

Toxic Effects of *Citrus maxima* Based Combinatorial Formulations on Important Metabolic Enzymes in Indian White Termite *Odontotermes obesus*

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Abstract

In the present study, *Citrus maxima* essential crude oil extract was used to prepare combinatorial formulations and workers of Indian white termite *Odontotermes obesus* were treated topically with 40% and 80% of 24 hr LD₅₀ values of these formulations. In subsequent bioassays levels of various enzymes *i.e.* alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase were evaluated to determine the anti-termite efficacy of *Citrus maxima* essential oil based combinatorial formulations. S-RET-A, S-RET-B and S-RET-C caused significant ($p > 0.05$) decrease in glutamate oxaloacetate transaminase *i.e.* 87.47%, 86.81% and 81.77% & 82.04%, 79.39% and 74.75% respectively at 16 h treatment. *In vivo* exposure of 40% and 80% of LD₅₀ of combinatorial formulations caused very significant ($p > 0.05$) reduction in all the test enzymes *i.e.* alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase except acetylcholinesterase levels after 16 h all tested treatments in comparison to control. Both dose-response and time period were found important in physiological alteration in levels of various enzymes. Combinatorial mixtures of Citrus essential oils have shown synergistic activity against termites. The research findings of the present study would help termite control in crop fields, gardens and houses in a sustainable way.

Keywords

Citrus maxima, Essential Oils, *Odontotermes obesus*, Enzymes, Inhibition, Termiticidal Action

1. Introduction

The plant essential oils are a complex mixture of various bio-organic constituents such as lectins, polypeptides, alkaloids, phenols, quinines, flavones, flavonoids, terpenes, tannins, coumarins, benzene derivatives, various hydrocarbons and linear compounds [1]. They have traditionally been used in folk medicine for thousands of years to treat various ailments [2] [3]. *Citrus maxima* belong to the family Rutaceae, its leaves, flowers and peels contain essential oils which contain various important biologically active compounds such as α/β -pinene, sabinene, β -myrcene, d-limonene, linalool, α -humulene, and α -terpineol belonging to the monoterpenes, monoterpene aldehyde/alcohol and sesquiterpenes group [4]. The above compounds also showed various health benefits and are used for the treatment of pain, fever, nausea, inflammation, infections, and nervous disorders [5]. Plants synthesize essential oils which possess thousands of active ingredients and their biological activities are still unknown [6] [7].

Most of these plant-derived compounds are used as an alternative to synthetic insecticides for controlling termites. These show effects at cellular and physiological levels, mainly protective effects [8]. Phytochemicals such as alkaloids, flavonoids and phenols are very useful in controlling insects, pests and pathogens in an environment-friendly way [9] [10]. Flavonoids are widely distributed as secondary metabolites produced by plants and have various potential biological benefits, including antioxidant, anti-inflammatory, anticancer, antibacterial, antifungal and antiviral activities [11]. Flavonoids provide defense against pathogens, and environmental contamination and are used as medicinal ingredients and food additives for therapeutic, aromatic and culinary purposes [12]. Flavonoids show antifeedant and repellent activity against termite *C. formosanus* [13]. Similarly, flavonoids were found in the leaf of *Rhazya stricta* Decne and *Lantana camara* also showed antifeedant activity against *Psammotermes hybostoma* [14] while *Callitris glaucophylla* possessed antifeedant activity against *C. formosanus* [15]. Seeds of *Withania somnifera*, *Croton tiglium*, and *Hygrophilia auriculata* stopped cellulose digestion inside termite gut while seed oil of *Azadirachta indica* showed the highest toxicity against *Macrotermes* spp. [16]. Chlorpyrifos insecticide controls all sucking, biting, chewing and soil pests along with termites [17]. This is used as a liquid termite repellent. Lindane is a liquid termiticide that acts as a repellent [18] Imidacloprid binds much more strongly to the termite's neuron receptors than to mammals. So this insecticide was more toxic to termites than to humans. The insecticides like fipronil, thiamethoxam, acetamiprid, bifenthrin, chlorantraniliprole, cyfluthrin and cypermethrin showed strong anti-termitic activity [19].

Termites are found in a range of habitats and live inside mounds, soil, wooden house, bark and wood of dead trees in forests. At the global level, heavy devastation is seen by dry wood termite *Cryptotermes brevis* both in tropical and sub-tropical countries. This is one of the most important wood structure pests in the world [20] [21]. Besides, as for their negative role, termites also play an im-

portant role in the decomposition of above-ground deadwood and mediate the uptake of floating deadwood into the soil [22]. These also have an important role in human life, and some tribes use huge termite mounds for funeral and spiritual purposes [23]. Essential oil components are used as synergists in the form of baits that successfully exploit termite feeding, tunneling [24] and reproductive behavior [25].

Termites are the major decomposers, have extraordinary ecological impacts on agricultural and non-agricultural ecosystems and are important in the global carbon cycle, degradation processes and mineralization of nutrient-rich cellulose [26]. Termite tunneling activity helps in the improvement of soil fertility, nutrient availability, water infiltration and crop production [27] [28] [29]. Moreover, termites are also used by human beings for various purposes like food and medicine, superstitious beliefs, arts and literature across the world. Despite their useful properties, they often cause great harm to humans, by damaging goods especially those containing cellulose, such as books, stored wood, wooden structures, buildings, stored grain products, crops, standing trees, forests, etc. [30]. Termites cause a huge economic loss of above 40 billion dollars per year worldwide [31] [32]. They are fed on the bark and underlying tissue and damage the agricultural and horticultural crops [33]. Termites are a kind of threat to the farming community as all major field crops such as sugarcane, cotton, tobacco, cereals, vegetables, fruits, legumes, oilseeds and ornamental plants are directly or indirectly affected by termites [34] [35].

The termite, *Odontotermes obesus* (Rambur), is a destructive polyphagous pest in eastern Uttar Pradesh. It causes heavy losses in rural and urban areas. It severely damages commercial wood, food crops, orchard plants and household goods. In the present investigation, *Citrus maxima* essential crude oil extract was used to prepare combinatorial formulations to visualize the effects on certain metabolic enzymes. The main aim is to control worker termites in an eco-friendly manner alternative to replacing synthetic termiticides with the inherent resistance by insects and environmental and health effects on humans.

2. Experimental

2.1. Preparation of Combinatorial Mixtures

Citrus maxima (Brum) Chakotra/Pomelo fruit or grapefruit belonging to the Rutaceae family were collected from the garden of Deen Dayal Upadhyaya Gorakhpur University, India U.P. It is a natural, non-hybrid, citrus fruit, and native to Southeast Asia. This specimen was authenticated by an expert in botany and help was taken from the Taxonomy of Indian Angiosperms. The herbarium specimen is healthy and preserved in the Botanical garden of Gorakhpur University for Future Reference. 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 a combinatorial mixture w/v. Fresh peel was weighed and the extract was prepared in distilled water in a powder

mixture and grinder. The extract obtained was dried in the rotatory evaporator and kept in the refrigerator for further use. All chemicals used in this study were purchased from CDH-laboratory chemicals suppliers in India supplied by Eastern Scientific Company, Gorakhpur. Clevenger apparatus was used for extraction of *Citrus maxima* peel essential oils and their bioactive compounds.

Citrus maxima peel and other ingredients were used in the preparation of combinatorial mixtures. The details of all combinatorial mixtures are mentioned in the following table.

2.2. In Vivo Determination of Enzymatic Parameters

To observe the effect of *Citrus maxima* essential oil and its combinatorial mixtures on enzymatic parameters adult termite workers (500 mg) were provided 40% and 80% of LD₅₀ with the diet. Insects were sacrificed at the 4 h interval up to 16 h for the measurement of various enzyme levels. Insects were homogenized in phosphate saline buffer (pH 6.9) in a glass homogenizer and centrifuged in cold for 25 minutes at 15,000 rpm. Supernatant was isolated in a glass tube and used for the estimation.

2.3. Determination of Alkaline Phosphatase (ALP)

The Alkaline phosphatase level was determined according to the method of Andrech and Szeypiaske and modified by Bergmeyer [36]. For this purpose 500 mg treated termites were homogenized in 1 ml ice-cold PBS buffer and centrifuged at 15,000 rpm for 15 min. For measurement of alkaline phosphatase level, 0.10 ml of supernatant was added to 1.0 ml of alkaline buffer substrate and incubated for 30 minutes at 37°C. Alkaline buffer substrate was prepared by addition of 375 mg glycine, 10 mg MgCl₂·6H₂O and 165 mg p-nitrophenyl phosphate sodium salt in 42 ml of 0.1 N NaOH. The reaction was stopped by adding an excess of alkali (5.0 ml of 0.02 N NaOH). The p-Nitrophenol formed after the hydrolysis of p-nitrophenyl phosphate gave a yellow color with NaOH. Optical density was measured at 420 nm. A standard curve was prepared by using different concentrations of p-nitrophenol. Enzyme activity was expressed as μmoles of p-nitrophenol formed/30 min/mg protein. Three replicates were set in each experiment and the data obtained was statistically analyzed by the ANOVA method.

2.4. Determination of Acid Phosphatase (ACP)

Acid phosphatase activity in termites was determined according to the method of Andrech and Szeypiaske and modified by Bergmeyer [36]. For the determination of acid phosphatase level, whole body extract of termites was prepared similarly as mentioned above. For this purpose, 0.1 ml of supernatant was added to 1.0 ml of acid buffer substrate solution (0.41 gm citric acid, 1.125 gm sodium citrate and 165 mg p-nitrophenyl phosphate sodium salt to 100 ml of double distilled water). Contents were mixed thoroughly and incubated for 30 minutes at 37°C. To this tube 4.0 ml of 0.10 N NaOH was added to stop the reaction. A yellow

low color was developed which was measured at 420 nm. The standard curve was prepared by using different concentrations of p-nitrophenol. Enzyme activity was expressed as the amount of p-nitrophenol formed/30 min/mg protein. Three replicates were set in each experiment and the data obtained was statistically analyzed by the ANOVA method.

2.5. Determination of Glutamic-Oxaloacetic Transaminase (GOT)

GOT activity was measured according to the method of Reitman and Frankel [37]. For this purpose 500 mg treated termites were homogenized in 2 ml ice-cold PBS buffer and centrifuged at 15,000 rpm for 15 min. For estimation of GOT 0.10 ml of supernatant was taken and 0.50 ml of GOT substrate was added to it. GOT substrate was prepared by adding 0.292 gm of α -ketoglutaric acid and 26.6 gm of DL-aspartic acid into a 1.0 liter volumetric flask. Contents were mixed thoroughly and 1 N NaOH was added slowly to the above solution. pH of the solution was adjusted to 7.4 by using PBS buffer. The total volume of the solution was maintained at 1000 ml by adding buffer (13.97 gm K_2HPO_4 and 2.69 gm KH_2PO_4 in 1000 ml water). To this tube, 0.50 ml of 2 - 4 dinitrophenyl hydrazine solution (0.198 gm of 2, 4-dinitrophenyl hydrazine was dissolved in 1 N HCl to make 1000 ml.) was added and kept standing for 15 minutes at room temperature. Then 5.0 ml of 0.4 N NaOH (1.6 gm NaOH dissolved in 100 ml distilled water) was added and mixed well. Now contents were left for 20 minutes at room temperature. Optical density was recorded at 505 nm by setting the blank with distilled water. The standard curve was prepared by using oxaloacetic acid as the standard. Enzyme activity was expressed in units of glutamate oxaloacetate transaminase/30 min/mg protein.

2.6. Determination of Glutamate-Pyruvate Transaminase (GPT)

GPT activity in whole body extract of termites was measured according to the method of Reitman and Frankel [37]. For this purpose worker termites (500 mg) were treated and homogenized after 2 hrs in 2 ml ice-cold PBS buffer and centrifuged at 15,000 rpm for 15 min. 0.10 ml of centrifuged supernatant was added to 0.50 ml of GPT substrate. GPT substrate was prepared by dissolving 0.292 gm of α -ketoglutaric acid and 17.8 gm of DL alanine in a 1.0 liter volumetric flask. 1 N NaOH was slowly added to the above mixture. It was mixed well until all solids dissolved completely. The pH of the substrate was adjusted to 7.4 by adding a sufficient volume of buffer and the total volume was maintained at 1000 ml. The buffer was prepared by dissolving 13.97 gm K_2HPO_4 and 2.69 gm KH_2PO_4 in 1000 ml distilled water. In the above supernatant, 0.5 ml of GPT substrate and 0.50 ml of 2 - 4 dinitrophenyl hydrazine solution (0.198 gm of 2, 4-dinitrophenyl hydrazine was dissolved in 1 N HCl to make 1000 ml) were added and kept for 15 minutes at room temperature. Now 5.0 ml of 0.4 N NaOH (1.6 gm NaOH dissolved in 100 ml distilled water) was added, mixed well and allowed to stand at room temperature for 20 minutes. The optical density was noted at 505 nm

and the blank was set with water to make the background absorbance zero. The standard curve was prepared by using oxaloacetic acid as the standard. The enzyme activity was expressed in units of glutamate-pyruvate transaminase activity/mg protein. Three replicates were set for each test and the control and data obtained were statistically analyzed by the ANOVA method.

2.7. Determination of Acetylcholinesterase (AChE)

Acetylcholinesterase activity was determined according to the method of Ellman [38]. For this purpose, 500 mg treated termites were homogenized in ice-cold PBS buffer for 5 minutes in a glass-glass homogenizer. It was centrifuged at 15,000 rpm in cold to get the supernatant. For estimation of AChE level 0.050 ml of supernatant was mixed with (10 mm path length cuvette) 0.10 ml freshly prepared acetyl cholinethiodide solution (5×10^{-4} M) and into it 0.05 ml DTNB (0.19818 gm/l) a chromogenic agent and 1.45 ml of PBS (pH 6.9) were added. The change in absorbance was recorded at 412 nm regularly for three minutes at 25°C. Enzyme activity was expressed in μ moles "SH" hydrolyzed per minute per mg protein.

2.8. Statistical Analysis

The LD₅₀ in termite workers was determined for each extract and combinatorial mixture by using Probit analysis. Mean, standard deviation, standard error, correlation and Student t-test were applied by the ANOVA program. The Chi-Square test was applied to establish the repellent activity [39].

3. Results

In this investigation, the toxic effects of *Citrus maxima* essential oil and its combinatorial mixtures on certain metabolic enzymes such as alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase were evaluated. Levels of these enzymes were determined in whole-body extracts of termites treated with 40% and 80% of LD₅₀ of *Citrus maxima* essential oil and its combinatorial mixtures separately for 4, 8, 12 and 16 h (Figures 1-19).

40% and 80% of LD₅₀ of combinatorial mixtures S-RET-A, S-RET-B and S-RET-C caused significant ($p > 0.05$) decrease in glutamate oxaloacetate transaminase *i.e.* 87.47%, 86.81% and 81.77% & 82.04%, 79.39% and 74.75% respectively at 16 h treatment (Figures 1-3). Similarly, 40% and 80% of LD₅₀ of Combinatorial mixture B-RET-A, B-RET-B and B-RET-C caused a significant decrease in acid phosphatase level at 16 h treatment in comparison to control, the level recorded 90.25%, 86.65% and 87.59% & 86.63%, 85.33% and 85.02% respectively (Figures 4-6).

When termites were treated with 80% of LD₅₀ of C-RET-A, C-RET-B and C-RET-C combinatorial mixtures caused maximum significant ($p > 0.05$) increase in acetylcholinesterase level at 16 h of treatment *i.e.* 103.02%, 109.34%

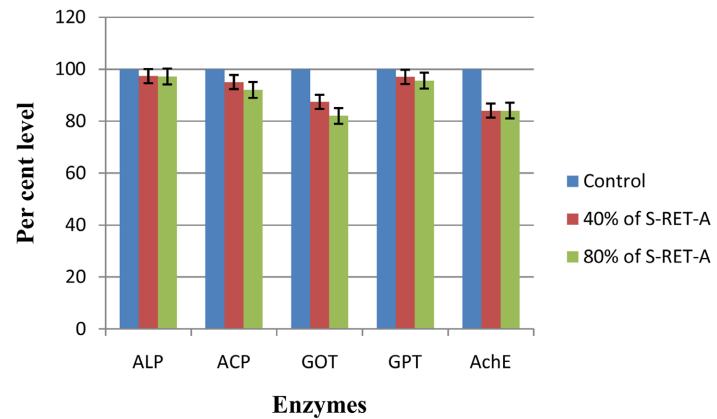


Figure 1. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of S-RET-A mixture at 16 h.

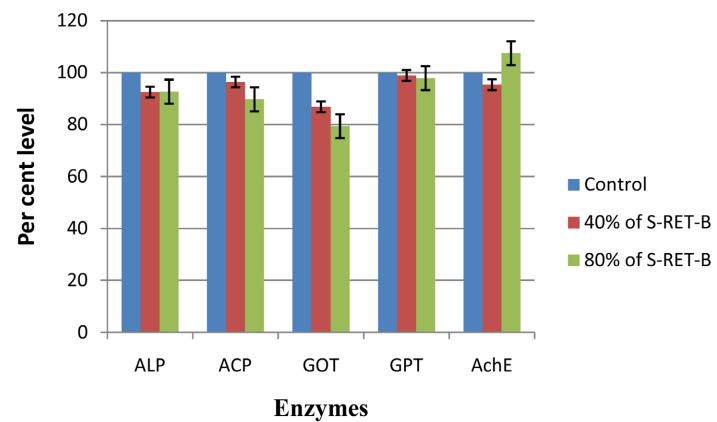


Figure 2. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of S-RET-B mixture at 16 h.

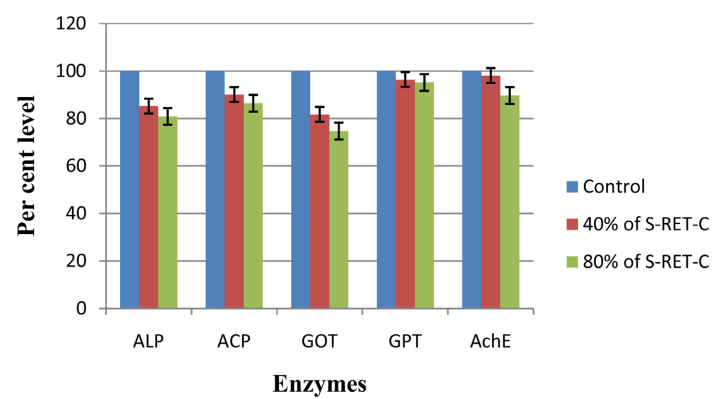


Figure 3. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of S-RET-C mixture at 16 h.

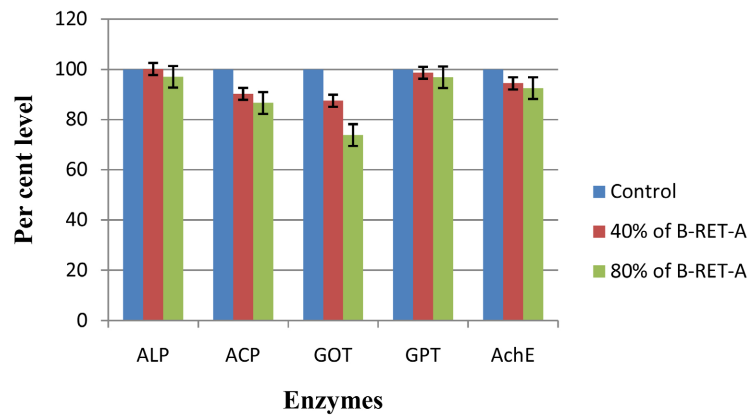


Figure 4. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of B-RET-A mixture at 16 h.

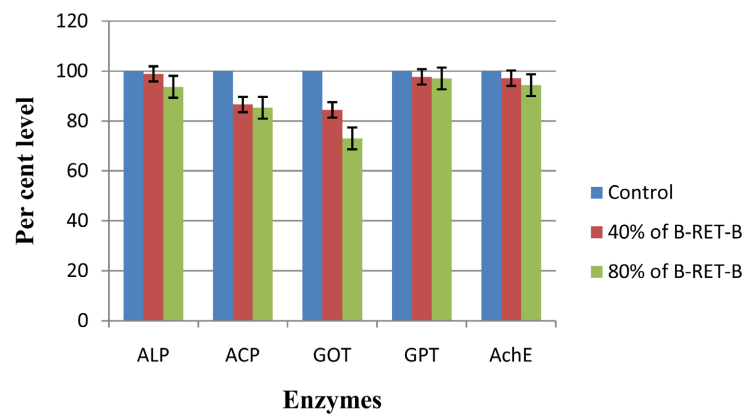


Figure 5. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of B-RET-B mixture at 16 h.

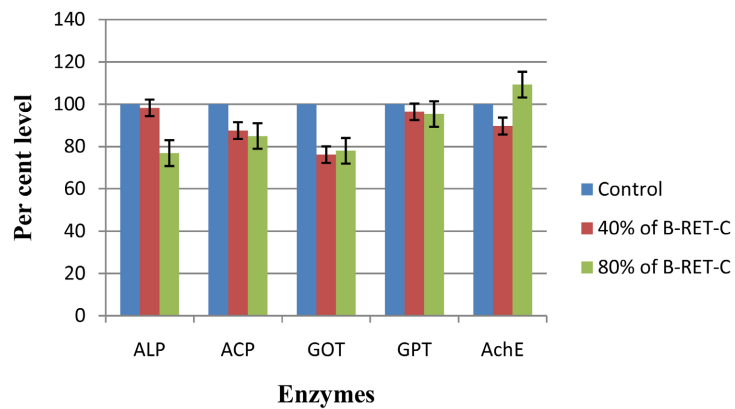


Figure 6. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of B-RET-C mixture at 16 h.

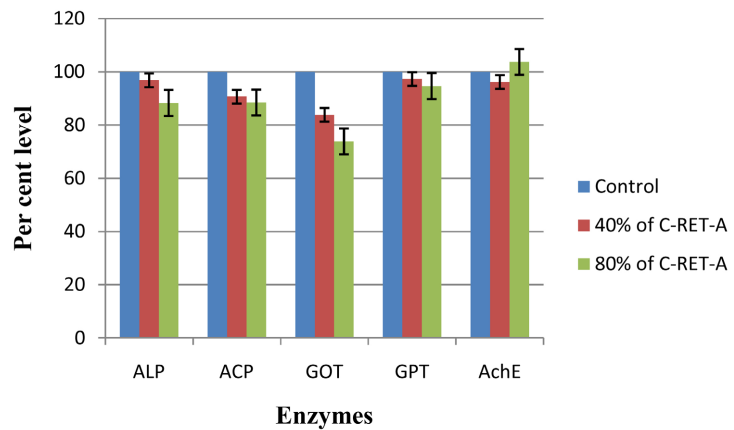


Figure 7. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of C-RET-A mixture at 16 h.

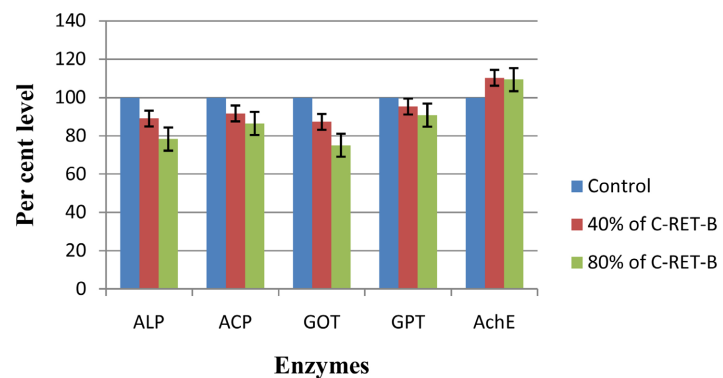


Figure 8. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of C-RET-B mixture at 16 h.

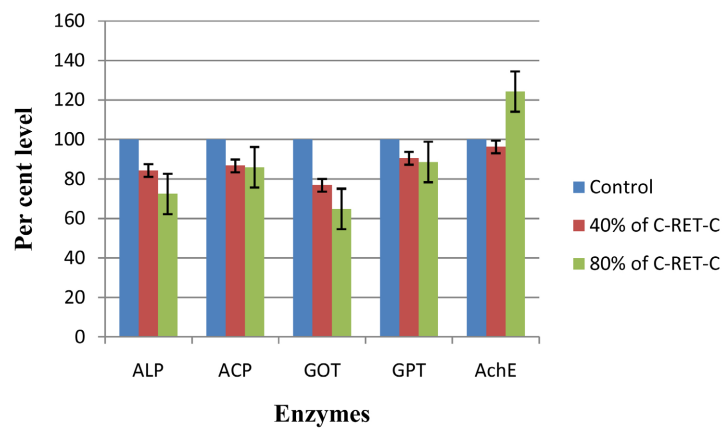


Figure 9. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of C-RET-C mixture at 16 h.

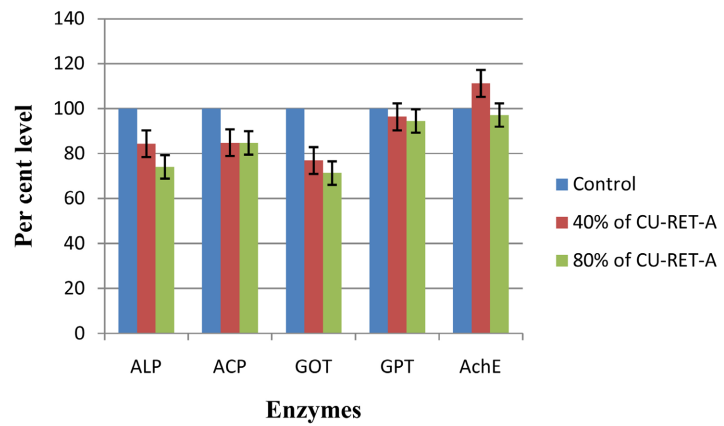


Figure 10. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of CU-RET-A mixture at 16 h.

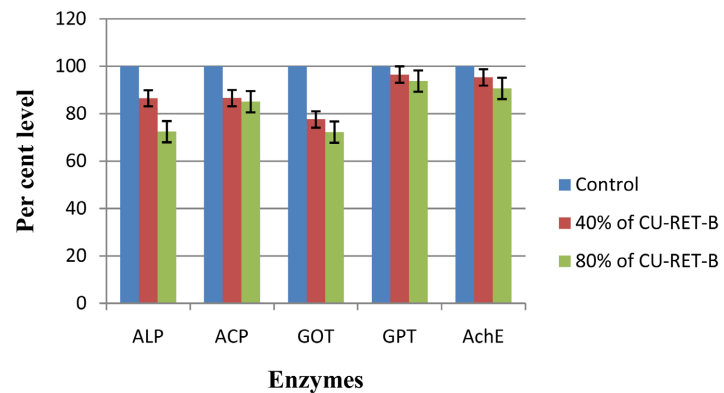


Figure 11. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of CU-RET-B mixture at 16 h.

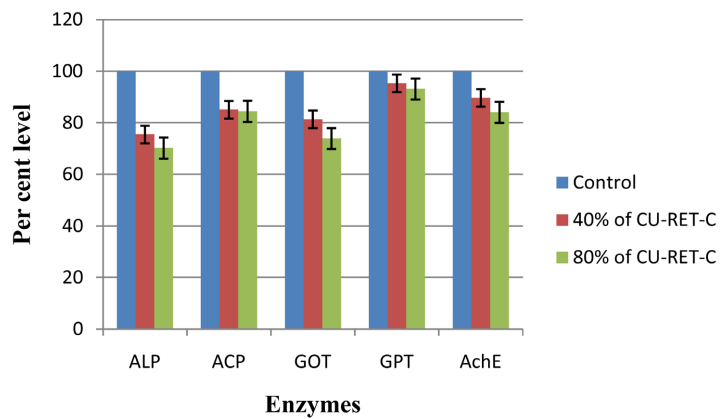


Figure 12. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of CU-RET-C mixture at 16 h.

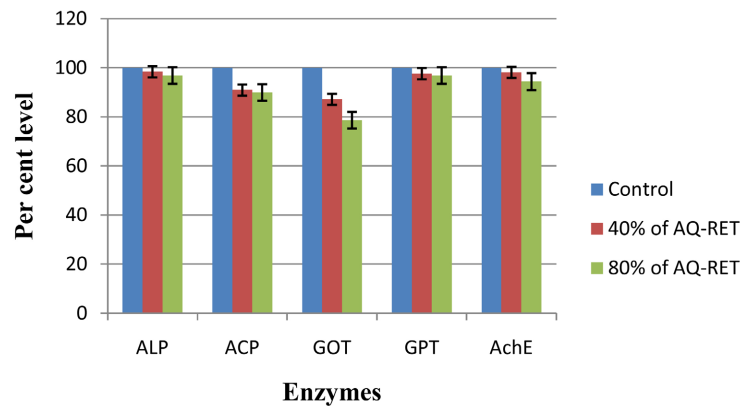


Figure 13. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of AQ-RET mixture at 16 h.

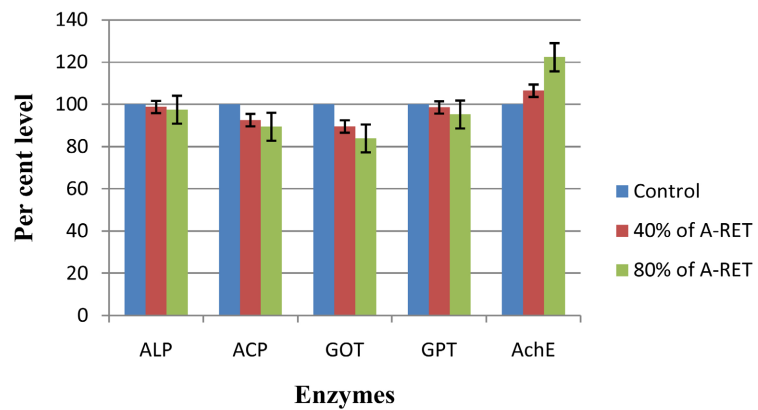


Figure 14. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of A-RET mixture at 16 h.

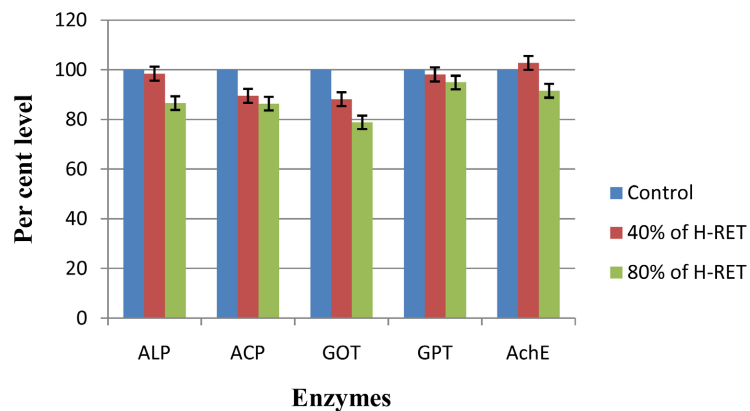


Figure 15. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of H-RET mixture at 16 h.

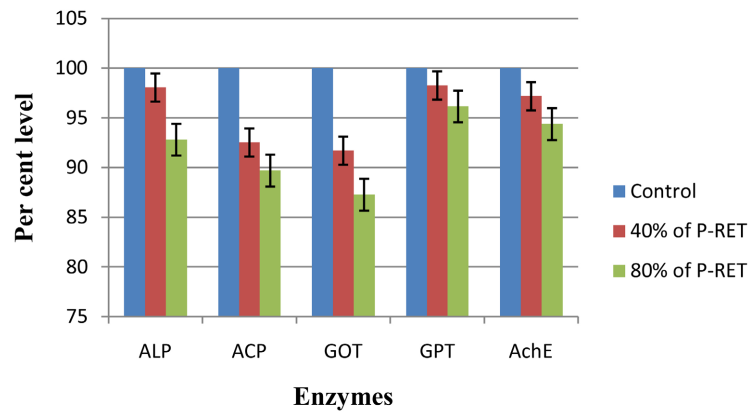


Figure 16. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of P-RET mixture at 16 h.

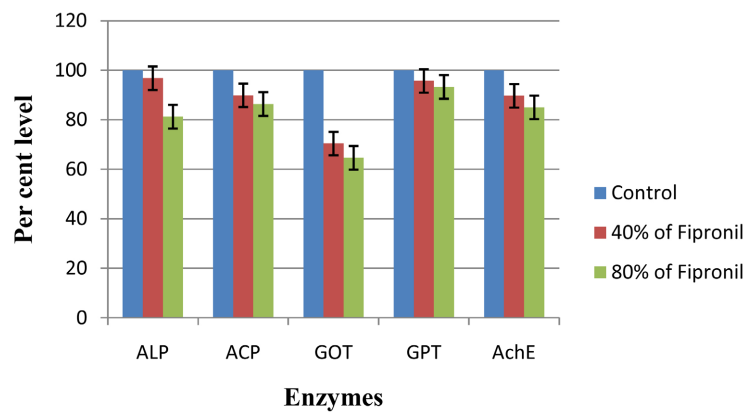


Figure 17. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of Fipronil at 16 h.

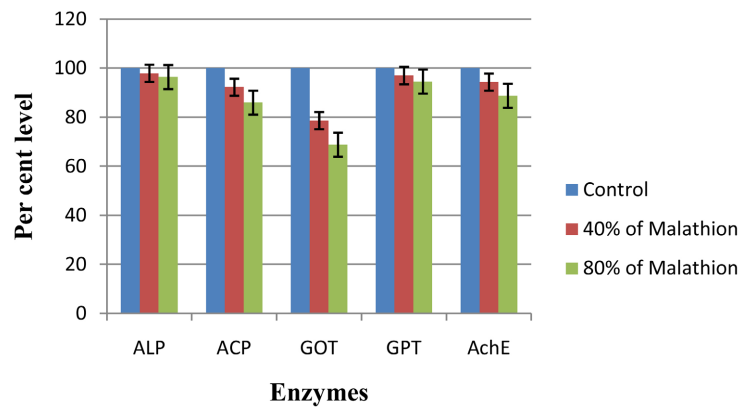


Figure 18. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of Malathion at 16 h.

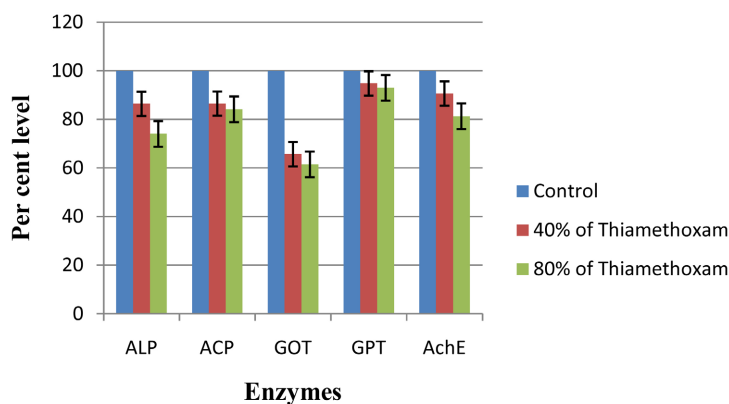


Figure 19. Comparison of alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase in termites treated with 40% and 80% of LD₅₀ of thiamethoxam at 16 h.

and 113.60% in comparison to control respectively (Figures 7-9), but in CU-RET-A, CU-RET-B and CU-RET-C level was found to be significantly ($p > 0.05$) decreased in comparison to control *i.e.* 97.19%, 90.65% and 84.11% respectively (Figures 10-12). Similarly, acetylcholinesterase level was found significantly ($p > 0.05$) decreased at 16 h of treatment in 80% of AQ-RET, H-RET and P-RET which were 94.39%, 91.58% and 94.39% respectively and slightly increased in A-RET *i.e.* 122.42% in comparison to control (Figures 13-16). Alkaline phosphatase levels in 80% of synthetic pesticides fipronil, malathion and thiamethoxam were 81.27%, 96.41% and 74.01% respectively after 16 h treatment (Figures 17-19). Glutamate pyruvate transaminase enzyme level was slightly decreased in all tested combinatorial mixtures as well as synthetic pesticides (Figures 1-19).

4. Discussion

For investigation of the anti-termite activity of *Citrus maxima* and its various combinatorial mixtures, the level of various enzymes such as alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and acetylcholinesterase were determined in termites. In this study levels of various enzymes were found to be altered that clearly indicated obstruction in their chemical pathways. These obstructions in their chemical pathways led to the formation of abnormalities in the insect's metabolism and make insects unable to survive. For this termites were treated with 40% and 80% of LD₅₀ of *Citrus maxima* and its various combinatorial mixtures and also with some synthetic pesticides (Table 1 & Table 2; Figures 1-19). Effects on enzyme level alteration were measured in whole-body extracts of termites. In combinatorial mixtures S-RET-A, S-RET-B, S-RET-C, B-RET-A, B-RET-B and B-RET-C mixtures have shown significant reduction in alkaline phosphatase and acid phosphatase levels which range was 97.20% to 76.90% and 92.10% to 85.20% respectively after 16 h treatment (Figures 1-6). While an elevation was measured in

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

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

*Synthetic pesticides.

acetylcholinesterase levels after 16 h treatment of 80% of C-RET-A, C-RET-B and C-RET-C *i.e.* 103.73%, 109.34% and 124.29% respectively (**Figures 7-9**). More specifically, in another experiment a similar dose of 80% of CU-RET-A, CU-RET-B and CU-RET-C mixture caused a slightly decrease in acetylcholinesterase levels after 16 h treatment in comparison to control termites *i.e.* 97.19%, 90.65% and 84.11% respectively (**Figures 10-12**). Alkaline phosphatase is an important membrane bound enzymes found in all body tissues. This is a lysosomal

Table 2. Showing LD₅₀ values after treatment of termites with various combinatorial fractions and pesticides.

S. No.	Name of Combinatorial Mixture	LD ₅₀ µg/gm	LD ₄₀ µg/gm	LD ₂₀ µg/gm	0.95 confidence limit UCL-LCL	Chi-Square value	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.

enzyme may have a role in autophagy. It also plays an important role in catabolism, pathological necrosis, autolysis and phagocytosis [40]. It mediates the transport of metabolites across the membrane and plays an important role in protein synthesis [41]. Its level may be increased due to the intoxication of body tissues and lysosomal disintegration that leads to the liberation of the enzyme from the membrane and its circulation from muscle [42]. Another reason for the increase in the level of serum acid phosphatase is toxicant-induced hypoxia [43]. Its inhibition may retard the protein synthesis in tissues and release excess free amino acids into the circulation, thereby, increasing amino acid level in the serum. In desert locusts, *Schistocerca gregaria* acid phosphatase (ACP) activity gets increased in the haemolymph 3-day post inoculation with the entomopathogenic fungus *M. anisopliae* [44]. An elevated level of ACP also indicates about the breakdown of haemocytes and a marked reduction in the proportion of plasmatocytes and coagulocytes [44]. The level of acid and alkaline phosphatase was noted very high in lufenuron-treated *Helicoverpa armigera* [45]. This

elevated level of detoxification enzymes can possibly lead to an increase in resistance development against synthetic chemical insecticides [45]. Acid phosphatase (ACP) and alkaline phosphatase (ALP) are hydrolytic enzymes, which hydrolyze phosphomonoesters under acid or alkaline conditions, respectively. ACP, as a lysosomal marker enzyme [46], is abundant in the decomposed tissues and organs subjected to cytolysis [47]. Similarly, phosphatase and transaminase activity are also disturbed due to exposure to certain insecticides in the grubs of the red palm weevil *Rhynchophorus ferrugineus* [48].

In vivo exposure of 40% and 80% of LD₅₀ of AQ-RET and A-RET mixtures caused very significant ($p > 0.05$) reduction in all the test enzymes i.e. alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase except acetylcholinesterase levels after 16 h all tested treatments in comparison to control (Figure 13 & Figure 14). Contrary to this, a similar dose of H-RET and P-RET caused a significant reduction in all the test enzymes i.e. alkaline phosphatase, acid phosphatase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase including acetylcholinesterase levels in comparison to control at 16 h treatment (Figure 15 & Figure 16). Alanine aminotransferase (ALT), also known as glutamic-pyruvic transaminase (GPT), is a cytosolic enzyme involved in gluconeogenesis that catalyzes the amination of α -ketoglutarate from alanine to produce pyruvate and glutamate [49]. Both enzymes glutamic oxaloacetic transaminase (GOT) and serum glutamic pyruvic transaminase (GPT) are found in the liver, heart cells, muscle tissue, pancreas and kidney. Alteration in levels of these enzymes displays damage to these tissues [50]. Glutamic oxaloacetic transaminase (GOT) and glutamine pyruvic transaminase (GPT) are also known as aspartic transferase (AST) and alanine transaminase (ALT) respectively. The transaminases are key enzymes in the formation of non-essential amino acids, gluconeogenesis, and metabolism of the nitrogen compound, and are associated with protein metabolism [51]. Various scientific researches proved that plant essential oil has a variety of volatile active components which showed strong insecticidal, termiticidal, repellent and toxic effects with least or no residual effect in non-target organisms [52]. Due to this, environment sustainability does not alter. On the other side, synthetic pesticides create various adversities in natural ecosystems as well as human beings [53].

Aqueous extracts of *Gloriosa superba* [54], *Paronia emodi* [55], *Corydalis incise* [56], *Artemisia annua* [57], *Teucrium royleanum* [58], *Andrache cardifolia* [59], *Angelica archangelica* and *Geranium sylvatica* efficiently altered various enzymes levels such as acetylcholinesterase, alkaline phosphatase and amino transferase of insects [60]. The 40% of LD₅₀ of synthetic pesticides fipronil, malathion and thiamethoxam caused a significant decrease in glutamate oxaloacetate transaminase levels i.e. 70.44%, 78.66% and 65.73% respectively at 16 h treatment, while 80% of LD₅₀ of fipronil, malathion and thiamethoxam caused a decrease in all the enzymatic parameters at 16 h treatment in comparison to

control (Figures 17-19). Similarly, certain alkaloids found in the Amaryllidaceae family plants inhibit acetylcholinesterase levels in various insect pests [61]. A large number of alkaloids are found abundantly in plants [62].

Above enzyme activity of *Citrus maxima* essential oil and its combinatorial mixtures were evaluated with comparison to a few inorganic insecticides like malathion, fipronil and thiamethoxam. It was found that *Citrus maxima* essential oil and its combinatorial mixtures have shown more significant termiticidal efficacy than synthetic pesticides. Phenolic compounds like phosphorus oxychloride showed acetylcholinesterase inhibition at sub-lethal doses in termite *C. formosanus* [63]. Contrary to this malathion also potentially inhibits acetylcholinesterase activity more than malaxon and isomalathion [64]. Commonly used organophosphorus insecticide shows genotoxicity in both *in vivo* and *in vitro* treatments [65].

It is a well-known fact that the presence of any pesticide causes stress in insects. In a state of stress, insects show significant induction in hydrolytic activities in the body tissues which elevate or reduced the acid and alkaline phosphatase levels. Acid and alkaline phosphatase are also found in the blood, liver, plasma and intestine of human beings [66] [67]. Similarly in the presence of a toxicant, the transamination of amino acids get increase, hence the level of glutamate pyruvate transaminase, and glutamate oxaloacetate transaminase get altered [68].

Toxic substances mainly pesticides affect acetylcholinesterase activity in mice [69]. Organophosphate (OP) pesticides are known as nerve agents, these are major neuro inhibitors of acetylcholinesterase (AChE) activity. Both carbamate and organophosphate poisons decreased AChE levels. Similarly, diazinon poisoning also influences acetylcholinesterase and butyrylcholinesterase level in affected animals and men [70]. In this investigation alteration in the level of certain enzymes in whole body extract of termites may be due to physiological alterations which are induced by different *Citrus maxima* essential oil and its combinatorial mixtures. However, elevation or reduction in enzyme levels is associated with metabolic alterations in insects. However, *Citrus maxima* essential oil and its combinatorial mixtures significantly change phosphatases, transaminase and esterase levels, which indicate very high toxic effects on the body tissues of termites. Synthetic pesticides cause short-term and long-term adverse effects on the health of animals and humans as well as also alter the ecological parameters in the surrounding environment [71]. The primary effects of synthetic pesticides are burning eyes, rashes, blisters, blindness, nausea, dizziness, and diarrhea and ultimately lead to death [72].

5. Conclusion

In the present study, the tested compounds *Citrus maxima* essential oil and its combinatorial mixtures disturb the activities of enzymes acid phosphatase (ACP), alkaline phosphatase (ALP), glutamate oxaloacetate transaminase

(GOT), glutamate pyruvate transaminase (GPT) and acetylcholinesterase (AChE) which play different roles in the insect body. It also displays obstruction in their chemical pathways and imposes an abnormal state in the termites at sub-lethal doses. These formulations make termite workers unable to survive because of the loss of physiological functions, such as growth, development, and reproduction, in the insects and ultimately resulting in death. So, *Citrus maxima* essential oil and its combinatorial mixtures could be alternatives for synthetic pesticides and can be used for termite control in a sustainable and eco-friendly manner. In conclusion, *Citrus maxima* essential oil possesses a potent compound for inhibiting the enzyme activities in termites.

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Authors' Contributions

Ravi Kant Upadhyay and Lokpriy Pandey were responsible for conception, experiments, writing and revising the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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