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# Imidacloprid impedes mitochondrial function and induces oxidative stress in cotton bollworm, *Helicoverpa armigera* larvae (Hubner: Noctuidae)

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**Abstract**
Neonicotinoids have high agonistic affinity to insect nicotinic acetylcholine receptors (nAChR) and are frequently used as insecticides against most devastating lepidopteran insect pests. Imidacloprid influenced dose-dependent decline in the state III and IV respiration, respiration control index (RCI), and *P/O* ratios, in vitro and in vivo. The bioassay indicated its $LD_{50}$ value to be 531.24 μM. The insecticide exhibited a dose-dependent inhibition on $F_0F_1$-ATPase and complex IV activity. At 600 μM, the insecticide inhibited 83.62 and 27.13% of $F_0F_1$-ATPase and complex IV activity, respectively, and induced the release of 0.26 nmoles/min/mg protein of cytochrome c. A significant dose- and time-dependent increase in oxidative stress was observed; at 600 μM, the insecticide correspondingly induced lipid peroxidation, LDH activity, and accumulation of $H_2O_2$ content by 83.33, 31.51 and 223.66%. The stress was the maximum at 48 h of insecticide treatment (91.58, 35.28, and 189.80%, respectively). In contrast, catalase and superoxide dismutase were reduced in a dose- and time-dependent manner in imidacloprid-fed larvae. The results therefore suggest that imidacloprid impedes mitochondrial function and induces oxidative stress in *H. armigera*, which contributes to reduced growth of the larvae along with its neurotoxic effect.

**Keywords** *H. armigera* · Mitochondria · Oxidative phosphorylation · Oxidative stress · Imidacloprid

## Introduction

Imidacloprid, the first commercial member of a new class of insecticides called neonicotinoids, has high potency and systemic action against piercing-sucking pests. It functions primarily as plant systemic with application to the seed moving to the growing top and offering prolonged protection from sucking pest. Even though neonicotinoids are largely ineffective against lepidopterous insects, crops expressing Bt endotoxin encourage their use as these types of pests not controlled by the endotoxin are often highly sensitive to neonicotinoids (Tomizawa and Casida 2005). The most devastating, polyphagous pest, *Helicoverpa armigera* (Noctuidae: Lepidoptera), commonly known as cotton bollworm/legume pod borer is a major constraint to crop production in Asia, Africa, Australia and Mediterranean Europe for the last four–five decades (Sharma 2005, Han et al. 2012). It represents a threat to agricultural production to 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 420 million (US$) annual loss globally worldwide (Carneiro et al. 2014, Akbar et al. 2012a, Ameta and Bunkner 2007). The field efficacy of the imidacloprid against *H. armigera* in Indian sub-continent found was to be around 97% (Yogeeswarudu and Venkata Krishna,

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2014). Imidacloprid along with other class of insecticides reported to be more effective against *H. armigera* and other lepidopteran insects (Regupathy et al. 2004; Zhu et al. 2015 and 2017).

Neonicotinoid insecticides showed more specific and high agonistic affinity to insect nicotinic acetylcholine receptors (nAChR) compared to vertebrates (Liu and Casida, 1993; Zhang et al. 2000). It has a similar mode of action as nicotine, a natural insecticide, acting as an agonist of nicotinyl acetylcholine receptor (Bai et al. 1991) with additional systemic and contact insecticidal activity (Stoughton et al. 2008, Suchail et al. 2000). It has very high potency and systemic action against homopteran pests and for some species of the order coleoptera, diptera and lepidoptera (Dikshit & Lal 2002). In agriculture, it is used for seed dressing or is directly applied to soil or foliage to control insect pests of corn, cotton, potatoes, rice, vegetables, and fruits (Overmyer et al. 2005; Gupta et al. 2002).

Extensive studies carried out on the effect of imidacloprid on fecundity, reproduction and survivorship of female in *H. armigera* (Ahmad et al. 2013) suggest reduction of the larval population up to 97% under field conditions (Yogeeswarudu and Venkata Krishna, 2014). However, in the present study, we evaluate the effect of the insecticide on the mitochondrial functioning to gain an understanding of different target sites apart from its interaction with nAChR in *H. armigera*.

## Materials and methods

### Chemicals

Bovine serum albumin (BSA), adenosine diphosphate (ADP), adenosine triphosphate (ATP), cytochrome c, dichlorophenol indophenol (DCPIP), decylubiquinol, Ethylenediaminetetraacetic acid (EDTA), phenazinemethosulfate (PMS), NADPH, NADH, trichloroaceticacid (TCA), sodium succinate were purchased from Sigma Aldrich (Mumbai, India). Sucrose was procured from Qualigens (Mumbai, India). Rallis India Limited, Bangalore, India, kindly provided Imidacloprid. All other chemicals used were commercial products and of analytical grade of the highest purity available.

### Insects

Chickpea-based semisynthetic diet was used to rear *H. armigera* larvae under laboratory conditions at 27 ± 1 °C, 65 ± 5% RH, and 12 h photoperiod, at Insect Rearing Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India.

### Bioassay of imidacloprid

The insecticide was incorporated into the artificial diet in different concentrations (0–600 μM) to perform the bioassay. The initial weights of third-instar *H. armigera* larvae were recorded before releasing them in the artificial diet. The larvae fed on diet without insecticide served as control. There were three replications for each treatment in completely randomized design, and 10 larvae in each replication. The larval weights and mortality were recorded 7 days after initiating the experiment (Akbar et al. 2012a).

### Assay of acetylcholine esterase (AChE)

About forty third- to fourth-instar larvae of *H. armigera* fed on the insecticide (400–600 μM) were homogenized in 20 mL phosphate buffer (20 mM, pH 7.4) containing 0.5% Triton X-100. The supernatants collected were filtered through moist muslin cloth, centrifuged at 15000×g for 30 min at 4 °C. The filtrates obtained were used as a source of crude enzyme, and the activity was measured using microplate reader at 412 nm. The total reaction volume of each well was 200 μL, containing 100 μL of enzyme, 10 μL DTNB (50 mM), 10 μL acetylcholine chloride (100 mM) and 80 μL phosphate buffered saline (pH 7.4) (Shang et al. 2007). Wells without the substrate were served as control. The assays were performed in three replicates.

### Isolation of mitochondria

The larvae were fed on a diet containing various concentrations of imidacloprid (400–600 μM) for 24 h. The insecticide-fed and -unfed larvae were starved for 3 h, washed with distilled water and then homogenized in sucrose solution (0.25 M) containing 0.1% defatted BSA. The homogenates were filtered through a moist muslin cloth, and centrifuged at 800×g rpm for 10 min at 4 °C. The supernatants were re-centrifuged for 10 min at 8000×g to obtain mitochondrial pellet (Akbar et al. 2012a). The pellet was re-suspended in the isolation solution and the protein concentration was measured using BSA as a standard according to Lowry's method (Lowry et al. 1951).

### Effect of imidacloprid on the mitochondrial respiration and respiratory enzyme complexes

For in vitro studies, mitochondria were isolated from third instar larvae, incubated with imidacloprid (400–600 μM) for exactly 2 min and oxygen consumption rate was evaluated using oxygraph (Hansatech Instruments Limited, Bachofer, Reutligen 72,734, Germany) fitted with Clark-type oxygen electrode (Akbar et al., 2012a, b). The assay medium contained sucrose (50 mM), KCl (100 mM), $MgCl_2$(5 mM) in HEPES buffer at pH 7.2 (5 mM) and freshly isolated mitochondria in a total reaction volume of 1.5 ml. The rate of state III respiration was measured by adding ADP (100 mM) to the substrate, sodium succinate (10 mM, pre-incubated for 1 min.

With the mitochondrial extract. State IV respiration was measured in the absence of ADP (Akbar et al. 2012a).

To evaluate the in vivo effect of imidaclorid on mitochondrial respiration, the third- and fourth-instar larvae were fed on an artificial diet containing varying concentrations of imidacloprid (400–600 μM) for 24 h. Mitochondria isolated from these larvae were evaluated for mitochondrial respiration and enzyme complex assays.

## Mitochondrial enzyme complex assays

The NADH:ubiquinone oxidoreductase activity (complex I) was measured using ferricyanide as an electron acceptor. The rate of disappearance of potassium ferricyanide at 420 nm was measured spectrophotometrically. The reaction was initiated by treating the mitochondrial extract (0.46 mg protein) with NADH (0.02 mM) in a reaction mixture containing KCN (0.25 M), $MgCl_2$ (0.005 M) and potassium ferricyanide (1 mM) in sodium phosphate buffer (50 mM), pH 7.2 (Akbar et al. 2012a). NADH:ferricyanide reductase (complex II) activity was measured using succinate as a substrate. The enzyme activity was started by adding the mitochondrial extract (0.14 mg) to the reaction mixture containing succinate (5 mM), and phenazine methosulphate (PMS) (1 mM). The rate of reduction of 2, 6-dichlorophenol indophenol (DCPIP) (0.1 mM) at 600 nm was measured spectrophotometrically. NADH:decyclubiquinol/ferricytochrome oxidoreductase (complex III) activity was measured as the rate of increase in the absorbance of reduced cytochrome c at 550 nm in a reaction mixture containing cytochrome c (50 μM) in sodium phosphate buffer (50 mM), pH 7.2, and sodium azide (3 mM). The reaction was started by adding the mitochondrial extract (0.1 mg) and decylubiquinol (30 μM) to the assay mixture. The activity was calculated using extinction coefficient 19.1 $mM^{-1}$ $cm^{-1}$ for reduced cytochrome c (Paradies et al. 2001). Cytochrome c oxidase (complex IV) activity was initiated by adding the mitochondrial extract (0.15 mg) to 1 ml reaction mixture 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 (Gassner et al. 1997; Mehta et al. 2009). 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 et al. 1967).

## Detection of cytochrome c release

Mitochondria (3 mg) isolated from the third-instar larvae were incubated in 50 mM phosphate buffer, pH 7.2, containing varying concentrations of imidacloprid (400–600 μM) for 10 min, in the presence of 100 μM $Ca^{2+}$. The mixtures were centrifuged at 12,000×g for 10 min at 4 °C to obtain the mitochondrial pellet and the supernatants re-centrifuged to eliminate the mitochondrial fragments (Akbar et al. 2012b). The supernatants obtained were used to quantify cytochrome c release spectrophotometrically at 550 nm. Mitochondriawere incubated with 0.01% Triton X-100 in 50 mM potassium phosphate, at pH 7.2 served as control (Chamberlin 2007).

## Effect of imidacloprid on the oxidative stress in *H. armigera*

The third- and fourth-instar larvae fed on an artificial diet containing varying concentrations of imidacloprid (400–600 μM) for 24 h were dissected to remove guts, and homogenized in 50 mM sodium phosphate buffer at pH 7.2. The homogenate was centrifuged at 8000×g for 20 min, and the supernatant served as source to measure the oxidative stress parameters and antioxidant enzymes.

## Lipid peroxidation

Lipid peroxidation was estimated as per Dua and Gill (2001) by estimating the malondialdehyde formed by the reaction with thiobarbituric acid forming pink-colour complex which was measured at 532 nm. The nonspecific absorbance was subtracted by measuring the absorbance at 600 nm. The amount of MDA was calculated using $1.56 \times 10^5$ M/cm as extinction coefficient and was expressed as μmol of MDA/mg of protein.

## Lactate dehydogenase leakage

Lactate dehydrogenase activity was measured in larval homogenates by measuring the decrease in NADH content at 340 nm. In brief, 0.5 mg of enzyme protein was taken in 1 mL of assay mixture containing sodium pyruvate (5 mM) in Tris-HCl buffer (10 mM, pH 7.8). The reaction was started by adding 0.26 mM of NADH. The enzyme activity was expressed as mM/min/mg of protein (Crabtree and Newsholme, 1972).

## Quantification of $H_2O_2$

The $H_2O_2$ content was determined according to Nooren and Ashraf (2009). In brief, insecticide-fed larvae were homogenized in 0.1% trichloroacetic acid (TCA), centrifuged at 8000×g for 15 min. From the homogenates, 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 μmoles of $H_2O_2$ $mg^{-1}$ of protein.

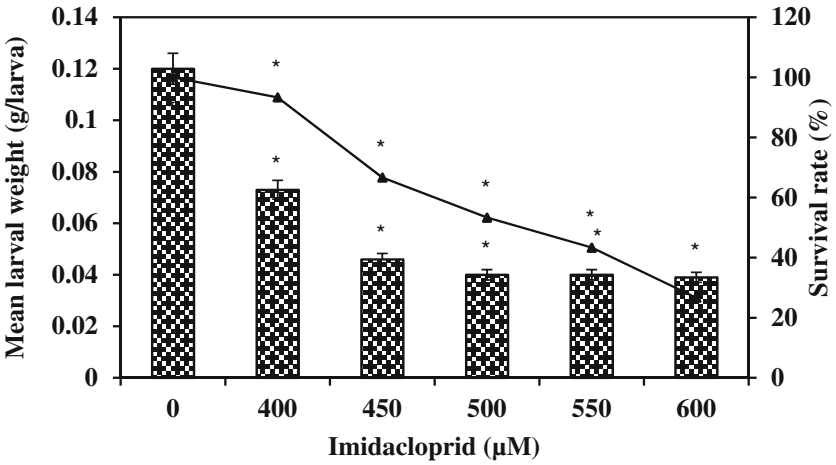


**Fig. 1** Bioassay for imidacloprid. Third-instar larvae were fed on artificial diet containing varying concentrations of imidacloprid (0–600 μM). The larvae fed on diet containing no insecticide served as control. The data represents the mean ± SD ($n = 3$) (significantly different from control at $^*p < 0.05$)

### Antioxidant enzymes assay

The catalase activity was measured spectrophotometrically by monitoring the rate of decomposition of hydrogen peroxide at 240 nm and was calculated using extinction coefficient, 43.6 $M^{-1}$ $cm^{-1}$. One unit of catalase (CAT) activity was equivalent to 1 μM of $H_2O_2$ decomposed per min/mg of protein (Olgun and Misra 2006). The superoxide dismutase (SOD) activity was measured as a reduction in the rate of inhibition of NBT with the superoxide ions (Zhou and Prognon 2006).

### 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 $LD_{50}$ value for the insecticide was determined using EPA PROBIT analysis program (Version 1.5).

## Results

### Bioassay of insecticide

Imidacloprid didnot affected the larval growth at lower concentrations (< 400 μM), but significantly inhibited the growth of *H. armigera* larvae in a dose-dependent manner beyond 400 μM concentration. At 600 μM, there was ~67.5% larval growth inhibition and the survival rate was reduced by 73.33%. The $LD_{50}$ value for the insecticide was found to be 531.24 μM (95% confidence limit) (Fig. 1).

### In-vivo effect of imidacloprid on AChE enzyme

Significant dose- and time-dependent inhibition of AChE was observed in larvae fed on imidacloprid. The insecticide potentially inhibited 80.35% of AChE activity (0.038 μM/min/mg

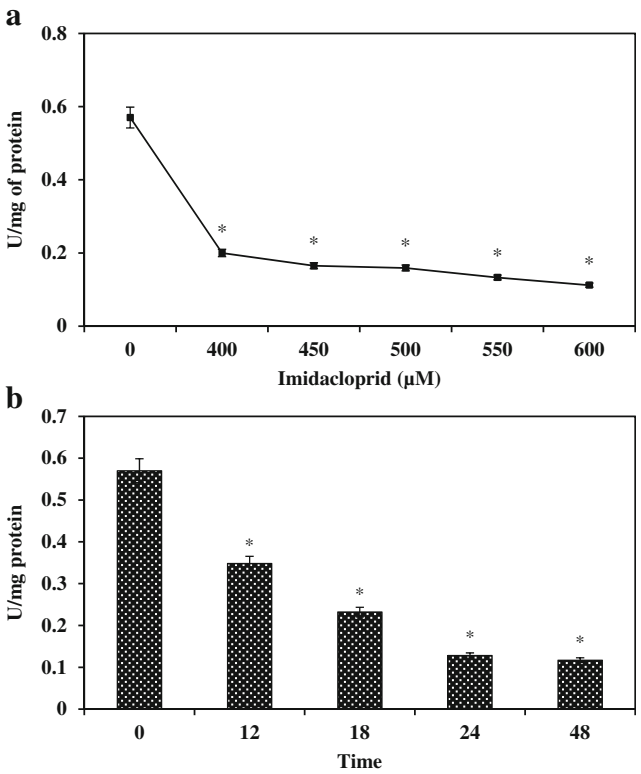


**Fig. 2** Effect of imidacloprid on AChE activity. Conditions for AChE activity are described in Materials and Methods. (**a**) Dose-dependent response of AChE activity in *H. armigera* fed on diet containing varying concentrations of imidacloprid (400–600 μM). (**b**) Time-dependent response of AChE activity in *H. armigera* fed on diet containing $LD_{50}$ concentration (531.24 μM) of imidacloprid. The larvae fed on insecticide-untreated diet served as control. The data points represent the mean ± S.D. ($n = 3$) $^*p < 0.05$)

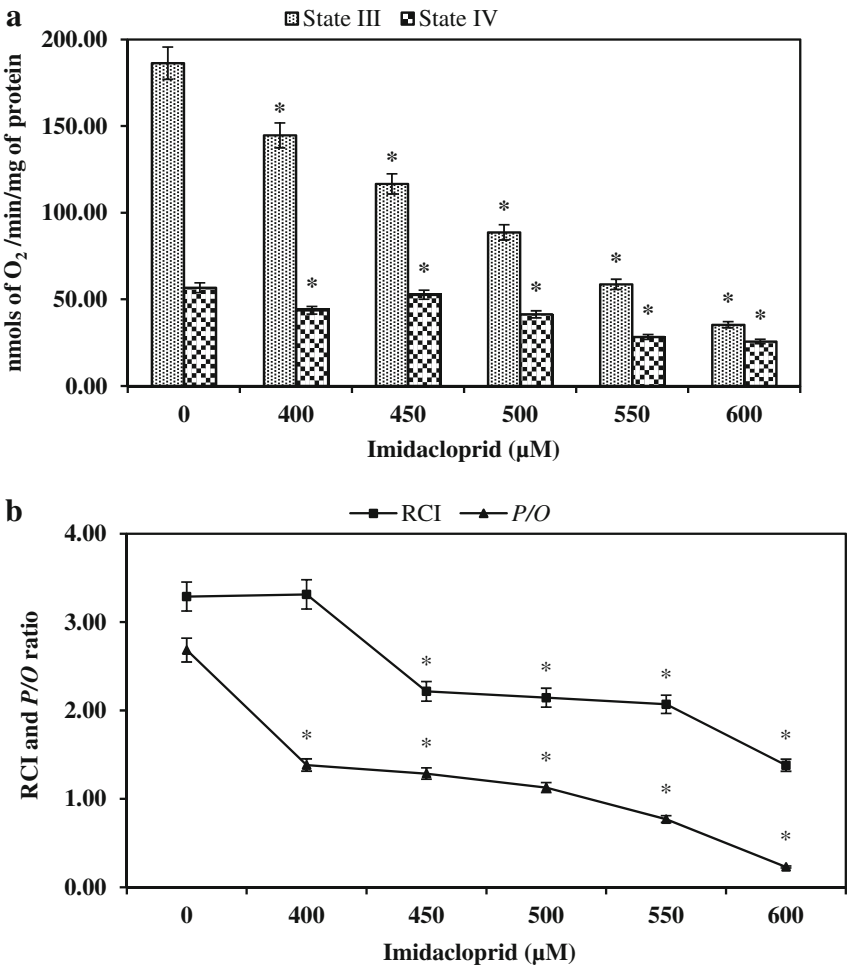


**Fig. 3** In vitro effect of imidacloprid on mitochondrial respiration when succinate was the oxidisable substrate. (**a**) Mitochondrial oxygen uptake in the presence (state III respiration) and absence of ADP (state IV respiration), (**b**) RCI (state III/state IV respiration) and *P/O* ratio (nmoles of ADP added/nmoles of $O_2$ consumed) for the isolated mitochondria at varying concentrations of imidacloprid (400–600 μM). The mitochondria isolated from the insecticide-untreated larvae served as control. The data represents the mean ± S.D. ($n = 3$) (significantly different from control at $^*p < 0.05$)

protein) at 600 μM concentration compared to the enzyme activity in control larvae (0.57 ± 0.001 μM/min/mg protein) (Fig. 2a). At $LD_{50}$ concentration (531.24 μM), the insecticide inhibited 77.54 and 79.47% of enzyme activity at 24 and 48 h, respectively (Fig. 2b).

## In-vitro effect of imidacloprid on mitochondrial respiration

Significant inhibition of state III and IV respiration were observed in a dose-dependent manner. At 600 μM concentration, imidacloprid inhibited 81.04 and 54.70%, respectively. For control larvae, the state III and IV respiration values were found to be 186.33 ± 8.66 and 56.67 ± 5.69 nmoles of $O_2$/min/mg protein, respectively. The respiration control index (RCI) and *P/O* ratios were also inhibited in a dose-dependent manner. The RCI and *P/O* ratio were inhibited by 58.13 and 91.56%, respectively, at 600 μM of imidacloprid. For control mitochondria, the RCI and *P/O* ratio were 3.29 ± 0.68 and 2.68 ± 0.88, respectively, for succinate oxidation (Fig. 3a and b).

## In-vivo effect of imidacloprid on mitochondrial respiration

For control larvae, the state III and IV respiration were found to be 186.33 ± 4.46 and 56.67 ± 0.68 nmoles of $O_2$/min/mg protein, respectively, and the respiration control index (RCI) and *P/O* ratio were 3.29 ± 0.68 and 2.68 ± 0.90, respectively, for succinate oxidation. Imidacloprid significantly inhibited all the respiratory parameters in a dose-dependent manner. At 600 μM concentration, imidacloprid inhibited 72.98 and 31.76% of state III and IV respiration, respectively. The RCI (3.29 ± 0.016) and *P/O* (2.68 ± 0.054) ratio was inhibited by 60.48 and 63.43%, respectively (Fig. 4a and b).

## In-vitro effect of imidacloprid on the mitochondrial enzyme complexes

Complexes I, II and III were unaffected to different concentrations of insecticide, whereas significant reduction was observed in the $F_0F_1$-ATPase and complex IV activities in a dose-dependent manner. At 600 μM, imidacloprid inhibited 91.58% (0.16 ± 0.001 μM/min/mg protein) of $F_0F_1$ ATPase

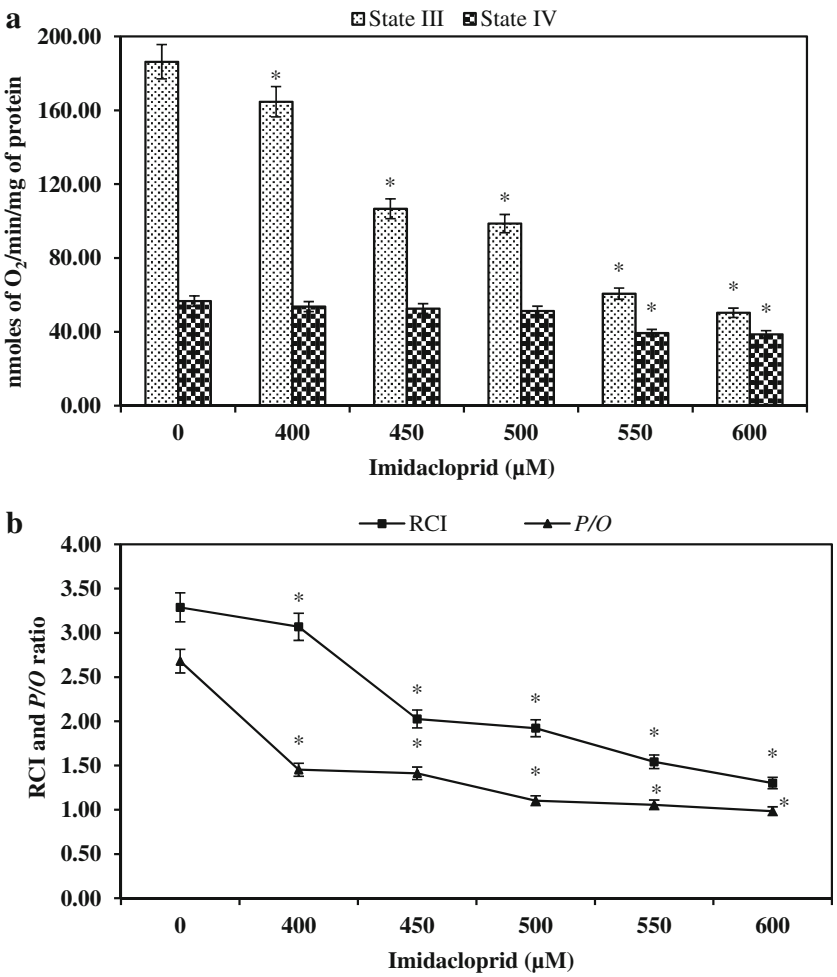


**Fig. 4** In vivo effect of imidacloprid on mitochondrial respiration for the succinate oxidation. (**a**) Oxygen consumption in the presence (state III), and absence of ADP (state IV), (**b**) RCI and *P/O* ratio measured in larvae fed on diet containing 400–600 μM of imidacloprid compared to control. The mitochondria isolated from larvae fed on diet containing no insecticide served as control. The data represents the mean ± S.D. ($n = 3$). (significantly different from control at $^*p < 0.05$)

activity and complex IV by 62.73% (1.06 ± 0.001 μM/min/mg protein) (Fig. 5a).

## In-vivo effect of imidacloprid on the mitochondrial enzyme complexes

Imidacloprid exhibited significant inhibition on $F_0F_1$-ATPase activity in a dose-dependent manner compared to control (1.94 ± 0.0185 μM/min/mg protein). At 600 μM, imidacloprid inhibited 83.62% (0.32 ± 0.0015 μM/min/mg protein) of $F_0F_1$ ATPase activity. The activity of complex IV was reduced by 27.13% at 600 μM compared to the mitochondria isolated from control (3.17 ± 0.0044 μM/min/mg protein) larvae. Complex I showed enhanced activity of 91.9%, whereas complexes II and III showed no significant changes in their activity (Fig. 5b).

## In-vitro release of cytochrome c

Dose-dependent response tothe release of cytochrome c was observed in the mitochondria isolated from *H. armigera* larvae when incubated with imidacloprid. A significant increase in the release of cytochrome c levels was observed at >550 μM concentrations which is above the $LD_{50}$ level. At 600 μM, imidacloprid induced the release of 0.26 nmoles/min/mg of protein of cytochrome c, whereas on incubation with Triton X-100 it was found to be 0.91 ± 0.053 nM/mg of protein (Fig. 6).

## Influence of imidacloprid on oxidative stress

A significant dose- and time-dependent increase in the accumulation of $H_2O_2$ content, lipid peroxidation, and LDH activity was observed in imidacloprid-fed larvae. At 600 μM, the insecticide induced $H_2O_2$ content by 223.66%, compared with the control larvae (0.49 ± 0.0015 μM/mg protein). At 48 h of insecticide treatment, $H_2O_2$ content was increased by 189.80% (Fig. 7a and b). At 600 μM, the insecticide induced lipid peroxidation and LDH leakage by 83.33% and 114.85% compared with the control (0.36 ± 0.041 μM MDA/mg of protein and 0.202 ± 0.057 nmoles/min/mg of protein), respectively.

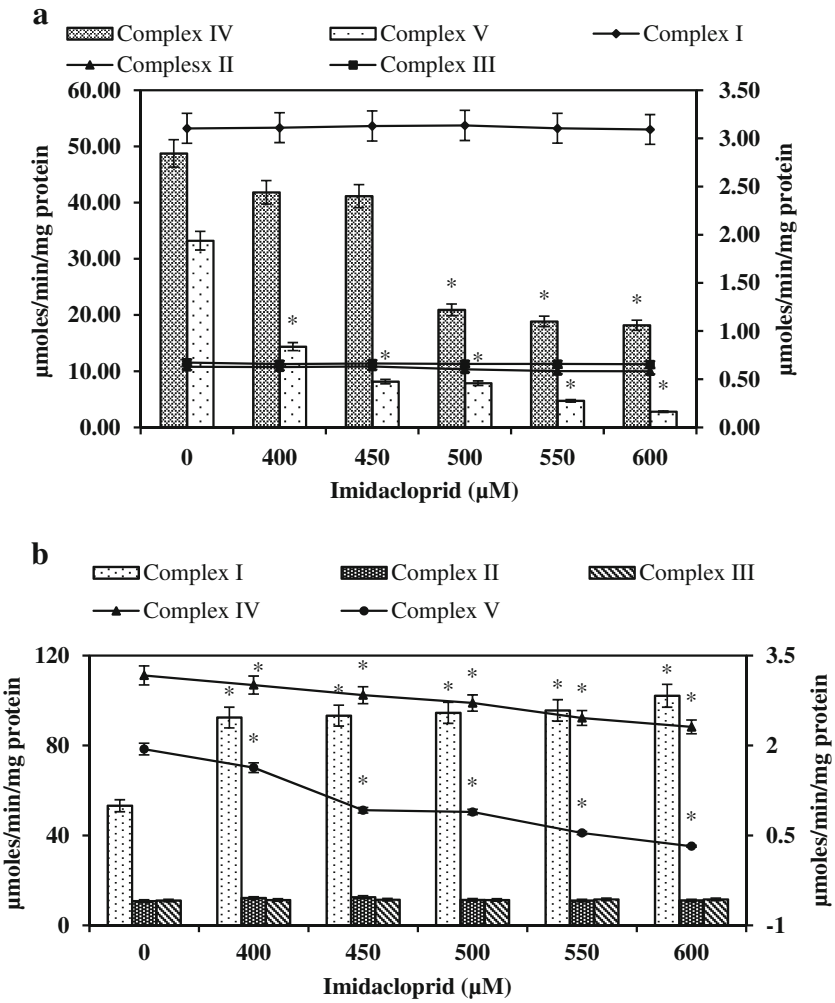


**Fig. 5** **(a)** In vitro and (**b**) In vivo effect of imidacloprid on mitochondrial respiratory enzymes. NADH:ubiquinone oxidoreductase, NADH:ferricyanide reductase, NADH:decyclubiquinol/ ferricytochrome c oxidoreductase, cytochrome c oxidase, and $F_0F_1$-ATPase activities were measured in larvae fed on diet containing varying concentrations of imidacloprid (400–600 µM). The mitochondria untreated with insecticide served as control. The data points represent the mean ± S.D. (n = 3) *$p < 0.05$)

Oxidative stress was found to be maximum at 48 h of insecticide treatment, wherein, lipid peroxidation, and LDH activity were increased by 35.27% and 91.58%, respectively, compared to control larvae (Fig. 8a and b).

## Influence of imidacloprid on antioxidant enzymes

A significant reduction was observed in the CAT and SOD activity in a dose- and time-dependent manner in

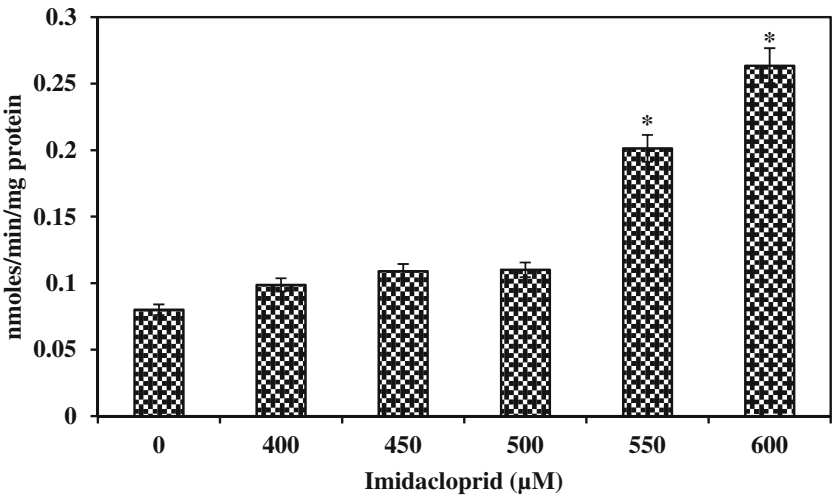


**Fig. 6** Quantification of cytochrome c released. Cytochrome c release was estimated by incubating mitochondria (2.5 mg of protein) isolated from *H. armigera* larvae in the presence of different concentrations of imidacloprid (400–600 µM) for 10 min. Mitochondria incubated with 0.01% Triton X-100 served as positive control. The data points represent the mean ± S.D. (n = 3) *$p < 0.05$)

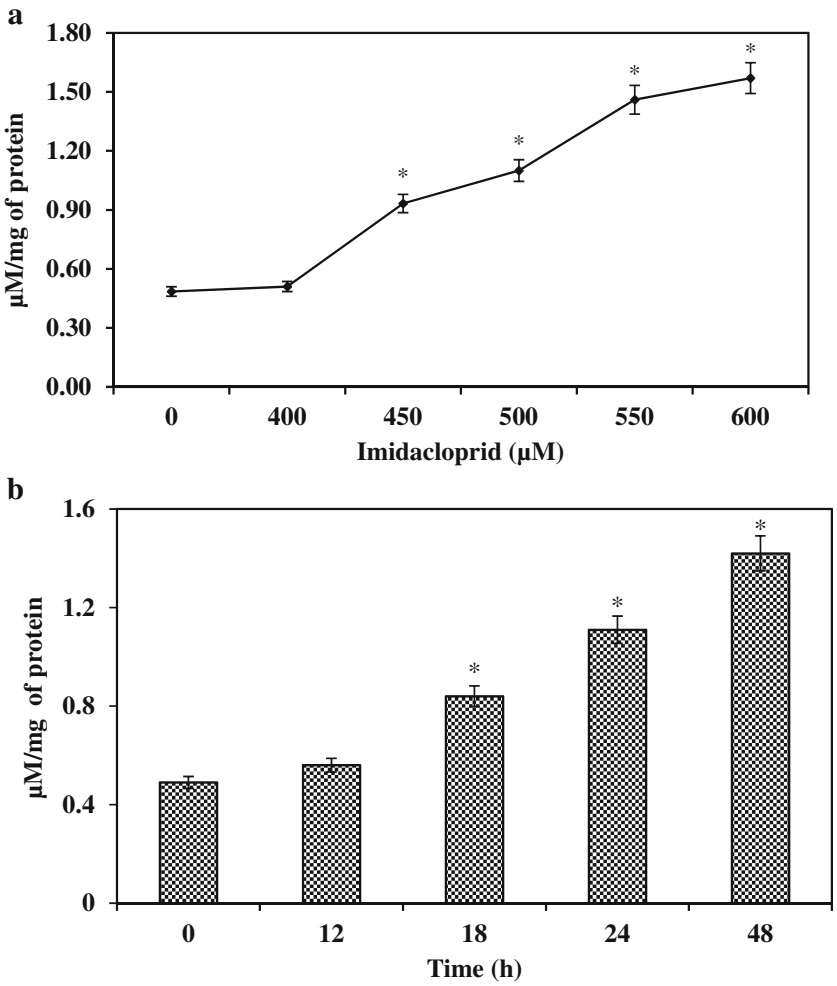


**Fig. 7** Effect of imidacloprid on accumulation of $H_2O_2$. (**a**) Dose-dependent response to $H_2O_2$ accumulation in *H. armigera* larvae fed on diet containing varying concentrations of imidacloprid (400–600 μM). (**b**) Time-dependent response for $H_2O_2$ accumulation in *H. armigera* larvaefed on diet containing $LD_{50}$ concentrations (531.24 μM) of imidacloprid. The larvae fed on diet containing no insecticide served as control. The data points represent the mean ± S.D. (n = 3) *$p < 0.05$

imidacloprid-fed larvae. The enzyme activities were 59.5 ± 4.50 and 2.32 ± 0.079 U/mg protein in control larvae. At 600 μM, the activities were inhibited by 26.30 and 33.53%, and at 48 h, the reduction was observed to be 29.36% and 54.74%, respectively (Fig. 9a and b).

## Discussion

The primary function of the mitochondria is to produce cellular ATP molecules through respiration apart from other biochemical processes. To understand the effect of imidacloprid on mitochondria, respiratory parameters (state III, state IV respiration, RCI and *P/O* ratio) were studied in relation to the insecticide. All the respiratory parameters for succinate oxidation were inhibited in a dose-dependent manner with increase in the insecticide concentration, in vitro. The significant inhibition of $F_0F_1$-ATPase and a moderate decline in the complex IV activity may be due to direct interaction of the insecticide with the enzyme complexes in mitochondria, which had inhibited oxygen consumption thereby affecting the state III and state IV respiration. Decreased level of $F_0F_1$-ATPase activity is the evidence of inhibition of phosphorylation efficiency. The reduced RCI values for mitochondria isolated from the larvae fed on insecticide indicate the mitochondrial injury. Similar observations were made in honeybee (*Apis mellifera*) mitochondria exposed to neonicotinoids, fipronil, imidacloprid (Nicodemo et al. 2014) and the components of pristine® (Campbell et al. 2016). Decreased ATP concentration and mitochondrial transition from active (state III) to resting (state IV) respiration are the result of inhibition of mitochondrial $F_0F_1$ ATPase activity (Johnson et al. 2006).

The terminal enzyme of the electron transport chain, complex IV, catalyzes the reduction of molecular oxygen to water. The biological energy conversion in mitochondria is because of the reduction of oxygen by this enzyme coupled to the pumping of four protons across the membrane. Since the movement of electrons was subsequently coupled to ATP synthesis, the moderate inhibition of complex IV and potential inhibition of $F_0F_1$ ATPase enzyme activity are positively correlated (Martinez-Cruz et al. 2012). The moderate decline in the complex IV activity demonstrated an insecticide-induced decline in flux through substrate oxidation system. Decreasing

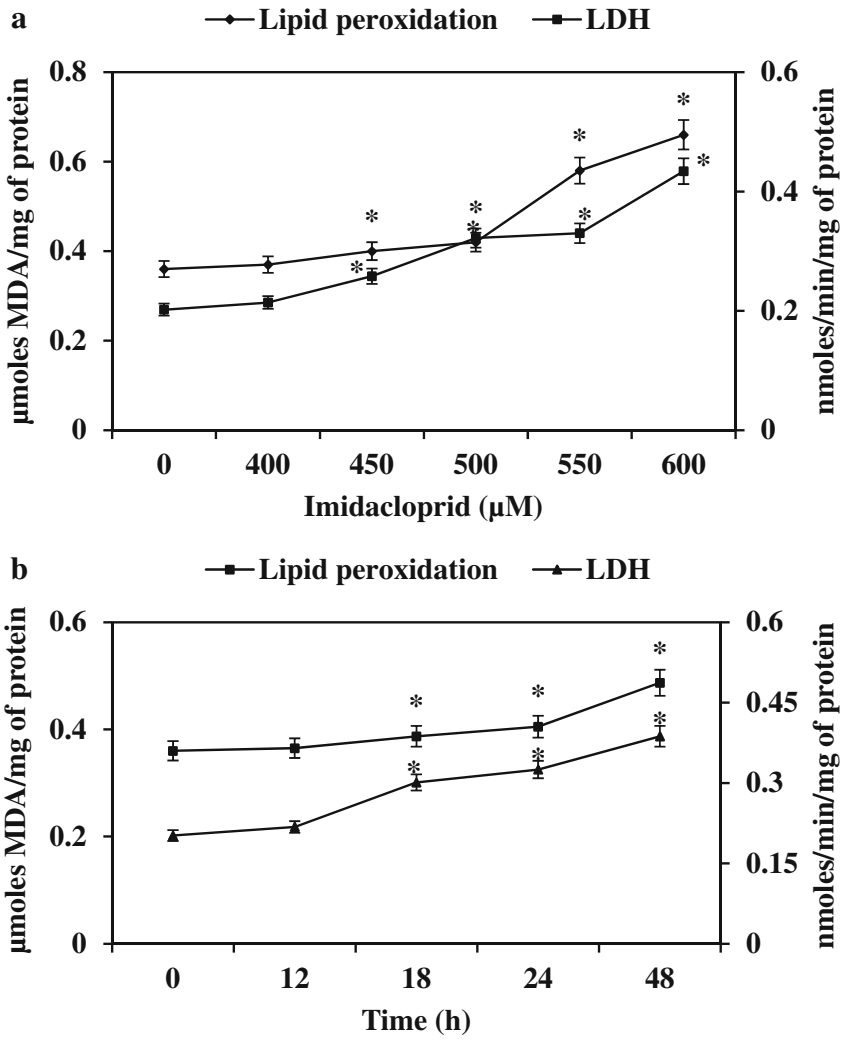


**Fig. 8** Influence of imidacloprid on lipid peroxidation and LDH. (**a**) Dose-dependent, and (**b**) time-dependent response for lipid peroxidation, and lactate dehydrogenase in *H. armigera* fed on diet containing different concentrations (400–600 μM) and $LD_{50}$ concentration (531.24 μM) of imidacloprid, respectively. The larvae fed on diet containing no insecticide served as control. The data points represent the mean ± S.D. (n = 3) *$p < 0.05$)

pattern in the cytochrome c oxidase activity is associated with reduced state III respiration (Petrosillo et al. 2013). A moderate decrease in the complex IV activity, as observed in the mitochondria isolated from imidacloprid-fed larvae (0–600 μM), shows reduced oxygen consumption, inferring the potentiality of the insecticide interfering with the insect's phosphorylation efficiency. Increasing respiration rates in isolated mitochondria from rat brain show exceeding levels of complex IV, suggesting that the activity of this enzyme complex is unlikely to have an impact on mitochondrial respiration (Davey and Clark, 1996; Petrosillo et al. 2013). However, in the present study, complex I showed higher activities compared to treated insects suggesting insecticide-mediated change in the microenvironment around the protein. Complexes I and III are thought to be functionally intact and act as a single enzyme in most of the studies (Bianchi et al. 2004; Chamberlin 2007). Asignificant increase in complex I activity and unaltered complex III activity implicates the impaired mitochondrial activity due to imidacloprid.

The release of cytochrome c from mitochondria along with transmembrane proteins is a key initiative in the apoptotic process, where a cascade of caspases gets activated (Desagher et al. 1999; Li et al. 1997). At 600 μM of imidacloprid, 0.26 nmoles/min/mg of cytochrome c was effluxed when incubated with the mitochondria isolated from *H. armigera* larvae. The increasing levels of cytochrome c releasedintocytosol after incubating mitochondria with increasing concentrations of imidacloprid implicate the possibilities of apoptosis in the present study. OPs and anti-inflammatory drug, piroxicam, induced the release of cytochrome c in mitochondria isolated from *H. armigera,* and Chinese hamster lung fibroblast V79 cell lines, respectively (Akbar et al. 2014; Chakraborty et al., 2007).

As the rate of electron transfer reduces, the mitochondrial enzymes may leak electrons to molecular oxygen promoting the formation of superoxide ions. Mitochondrial electron transport chain (ETC) has been known as a major intracellular source of ROS (Chance et al. 1979). Most of the organophosphates, carbamates, pyrethroids insecticides and some

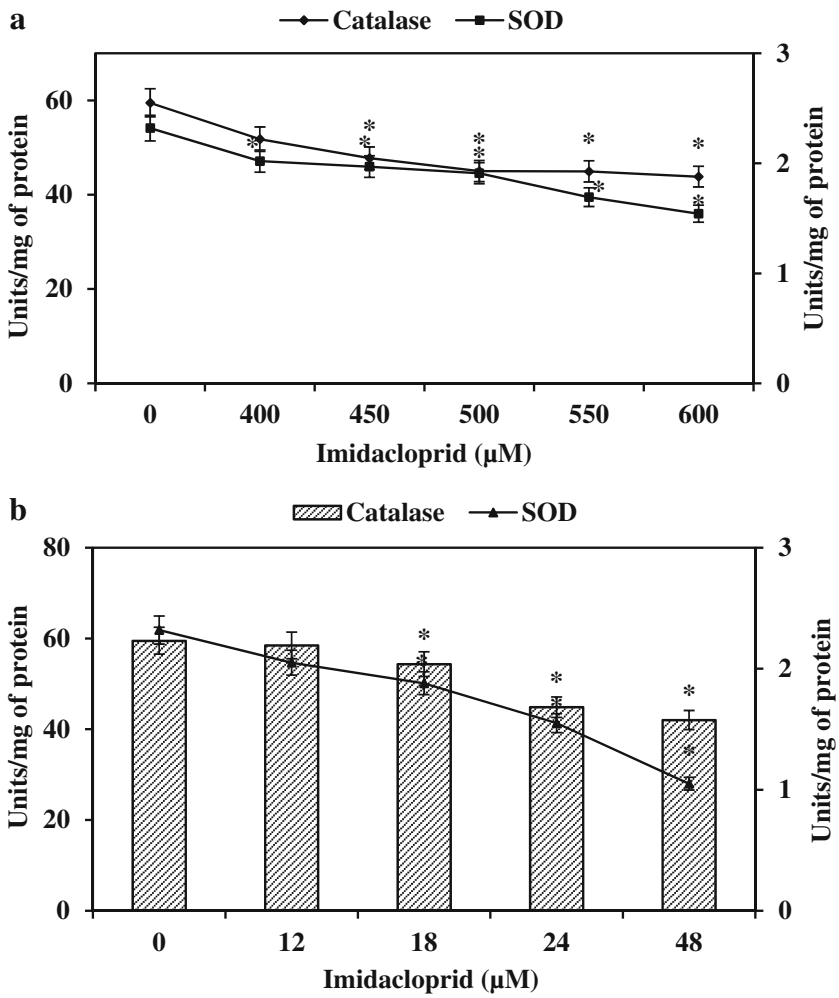


**Fig. 9** Effect of imidacloprid on antioxidant enzymes. (**a**) Dose- and (**b**) time-dependent response for catalase and SOD activity in *H. armigera* fed on diet containing varying (400–600 μM) and $LD_{50}$ concentration (531.24 μM) of imidacloprid, respectively. The larvae fed on diet containing no insecticide served as control. The data points represent the mean ± S.D. (n = 3) *$p < 0.05$)

herbicides induce oxidative stress through ROS (Mehta et al. 2009, Kamboj et al. 2008, Akbar et al. 2012a, Kumar et al. 2015). Complexes I and III are reported to be the sources of ROS generation in rat heart mitochondria and bovine heart submitochondrial particles, which then act as intercellular mediators of apoptosis (Korshunov et al. 1999; Paradies et al. 2001). In the present study, imidacloprid- induced oxidative stress in *H. armigera* larvae in a dose- and time-dependent manner as evidenced by increased levels of $H_2O_2$ content, and lipid peroxidation in insecticide-fed larvae. Increased extracellular activity of LDH enzyme under oxidative stress condition was consequently observed due to the loss of cell integrity during the lipid peroxidation process (Jovanović et al. 2010). In general, antioxidant enzymes are the potent scavengers of ROS, but the inhibition observed in the activities of CAT and SOD enzymes may be because of direct interaction of the insecticide with the enzymes, thereby resulting in increased levels of $H_2O_2$ content and lipid peroxidation. Catalase activity was unaffected due to insecticides in thymocytes and insects (Akbar et al., 2012a, b; Olgun and Misra 2006). In contrast, organochlorine insecticide reduced the catalase activity (Janqueira et al. 1986). In the present investigation, imidacloprid induced a dose-dependent decline in the body weights in *H. armigera* larvae, which is due to the cessation of feeding behaviour and interaction between the insecticide and AChR as evidenced by a decreasing trend in the AChE activity with an increase in the insecticide concentration. Interaction of imidacloprid with AChR alters the AChE activity affecting the neural activity, thereby resulting in the mortality of the larvae. The *H. armigera* not only responded to contact stimuli but also resulted in rapid cessation of the feeding behaviour when fed on the insecticide. Yogeswarudu et al. (2014) reported similar observations in *H. armigera* when treated with imidacloprid.

## Conclusion

The toxic effects of imidacloprid on the growth and development of *H. armigera* larvae were due to induced mitochondrial dysfunction and oxidative stress, apart from interaction with AChR.

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