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Efficacy of a Combined Treatment of Neem Oil Formulation and Endosulfan Against *Helicoverpa armigera* (Hub.) (Lepidoptera: Noctuidae)

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ABSTRACT: Efficacy of the combined treatment of a neem oil formulation and endosulfan on feeding and midgut enzyme activities of *Helicoverpa armigera* larvae was studied. The antifeedant activity was recorded at 24 h after treatment and the activities of midgut digestive (total serine protease and trypsin) and detoxifying (esterase and glutathione-S-transferase) enzymes were estimated at 72 h after treatment. The antifeedant activity in endosulfan + neem oil formulation (endosulfan 0.01% and neem oil formulation 1% at 1:1 ratio) was 85.34%, significantly greater than in individual treatments. Midgut digestive enzymes and EST activities were significantly reduced and the GST activity significantly increased in the combined treatment of endosulfan + neem oil formulation, thus showing increased effect of the combined treatment of the two pesticides. These results suggest that neem oil can be used in combination with endosulfan to reduce its quantity.

KEYWORDS: neem oil, endosulfan, antifeedant activity, detoxifying enzymes

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Introduction

Indiscriminate and injudicious use of insecticides has led to a number of adverse effects in the environment.[1] The undesirable effects of these chemical insecticides used against insect pests in crops warrants the development of strategies that could eliminate or reduce the involvement of insecticides for controlling insect pests.[1] Plant-derived products are considered practical and safe to the environment, because of their easy availability, biodegradability and target-specific nature.[2] Insects have developed resistance to almost all classes of chemical pesticides by detoxification mechanisms, which involve a number of detoxifying enzymes, the important ones being esterases (ESTs), glutathione-S-transferase (GSTs) and mono-oxygenases.[3,4] The detoxification mechanism in insects can be studied by measuring the detoxifying enzymes produced in the insects against pesticides.[3]

Serine protease and trypsin are the important digestive enzymes in insects, involved in protein digestion, and are the main targets of insecticides and plant allelochemicals.[5] The GSTs play an important role in the resistance of insects to a number of insecticides.[4,6,7] Esterases are important detoxifying enzymes, involved in detoxification and sequestration of many insecticides.[8]

Cotton bollworm/legume pod borer, *Helicoverpa armigera* (Hub.) is a notorious polyphagous pest and an important constraint to crop production in Asia, Africa, Australia and Mediterranean Europe. It attacks more than 200 different species of plants.[9] Due to the high population buildup and

enormous crop losses, insecticides are frequently used for controlling this pest. *H. armigera* has developed resistance to several insecticides.[10]

The present study was carried out to determine the efficacy of a neem oil formulation, prepared from neem and karanj oils, and endosulfan in terms of feeding deterrence and activity of midgut digestive (serine protease and trypsin) and detoxifying enzymes (EST and GST) of *H. armigera*.

Materials and Methods

Chemicals. Agar agar, Methyl-*p*-hydroxybenzoate, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin (BSA), 1-napthyl acetate, Fast Blue B Salt, 1-napthol, yeast extract, Auromycin powder and ethylenediaminetetraacetic acid (EDTA) were obtained from HiMedia Laboratories, Mumbai, India. Sorbic acid, ascorbic acid, cholesterol and formaldehyde were procured from Sisco Research Laboratories, Mumbai, India. Endosulfan was obtained from Bayer Crop Science Ltd., Mumbai.

***Helicoverpa armigera* culture.** Larvae of *H. armigera* were obtained from laboratory culture at the Entomology Research Institute, Loyola College, Chennai, India. The *H. armigera* larvae were reared on a chickpea-based, semi-synthetic artificial diet.[11] The ingredients of the artificial diet were weighed separately (Table 1). Measured quantities of Part A were mixed. Agar-agar was added to water in a separate container and boiled for 5 min (Part B). The ingredients of Part A and Part B were mixed thoroughly in a blender to get an even consistency. The diet was poured into small plastic cups and allowed to cool under a laminar flow for 1 to 2 h.

Neem oil formulation. The neem oil formulation was prepared using neem oil (45%), karanj oil (45%), azadirachtin technical (0.05%) and karanjin technical (0.05%) and the emulsifier 1-(Dimethylamino)-2-nitroethylene (DMA-NE) (7.8%).

Table 1. Composition of semi-synthetic diet for *Helicoverpa armigera*.

DIET PARTS	INGREDIENTS	QUANTITY (g) PER 1,000 ml DIET
Part A	Chickpea flour	300
	Sorbic acid	3.0
	Methyl-p-hydroxy benzoate	5.0
	Ascorbic acid	4.7
	Yeast	48
	Auromycin powder	11.5
	Cholesterol	1.5
	Formaldehyde (1%)	20 ml
	Multivitamin solution (A,B,D,E,C) drops	10 µl
	Water	450 ml
Part B	Agar-agar	17.3
	Water	800 ml

The ingredients were added in a mixer and stirred for 30 minutes continuously using an electric stirrer.

Treatments. The following treatments were given to *H. armigera*:

Treatment 1: neem oil formulation 0.2%. Treatment 2: neem oil formulation 1%. Treatment 3: endosulfan 0.01%. Treatment 4: azadirachtin (40.86% purity from EID Parry) 5 ppm. Treatment 5: endosulfan (0.01%) + neem oil formulation (1%) at 1:1 ratio. Treatment 6: untreated control.

Antifeedant bioassay. The antifeedant activity was studied by the no-choice method of Bentley et al.[12] Cotton leaves were first washed with tap water then dried, and leaf discs (4 cm dia.) were cut with a cork borer. The leaf discs were dipped separately in each treatment. Leaf discs treated with distilled water served as control. Leaf discs treated with azadirachtin were used as reference control. One treated leaf disc was put in a Petri dish and a third instar *H. armigera* larva, pre-starved for 3 h was released on it. In each Petri dish, wet filter paper was placed to avoid desiccation of the leaf discs. After 24 h of treatment the unfed leaf area was measured by a leaf area meter (Delta-T devices, serial No. 15736 F96, UK).

The percent antifeedant activity was calculated using the formula of Bentley et al.[12]

$$\text{Antifeedant activity} = \frac{\text{Leaf area consumed in control} - \text{area consumed in treated leaf}}{\text{Leaf area consumed in control}} \times 100$$

Total serine protease assay. The larvae were dissected after 24 h and midgut was extracted in 0.2 M sodium phosphate buffer (pH 7.5). The midguts were removed and homogenized in 0.1 M glycine-NaOH buffer, pH 10, containing 1 mM EDTA. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used as enzyme for serine protease and trypsin activities. To estimate the serine protease activity of insect midgut, the method followed by Hegedus et al[13] was followed using azocasein as a substrate. The midgut supernatant 0.04 mL was put in a test tube and 0.3 mL of 1% azocasein solution (prepared in 0.05 M glycine-NaOH buffer, pH 10) was added to it. After incubation for 15 min at 28°C, 0.34 mL of 10% TCA was added to it. The reaction mixture was incubated for 1 h at room temperature and centrifuged at 12,000 rpm for 10 min. The supernatant was collected and 0.68 mL of 1 M NaOH was added to it. Absorbance was read at 495 nm on a HITACHI UV-2010 spectrophotometer.

The serine protease activity (SP) was calculated by subtracting the azocasein blank absorbance from sample absorbance, divided by incubation time in min, multiplied by 1000.

$$\text{SP} = \frac{\text{Abs}_{(\text{sample})} - \text{Abs}_{(\text{blank})}}{\text{Incubation time (min)}} \times 1000$$

Units are tryptic activity (mu) per min of incubation per mg protein (mu min^{-1} mg^{1} protein).

Trypsin assay. Trypsin activity was estimated as per the method of Perlmann and Lorand.[14] The supernatant (0.15 mL) was added to 1 mL of 1 mM BApNA (in 0.2 M glycine—NaOH buffer, pH 10). After incubation at 37°C for 10 min, 0.2 mL of 30% acetic acid was added to terminate the reaction. Absorbance was read at 410 nm and the enzyme activity was expressed as (µmol min^{+1} mg^{-1} protein).

Esterase assay. Esterase activity was determined according to the method of Van Asperen[15] with slight modifications. To 2 mL of 1.5 mM 1-napthyl acetate solution, 100 µL of diluted enzyme sample (10 times with 0.1 M sodium phosphate buffer) was added. This mixture was incubated at 25°C for 30 min. The reaction was stopped by addition of Fast Blue B (in 5% SDS) staining solution. The reaction mixture was incubated for 15 min and absorbance was recorded at 490 nm. The concentration of hydrolyzed substrate was determined from standard curve of 1-napthol. Specific activity was expressed as µmol of 1-napthol formed/min/mg protein.

Glutathione-S-transferase (GST) assay. The GST activity was determined using CDNB and reduced GSH as substrates, according to the methods of Habig et al[16] with slight modifications. To 1 mL of phosphate buffer (pH 7.5), 100 µL of CDNB (25 mM) and 1.6 mL of distilled water were added. The reaction was started by adding 100 µL of diluted enzyme solution. (The stock solution was diluted 10 fold with 0.1 M sodium phosphate buffer, pH 7.5). The reaction mixture was incubated at 37°C for 5 min and 100 µL of 20 mM GSH was added. Optical density at 340 nm was recorded at 30 s intervals for 3 min. Activity was calculated with an extinction coefficient of 9.6 mM cm^{-1} for CDNB. Specific activity was expressed as nmol of CDNB conjugate formed min^{-1} mg^{-1} protein.

Protein assay. Total protein content of the enzyme extract was determined by the Bradford method,[17] using Bovine Serum Albumin (BSA) as the standard.

Statistical analysis. The data was analyzed by analysis of variance (ANOVA) using SPSS (15.1). Tukeys/multiple comparison tests were used to separate the means, when the treatment effects were statistically significant ($p \leq 0.05$).

Results

Antifeedant activity. The combined treatment of neem oil formulation and endosulfan in 1:1 ratio showed significantly higher antifeedant activity (85.34 ± 8.8%, $F_{(4,14)} = 23.5$, $P \leq 0.001$) than those of the individual treatments of endosulfan 0.01% (43.6 ± 5.1%), neem oil formulation at 0.2% (57.2 ± 6.0%), and neem oil formulation at 1% (68.7 ± 5.9%) (Fig. 1). The azadirachtin at 5 ppm showed 49.5 ± 7.1% antifeedant activity against *H. armigera*.

Serine protease activity. The *H. armigera* larvae fed on leaves treated with endosulfan and neem oil formulation showed reduced serine protease activity (Fig. 2). Serine protease activity of the larvae fed on leaves treated with neem oil formulation and endosulfan in 1:1 ratio was significantly lower ($F_{(4,14)} = 16.3$, $P \leq 0.001$) as compared to that of larvae fed on leaves treated with endosulfan (0.01%), neem oil formulation (0.2 and 1%), azadirachtin (5 ppm) and untreated control leaves. There was no significant difference in serine protease activity of the larvae fed on leaves treated with neem oil formulation (0.2 and 1%) and endosulfan (0.01%).

Trypsin activity. The trypsin activity of the larvae fed on leaves treated with the combination of neem oil formulation and endosulfan at 1:1 ratio was more strongly reduced

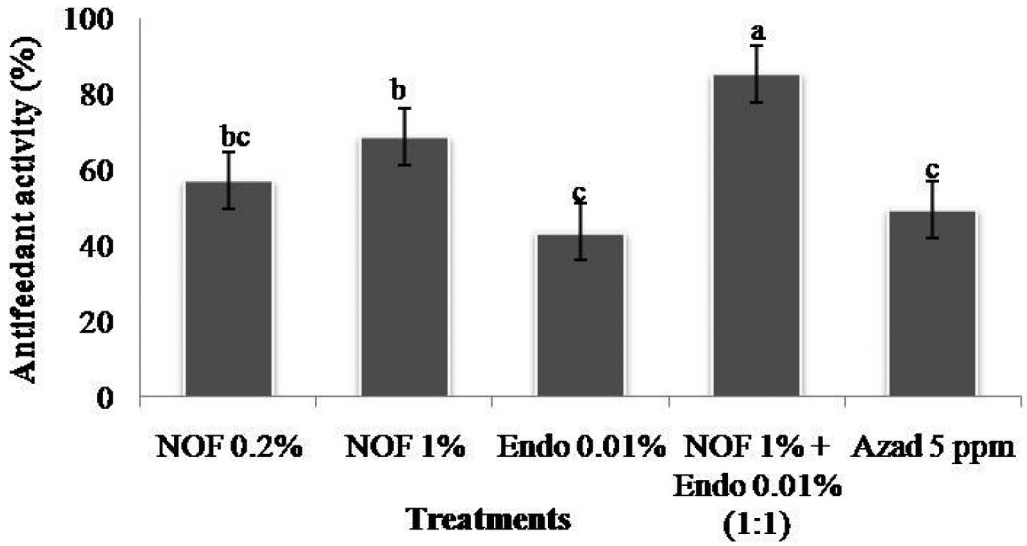

Figure 1. Antifeedant activity (%) of neem oil formulation, endosulfan and azadirachtin against *Helicoverpa armigera*. Bars (Mean ± SD) with similar letters are not statistically different by Tukey's test ($p \leq 0.05$).
Abbreviations: NOF, neem oil formulation; Endo, endosulfan; Azad, azadirachtin.

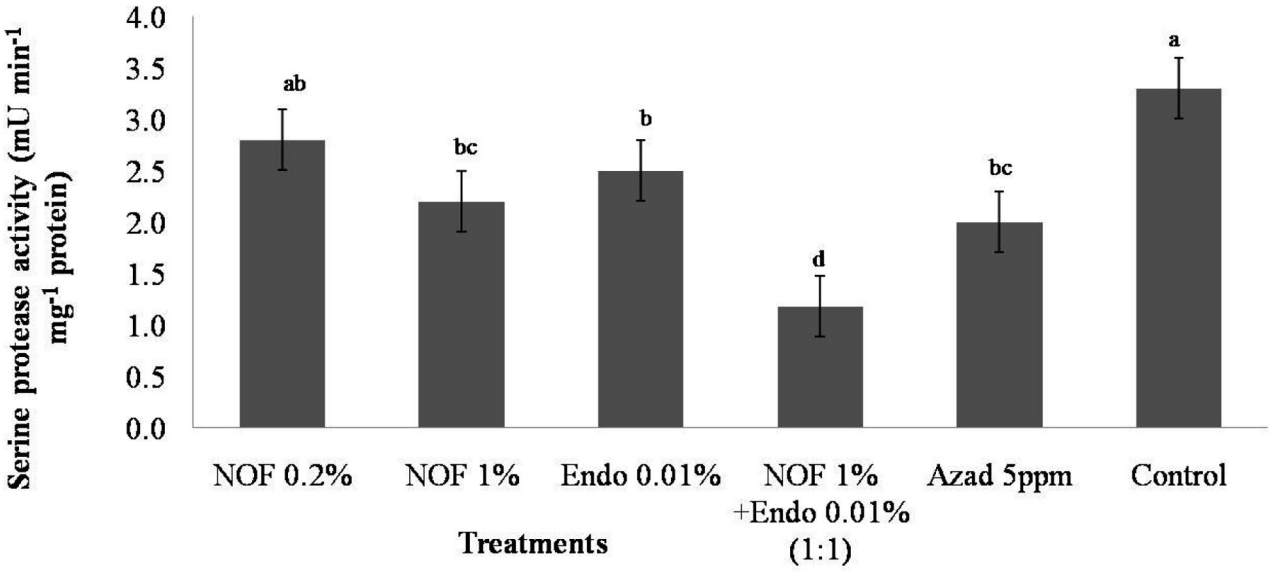

Figure 2. Serine protease activity (mU min^{-1} mg^{-1} protein) of *Helicoverpa armigera* larvae fed on treated leaves. Bars (Mean ± SD) with similar letters are not statistically different by Tukey's test ($p \leq 0.05$).
Abbreviations: NOF, neem oil formulation; Endo, endosulfan; Azad, azadirachtin.

($F_{(4,14)} = 12.0$, $P \leq 0.05$) than that of the larvae fed on leaves treated individually with endosulfan (0.01%), neem oil formulation (0.2% and 1%), azadirachtin (5 ppm) and the untreated control leaves (Fig. 3).

Glutathione-S-transferase activity. A slight increase in GST activity was observed in the larvae fed on treated leaves (Fig. 4). However, the differences were significantly higher ($F_{(4,14)} = 7.8$, $P \leq 0.05$) in the larvae fed on the leaves with combined treatment of neem oil formulation and endosulfan at 1:1 ratio. No significant differences were recorded in the larvae fed on the leaves with rest of the treatments.

Esterase activity. A significant reduction in esterase activity was observed in the larvae fed on leaves treated with neem oil formulation and endosulfan at 1:1 ratio and with neem oil formulation (1%) than those fed on leaves treated with neem oil formulation (0.2%), endosulfan (0.01%), azadirachtin (5 ppm) and the untreated control leaves ($F_{(4,14)} = 10.7$, $P \leq 0.05$) (Fig. 5).

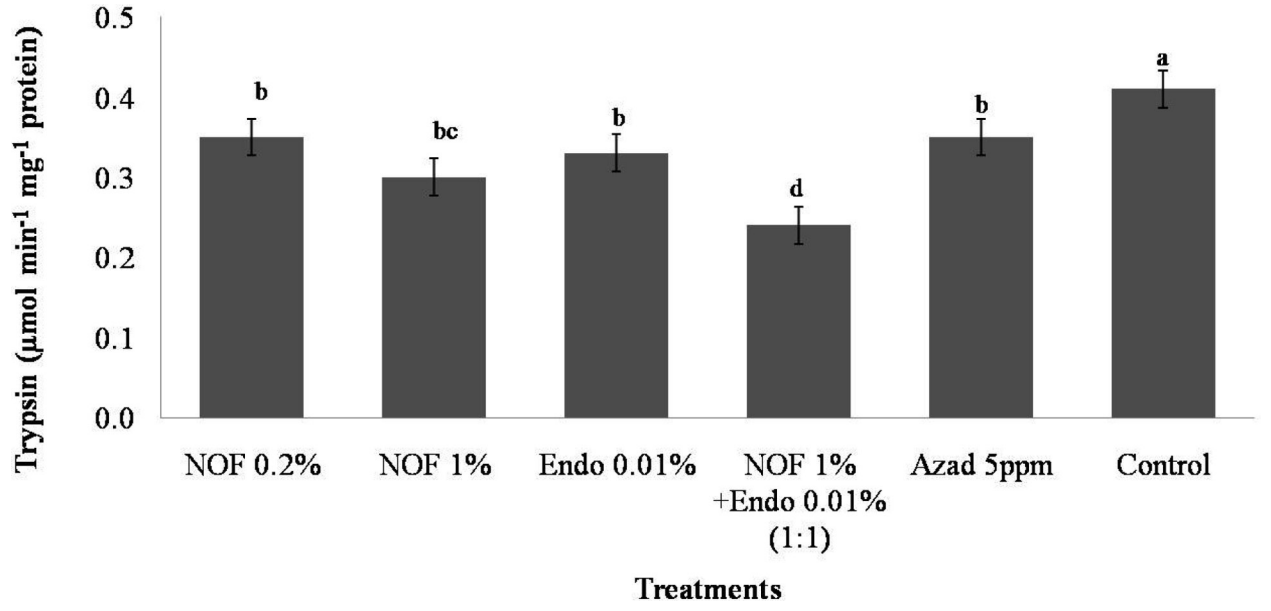

Figure 3. Trypsin activity (µmol min^{-1} mg^{-1} protein) of *Helicoverpa armigera* larvae fed on treated leaves. Bars (Mean ± SD) with similar letters are not statistically different by Tukey's test ($p \leq 0.05$).
Abbreviations: NOF, neem oil formulation; Endo, endosulfan; Azad, azadirachtin.

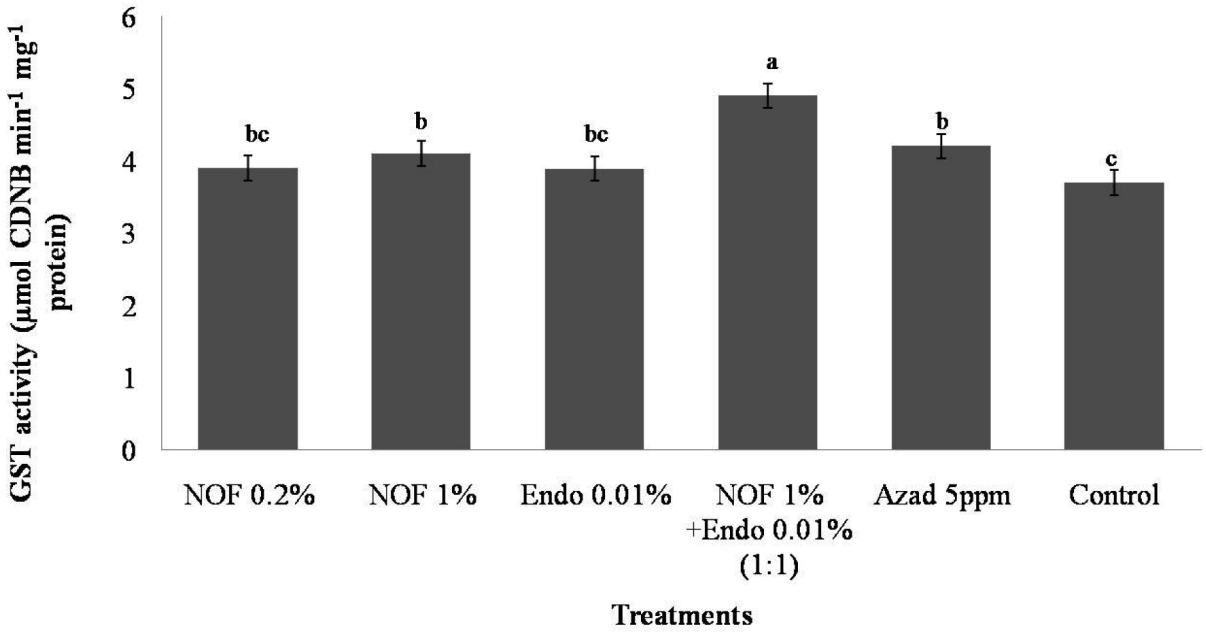

Figure 4. Glutathione-S-transferase (GST) activity (µmol CDNB min^{-1} mg^{-1} protein) of *Helicoverpa armigera* larvae fed on treated leaves. Bars (Mean ± SD) with similar letters are not statistically different by Tukey's test ($p \leq 0.05$).
Abbreviations: NOF, neem oil formulation; Endo, endosulfan; Azad, azadirachtin.

Protein content. The overall protein content showed significant reduction in larvae fed on leaves with neem oil formulation and endosulfan at 1:1 ratio ($F_{(4,14)} = 19.4$, $P \leq 0.05$), compared with the protein content of the larvae fed on leaves treated with endosulfan (0.01%), neem oil formulation (0.2 and 1%), azadirachtin (5 ppm), and the untreated control leaves (Table 2).

Discussion

Plant-based insecticides have been playing a promising role in insect pest control. Many authors have reported the antifeedant activity of various plant extracts against *H. armigera*,[18,19] *Helicoverpa zea* Bod.,[20] and *Spodoptera litura* (Fab.).[21,22] Our results showed significantly higher antifeedant activity in the combined treatment of the two insecticides (neem oil

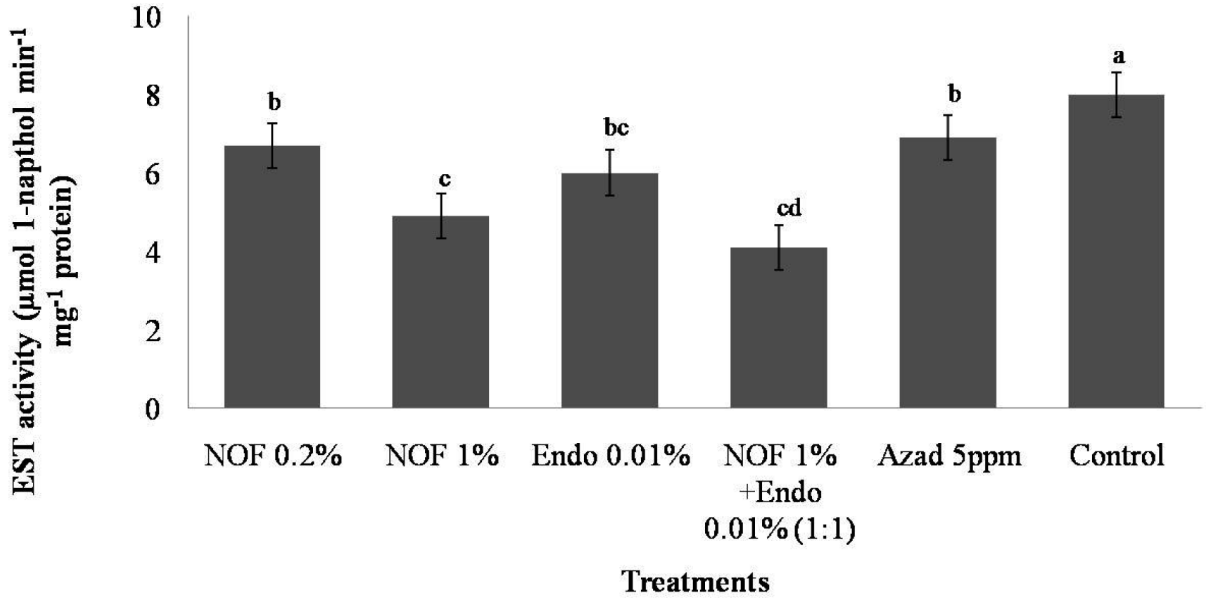

Figure 5. Esterase (EST) activity (µmol 1-napthol min^{-1} mg^{-1} protein) of *Helicoverpa armigera* larvae fed on treated leaves. Bars (Mean ± SD) with similar letters are not statistically different by Tukey's test ($p \leq 0.05$).
Abbreviations: NOF, neem oil formulation; Endo, endosulfan; Azad, azadirachtin.

Table 2. Protein content (mg ml^{-1}) of *Helicoverpa armigera* larvae after treatment with neem oil formulation, azadirachtin and endosulfan.

TREATMENT	PROTEIN CONTENT (mg ml^{-1})
NOF 0.2%	10.1 ± 2.1^{b}
NOF 1%	8.4 ± 1.5^{c}
Endo 0.01%	8.7 ± 1.9^{c}
NOF 1% + Endo 0.01% (1:1)	6.5 ± 1.2^{d}
Azad 5 ppm	11.7 ± 1.9^{b}
Control	13.6 ± 2.4^{a}

Values (Mean ± SEM) carrying same alphabet(s) within a column are not significantly different by Tukey's test ($p \leq 0.05$).
Abbreviations: NOF, Neem oil formulation; Endo, Endosulfan; Azad, Azadirachtin.

formulation and endosulfan at 1:1 ratio) compared to their individual treatments. Botanical pesticides have been found highly toxic when combined with chemical compounds.[23–25] Furthermore, insecticides used in combinations show enhanced toxicity over the results when they are used individually.[26]

Serine proteases and trypsin are the important digestive enzymes in insects, involved in digestion of proteins. Any imbalance in their activity will have drastic effects on insect growth and development, as the insect will be devoid of essential amino acids. The present study showed that the combined treatment of endosulfan and neem oil formulation at lower concentrations significantly reduced the activity of these enzymes. A number of reports have shown a reduction in the activities of insect digestive enzymes by synthetic insecticides and plant allelochemicals.[5,27]

An increase in GST activity was observed in insects fed on treated leaves, and the combined treatment of neem oil formulation and endosulfan in 1:1 ratio had a more significant effect than the rest of the treatments. This might be due to the fact that the insecticide/neem oil formulation toxicity induces a stress causing increase of the GST enzyme to resist oxidative damage. Our results correlate with the findings of War et al,[24] who observed greater levels of GST in *S. litura* treated with combined treatment of neem oil and endosulfan. Increased GST activity in *Plutella xylostella* L. in combined treatment with sesame oil and cypermethrin has been reported.[28] Furthermore, War et al[24] observed the synergistic effect of neem oil formulation and endosulfan against *S. litura* and reported an increase in GST activity following combined treatment with endosulfan and neem oil formulation.

Esterase activity was inhibited in all the treated larvae, and more inhibition was found following the combined treatment with the two pesticides. This may be attributed to the additive effect of the combined treatment of the two insecticides. Esterases are important detoxifying enzymes in insects, so their inhibition can contribute significantly to controlling insect pests. *Leptinotarsa decemlineata* (Say.) larvae, when fed on potato leaves treated with F18, showed reduced esterase activity.[8] It has been found that the secondary metabolite hydroxamic acid DIMBOA inhibits esterase activities in insects.[29,30] Senthil Nathan et al[27] reported the inhibition of acetylcholinesterase activity in *Nilparvata lugens* (Stal.) on treatment with azadirachtin. War et al[24] reported the synergistic activity of neem oil formulation with endosulfan against *S. litura* and observed that combined treatment of the two significantly inhibited the EST activity of the insect pest. This additive effect of neem oil formulation and endosulfan on the inhibition of the insect's detoxification enzymes may be due to the higher toxicity of the combined treatment.[30,31] The combined treatment of neem oil and endosulfan resulted in significant reduction of total protein content in *H. armigera* larvae as compared to the rest of the treatments. This decrease in protein content can be attributed to the higher toxicity of the combined treatment and the reduction in the production of the enzymes and other protein-based compounds. Since proteins are important for insect growth and development, lower levels of proteins will negatively affect the insect pest. Toxicity of insecticides has been found to reduce the protein content in insect pests.[27]

In conclusion, our results indicated that the combined treatment of neem oil formulation and endosulfan at 1:1 ratio showed high antifeedant activity and affected the activity of midgut enzymes in insects drastically. Reducing the midgut digestive enzymes will affect the insects' growth and development, and affecting the detoxifying enzymes will prevent the insect from developing resistance. This gives strong evidence that neem oil formulation could be used as an additive with endosulfan to reduce the quantity of harmful synthetic insecticides used. If this oil formulation acts synergistically with other insecticides as well, farmers can reduce the use of insecticides and increase their effectiveness by the addition of this oil formulation.

Author Contributions

Conceived and designed the experiments: ARW, MGP, SI. Analyzed the data: ARW, MYW, BH, MGP. Wrote the first draft of the manuscript: ARW, MGP. Contributed to the writing of the manuscript: BH, MYW, MGP, TA. Agree with manuscript results and conclusions: ARW, MGP, MYW, BH, SI. Jointly developed the structure and arguments for the paper: ARW, MGP, MYW, BH, SI, TA. Made critical revisions and approved final version: SI, MGP. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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