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# Toxicology and Biochemical Basis of Cantharidin Effects on *Helicoverpa armigera* (Hub.) (Lepidoptera: Noctuidae)

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**Abstract.-** Toxicity of cantharidin, a well known natural compound produced by beetles of family Meloidae and Oedemeridae, was examined on *Helicoverpa armigera*. Furthermore, its effect on different metabolic enzymes, responsible for resistance towards insecticides, was also investigated. Bioassay results showed that $LC_{50}$ value of cantharidin was 0.068 mg/g, using diet incorporation bioassay under laboratory conditions. Body weight of insects was also significantly reduced by cantharidin treatment. The $LC_{10}$ concentration of cantharidin, 0.01mg/g was tested to see its effect on metabolic enzymes such as; alkaline phosphatase (ALP), glutathione *S*-transferase (GST), cytochrome P450s (P450s) and carboxylesterase (Car-E). Our results showed that cantharidin significantly inhibited the activity of ALP after 48 h, whereas activity of GST was significantly inhibited after 24 h. Cytochrome P450s activity remained significantly high at 12 to 48 h after treatment. Statistically, no significant change was recorded in Car-E activity. It may be concluded from the results that ALPs and GSTs may be target of cantharidin, whereas P450s may be involved in detoxification of cantharidin in insects. In light of our results, we suggest cantharidin as potential candidate insecticide precursor and its scope in pest management needs to be explored further to solve the problem of insecticide resistance.

**Keywords:** Cantharidin, *Helicoverpa armigera,* alkaline phosphatase, glutathione *S*-transferase, P450, carboxylesterase.

## INTRODUCTION

*Helicoverpa armigera* Hüb. (Lepidoptera: Noctuidae) commonly known as cotton bollworm or American bollworm, is distributed worldwide and considered a serious pest of many economic crops. Its presence has been reported from Asia, Europe, Australia and Africa (EPPO, 1996). This pest is responsible for losses of US $2 billions to crops annually (ICRISAT, 2003). Amongst cotton pests, *H. armigera* is regarded as major pest infesting cotton. Other than cotton, this pest is also responsible for losses to legumes and many other crops (Bhatnagar *et al.*, 1982).

*H. armigera* attained the status of primary pest of cotton due to the indiscriminate and injudicious use of insecticides especially in the 1980s and 1990's. Management of this pest has never been easy due to the development of resistance to several classes of insecticides. Field population of *H. armigera* has shown moderate to high level of insecticide resistance to organophosphate and pyrethroide insecticides (Ahmed *et al.,* 1995). One of the reasons for rapid resistance development has been due to the excessive use of a particular class of the insecticide group (Ramasubramanian and Regupathy, 2004).

There is enough evidence that injudicious use of pesticide has caused the rise of many problems in agricultural crops, *e.g*., increased infestation level of whiteflies in many crops, plant hoppers in rice and resurgence of cotton bollworm in cotton growing areas, etc. The problem of pest resurgence, outbreaks of secondary pests and insecticide resistance is due to the elimination of natural enemies in agro-ecosystem (Van den Bosch, 1978).

Several mechanisms of insecticide resistance have been attributed to resistance in *H. armigera*. These mechanisms include increased rate of insect metabolism (Ahmed and McCaffery, 1991), decreased nerve sensitivity (West and McCaffery, 1992) and mechanism that reduces the penetration

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of insecticides (Gunning *et al.*, 1991; Armes *et al.*, 1992; Kennaugh *et al.*, 1993). Metabolic resistance includes the action of insect metabolic enzymes such as alkaline phosphatase, glutathione *S*-transferase, carboxylesterase and cytochrome P450s. The action of these enzymes turns chemical compounds into less toxic metabolites. Generally, enzymes from three families, cytochrome P450s, GSTs and esterases are involved in resistance towards insecticides. Increased rate of metabolism can result from change in enzyme form or increased rate of production of detoxification enzymes, which on the other hand is produced in much less quantities in susceptible insects (Hemingway *et al.*, 1999; Siegfried and Scharf, 2001). Pyrethroids resistant *H. armigera* from Australia have shown enhanced esterase activity. In Indian strain of *H. armigera* glutathion S-transferases (GSTs) are involved in pyrethroids resistance (Gunning *et al.*, 1996).

Biopesticides with mode of action other than the conventional insecticides may reduce the risk of insecticide resistance and pest resurgence problems while comparatively safe and ecologically acceptable. In early studies, our candidate insecticide, cantharindin EC was found to have low toxicity against quail, ladybird beetles and soil microorganisms in China (Feng-Li *et al.*, 2009) The insecticidal and antifeedant activities of cantharidin are well-established fact on armyworm and diamond moth (Ya-Lin *et al.*, 2003).

It is clear from the above review that metabolic enzymes play important role in detoxification of conventional insecticides and development of resistance however, these studies are lacking in providing information about their interaction with our candidate insecticide. We report here the toxicity of cantharidin to *H. armigera* and the enzymatic response of alkaline phosphatase (ALP), glutathione *S*-transferase (GST), cytochrome P450 monooxygenases and carboxylesterase (Car-E). The major objectives of our study are to understand the effect of cantharidin, as model insecticide on American bollworm using toxicological and biochemical methods.

## MATERIALS AND METHODS

*Reagents and chemicals*

P-Nitroanisol (BODI), Fast blue B salt (Urchem) 1-Naphthol (Kermel), L-Glutathione reduced (Wolsen), 1-Naphthyl acetate (SCR), Sodium darbital (ZIBO) 1-Chloro-2, 4-dinitrobenzene (ABCR), p-NPP (Amresco), NADPH (Wolsen). Other chemicals used in the experiments were of commercial grade.

*Laboratory extraction of cantharidin*

Meloide beetles were weighed and placed in triple neck round bottom flask after fine grinding. The finely ground beetles were submerged in 3M HCI. The bottle was placed on water bath and a stirrer rod inserted into the middle neck hole, whereas thermometer and condensation tube was inserted into the other two neck holes, respectively. The ingredients are heated for 10 hours at 80°C. The fine grounded paste was transferred to funnel vacuum filtration. Filtrate and the residue both were collected for further processing. Put the filtrate into the Craig apparatus and residues back to the triple neck flask and add 3M HCI again. Cantharidin's extraction was started with addition of dichloromethane ($CH_2Cl_2$) in the Craig apparatus and mixed by inversion with hands for five minutes and the liquid was allowed to separate until two clear phases are seen. Organic phase was collected and dichloromethane was added in the upper layer and the process was repeated for 4-5 times. All the organic phase was collected after 4-5 repeats and was placed into the round bottom flask for distillation. Crystals were recovered in the bottom after evaporation of solvent ($CH_2Cl_2$) and washed with petroleum ether, ethanol and acetic acid until the crystals became colorless. Finally, the colorless crystals were dissolved in acetone for final crystallization.

*Rearing of* H. armigera

*H. armigera* larvae were procured from Henan Jiyuan Baiyun Industry Co., Ltd. China and reared until F1 for use in bioassay. Group of 24 larvae were placed into 24 chamber plastic box obtained from the company. The boxes were placed in an incubator at 27+1°C and 40 to 50% RH with 12 h photoperiod. After pupation pupae were collected and placed in plastic jar having cotton

cloth on both sides and vial was placed in the middle with 10% sugar solution dispensed through cotton. Eggs were collected on the lower and upper cotton cloth and placed in transparent plastic bags for emergence.

*Artificial diet*

Artificial diet for rearing of cotton bollworms was prepared in the laboratory following the method and ingredients used by Ahmed and McCaffery (1991).

*Bioassay*

Diet incorporation bioassay was used to determine the toxicity of cantharidin. Batches of healthy homogeneous third instar larvae were selected for bioassay. Cantharidin was added to the semi solid artificial diet at the rate of 0.05mg/g, 0.06mg/g, 0.07mg/g, 0.1 mg/g and 0.125mg/g, respectively, using acetone and mixed well. Each gram of artificial diet was added 50ul of each concentration. Acetone was allowed to evaporate for 60 min before allowing insects to feed. One larva of third instar was introduced to each cell of 24 cell plastic bioassay tray. The mortality data was recorded until five days. Effect of cantharidin, 0.031mg/g on body weight was also calculated on third instar larvae of *H. armigera*. Data were recorded at 12, 24, 36, 48 and 56 h after treatment.

*Experimental design and statistical analysis*

The laboratory bioassay experiment was carried out in Randomized Complete Block Design (RCBD) with three replicates per treatment. The Analysis of variance (ANOVA) was carried with SPSS 17.0 to compare effect of different doses of cantharidin on mortality at different time intervals. Means were separated by the least significant differences (LSD) test. The probability value, $P \leq 0.05$ was considered significant.

Probit analysis was used to determine the different LC values for cantharidin after acquiring data from bioassay experiment (Finney, 1971). SPSS 17.0 software was used for analysis of photometric data (SPSS Inc., Chicago, IL). Significance of the effect of cantharidin on enzymes specific activity was determined by independent sample *t-test*. Means were considered significantly different at $P \leq 0.05$.

*Estimation of enzyme activity*

*Insect treatment for enzyme assay*

Laboratory prepared artificial diet as mentioned above was mixed with 0.01mg/g of cantharidin, dissolved in acetone. The acetone was allowed to evaporate for one hour before introduction of larvae into it. Larvae of early third instar, after being starved for eight hours, were introduced to the cantharidin-treated artificial diet. Artificial diet with acetone was used as a control. Samples were collected from treatment and control groups at 12, 24, 36 and 48 h for determination of enzyme activity. Collected larval samples were flash frozen and stored at -80°C just after collection. Five larvae were used per replication both in treatment and control groups. All the experiments were replicated three times.

*Protein determination*

Protein contents within homogenates were determined using bovine serum albumin as standard (Bradford, 1976).

*Specific activity of ALP*

Method of Bessey (1964) was used for determination of ALP specific activity. The rate of formation of yellow color of *p*-nitrophenol produced by the hydrolysis of p-nitrophenyl phosphate in alkaline solution was measured spectrophotometrically at 405 nm. Tissue homogenates were prepared by homogenizing larvae with glass homogenizer in ALP buffer containing 0.824g sodium barbital, 0.35ml of 0.2 M HCl mixed in 100 ml of water. Tissue homogenates of the larvae were subjected to centrifugation at 10000 rpm at 4°C for 15 min. The supernatants obtained were used as enzyme test solutions. Reaction was initiated with addition of 2ml of ALP buffer and 0.5ml of 0.0075 M *p*-NPP to the enzyme test solution and placed for incubation at 37°C for 30 min. Finally, 2ml of 0.5 M NaOH was used for termination of and ALP activity was determined spectrophotometrically at 405nm after five minutes.

*Specific activity of GST*

Method of Booth *et al.* (1973) was followed for specific activity of GST. Larvae were

homogenized in 0.1 M phosphate buffer of pH 7.4 on ice using glass homogenizer. The homogenates were subjected to centrifugation at 10,000 rpm for 15 min. at 4°C. The supernatants obtained were used as enzyme test solutions. Enzyme solution of 0.11ml was mixed with 1.4ml 0.1M of pH 8.9 (containing 10mM glutathione) and incubated at 25°C for 10 min. and added 60uL 30mM CDNB. Enzyme activity was measured spectrophotometrically at 340nm. Extinction coefficient of CDNB, 9.6 $mM^{-1}$ $cm^{-}1$ was used for calculation of specific activity.

*Specific activity of cytochrome P450 O-demethylase*

The activity of p-nitroanisole *O*-demethylase was determined followed by Hansen and Hodgson 1971. Larvae were homogenized by glass homogenizer in pre-cooled 0.1M PBS of pH 7.2 on ice. Tissue homogenates of larvae were subjected to centrifugation at 10,000 rpm at 4°C for 15 min. The Supernatants obtained were used as enzyme test solutions. Enzyme test solutions of 0.5 ml containing 0.1M sodium phosphate (pH 7.8) and 0.005M NADPH was prepared. Reaction was initiated by the addition of 10uL 0.002M nitroanisole and placed for incubated in water bath at 25°C with shaking for 30 min. The reaction was terminated by the addition of 0.5ml of IM HCl. The product *p*-nitrophenol was extracted with $CHCl_3$ and then centrifuged to get two fractions. The $CHCl_3$ fraction was back extracted with 0.5M NaOH. The optical density of NaOH solution was determined at 400nm and the product was quantified using the experimentally determined curve.

*Specific activity of Car-E*

Car-E activity was determined followed by Van, 1962. The general buffer was 0.04 M, pH 7.0 phosphate buffer. 1-Naphthyl acetate ($3x\ 10^{-4}M$) was used as substrate. Standard curve was determined by different concentration of α-naphthol phosphate mixed with PBS. The enzyme substrate mixtures were placed for incubation at 37°C for 30 min in water-bath. Fast Blue B salt-sodium dodecyl sulphate solution of 1ml was added for termination of reaction. Enzymatic activity was measured spectrophotometrically at 600nm after 30 min.

## RESULTS

*Bioassays*

Bioassay results showed that our used concentrations of cantharidin affected *H. armigera* and caused low to high mortality depending on the concentration and time. The mortality of cantharidin at higher dose of 0.125 mg/g after different intervals was significantly high. Significantly high mortality was recorded at all the concentrations after 120 h ($P \leq 0.05$) (Fig.1). Different LC values were calculated to know the lethal and sub lethal doses. $LC_{50}$ of cantharidin was found to be 0.068 mg $g^{-1}$. Furthermore, $LC_{10}$ (0.01 mg/g) as sub lethal dose was used for enzyme assay (Table I).

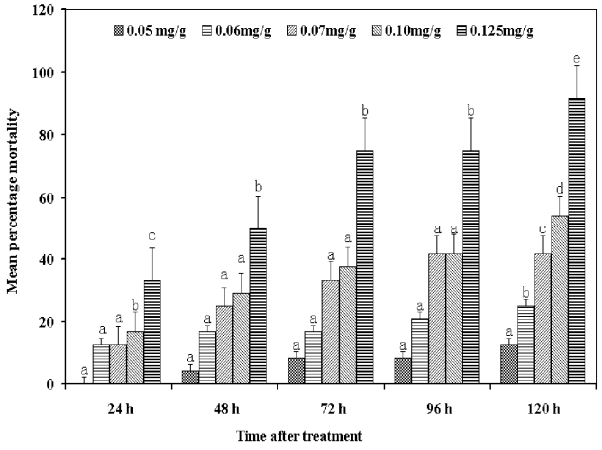


Fig. 1. Mortality of *Helicoverpa armigera* larvae at different intervals after treatment. Error bars indicate Standard Error (SE). Each bar indicates mean value. Means were compared by one way ANOVA. Bars sharing the same letters at a time interval are not significantly different among one another.

*ALP activity*

No significant increase in ALP activity was seen at 12 h after treatment but it started to decline after that highly significant inhibition of ALP was observed at the end of 48 h *($t=360$, $df=2$, $P \leq 0.01$)* (Fig. 2A).

*GST activity*

GSTs activity was slightly higher in treatment as compared to control at 12 h. At the end of 24 h

interval enzyme activity in the treatment was significantly lower (*t=55.17, df=4, P≤0.05*) compared to control. Highly significant activity inhibition trend was also seen at 36 *(t=4.45, df= 4, P≤0.01)* and 48 h *(t=207.8, df=4, P≤0.01)* intervals, respectively (Fig. 2B).

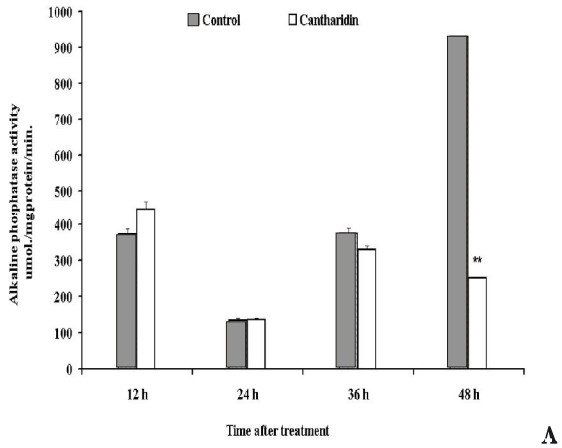


Fig. 2. Specific activity of alkaline phosphatases (µmole $mg^{-1}$ $min^{-1}$) (A), glutathione *S*-transferases (µmole $mg^{-1}$ $min^{-1}$) (B), And cytochrome P450s (C) at different intervals after treatment. Asterisks show significant difference between control and treatment by independent *t-test* at α 0.05 level.

*Cytochrome P450 O-demethylase activity*

Cytochrome P450 *O*-demethylase activity remained high through the assay. At 24 h after treatment activity remained significantly lower *(t=-8.6, df=4, P≤0.05)* than control. P450 *O*-demethylase activity was seen highly significant in treatment at 12 *(t=-3.5, df=4, P≤0.01)*, 36 *(t=-27.71, df=4, P≤0.01)* and 48 h *(t=11.02, df=4, P≤0.01)*, respectively, compared to control (Fig. 2C).

*Car-E activity*

Statistically, no significant change in Car-E activity was observed at 12 to 48 h after treatment. However, at 12 and 24 h intervals, the activity remained slightly low in treatment as compared to control. But after 36 and 48 h interval the activity remained slightly high compared to control.

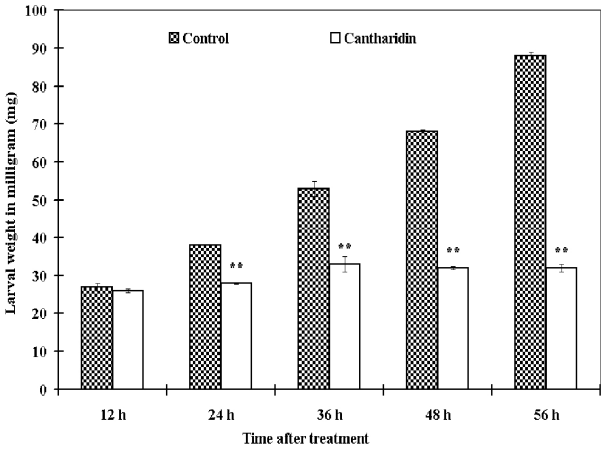


Fig. 3. Effect of cantharidin treatment on the body weight of *H. armigera* larvae exposed to artificial diet containing 0.031mg/g cantharidin at different intervals after treatment. Asterisks show significant differences between control and treatment.

*Body weight*

Cantharidin treatment of larvae showed retarded growth. Body weight of larvae feeding on

artificial diet containing 0.031mg/g cantharidin was significantly lower than those feeding on untreated artificial diet (Fig. 3). No significant weight loss was recorded at 12 h *(t=1.2, df=6, P≤0.05)* after treatment. However, significant body weight loss was recorded at 24 to 56 h *(t=37.45, df=6, P≤0.05)* after treatment, compared to control.

**Table I.- LC values of cantharidin for *Helicoverpa armigera* Hub.**

| LC values | Dose | Upper limit* | Lower limit* | Heterogeneity | Chi-Square | Df |
|---|---|---|---|---|---|---|
| $LC_{10}$ | 0.011mg $g^{-1}$ | 0.034 | -0.044 | 0.54 | 1.64 | 3 |
| $LC_{50}$ | 0.068mg $g^{-1}$ | 0.080 | 0.051 | | | |
| $LC_{90}$ | 0.012mg $g^{-1}$ | 0.165 | 0.107 | | | |

* Upper and lower limits of LC values at α 0.05

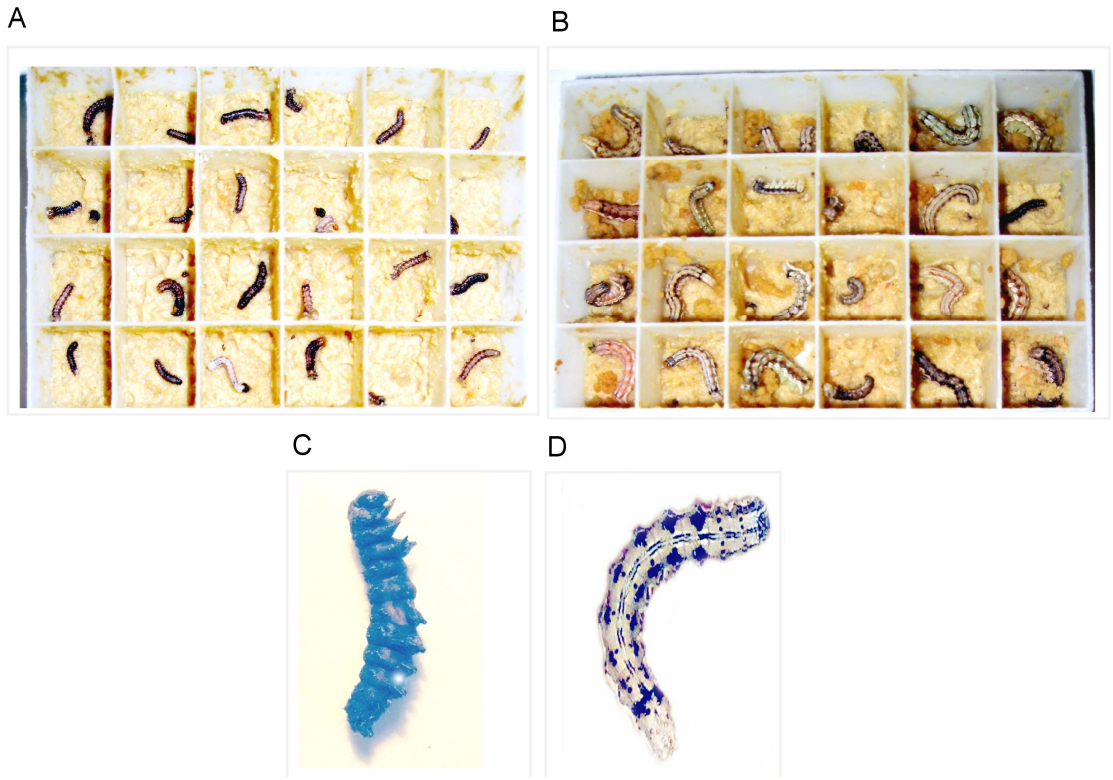


Fig. 4. Morphological structure of *Helicoverpa armigera*. (A) Larvae treated with cantharidin in 24 cell plastic box (B) Control larvae in 24 cell plastic box (C) Dead larva after treatment with cantharidin (D) control larvae.

*Morphological effect on* H. armigera

Insects fed on cantharidin containing artificial diet presented dark brownish necrotic look after death. However, no rupture of tissue or fluid was seen on the treated larvae. The size of the larvae also remained small compared to control *(t=37.45, df=6, P≤0.05)* (Fig.4).

## DISCUSSION

Cantharidin was incorporated into the artificial diet and third instar *H. armigera* were allowed to feed on it for five days. As shown in Fig.1 cantharidin caused mortality in concentration dependent manner. Exposure to cantharidin also reduced activity of ALP and GST whereas, Car-E activity was not influenced. However, P450s activity remained significantly high.

Earlier studies showed that the toxicity of cantharidin has been its binding to phosphoprotein 2A (PP2A). Other than PP2A detailed physiological and biochemical effects of cantharidin and its mechanism of action remains widely unknown (Graziano and Casida, 1987; Kawamura *et al.,* 1990; Graziano *et al.,* 1987; Decker 1968; Bagatell *et al.,* 1969). In our experiment, cantharidin treatment of sub lethal dose significantly reduced level of ALP, in vivo. Reduced level of ALP suggested that energy metabolism was affected by reduction in level of phosphorus liberation that in turn decreased metabolism as well as transport of metabolites with decreased rate, could be the direct effect of cantharidin on enzyme regulation. Similar effects of azadirachtin and nucleopolyhedrovirs (NPV) were observed on ALP and other digestive enzymes (Huang *et al.,* 2004; Nathan *et al.,* 2005).

Cantharidin in our study also reduced weight of larvae. Decrease in larval weight could be because of the decreased level of enzyme activity and indicate general disturbance in metabolism, in cantharidin treated insects.

In previous studies, high level of esterases and phosphatases in resistant insects compared to susceptible insects has shown their contribution to the resistance mechanism. And it has been suggested to be involved in increased metabolism and development of resistance towards insecticides (Srinivas *et al.,* 2004). The suppression of ALP activity by cantharidin could be one of the reasons for its toxicity mechanism in our experiment. Similarly, the involvement of GST has also been reported by many authors as involved in insecticide metabolism and its increased level has been attributed to the mechanism of resistance in several pests (Balabaskaran *et al.,* 1989; Yu and Ngugen 1992; Mohan and Gujar, 2003). Inhibitory effect of cantharidin on GST suggested its inability to metabolize cantharidin.

Activity of cytochrome P450s remained largely high, showing its possible involvement in metabolism of cantharidin. The significantly high level could be the cause of delayed toxicity of cantharidin in our experiment. Insecticide metabolism is catalyzed by cytochrome P450s, an important enzyme family. Enhanced levels of P450s have been reported in resistant insects such as cotton bollworm, diamondback moth and are regarded as major resistance mechanism to pyrethroids. For example, azinphosmethyl, heptachlor, aldrin and diazinon are metabolized by CYP6A1 in *Musca domestica* (Guzov *et al.,* 1998). In our experiment the increased level of P450s activity elucidate similar mechanism of detoxification by *H. armigera* with the above mentioned studies. Many previous studies have shown that over expression or elevated levels of cytochrome P450s were seen in insecticide resistant strains of insects. Pyrethroids resistant field population of *H. armigera* from Australia was reported with over expressed levels of CYP 6B7 (Ranasinghe *et al.,* 1998). In China, main resistance mechanism of resistance towards pyrethroids has been documented as enhanced oxidative (Shen and Wu 1995; Yang *et al.,* 2005). The high activity of P450s in present experiment showed that the Chinese strain of *H. armigera* showed similar mechanism of detoxification towards cantharidin as Australian strain.

In conclusion, cantharidin was found to be toxic against *H. armigera* in used concentrations. Lower activity of ALP and GST indicated inability of these enzymes to metabolize cantharidin and may be the possible target of cantharidin. Only P450s were found to be active against cantharidin showing their possible involvement in cantharidin metabolism. We found cantharidin very toxic to *H. armigera* in the present study. However, we do not suggest its use as a large scale pest management program tactic, because of its high cost of production and environmental consequences. However, its scope as insecticide could still be explored further by synthesizing new improved cost effective analogues with little adverse effects on environment. Furthermore, its use on small scale in controlled environment could however be suggested to counter insecticide resistance problem for effective pest management.

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