetic pesticides. Limonoids are a group of highly oxygenated terpenoid secondary metabolites found mostly in the seeds, fruits, and peel tissues of citrus fruits. Limonin, the aglycones, and glycosides of limonoids show insecticidal activity.[47,48] This plant chemical diversity is not only used to control termites but also used to control other insect pests. However, for controlling termite infestation, there is an immense need to discover and use effective, environment friendly, and safe termite control agents with minimal mammalian toxicity.[49] These plant origin natural pesticides control wide range of insect pests population even applied in very low quantity.[50] These are much safer, low cost, and easily biodegradable in the medium and show no residual effect.

CONCLUSION

Termites are the highly destructive polyphagous insect pests which inhabit in high humidity zones and severely infest and invade forest and garden tress and field crops. Although, several methods are used to control termite population in the infield. From the results, various combinatorial mixtures of *C. maxima* were found highly effective against termites. These were tested garden soil, trees, and various field experiments. These have provided enormous protection against termite infestation in seasoned wood sticks; these significantly have cut down tunneling and mud-plastering in termites. These have provided protection to seedlings and plant foliages in maize and millets crops in the agriculture crop field. From results, it is clear that *C. maxima* and its combinatorial mixtures significantly reduced the wood consumption/infestation by termites that controlled wood weight loss. In test wood sticks, percent termite infestation was significantly reduced due to action of ingredients. This reduction was found to be concentration and time dependent. This activity of ingredients was remain up to 6 months, though it was little bit reduced later on.

This termite activity in essential oils of various *Citrus* species is contributed by highly effective bio-organic components such as monoterpene, limonene, β-pinene, geranyl acetate, and verbenone, citronellal and citronellol, germacrene isomers, pinene, linalool dimmer, bornane, citral, anethole, anisole, safrole, and demitol organic constituents. The presence of anti-termite or insecticidal activity in laboratory and field conditions, it can be said that *C. maxima* have these active components found in peel of *C. maxima,* can be used for the development of a new termiticidal formulations and preservative of commercial wood.

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