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Synergistic Activity of Endosulfan with Neem Oil Formulation Against Tobacco Caterpillar *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae)

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ABSTRACT

Indiscriminate use of synthetic insecticides for insect pest management has resulted in many harmful effects like, insect resistance, effects on the beneficial organisms etc. There is a need to use alternative methods for insect pest control to reduce the frequency of insecticides utilized for pest management. Furthermore, there is now more emphasis on the ban of endosulfan due to its relatively higher toxicity to non-target organisms. Series of experiments were conducted to find out the synergistic activity of a neem oil formulation with endosulfan against *Spodoptera litura*. Antifeedant activity and effect on detoxifying enzyme activities, viz., esterase and Glutathione-S-Transferase (GST) were studied. Antifeedant activity was studied by no-choice method using castor leaf discs. Esterase and GST activities were estimated spectrophotometrically. Antifeedant activity was significantly greater (85.34%) in neem oil formulation+endosulfan (endosulfan 0.01% and neem oil formulation 1% at 1:1 ratio) treatment than in individual treatments. Esterase activity was significantly lower and GST activity slightly higher in neem oil formulation+endosulfan (endosulfan 0.01% and neem oil formulation 1% at 1:1 ratio) treatment. A considerable influence of the combined treatment was observed on esterase and GST activities. Present study reveals that using little amounts of synthetic insecticides along with neem oil could be effective in controlling insect pests. Therefore, this neem oil formulation could be used as a synergist with endosulfan to reduce the quantity of synthetic insecticides for insect pest control.

Key words: Antifeedant activity, detoxifying enzymes, esterase, glutathione-S-transferase

INTRODUCTION

Fifty years of sustained struggle against harmful insects using synthetic pesticides has produced negative secondary effects. These include mammalian toxicity, pesticide resistance in insects, pest resurgence, elimination of natural enemies and loss of biodiversity (Regnault-Roger, 1997). Because of the problems associated with the use of toxic synthetic chemical insecticides, a search is underway to discover new, alternative methods of insect pest management that are sustainable, economical and environment friendly (Isman, 2006). Among the alternative strategies, plant products have been found to be quite effective, socially acceptable, biodegradable and target specific (Leatemia and Isman, 2004; Khatter, 2011; Baskar *et al*., 2009). Plant derived insecticides have a wide range of mode of actions, such as, feeding deterrents, insecticides, ovicidal, oviposition deterrents and growth inhibitors (Isman, 2006; Ikbal *et al*., 2007; Abdullahi *et al*., 2011). Insects have developed resistance to almost all the classes of chemical pesticides either through

detoxification or less sensitivity (Gunning *et al*., 1999). Botanical pesticides are widely used for the control of insect pests (Iloba and Ekrakene, 2006; Curzio *et al*., 2009; Abdullahi *et al*., 2011). However, combination of botanicals have been found more promising against insect pests, for example, combination of neem oil and endosulfan reduced the number of jassids and their damage in Okra (Mandal *et al*., 2007). There are number of reports where effective synergistic activity of biological control agents in combination with chemical insecticides have been observed against insect pests (Mandal *et al*., 2007; Morales-Rodriguez and Peck, 2009; Koppenhofer and Fuzy, 2003).

Glutathione-S-transferase (GST: E.C. 2.5.1.18) and esterases (E.C. 3.3.3.7) are important detoxifying enzymes involved in the metabolism of a broad range of foreign and endogenous compounds in insects (Conyers *et al*., 1998; Francis *et al*., 2001). GST's are multifunctional detoxifying enzymes that catalyze the conjugation of reduced Glutathione (GSH) (Armstrong, 1991) and plays an essential role in detoxification of insecticides, thereby rendering them less or non toxic (Rufingier *et al*., 1999). Esterases constitute a widely distributed family of enzymes in insects. They hydrolyze carboxyl ester, amide and thioester bonds in a variety of compounds (Mukanganyama *et al*., 2003). Esterases are important enzymes involved in resistance to many insecticides (Conyers *et al*., 1998).

The tobacco caterpillar, *Spodoptera litura* (Fab.) is an economically important polyphagous pest of seasonal crops in many countries including India, Japan, China and Southeast Asia (Sahayarij *et al*., 2007). It attacks more than 200 different species of plants, of which 40 species are grown in India. The main host plants are; crucifers, cucurbits, groundnut, maize, tea, tobacco and various ornamentals. It has developed resistance to a number of synthetic insecticides (Sahayarij *et al*., 2007).

Broad spectrum mechanism of action makes neem oil an important candidate in controlling wide range of insect pests (Isman, 2006; Nderitu *et al*., 2008; Ogbuewu *et al*., 2011) and is used against many agricultural and medical pests (Dua *et al*., 2009). Neem oil has been widely used in combination with chemical insecticides, as a promising alternative for insect pest management both economically and ecologically (Koppenhofer and Fuzy, 2003; Baki *et al*., 2005; Morales-Rodriguez and Peck, 2009). Neem oil formulations have been found to synergize the activity of malathion, quinalphos and monocrotophos against *Tribolium castaneum* (Parmar and Dutta, 1986). Although, the synergism of neem oil and synthetic insecticides has been well studied against insect pests, however the studies on effect of combined treatment on detoxifying enzymes are limited. The present study was carried out to find out the synergistic activity of endosulfan with a neem oil formulation on antifeedant activity and on the activities of midgut detoxifying enzymes (esterase and GST) of *S. litura*.

MATERIALS AND METHODS

The study was carried out at Entomology Research Institute, Loyola College, Chennai, during 2008-2009.

Insect culture: Third instar larvae of *S. litura* were obtained from the stock culture maintained on artificial diet under laboratory conditions (26±1°C; 11±0.5 h photoperiod and 75±5% relative humidity) from the insectary of the Entomology Research Institute, Loyola College, Chennai, India.

Chemicals: Reduced Glutathione (GSH), 1-chloro-2, 4-dinitrobenzene (CDNB), Bovine Serum Albumin (BSA), Sodium Lauryl Sulphate (SDS), 1-napthyl acetate, Fast blue B salt, 1-napthol and

EDTA were obtained from Himedia Lab. Pvt. Ltd., Mumbai, India. Endosulfan was procured from Bayer Crop Science Ltd., Mumbai, India.

Preparation of neem oil formulation: The neem oil formulation was prepared using neem oil (45%), karanj oil (45%), azadirachtin technical (0.05%), karanjin technical (0.05%) and emulsifier (DMA-NE) (7.8%). Neem oil, Karanj oil, Azadirachtin and karanjin were gifted by Nimbion Organics, Chennai. The ingredients were added in a mixer and stirred for 30 min continuously using an electric stirrer.

Treatments: Insects were divided into five groups with 20 insects in each. The treatments used were; neem oil formulation (0.2 and 1%), endosulfan (0.01%), azadirachtin (40.86% purity) 5 ppm and Neem oil formulation (1%) + endosulfan (0.01%, 1:1 ratio) and water control. However, azadirachtin treatment was used only for comparison in antifeedant activity.

Antifeedant bioassay: The antifeedant activity was studied by no-choice method. Castor leaves were washed with tap water, dried and leaf discs (4 cm dia.) were cut with a cork borer. The leaf discs were dipped separately in neem oil formulation (0.2 and 1%), endosulfan (0.01%), azadirachtin 5 ppm and Neem oil formulation (1%)+endosulfan (0.01%, 1:1 ratio). Leaf discs treated with distilled water served as a control. Leaf discs treated with azadirachtin (40.86% purity, from EID Parry) were used as a reference control. One treated leaf disc was placed in each Petri dish with a third-instar *S. litura* larva, prestarved for 3 h. Wet filter paper was placed on the lid of each Petri dish to avoid drying 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) and the larvae were transferred to fresh untreated castor leaves and fed on alternate days.

The percent antifeedant activity was calculated using the formula of Bentley *et al.* (1984).

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

where, Lf is leaf area

Preparation of enzyme extract: The larvae were dissected in 0.1 M sodium phosphate buffer (pH 7.5), midguts were removed and homogenized in 0.1M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA. The homogenate was centrifuged at 15, 000 rpm for 15 min at 4°C and the supernatant collected was used as enzyme source. All spectrophotometric analyses were carried out on HITACHI UV-2010 spectrophotometer.

Esterase assay: Esterase activity was determined according to the method of Van Asperen, 1962) with slight modification. The concentration of the hydrolyzed substrate was determined from standard curve of 1-napthol. Specific activity was expressed as μ mol of 1-napthol formed min^{-1} mg^{-1} protein.

Glutathione-S-transferase assay: Glutathione-S-transferase activity was determined using CDNB and reduced GSH as substrates according to Habig *et al.* (1974). GST activity was calculated with an extinction coefficient of 9.6 mM cm^{-1} for CDNB and was expressed as nmol of CDNB conjugate formed min^{-1} mg^{-1} protein.

Protein determination: Protein concentration was determined as per Bradford (1976), with Bovine serum albumin as standard.

Statistical analysis: The data obtained for antifeedant activity and enzyme assays were subjected to analysis of variance (one way ANOVA) followed by Tukey's multiple range test using SPSS .

RESULTS AND DISCUSSION

Synergistic combination of biological and chemical insecticides yield a promising alternatives for insect pest management (Morales-Rodriguez and Peck, 2009; Koppenhofer and Fuzy, 2003). Botanicals play an important role as synergists in insect pest management both economically and ecologically (Baki *et al.*, 2005). Antifeedant activity of neem oil formulation+endosulfan was significantly greater (85.3%) against *S. litura* larvae than the individual treatments (Fig. 1). This was followed by neem oil formulation at 1% (68.72%) and 0.2% (57.24%). Least antifeedant activity was observed on the leaf discs treated with endosulfan at 0.01% (43.6%) and azadirachtin (49.5%). Synergism between botanicals and chemical insecticides against insect pests has been well documented. Combination of neem cake+neem oil+endosulfan reduced the number of jassids and their damage in okra (Mandal *et al.*, 2007). Azadirachtin in combination with half doses of malathion, decamethrin and Bt were found effective against *S. litura* (Jat and Bhardwaj, 2005). Baki *et al.*, 2005 reported the synergism between *Wedelia calendulacea* plant extracts and lambda cyhalothrin on adult red flour beetle *Tribolium castaneum*. Synergistic activity of *Melia toosendan* extract with malathion against *S. litura* was investigated by Feng *et al.* (1995). Rao and Dhingra, (2000) reported reduced feeding in the combined treatment of neem oil and synthetic pyrethroids against *S. litura*. The present results illustrated that botanicals can be used to increase the effectiveness of synthetic insecticides (Baki *et al.*, 2005). However, antagonistic effect of Azadirachtin on cyfluthrin and permethrin against *Blattella germanica* (L.) was observed by Salehzadeha and Mahjub (2011).

Detoxifying enzymes: Detoxifying enzymes play an important role in development of insect resistance to insecticides by detoxifying the pesticide compounds through intrinsic detoxification.

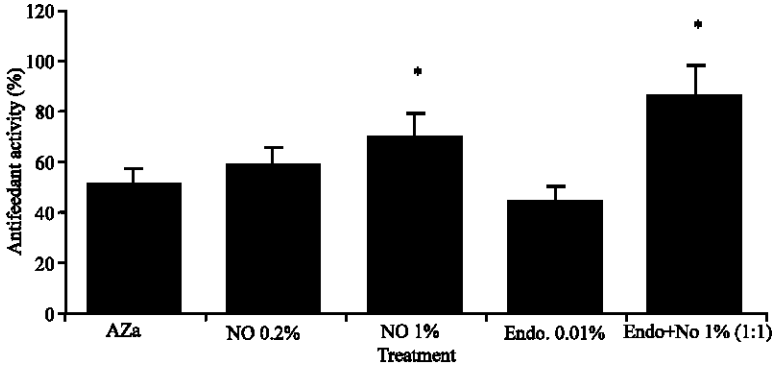

Fig. 1: Percentage antifeedant activity of neem oil formulation and endosulfan against larvae of *S. litura*. Aza: Azadirachtin (5 ppm), NO: Neem oil formulation, Endo: Endosulfan, *p≤0.05

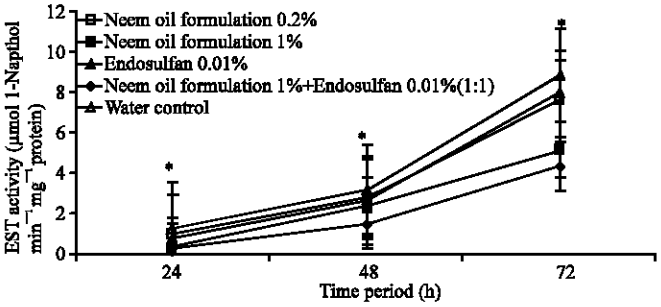

Fig. 2: Esterase activity (µmol 1-napthol formed min^{-1} mg^{-1} protein) of *S. litura* larvae after treatment with neem oil formulation and endosulfan. *$p \leq 0.05$

The role of insect detoxification enzymes in the metabolism of insecticides, allelochemicals and other xenobiotics has been well established (Conyers *et al.*, 1998; Ortego *et al.*, 1999). The enzymatic and non-enzymatic processes in insects can be affected by various factors such as diet, chemicals, etc. Although, esterase activity showed an increasing trend with time, but when comparing the treatments, significantly less activity was observed in the combined treatment of neem oil formulation+endosulfan across the test period (Fig. 2). After 72 h of treatment, lowest activity was recorded in the larvae fed on leaf discs treated with neem oil formulation (1%)+endosulfan (0.01%) (3.5 µmol 1-napthol formed min^{-1} mg^{-1} protein) followed by those fed on leaf discs treated with neem oil formulation (1%) (5.09 µmol 1-napthol formed min^{-1} mg^{-1} protein). Least reduction was found in individual treatment of endosulfan (0.01%) (7.97 µmol 1-napthol formed min^{-1} mg^{-1} protein) and neem oil formulation at 0.2% (7.65 µmol 1-napthol formed min^{-1} mg^{-1} protein). Larvae fed on water treated control leaf discs showed higher esterase activity (8.76 µmol 1-napthol formed min^{-1} mg^{-1} protein). Moreover, reduction in esterase activity of larvae fed on leaf discs treated with neem oil formulation (1%)+endosulfan (0.01%) as compared to control was 2.4 folds, both at 48 and 72 h after infestation. Esterases are involved in the metabolism of insecticides in insects (Conyers *et al.*, 1998; Rufingier *et al.*, 1999). Reduction in their activity due to combined treatment of neem oil formulation (1%) and endosulfan (0.01%) at 1:1 ratio could result in increased susceptibility of *S. litura*. Inhibition of acetylcholine esterase activity by azadirachtin has been studied in *Nilaparvata lugens* (Nathan *et al.*, 2008). From the above results, it is evident that esterase activity was inhibited in all the treatments and the least activity was observed in insects fed on leaf discs treated with neem oil formulation+endosulfan. This may be due to the synergistic effect of two insecticides (Scott *et al.*, 2002). Our results are supported by many earlier reports. For example, Feng *et al.* (1995) observed the synergistic activity of *M. toosendan* with malathion through inhibition of esterase activity in *S. litura*. Ingestion of neem oil significantly reduced the esterase activity in larvae and adults of *Choristoneura rosaceana* (Smirle *et al.*, 1996). Furthermore, there are many reports where inhibition of esterase activity by plant products has been recorded (Mukanganyama *et al.*, 2003; Nathan *et al.*, 2008; Caballero *et al.*, 2008). Allelochemicals could either reduce or inhibit detoxification mechanisms and possibly increase the susceptibility of insects to insecticides (Yu and Abo-Elghar, 2000).

GST catalyzes the nucleophilic reactions of GSH with a number of electrophilic compounds and plays an important role in drug metabolism, exogenous substance biotransformation and protection of organisms against oxidative damage (Jiang *et al.*, 2003). At 72 h after infestation, the GST activity was higher in larvae fed on leaf discs treated with neem oil formulation (1%)+endosulfan

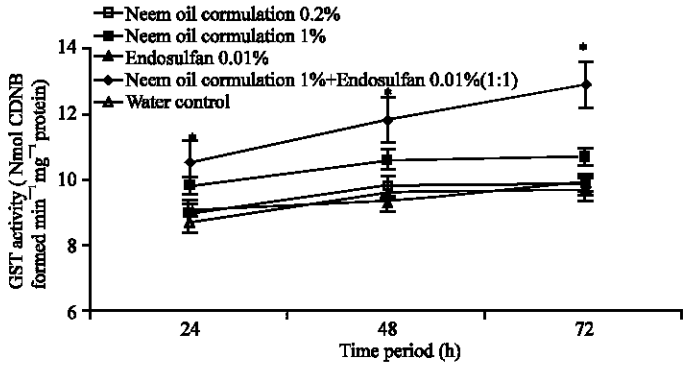

Fig. 3: GST activity (nmol CDNB-conjugate formed min^{-1} mg^{-1} protein) of *S. litura* larvae after treatment with neem oil formulation and endosulfan. * $p \leq 0.05$

(0.01%) (12.9 nmol CDNB-conjugate formed min^{-1} mg^{-1} protein) followed by those fed on leaf discs treated with neem oil formulation (1%) (10.69 nmol CDNB-conjugate formed min^{-1} mg^{-1} protein). No significant differences were observed among the larvae fed on leaves with other treatments (Fig. 3). Larvae fed on leaf discs treated with endosulfan (0.01%), neem oil formulation 0.2% and on water treated controls showed the activity of 9.86, 9.87 and 9.69 nmol CDNB-conjugate formed min^{-1} mg^{-1} protein, respectively. Similar results were obtained for 24 and 48 h after treatment. An increase of 1.2 fold in GST activity was observed in larvae fed on leaf discs treated with neem oil formulation (1%)+endosulfan (0.01%) as compared to the control, both at 24 and 48 h after infestation. The higher oxidative stress due to the combined toxicity of neem oil formulation and endosulfan might have resulted in increased GST activity to resist the oxidative damage. Plant products have been reported to induce the GST activities in many insect pests. Yin *et al.* (2008), observed an increase in GST activity in *Oxya chinensis* on treatment with 5-Aminolevulinic Acid (ALA). Induction of GST activity in *S. litura* on treatment with a mixture of limonoids F18 (1, 7-di-O-acetylhavanensin and 3, 7-di-O-acetylhavanensin) was recorded by Caballero *et al.* (2008). The present results are supported by the findings of Vinseton *et al.* (2003), who observed increased GST activity in *Plutella xylostella* on combined treatment of sesame oil and cypermethrin.

Protein concentration: Decrease in protein content was observed in the larvae fed on treated leaf discs than the larvae fed on control leaf discs (Table 1). Greater reduction in protein content throughout the test period was observed in the larvae fed on leaf discs treated with neem oil formulation (1%)+endosulfan (0.01%) ($p \leq 0.001$, 0.01 and 0.01 at 24, 48 and 72 h, respectively) as compared to the larvae fed on control leaf discs. No significant difference was recorded in protein content between the larvae fed on leaf discs treated with endosulfan (0.01%) and on control leaf discs ($p \leq 0.05$) throughout the test period. Reduction in protein concentration is a common phenomenon in insects after treatment with toxic compounds (Smirle *et al.*, 1996; Nathan *et al.*, 2008). Greater reduction in protein content in larvae fed on leaf discs treated with neem oil formulation and endosulfan manifests the toxicity of the combined treatment of the two insecticides. Our results are supported by a number of reports where toxicity of insecticides lead to reduced protein content of insects (Smirle *et al.*, 1996; Mukanganyama *et al.*, 2003; Nathan *et al.*, 2008; Caballero *et al.*, 2008).

Table 1: Protein concentration (mg mL^{-1}) of *S. litura* larvae after treatment with neem oil formulation and endosulfan

	Time after treatment (h)		
Treatment	24	48	72
NO 0.2%	7.2±1.3^{a}	9.1±1.4^{ab}	11.5±2.2^{a}
NO 1%	5.1±1.0^{bc}	6.3±1.2^{c}	7.8±2.1^{c}
Endo. 0.01%	6.4±2.1^{ab}	8.4±2.1^{bc}	9.9±1.7^{b}
Endo. (0.01%) + NO 1% (1:1)	2.9±0.8^{d}	5.2±1.4^{d}	5.9±0.9^{d}
Water	7.8±1.1^{a}	10.5±2.3^{a}	12.2±2.4^{a}

Values (Mean±SEM) carrying same alphabet (s) within a column are not significantly different by Tukey's test ($p \leq 0.05$). NO: Neem oil formulation, Endo: Endosulfan

CONCLUSION

Leaf discs treated with neem oil formulation+endosulfan not only showed high antifeedant activity but also affected the activity of esterases and GST besides reducing total protein content. Esterases appeared to be more sensitive to the inhibitory effect combined of treatment of neem oil formulation + endosulfan than GST. Therefore, this neem oil formulation could be used as a synergist with endosulfan to reduce the quantity of synthetic insecticides and increase their effectiveness in insect pest management. However, further study is needed to develop more and more synergists that could be utilized in insect pest management programmes to reduce the indiscriminate use of harmful chemical insecticides.

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