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ARTICLE

Evaluation of flubendiamide-induced mitochondrial dysfunction and metabolic changes in *Helicoverpa armigera* (Hubner)

Bharat Nareshkumar[1] | **Shaik Mohammad Akbar**[2] | **Hari Chand Sharma**[2] | **Senigala K. Jayalakshmi**[3] | **Kuruba Sreeramulu**[1]

[1]Department of Biochemistry, Gulbarga University, Gulbarga, Karnataka, India

[2]Entomology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana State, India

[3]Department of Plant Pathology, Agriculture Research Station (University of Agricultural Sciences-Raichur), Gulbarga, Karnataka, India

Correspondence
Dr. Kuruba Sreeramulu, Department of Biochemistry, Gulbarga University, Kalaburagi-585 106, India.
Email: ksramu@rediffmail.com

Abstract

Phthalic acid diamide insecticides are the most effective insecticides used against most of the lepidopteran pests including *Helicoverpa armigera*, a polyphagous pest posing threat to several crops worldwide. The present studies were undertaken to understand different target sites and their interaction with insect ryanodine receptors (RyR). Bioassays indicated that flubendiamide inhibited the larval growth in dose-dependent manner with LD_{50} value of 0.72 μM, and at 0.8 μM larval growth decreased by about 88%. Flubendiamide accelerated the Ca^{2+}-ATPase activity in dose-dependent trend, and at 0.8 μM, the activity was increased by 77.47%. Flubendiamide impedes mitochondrial function by interfering with complex I and F_0F_1-ATPase activity, and at 0.8 μM the inhibition was found to be about 92% and 50%, respectively. In vitro incubation of larval mitochondria with flubendiamide induced the efflux of cytochrome c, indicating the mitochondrial toxicity of the insecticide. Flubendiamide inhibited lactate dehydrogenase and the accumulation of H_2O_2, thereby preventing the cells from lipid peroxidation compared to control larvae. At 0.8 μM the LDH, H_2O_2 content and lipid peroxidation was inhibited by 98.44, 70.81, and 70.81%, respectively. Cytochrome P450, general esterases, AChE, and antioxidant enzymes (catalase and superoxide dismutase) exhibited a dose-dependent increasing trend, whereas alkaline phosphatase and the midgut proteases, except amino peptidase, exhibited dose-dependent inhibition in insecticide-fed larvae. The results

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suggest that flubendiamide induced the harmful effects on the growth and development of *H. armigera* larvae by inducing mitochondrial dysfunction and inhibition of midgut proteases, along with its interaction with RyR.

KEYWORDS
cytochrome c, flubendiamide, *Helicoverpa armigera*, mitochondrial dysfunction, proteinases

1 | INTRODUCTION

Helicoverpa armigera (Noctuidae: Lepidoptera), commonly known as cotton bollworm/legume pod borer, is a devastating polyphagous pest and a major constraint to crop production in Asia, Africa, Australia, and Mediterranean Europe for the last four to five decades (Han et al., 2012; Sharma, 2005). It represents a challenge for agricultural production worldwide because of its feeding on more than 200 different species of economically important crops, such as soybeans, cotton, sorghum, corn, sunflower, peanuts, chickpea, pigeon pea, groundnut, maize, and vegetables like beans, tomatoes, peppers, and even forest trees, causing an average of $10,000 million annual loss globally (Akbar, Sharma, Jayalakshmi, & Sreeramulu, 2012b; Ameta & Bunker, 2007; Carneiro et al., 2014). Most of the lepidopteran insects including *H. armigera* have developed resistance against organophosphates (OPs), carbamates, and pyrethroids (Akbar, Aurade, Sharma, & Sreeramulu, 2014; Armes, Jadhav, and DeSouza, 1996; Srinivas, Udikeri, Jayalaxmi, & Sreeramulu, 2004). Since it has developed resistance to almost all class of insecticides, a new class of insecticide with different mode of action has emerged. One among such insecticide group is pthalic acid diamide insecticide.

Flubendiamide (N^2-[1,1-dimethyl-2-methyl sulphonyl ethyl]-3-iodo-N^1-2-methyl-4-{1,2,2,2-tetrafluro-1-(trifluromethyl) ethyl} phenyl), a phthalic acid diamide insecticide, belongs to benzene dicarboxamide group of insecticides, which has action on the insect ryanodine receptors (RyR) leading to calcium release resulting in uncoordinated muscular contraction (Tohnishi, Nishimatsu, Motoba, Hirooka, & Seo, 2010). The minimal effect of this compound on the beneficial arthropods and natural enemies made it valuable in integrated pest management (IPM) programs (Tohnishi et al., 2010). The field evaluation of flubendiamide on *H. armigera* showed potent antifeedent and larvicidal action with no significant effect on natural enemies like *Coccinella* and *Chrysoper lacarnea* (Ameta &Bunker, 2007). Insecticides induce oxidative stress leading to the cellular toxicity and tissue damage (Agrawal & Sharma, 2010). Mitochondria are more susceptible to pharmacological and pesticides compounds and are the best source to study biomembrane interactions (Chakraborty, Chakraborty, Raha, Mandal, & Sarkar, 2007). Studies were carried out to evaluate the effects of methyl parathion (OP) and carbofuran (carbamate) on *H. armigera* mitochondrial bioenergetics (Akbar, Sharma, Jayalakshmi, & Sreeramulu, 2012a; Carneiro et al., 2014; Srinivas et al., 2004). Pyrethroids were found to induce mitochondrial dysfunction in rat liver mitochondria (Gassner, Wüthrich, Schooltysik, & Solioz, 1997; Kamboj, Kumar, Kamboj, & Sandhir, 2008; Kumar et al., 2015; Mehta, Verma, & Srivastava, 2009).

The phthalic acid diamide insecticides interact with RyR and modulate the Ca^{2+} channel, thereby inducing release of Ca^{2+} during muscle contraction (Ebbinghaus-Kintscher et al., 2006; Masaki et al., 2006; O-Uchi et al., 2013). Mitochondrial Ca^{2+} release is a key tool to access various cellular functions including energy metabolism, reactive oxygen species (ROS) generation, cell growth, and apoptosis (O-Uchi et al., 2013). The contradictory observation with Ca^{2+} is its ability to stimulate ATP synthesis and mitochondrial dysfunction as a result of loss of cellular Ca^{2+} homeostasis (O-Uchi et al., 2013). Flubendiamide also has larvicidal action as a stomach poison and an oral intoxicant (Takkar, Sahoo, Singh, Battu, & Singh, 2012). Insecticides interfering with mitochondrial respiration induce generation of ROS such as free radicals (FR), hydrogen peroxides (H_2O_2) and peroxynitrite anions which interact with the cellular organic matters such as membranes, lipids and proteins, and nucleic acids leading to the oxidative damage. The FR/ROS

scavenging enzymes, catalase (CAT), and superoxide dismutase (SOD) terminate the oxidation followed by chain of reactions leading to apoptosis in cells by indigenous antioxidant defense system (Agrawal & Sharma, 2010). Synergistic role of detoxifying enzymes against OPs and other group of insecticides has been reported earlier (Akbar et al., 2014; Han et al., 2012; Srinivas et al., 2004). However, little information is available on the effect of flubendiamide in insects. The present studies were undertaken to evaluate the different target sites of flubendiamide in *H. armigera* other than RyR in insect muscle cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Flubendiamide was kindly provided by Rallis India Limited, Bangalore, India. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), azocasein, bovine serum albumin (BSA), cytochrome c, dichlorophenol indophenol (DCPIP), decylubiquinol, *N* α-benzoyl-DL-arginine *p*-nitroanilide, *N*-glutaryl-L-phenylalanine *p*-nitroanilide, phenazine methosulfate (PMS), NADH, NADPH, were purchased from Sigma Aldrich (Mumbai, India). Sucrose was procured from Qualigens (Mumbai, India). All other chemicals used were commercial products and of analytical grade of highest purity available.

2.2 | Insects

The *H. armigera* larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions at 27 ± 1°C, 65 ± 5% RH, and 12 h photoperiod, obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India.

2.3 | Bioassay of flubendiamide

The bioassay was performed by incorporating the insecticide into the artificial diet in different concentrations (0–0.8 μM). The initial weights of third-instar *H. armigera* larvae were recorded before releasing them into the artificial diets. There were three replications for each treatment in completely randomized design, and 10 larvae in each replication. The larval weights were recorded 7 days after initiating the experiment (Akbar et al., 2012a).

2.4 | Assay of Ca^{2+}-ATPase activity

Muscle membrane preparation was obtained by dissecting ventral and longitudinal muscle tissues from fifth-sixth instars of *H. armigera* larvae fed on diet containing varying concentrations of flubendiamide (0–0.8 μM) under stereoscopic microscope. Ca^{2+}-ATPase assays were performed according to Masaki et al., (2006). In brief, 1 mg of membrane preparation was suspended in 1 ml of reaction mixture containing 40 μM free Ca^{2+}, 100 mM KCl, 6 mM $MgCl_2$, 0.8 mM of EGTA, and 50 mM Tris/HCl; pH 7.4. The reaction was initiated by adding 1 mM of ATP at 25°C, and the liberated inorganic phosphate was measured colorimetrically.

2.5 | Isolation of mitochondria

H. armigera larvae were fed on diet incorporated with various concentrations of flubendiamide (0–0.8 μM) for 24 h. The insecticide-treated and -untreated larvae were starved for 3 h, washed in distilled water, homogenized in dounce homogenizer under cold conditions in 0.25 M sucrose containing 0.1% defatted BSA. The homogenates were filtered through a moist muslin cloth and the filtrates centrifuged at 1600 × *g* for 10 min at 4°C. The supernatant were re-centrifuged at 8000 × *g* for 10 min to get mitochondrial pellet at 4°C (Akbar et al., 2012a). The mitochondrial pellet obtained was re-suspended in the isolation solution and the protein concentration was determined using BSA as a standard (Lowry, Rosebrough, Farr, & Randal, 1951).

2.6 | Mitochondrial enzyme complex assays

The NADH:ubiquinone oxidoreductase activity (complex I) was measured using ferricyanide as electron acceptor, in which the rate of disappearance of potassium ferricyanide (420 nm) was measured spectrophotometrically. The reaction was started by adding 0.02 mM NADH to mitochondrial protein (0.44 mg) in a reaction mixture comprising of KCN (0.25 M), $MgCl_2$ (0.005 M), and potassium ferricyanide (0.001 M) in sodium phosphate buffer (50 mM); pH 7.2 (Akbar et al., 2012a). NADH:ferricyanide reductase (complex II) activity was detected by measuring the rate of reduction of 0.1 mM 2,6-dichloro phenol indophenol (DCPIP) at 600 nm. Enzyme activity was determined using 5 mM succinate as substrate, along with 1 mM PMS in the same reaction mixture used to assay complex I (Akbar et al., 2012a). NADH:Decyclubiquinol/ferricytochrome c oxidoreductase (complex III) activity was measured as the rate of increase in the absorbance of reduced cytochrome c in a reaction mixture containing cytochrome c in 50 mM sodium phosphate buffer, pH 7.2, at 550 nm. The activity was calculated using extinction coefficient 19.1 mM^{-1} cm^{-1} for reduced cytochrome c. Cytochrome c oxidase (complex IV) activity was initiated by adding mitochondrial protein making 1 ml reaction volume containing 10 mM reduced cytochrome c in 50 mM phosphate buffer, pH 7.2, and the oxidation of cytochrome c was measured at 550 nm (Akbar et al., 2012a; Gassner et al., 1997). The mitochondrial F_0F_1-ATPase activity was determined colorimetrically at 660 nm by quantifying inorganic phosphate released from ATP in 50 mM Tris–HCl buffer, pH 7.4 (Baginski, Foa, & Zak, 1967).

2.7 | Estimation of cytochrome c content

The mitochondria isolated from third instar larvae were incubated for 1 h with 50 mM phosphate buffer, pH 7.2, containing varying concentrations of flubendimide in presence of 100 μM Ca^{2+}. The mixtures were centrifuged at 8000 × *g* for 10 min at 4 °C and cytochrome c content was measured in the supernatant (Akbar et al., 2014). Mitochondria incubated with Triton X-100 for the release of total cytochrome content served as positive control (Chamberline, 2007).

2.8 | *In vivo* effect of flubendiamide on the oxidative stress in *H. armigera*

The third instar larva fed with insecticide for 24 h was homogenized in 100 mM Tris–HCl buffer (pH 7.8) containing 5 mM EDTA. The homogenates were centrifuged at 8000 × *g* for 30 min (Freitas et al., 2007), the supernatant was used for measuring oxidative stress parameters as follows.

2.8.1 | Lipid peroxidation

Lipid peroxidation was assayed by measuring the amount of malondialdehyde (MDA) formed by the reaction with thiobarbituric acid forming pink colored complex which is measured at 532 nm. The nonspecific absorbance was subtracted by measuring the absorbance at 600 nm. The amount of MDA was calculated using 1.56×10^5 M^{-1} cm^{-1} as extinction coefficient and the activity was expressed as micromoles of MDA per milligram of protein (Wills, 1966).

2.8.2 | Lactate dehydrogenase assay

Lactate dehydrogenase activity was measured in larval homogenates by measuring the decrease in NADH content at 340 nm. In brief, 0.20–0.35 mg of enzyme protein was dissolved in 1 ml of reaction mixture containing 5 mM sodium pyruvate in 10 mM Tris–HCl buffer, pH 7.8. The reaction was initiated by adding 0.26 mM NADH. The enzyme activity was expressed as mM min^{-1} mg^{-1} of protein (Crabtree & Newsholme, 1972).

2.8.3 | Quantification of H_2O_2 content

Insecticide-fed larvae were homogenized in 0.1% trichloro acetic acid (TCA), centrifuged at 8000 × *g* for 15 min. From the homogenate, 0.5 ml of the supernatant was mixed with 0.5 ml of 50 mM phosphate buffer, pH 7.2, and 1 ml potassium iodide (1 M). The change in absorbance was measured spectrophotometrically at 390 nm. The H_2O_2 content was

determined using extinction coefficient 0.28 μM^{-1} cm^{-1} and expressed as micromoles of H_2O_2 per milligram of protein according to Noreen and Ashraf (2009).

2.9 | Antioxidant enzymes assay

CAT activity was measured spectrophotometrically as the rate of decomposition of H_2O_2 at 240 nm and the activity was calculated using extinction coefficient, 43.6 M^{-1} cm^{-1}. One unit of CAT activity was equivalent to 1 μM of H_2O_2 decomposed per minute per milligram of protein (Olgun & Misra, 2006). The SOD activity was measured as reduction in the rate of inhibition of NBT with the superoxide ions (McCord & Fridovich, 1969).

2.10 | Effect of flubendiamide on detoxifying enzymes

Glutathione S-transferase was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate where change in the concentration of conjugated product 5-(2,4-dinitrophenyl)-glutathione was measured at 340 nm (Habig, Pabst, & Jakoby, 1974). Activities of acetylcholinesterase and non-specific esterase were measured in insecticide-fed larvae according to Han et al. (2012), whereas cytochrome P450 was measured as per Rose, Barbhaiya, Roe, Rock, and Hodgson (1995).

2.11 | Alkaline phosphatase assay

Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate where change in the absorbance was measured at 405 nm (Dean, 2002).

2.12 | Effect of flubendiamide on gut proteinases

Trypsin and chymotrypsin were measured spectrophotometrically using *N*-α-benzoyl-DL-arginine-*p*-nitroanilide and *N*-glutaryl-L-phenylalanine *p*-nitroanilide as substrates, respectively (Lomate & Hivrale, 2013; Vinod, Sharma, & Kachole, 2010). Aminopeptidase and elastase were measured by incubating the gut extract with leucine *p*-nitroanilide and *N*-succinyl-alanine-alanine-alanine *p*-nitroanilide substrates, respectively (Li et al., 2004; Visweshwar, Sharma, Akbar, & Sreeramulu, 2015). Change in absorbance was measured at 410 nm. One unit of enzyme activity was expressed as μM of *p*-nitroanilide (pNA) liberated min^{-1} ml^{-1} of enzyme (ε for pNA is 8.8 mM^{-1} cm^{-1}). Total protease activity was measured using 1% azocasein as substrate (Visweshwar et al., 2015).

2.13 | Statistical analysis

Data were subjected to One-way Analysis of Variance (ANOVA) using Genstat (14th edition, Version 14.1.0.5943, VSN International Ltd., United Kingdom) software to judge the significance of differences between the treatments by *F*-test, while the treatment means were compared by least significant difference (LSD) at $P < 0.05$. Ducan's Multiple Range Test (DMRT) was used to know the differences between treatments. The LD_{50} values were determined by using EPA PROBIT analysis program (Version 1.5).

3 | RESULTS

3.1 | Bioassay of insecticides

Flubendiamide inhibited the growth of *H. armigera* larvae in a dose-dependent manner. At 0.8 μM concentration there was ~73.7% larval growth inhibition. LD_{50} value for the insecticide was found to be 0.72 μM (95% confidence limit) (Figure 1A).

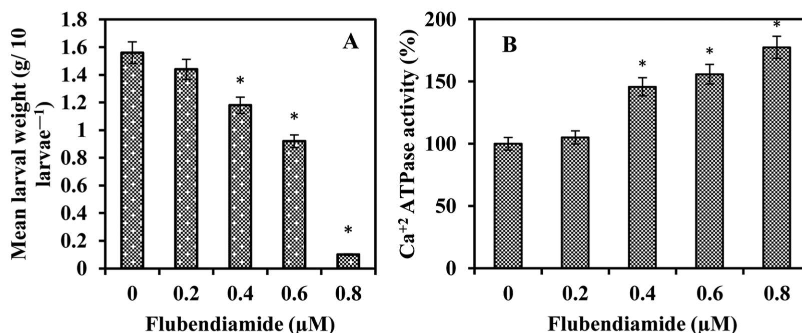

FIGURE 1 (A) Bioassay for flubendiamide. Third instar larvae were fed on an artificial diet containing varying concentrations of flubendiamide (0–0.8 μM); (B) Effect of flubendiamide on Ca^{2+}-ATPase activity. Activity of Ca^{2+} transporter was measured in insectiicde-fed larvae as described in text. The data represents the mean ± SD ($n = 3$) (*significantly different from control at $P < 0.05$)

3.2 | Influence of flubendiamide on Ca^{2+}-ATPase

The Ca^{2+}-ATPase activity increased in dose-dependent manner in *H. armigera* larvae fed on diet incorporated with flubendiamide. At 0.8 μM, Ca^{2+}-ATPase activity was increased by 77% (Figure 1B).

3.3 | In vivo effect of flubendiamide on the mitochondrial enzyme complexes

Flubendiamide exhibited significant inhibition on NADH:ubiquinone oxidoreductase (complex I) and F_0F_1-ATPase activity, in a dose-dependent manner, in vivo. At 0.8 μM, flubendiamide inhibited about 92 and 50% of complex I and F_0F_1-ATPase, respectively (Figure 2A). Complex III activity was reduced by 22% in insecticide-fed larvae (at 0.8 μM) as compared to the control larvae (11.8 ± 0.05 μM min^{-1} mg^{-1}). Complex II (0.020 ± 0.009 μM min^{-1} mg^{-1}) and IV (4.23 ± 0.327 μM min^{-1}mg^{-1}) were unaffected in insecticide-fed larvae (Figure 2B). However, complex II and complex III were overstimulated at lower concentrations (< 0.6 μM) but inhibited at higher concentrations (> 0.8 μM) (Figure 2B).

3.4 | Cytochrome c release

Increasing concentration of flubendiamide had significant effect on the efflux of cytochrome c of 0.87–2.83 nM mg^{-1} of protein, in mitochondria isolated from *H. armigera* larvae. However, the total cytochrome c content measured by incubating Triton X-100 was found to be 3.64 ± 0.0223 nM mg^{-1} (Figure 3a).

3.5 | Effect of flubendiamide on oxidative stress in *H. armigera*

Significant dose-dependent inhibition was observed for lipid peroxidation, lactate dehydrogenase, and H_2O_2 content. The inhibition was measured to be 40.20–67.18, 74.35–98.45, and 34.37–70.82% for lipid peroxidation, lactate dehydrogenase, and H_2O_2 content, respectively (Figures 3B and 3C).

3.6 | Antioxidant enzymes

The catalase and SOD activities were increased in a dose-dependent manner in flubendiamide-fed larvae. At 0.8 μM, the catalase activity was increased to 20.41 ± 2.08 U mg^{-1} when compared to control (12.25 ± 0.61 U mg^{-1}) and SOD activity was enhanced to 0.121 ± 0.001 U mg^{-1} when compared to control (0.065 ± 0.0032 U mg^{-1}) (Figure 3D).

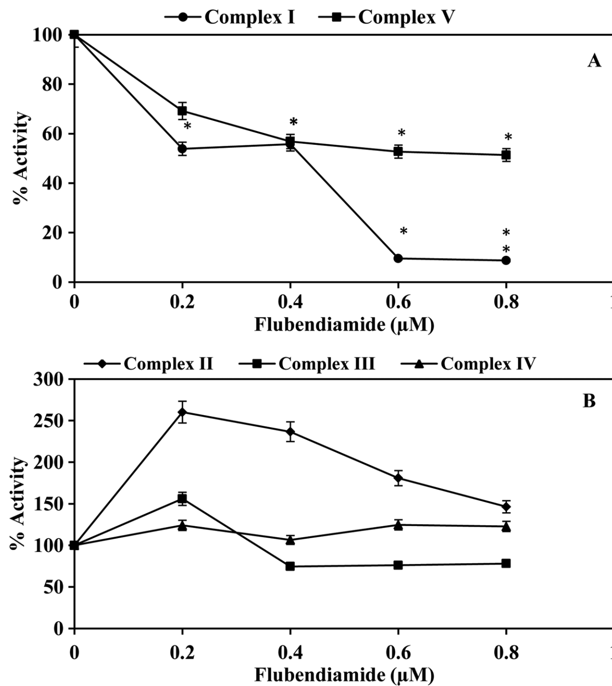

FIGURE 2 (A) Effect of flubendiamide on NADH:ubiquinone oxidoreductase and F_0F_1-ATPase activity; (B) NADH:ferricyanide reductase, NADH:decyclubiquinol/ferricytochrome c oxidoreductase and cytochrome c oxidase in mitochondria isolated from *H. armigera*. Enzyme activities were assessed as described in text. The data points represent the mean ± S.D. ($n = 3$) (* significantly different from control, $P < 0.05$)

3.7 | Detoxification enzymes

Cytochrome P450 (0.600 ± 0.012 U mg^{-1}) and AChE (323 ± 1.72 U mg^{-1}) significantly increased in larvae fed on diet containing flubendiamide (0.8 μM) compared to control larvae (0.067 ± 0.014 and 122.5 ± 0.0003 U mg^{-1}, respectively). However, non-specific esterase activity remained unaffected in flubendiamide-fed larvae compared to control larvae (Table 1). Significant dose-dependent reduction was observed for alkaline phosphatase activity in the larvae fed on diet containing increased concentration of flubendiamide (0–0.8 μM). The decline was measured to be 39.67 ± 0.003–4.427 ± 0.0036 μM min^{-1} mg^{-1} of protein (Figure 4A).

3.8 | Midgut proteases

Protease profile of larvae fed on diet containing insecticide for up to 3 days exhibited dose-dependent inhibition response when compared to control larvae. Trypsin and total protease was reduced by 2.5-fold, chymotrypsin and elastase activity was inhibited by 9.0- and 11.5-fold in larvae fed on flubendiamide (0.8 μM) containing diet for 3 days, whereas aminopeptidase activity was unaffected (Figure 4B).

4 | DISCUSSION

The *H. armigera* larvae are known for voracious feeding and their ability to adapt to the kind of food available. Flubendiamide is a novel class of insecticide, which exhibits an excellent insecticidal activity toward a broad spectrum of lepidopteran insects. Flubendiamide is a RyR agonist in insects which stimulates Ca^{2+} pump, causing severe muscle contraction, resulting in insect mortality. The Ca^{2+} transport is stoichiometrically coupled to Ca^{2+}-ATPase activity

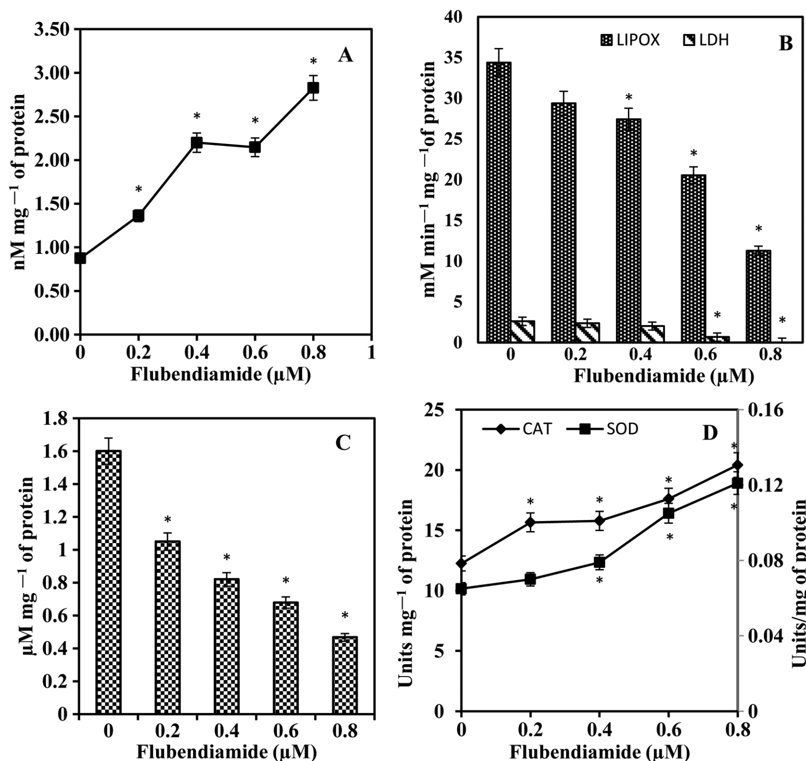

FIGURE 3 (A) Quantification of cytochrome c released. Cytochrome c release was estimated by incubating mitochondria (1 mg of protein) isolated from *H. armigera* larvae in the presence of different concentrations of flubendiamide for 1 h; (B) Dose dependent response of lipid peroxidase (LIPOX) and lactate dehydrogenase (LDH) in *H. armigera* fed on diet containing flubendiamide; (C) Quantification of H_2O_2 content in *H. armigera* fed on diet containing flubendiamide; (D) Dose-dependent response for catalase and superoxide dismutase (SOD) activities in the larvae fed on diet containing flubendiamide. The data points represent the mean ± S.D. ($n = 3$) (*significantly different from control $P < 0.05$)

TABLE 1 Dose dependent response of detoxificative enzymes in *H. armigera* fed on diet containing flubendiamide. The values were represented as mean ± SE ($n = 3$)

Insecticide (μM)	Cytochrome P450 (U mg^{-1} Protein)	AChE (U mg^{-1} Protein)	Esterase (U mg^{-1} Protein)	GST (U mg^{-1} Protein)
0	0.067 ± 0.014a	122.5 ± 0.0003a	2.177 ± 0.043a	66.2 ± 6.89a
0.2	0.157 ± 0.025a	151.56 ± 0.96ab	2.957 ± 0.223b	66.8 ± 8.18a
0.4	0.237 ± 0.026a	228 ± 0.96bc	4.73 ± 0.401c	65.5 ± 2.70a
0.6	0.177 ± 0.027a	323 ± 1.72c	5.86 ± 0.232d	66.1 ± 1.73a
0.8	0.600 ± 0.012b	279.1 ± 0.74c	9.957 ± 0.293e	53.4 ± 6.31a

Column values followed by same letters are not significantly different from each other at $P < 0.05$.

for maintaining balanced levels of intracellular Ca^{2+}. The dose-dependent decline in the body weight observed in *H. armigera* larvae fed on flubendiamide containing diet (correlation coefficient, $r = -0.94$) was due to the cessation of feeding behavior, interaction between the insecticide and RyR as evidenced by increased Ca^{2+} ATPase activity with increase in the insecticide concentration ($r = 0.50$). Interaction of flubendiamide with RyR alters the Ca^{2+} ATPase activity resulting in uncontrolled release of Ca^{2+} which finally leads to the mortality of the larvae (Masaki et al., 2009). LD_{50} value determined by bioassays for the insecticide was found to be 0.72 μM. *H. armigera* although responded to contact stimuli but without integral movements, resulted in thickening and shorting of the body and rapid cessation of feeding

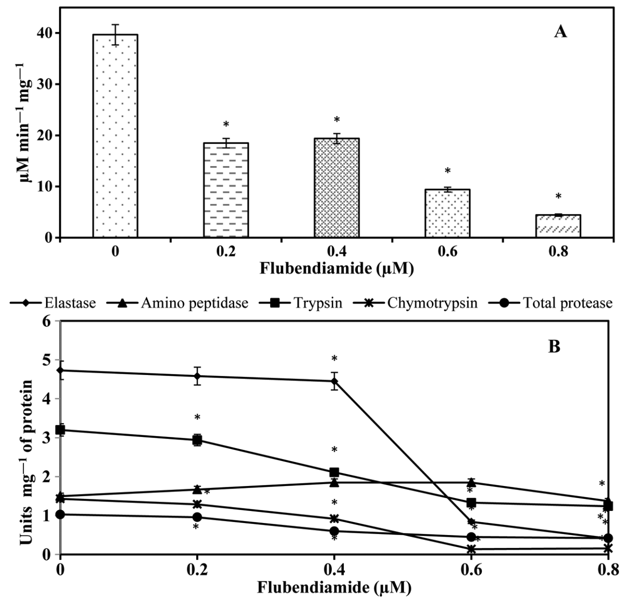

FIGURE 4 (A) Dose dependent response of alkaline phosphatase in *H. armigera* fed on diet containing flubendiamide. The values were represented as the mean ± S.D. ($n = 3$) (*significantly different from control, $P < 0.05$); (B) Dose dependent response of proteases in *H. armigera* fed on diet containing flubendiamide. Dose dependent response of alkaline phosphatase in *H. armigera* fed on diet containing flubendiamide. The activity was calculated as unit (U) per mg of protein and the data points represent the mean ± S.D. ($n = 3$) (least significant difference (LSD) was used to test significances between the means for the treatments and the untreated control at $^*P < 0.05$)

behavior when fed on the insecticide. Similar observations were also reported in *Spodoptera litura* when treated with EC_{50} concentration of flubendiamide (10 nM) (Masaki, 2008).

Flubendiamide inhibited mitochondrial enzyme complex I and F_0F_1-ATPase in vivo exhibiting significant and negative correlation (r = –0.88 to –0.94). Tumor necrosis factor (TNF)-induced inhibition of complex I in adult female rats was due to the interference of the insecticide to the complex resulting in conformational changes (Higuchi, Proske, & Yeh, 1998). Inhibition of F_0F_1-ATPase activity due to phytochemicals was due to dislocation of F_0 and F_1 in myelogenous leukemia cell lines (Zheng & Ramirez, 2000). Inhibition of complex I corresponds to the increased concentration of succinate in Kreb's cycle resulting in rapid electron flow through complexes II, III, and IV to compensate for reduced ATP levels (Higuchi et al., 1998). Complex II and IV were positively but non-significantly correlated with the increasing dose of insecticide (r = 0.032 to 0.631). However, complex III was negatively but non-significantly correlated to the fed insecticide (r = –0.56). Flubendiamide induced the efflux of cytochrome c when incubated with the isolated mitochondria (r = 0.96). Cytochrome c is released due to the inhibition of mitochondrial respiratory chain complex I by TNF in mammalian cell system (Higuchi et al., 1998). Inhibition of enzyme complexes of electron transport chain and efflux of cytochrome c thus indicates the toxicity of insecticide to the mitochondrial respiration.

Most of the OPs, carbamates, pyrethroid insecticides, and some herbicides have reported to induce oxidative stress by the production of ROS which targets cellular biomolecules in arthropods and mammals (Akbar et al., 2012a; Kamboj et al., 2008; Kumar et al., 2015; Mehta et al., 2009). However, in the present study, flubendiamide did not induce oxidative stress in *H. armigera* larvae as evidenced by dose-dependent decline in H_2O_2 content and lipid peroxidation in insecticide-fed larvae (r = –0.96). CAT and SOD were enhanced in insecticide-fed larvae in dose-dependent manner and were positively correlated (r = 0.966 to 0.967). Antioxidant enzymes are potent scavengers of ROS, therefore the increase in CAT and SOD activity has prevented the accumulation of H_2O_2 and other FRs in turn resulting in reduced lipid peroxidation in flubendiamide-fed larvae (r = –0.97). CAT and GSH peroxidase prevent the peroxidation of unsaturated lipids in mitochondrial suspension (Zimmermann, Flohe, Weser, & Hartmann, 1973). The inhibition

of GSH-induced lipid peroxidation by SOD in mitochondrial inner membranes isolated from rat liver has also been reported (Zimmermann et al., 1973). Although LDH is a sensitive parameter for evaluation of tissue damage and cytotoxicity, the enzyme was inhibited in *H. armigera* larvae in dose-dependent manner (r = –0.96). The inhibition in LDH activity might be due to the structural resemblance of flubendiamide with NADH which binds at the active site of the enzyme. Gossypol, having structural resemblance with NADH, was a competitive inhibitor of binding of NADH to LDH (Gómez-Diaz et al., 1997).

Several biochemical defensive mechanisms are involved in detoxification when a biological system is exposed to a toxicant, including cytochrome P450, GST, esterases, and proteases. *H. armigera* larvae exhibited increased levels of cytochrome P450 and general esterases in relation to the concentration of flubendiamide fed (r = 0.83 to 0.95). Activity of GST was unaffected in flubendiamide-fed larvae, which suggests that the enzyme was not involved in the detoxification mechanism. Although cytochrome P450 and general esterases are involved in insecticide tolerance in insects, the increased levels of these enzymes in the present study did not exhibit resistance to flubendiamide, which might be due to the novel mode of action of insecticide, i.e., interaction with RyR. In contrast, a chlorantraniliprole-resistant strain of diamondback moth, *Plutella xylostella* (L.) (48.17-fold) showed cross-resistance to flubendiamide (7.29-fold) and exhibited 4.26 times higher cytochrome P450 activity compared to susceptible strain, with no differences in the levels of GST and esterase (Liu, Wang, Ning, Qiao, & Wang, 2015). Alkaline phosphatase has been involved in insecticide resistance in organophosphate compounds (Srinivas et al., 2004). Levels of alkaline phosphatase are related to the physiological condition of insects, and reflect digestion, absorption, and positive transport of nutrients in the midgut (Miao, 1988). Alkaline phosphatase activity was significantly reduced in flubendiamide-fed larvae (r = –0.97). Similar results were reported in the midgut of NaF-treated silkworms after 48 h of treatment compared to the control (Miao, Jiang, & Bharathi, 2005). Neurotoxic potential of insecticides is judged by evaluating AChE activity (Kaizer et al., 2005). Increased AChE activity with a dose-dependent response to increase in concentration of flubendiamide (r = 0.91) in *H. armigera* thus indicates the overstimulation of neural activity subsequently resulting in insect mortality. Fahmy and Dahi (2009) recorded a significant increase in AChE activity by 4.94 and 18.65% in spinetoram-treated *S. littoralis* larvae of Kalyobia and Behiara, respectively. In contrast, *Drosophila melanogaster* exposed to the flubendiamide exhibited dose-dependent inhibition trend in the activity of AChE (Sarkar, Dutta, & Roy, 2015). Time-dependent response of proteases at LD_{50} concentration (0.72 μM) for 72 h exposure indicated that trypsin, chymotrypsin, elastase, and total protease were significantly inhibited, indicating that flubendiamide induced antifeedant activity through inhibition of proteases.

In conclusion, flubendiamide impedes mitochondrial function by inhibiting enzyme complex I and F_0F_1 ATPase of mitochondrial electron transport chain. The toxic effects of flubendiamide thus imposed on the growth and development of *H. armigera* larvae were however not due to oxidative stress but by inducing mitochondrial dysfunction and inhibition of midgut proteases, apart from its interaction with RyR.

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