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Toxicity of cypermethrin and enzyme inhibitor synergists in red hairy caterpillar *Amsacta albistriga* (Lepidoptera: Arctiidae)

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Abstract

Background: The frequent usage of broad-spectrum insecticides like cypermethrin in agriculture activities could lead to the development of resistance in insects like *Amsacta albistriga*.

Objectives: The present study was conducted to understand the toxicity of cypermethrin with and without a combination of three enzyme inhibitors (PBO, DEM, and TPP) on *A. albistriga* using a topical bioassay.

Methodology: The in vitro and in vivo studies were conducted to understand the effect of three enzyme inhibitors such as piperonyl butoxide (PBO) and diethyl maleate (DEM) and triphenyl phosphate along with six different concentration of cypermethrin on the activities of acetylcholinesterase (AChE), esterase (EST), glutathione S-transferase (GST), and mixed-function oxidase (MFO) of *A. albistriga*.

Results: Bioassay shows elevated LC_{50} for cypermethrin (63.32 ppm) whereas in a combination of PBO cypermethrin LC_{50} values were reduced into 12.039 ppm followed by TPP combination as 13.234 ppm. In vitro and in vivo inhibition analysis shows AChE inhibition by PBO $P < 0.01$, esterase inhibition by PBO, and TPP were less; GST inhibition by DEM was observed at $P < 0.001$. Native PAGE results revealed that the in vitro AChE isoenzyme inhibition could be possible by PBO synergism with cypermethrin in *A. albistriga*.

Conclusions: The overall results conclude that the PBO and TPP enzyme inhibitors could be fine synergist molecules when it mixed with cypermethrin insecticide to control and manage the insecticide-resistant *Amsacta albistriga* in the field.

Keywords: Enzyme inhibitors, In vitro inhibition, LC_{50}, AChE insensitivity, Electrophoresis

Background

In agriculture-based countries like India, pests and weed management are most important to get more yields. The fast-growing population of India leads to a shortage of food supplies to the people. Thus, resulting in frequent over usage of chemicals to the field to gain more yields. In growing countries, like India, 20–30% of agriculture yield reduction occurs each year due to the interruption

and activities of insects, pests, and improper weed removal. This leads to excess usage of insecticides, pesticides, and herbicides to the field (Mathiyazhagan and Natarajan 2013). This frequent application of pesticides or insecticide results in the resistant development in pests like *A. albistriga*. It is the most important problematic pest for oilseed crops such as castor (*S. indicum*), groundnut (*A. hypogaea*), sesame (*S. indicum*), sorghum (*S. bicolor*), cotton (*Gossypium*), and pigeon pea (*C. cajan*). Chemical insecticides like pyrethroid, organophosphates (OPs), and carbamate have been used to control this *A. albistriga* pest. The continuous use of

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these insecticides in the fields has led to the development of resistance in this pest (Muthusamy et al. 2015; Muthusamy and Shivakumar 2015). The evolution of insecticide resistance is a major problem in pest control. During the past three decades, more than 540 insect species have developed resistance against broad-spectrum insecticides (Gullan and Cranston 2005). Among the different resistance mechanisms, the metabolic breakdown of the pesticide is one of the major enzymatic systems involved in insecticide resistance. Many studies revealed that the development of insecticide resistance mechanisms in most of the insect species is mediated through increased activity of major detoxifying enzymes, such as esterase, glutathione S-transferase, and mixed-function oxidases (or) cytochrome P450 monooxygenases. Besides, the target site insensitivity such as insensitive acetylcholinesterase is also involved in multiple resistance mechanisms (Yu et al. 2003; Muthusamy et al. 2013).

To overcome the metabolic resistance mechanism among the insects, synergist molecules like enzyme inhibitors with chemical insecticides could be useful to control, manage, and delay resistant development in insects. Synergists like PBO, DEM, and TPP used as a potential inhibitor of cytochrome P450 enzymes (Pasay et al. 2009), glutathione S-transferase, and carboxylesterase have been used to increase the effectiveness of an insecticide as well as to monitor the resistance mechanisms (Wu et al. 2007; Yang et al. 2009).

Hence, the objective of the present study was to understand the synergistic effect of PBO, DEM, and TPP along with cypermethrin (Pyrethroid) insecticide on metabolic enzyme inhibition of *A. albistriga* by in vitro and in vivo analysis.

Material and methods
Insect source
The *Amsacta albistriga* larvae were collected from castor (*S. indicum*) and groundnut (*A. hypogaea*) field from Omalur and Attur towns of Salem district, Tamil Nadu, India. The collected *A. albistriga* was carefully transferred to the laboratory, and they were maintained for more than twenty generations under normal controlled conditions (25 °C ± 2 °C with 75 ± 5% relative humidity with a 14:10-h light: dark photoperiod) without exposure to any chemical insecticides.

Chemicals
The commercial grade cypermethrin 10% EC was obtained from Syngenta India, Ltd., Pune, and six concentrations were tested for bioassay ranging from 0.001 to 1.0 ppm. Synergist's piperonyl butoxide (PBO 90%) and diethyl maleate (DEM 90%) were purchased from Himedia Pvt. Ltd., Mumbai, and triphenyl phosphate (TPP 90%) was purchased from Sigma Chemicals, Ltd., Mumbai.

Bioassays
The topical application method was used to assess the toxicity of cypermethrin insecticide alone and along with synergistic compound by following the method of Yu and McCord (2007). Six concentrations of cypermethrin (0.001, 0.01, 0.05, 0.1, 0.5, and 1.0 ppm) were prepared in 1 mL of acetone, and doses of 1 μL of each concentration were applied on the ventral thoracic region of the early 3rd instar *A. albistriga* larvae using a 20-μL micropipette. Thirty larvae were taken for each concentration of treatment, triplicate analysis was performed for each concentration, and control larvae were treated with acetone alone. The treated as well as control larvae were maintained in the laboratory at 25 °C ± 2 °C with 75 ± 5% relative humidity with a 14:10-h light: dark photoperiod. The mortality was recorded after 24 h of treatment.

For the synergism experiment, a small group of a 2-day-old early 3rd instar larvae (25/synergist) was exposed to each synergist alone (serially diluted from 100 mM) to determine the maximum concentration that the insect could tolerate and remain alive for 24 h (data not shown). This primary experiment led us to select a concentration of 5.0 mM of each synergist used in combination with six different concentrations of cypermethrin to assess the synergism toxicity (Muthusamy et al. 2013). After treatment, the larvae were placed in the disposable empty plastic boxes (500 mL) and maintained as mentioned above. Treated larvae were provided with a castor leaf diet throughout the exposure. Mortality was observed after 24 h of treatment. Larvae were considered to be dead when touched with a small brush.

Biochemical analysis of enzyme inhibition assays
Preparation of tissue homogenate
Fifteen early 3rd instar larvae from the treatments of different batches including control larvae were separated and starved for 2 h to remove digested food particles. The insect whole body was homogenized in 2.5 mL phosphate-buffered saline (0.1 M phosphate-buffered saline, pH 7.6) on ice-cold condition. The homogenate was centrifuged at 4 °C, 10,000 rpm for 30 min; the solid debris and cellular materials were discarded; and the supernatant was collected into a sterile cryovial, placed on ice, and used immediately for assaying acetylcholinesterase (AChE), mixed-function oxidase (MFO), glutathione S-transferase (GST), and esterase assay (EST).

In vitro enzyme inhibition
The protein concentrations of the tissue homogenates were determined by Huang and Hanm (2007) method, and BSA was used as a standard protein. Thirty microliters of tissue homogenate supernatant (control larvae) was incubated with 5 mM of enzyme inhibitors (PBO, DEM, and TPP) and cypermethrin for 30 min at room

temperature. The experiments were performed in triplicates to minimize the error, and the enzyme assays were performed as follows.

Acetylcholinesterase assay

Acetylcholinesterase activity (AChE) was determined according to the method described by Nehare et al. (2010) using acetylthiocholine iodide as substrate. To 100 μL of insect homogenate (with and without inhibitors), 2.4 ml of 100 mM phosphate buffer (pH 7.4), and 0.1 ml mixture 90.075 M of acetylthiocholine-iodide and 0.01 M 5, 50-dithio-bis [2-nitrobenzoicacid] were incubated for 15 min at 27 °C. After incubation, the absorbance was recorded at 412 nm for every 10 s up to 5 min with UV–visible double beam spectrophotometer.

Esterase assay

Esterase activity was determined using alpha-naphthyl acetate as the substrate. The colorimetric formation of the diazo-dye complex with Fast Blue BB Salt was continuously assayed in a kinetic assay as described by Kranthi et al. (2005) with some modifications. Insect homogenate (with and without inhibitors) set up in triplicates, and 5 mL of substrate (10 mg of Fast Blue dissolved in 10 mL of 0.4 M phosphate buffer pH 6.8 plus 100 μL of 0.1 M α-naphthyl in acetone) was added. Change in absorbance was measured at 450 nm at 15-s interval for every 5 min in a time scan mode of UV–Vis double beam spectrophotometer.

Glutathione S-transferase

Quantification of GST was carried out by following the method of Yu and McCord, (2007) with slight modifications. The GST was reduced as glutathione (GSH) by developing a reaction with DCNB conjugate, and it was measured by spectrophotometer. Fifty microliters of 50 mM 2,4-dichloronitrobenzene (DCNB) and 150 μL of reduced glutathione (GSH) along with 2.79 mL phosphate-buffered saline (0.1 M pH 6.0 + 0.1 mM PTU) were added to insect homogenate (with and without inhibitors). The contents were gently shaken and incubated for 2–3 min at 20 °C and then transferred to a cuvette and kept at a sample slot of a UV-Vis spectrophotometer. The reaction mixture (3 mL) without enzyme was placed in the cuvette in the reference slot. Absorbance at 340 nm was recorded for 5–6 min by employing the time scan menu of the spectrophotometer. The GST activity was calculated as CDNB-GSH conjugate (μmole mg protein–1 min – 1) with a 9.6 mM/cm—extinction coefficient.

Mixed function oxidase assay

Mixed function oxidase activity was measured by following the method of Brogdon et al. (1994). The 3,3′,5,5′-tetramethylbenzidine (TMBZ) was used as a substrate.

Five hundred microliters of TMBZ (dissolved in 0.05 M Tris HCL pH – 7.0 containing 1.5% KCl), 250 μL potassium phosphate buffer 0.05 M, and 200 μL of 3% H_2O_2 were added to the insect homogenate (with and without inhibitors). After 5 min of incubation of the reaction mixture, the absorbance was taken at 630 nm in the UV-Visible spectrophotometer. The activity of the enzyme was read and expressed as nmol/min/mg proteins.

In vivo enzyme inhibition

In vivo enzyme inhibition experiments of AChE, esterase, GST, and MFO were carried out as per the output of the in vitro inhibition method (Johnson et al. 2006). The homogenate was taken from cypermethrin treatment with 5.0 mM concentrations of each three inhibitors given 1 h before the cypermethrin treatments. The experiments were performed in triplicates to minimize the error.

AChE isoenzyme inhibition

Non-denaturing polyacrylamide gel electrophoresis (nPAGE) was performed as per the protocol of Khan et al. (2013) with a discontinuous tris-glycine buffer system in a vertical electrophoresis unit by using a 7% and 4% separating and stacking gel to study the activity of AChE. The homogenate (4 μL contains 0.5 mg of protein) was mixed with sample loading dye (containing 4 μL of 8 g/500 ml TritonX-100/100 g/l sucrose and a pinch of bromophenol, blue) and loaded on to the gel. Electrophoresis was carried out at a constant voltage of 150 V in an ice-cold condition for 1 h.

For in vitro AChE inhibition, the gel was incubated in a 65-mL sodium phosphate buffer (0.1 M; pH 6.0) with 5 mM PBO inhibitor solution for 10 min at room temperature before staining. The gel was stained with 50 mg acetyl thiocholine iodide, 5 mL of 0.1 M sodium citrate, 10 mL of 30 mM copper sulphate, 10 mL of double-distilled water, and 10 mL of 5 mM potassium ferricyanide for AChE activity. The gel was placed in the destaining chamber with 7% acetic acid solution. The gel was visualized and photographed in the gel documentation system.

Statistical analysis

The mortality for cypermethrin against *A. albistriga* larvae was corrected using Abbott's formula (Abbott 1925), if the mortality in the control was more than 5%. Further, the data were subjected to probit analysis using SPSS software (Version 10.0 for windows, SPSS Inc., Chicago, USA) to determine median lethal concentrations (LC_{50}). The enzyme activities were performed in triplicate. Standard deviations (SD) were calculated using the GraphPad Prism version 16.0 for Windows (GraphPad Software), and data were expressed as a mean of

replicates ± SD. Significant differences between enzyme activity were analyzed by using Tukey's multiple comparison test at $p < 0.001$.

Results

Bioassay

The bioassay reports of the present study conclude that cypermethrin in combination with synergist was more toxic as compared to cypermethrin alone. The detailed toxicity effect of synergist mixed cypermethrin was tabulated in Table 1. LC_{50} value of cypermethrin alone was 63.326 ppm, whereas the combination of cypermethrin with synergists like PBO and TPP shows decrease LC_{50} value (Cyp + PBO = 12.039 ppm and Cyp + TPP = 13.234 ppm) and synergist DEM with cypermethrin (Cyp + DEM = 43.716 ppm) (Table 1).

In vitro and in vivo enzyme inhibition

The in vitro and in vivo inhibition of the present study shows that enhanced inhibition was noticed on AChE by cypermethrin + PBO treatment as 0.03 μmole/min/mg of protein; 0.015 μmole/min/mg of protein ($p < 0.01$) than cypermethrin and control (Fig. 1).

The activity of esterase and MFO enzymes is not significantly affected by the synergistic combination (Figs. 2 and 3). It might be the inefficiency of the synergists along with cypermethrin on the metabolic activity of *A. albistriga* due to the absence of suitable active binding sites on esterase and MFO enzymes for synergist. GST activity was less significantly inhibited by cyp + DEM combination 0.05 and 0.01 μmole/min/mg of protein (Fig. 4) with $p < 0.05$.

AChE isoenzyme inhibition

In vitro inhibition of AChE with cypermethrin on nPAGE shows better enzyme activity in lane 2 than 5 mM PBO inhibitor with cypermethrin which shows low activity band lane 3 (Fig. 5). However, the intensity of control ban lane 1 was high compared to other lanes, which confirms the inhibition of AChE activity.

Discussion

In the present study, the LC_{50} toxicity value of cypermethrin alone was 63.326 ppm, whereas the combination of

cypermethrin with synergists like PBO (12.039 ppm) and TPP (13.234 ppm) with low 5 mM concentration itself shows decrease LC_{50} value (than cypermethrin alone and synergist DEM with cypermethrin (Table 1). A similar type of synergistic effect of PBO has been studied in the pyrethroid resistance population of *Spodoptera litura*, fenvalerate resistance *H. armigera*, fipronil resistance *Chilo suppressalis*, and *Choristoneura rosaceana* (Ahmad 2009; Ashraf et al. 2010; Huang et al. 2010). Similarly, the synergistic effect of DEM and TPP on cypermethrin and fenvalerate insecticides has been reported in *Spodoptera litura* (Wu et al. 2007; Yang et al. 2009). A similar result was reported by Ahmad (2009) indicating good synergism by PBO in cypermethrin treatment in two field populations of *S. litura*. Johnson et al. (2006) and Khan et al. (2013) revealed high toxicity of λ-cyhalothrin and cypermethrin with PBO and DEM synergism in *Apis mellifera* and *Musca domestica*. Similarly, PBO and TPP synergism effect on λ-cyhalothrin toxicity were studied against *H. virescens*, *T. urticae*, and *Oligonyches pratensis* (Yang et al. 2001). Most of the study considered PBO at low concentration could influence the insecticidal effect in *Haematobia irritans irritans* (Diptera: Muscidae) (Li et al. 2007). Whereas our study shows high insecticidal activity at high concentrations of PBO tested, supporting our results PBO at high concentration is effective at high doses against whitefly, *B. tabaci*, and *Aleurothrixus floccosus* (Wang 2013). This variation in concentration of PBO may be due to the type of insect species and pesticide used. Moreover, the high concentration of PBO required for improved toxicity of cypermethrin in this study could be because of the nature of the insect's body that requires a high concentration of PBO to allow the penetration of cypermethrin in *A. albistriga*.

Generally, apart from AChE, the enzymes involved in resistance activity in insects like *A. albistriga* are esterase, MFO, and GST. The enhanced inhibition was noticed on AChE as 0.03 μmole/min/mg of protein with the treatment of cypermethrin + PBO than cypermethrin and control (0.015) ($p < 0.01$). Similar kinds of reports are reported by several authors on various insects with different synergist molecules. Espinosa et al. (2005) and Nehare et al. (2010) reported that the high synergism of

Table 1 Toxicity of cypermethrin (Cyp) and synergism with and without enzyme inhibitors PBO, DEM, and TPP in a field population of *Amsacta albistriga* 24 h post treatment

Treatment/larvae	LC_{50} (ppm)	Fudicial limits (95%)	Slope (±SE)	χ^2	df
Cypermethrin (Cyp)	63.326	48.022–78.630	0.41 (± 0.037)	0.099	4
Cyp + 5 mM PBO	12.039	4.360–33.240	0.31 (± 0.136)	0.011	4
Cyp + 5 mM DEM	43.716	33.89–53.53	0.37 (± 0.117)	0.049	4
Cyp + 5 mM TPP	13.234	5.439–32.340	0.30 (± 0.073)	0.143	4

Legend: *LC_{50}* lethal concentration for 50% of larva, *χ^2* chi^2, *df* degree of freedom, *ppm* parts per million, *SE* standard error
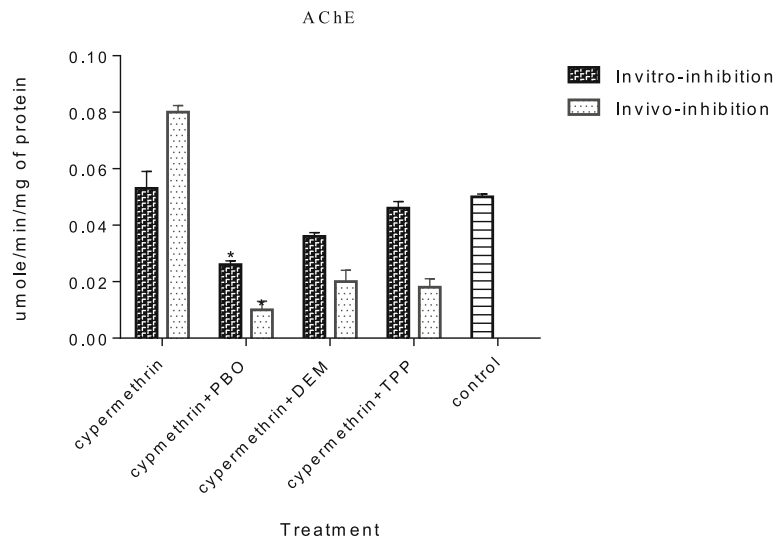
Legend: * indicates significant value *P*<0.01(Tukey's-multiple comparison test)
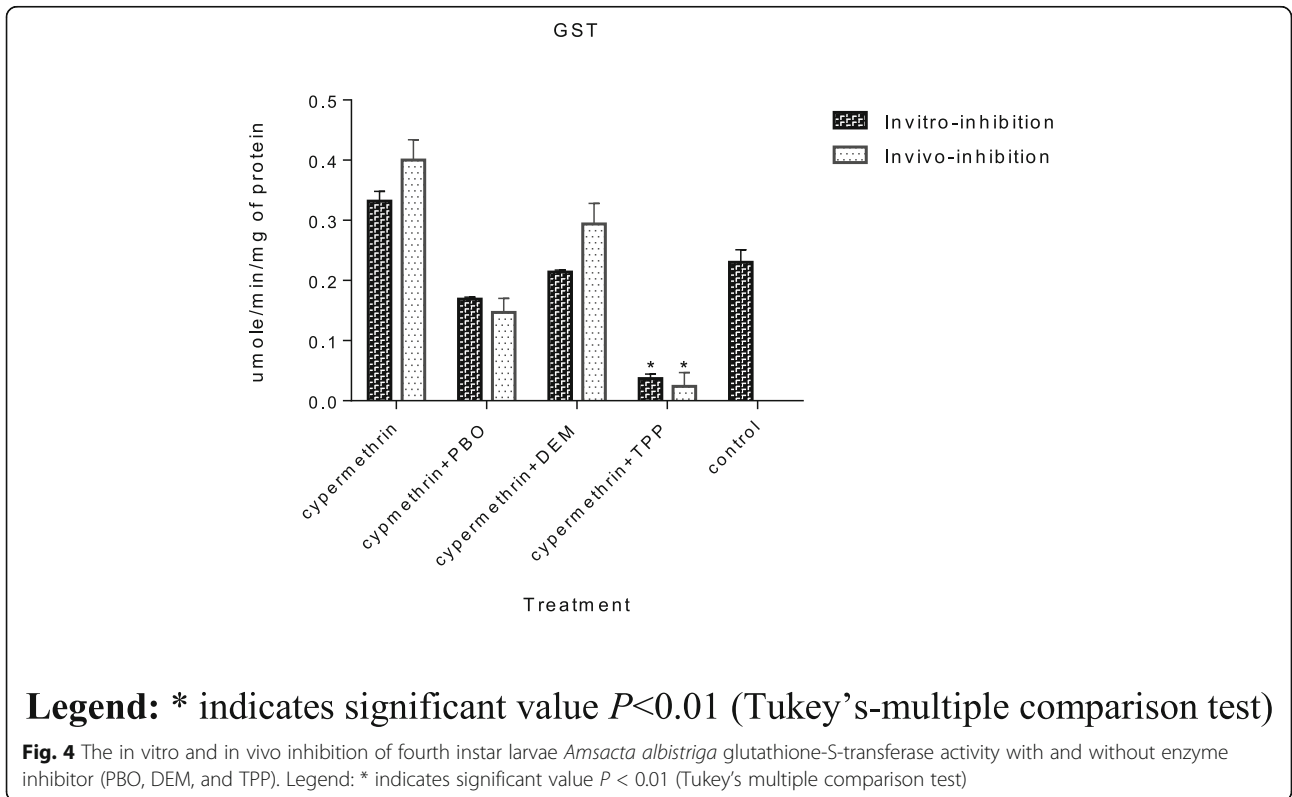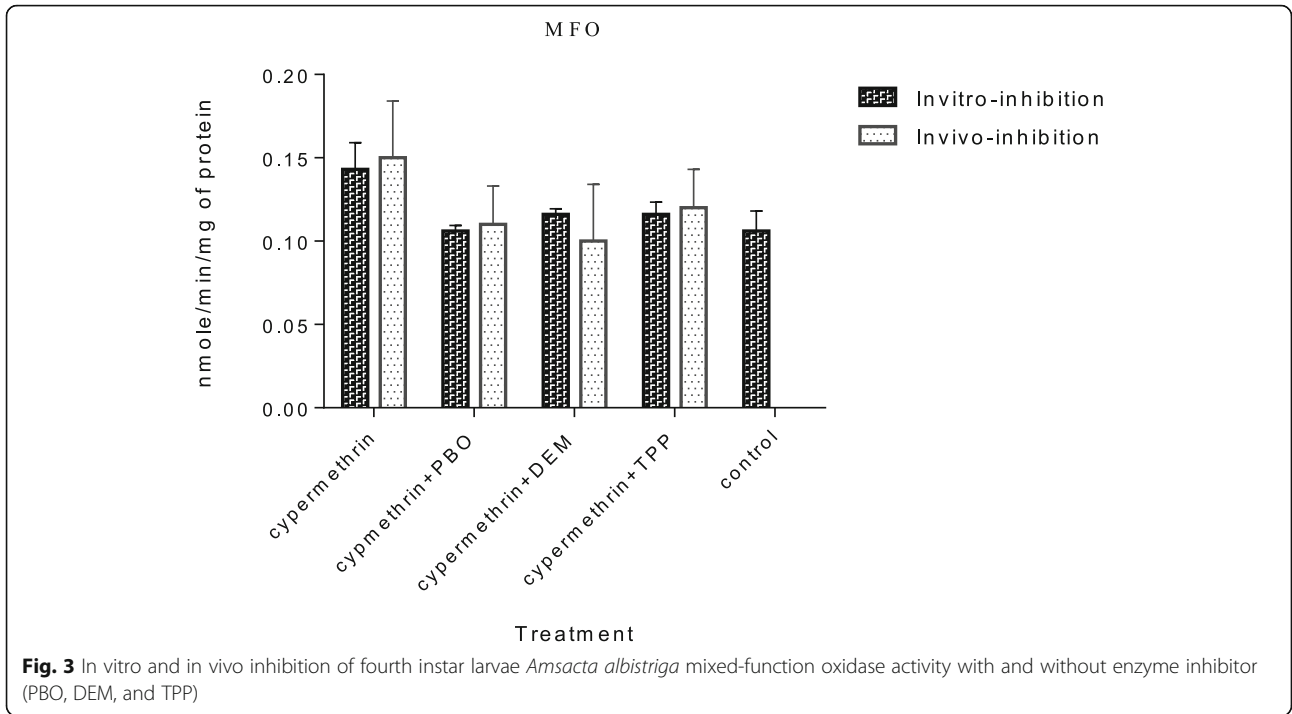
Fig. 1 In vitro and in vivo inhibition of fourth instar larvae *Amsacta albistriga* acetylcholinesterase activity with and without enzyme inhibitor (PBO, DEM, and TPP). Legend: * indicates significant value *P* < 0.01 (Tukey's multiple comparison test)
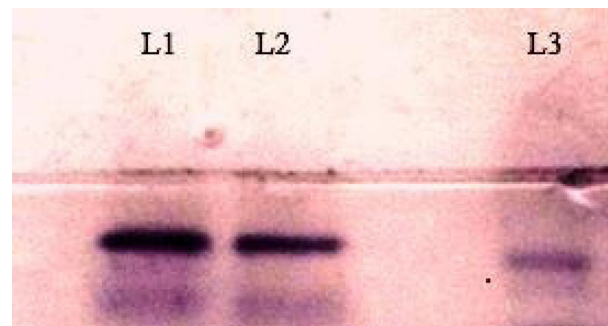
PBO with five insecticides in western flower thrips *Frankliniella occidentalis* by detoxification mediated through cytochrome P450 monooxygenases resistance mechanism in this pest (Gunning 2002). The effect of synergist on pesticide toxicity is studied mostly in the pest Noctuidae insect family. There are no reports on such studies in *A. albistriga*, which belongs to Arctiidae family. This study provides the first report on the synergism of PBO, DEM, and TPP on the toxicity of cypermethrin on *A. albistriga* larvae. It is important to study the combined effect of synergist on cypermethrin toxicity, because it is known that the synergistic effect may vary
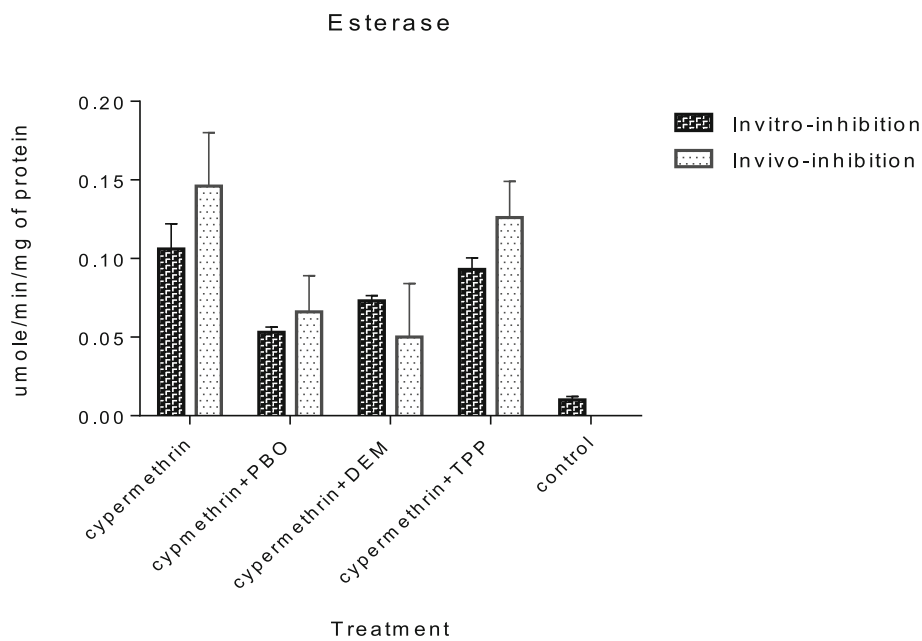
Fig. 2 In vitro and in vivo inhibition of fourth instar larvae *Amsacta albistriga* esterase activity with and without enzyme inhibitor (PBO, DEM, and TPP)

Fig. 3 In vitro and in vivo inhibition of fourth instar larvae *Amsacta albistriga* mixed-function oxidase activity with and without enzyme inhibitor (PBO, DEM, and TPP)

Legend: * indicates significant value *P*<0.01 (Tukey's-multiple comparison test)

Fig. 4 The in vitro and in vivo inhibition of fourth instar larvae *Amsacta albistriga* glutathione-S-transferase activity with and without enzyme inhibitor (PBO, DEM, and TPP). Legend: * indicates significant value $P < 0.01$ (Tukey's multiple comparison test)

Legend: Lane 1: control (without treatment); **Lane 2:** cypermethrin treatment; **Lane 3:** PBO inhibitor (5mM)

Fig. 5 In vitro inhibition of AChE isoenzyme, resolved by native polyacrylamide gel electrophoresis (PAGE), to 5 mM concentrations of PBO, before staining with acetyl thiocholine iodide (ATC) substrate. Legend: Lane 1: control (without treatment); Lane 2: cypermethrin treatment; Lane 3: PBO inhibitor (5 mM)

depending upon the insect species and type of synergists used (Wu et al. 2007; Young et al. 2006).

The activity of other enzymes namely esterase and MFO is not significantly affected by synergistic combination. It might be the inadequacy of the synergists along with cypermethrin on the metabolic activity of *A. albistriga* due to the absence of suitable active binding sites on esterase and MFO enzymes for synergist. Similarly, Wang et al. (2013) reported that the sublethal doses of (LD_{20}) of λ-cypermethrin together with synergist could decrease the carboxylesterase activity within 24 h. Significantly less GST activity was inhibited by cyp + DEM combination with $p < 0.01$. This shows the consistency of our results in both in vitro and in vivo studies. Nehare et al. (2010) reported DEM suppression of GST in indoxacarb resistant *P. xylostella*. Sheikh et al. (2012) reported that GST-mediated detoxification in cypermethrin resistance in a field population of *Spodoptera littoralis*. Most reports considered PBO as inhibitors of MFO; it has a range of effects on different living organisms.

The low-intensity band was noticed on in vitro inhibition of AChE experiment with cypermethrin and 5 mM of PBO on PAGE than pesticide alone. It confirms the inhibition of AChE of *A. albistriga*. This could be because of the effective binding of PBO with cypermethrin on the active site of the AChE enzyme. Similarly, AChE inhibition by methomyl insecticide revealed alteration in target site sensitivity in *H. armigera* (Gunning 2006). Zewen et al. (2003) reported imidacloprid resistance in a field population of brown planthopper *Nilaparvata lugens*, revealed by PBO synergism, suggests that target site resistance may also involve in MFO-mediated resistance. Wu et al. (2007) found significant inhibition of AChE activity in the field population of *P. xylostella, P. striolata, D. rapae*, and *C. plutellae*.

Conclusion

The emerging insecticide resistance among the insects for various insecticides or pesticides leads to difficult tasks in pest management in the agriculture field. Hence, finding a suitable synergist compound to enhance the toxicity of pesticides on insects is the need of the hour. The overall findings of the present study revealed that the suitable enzyme inhibitors such as PBO (12.039 ppm) followed by TPP (13.234 ppm) act as a good synergist molecule with cypermethrin insecticide against various enzymes such as AChE, EST, GST, and MFO of *A. albistriga*. Further, the data obtained in this study provide useful information for resistance monitoring, design, and develop an insecticide management strategy for control of *A. albistriga* in the field.

Availability of data and materials
The detailed methodology and analytical data of the present findings are available from the corresponding author on reasonable request.
The methodology and result details of the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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