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TOXICOLOGICAL EFFECT OF CHLORANTRANILIPROLE ON FAT BODY AND HAEMOLYMPH METABOLISM IN THE FINAL INSTAR LARVAE OF SILK INSECT, *BOMBYX MORI* L.

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ABSTRACT
Pesticides are substances that are used to kill undesirable organisms, repel or control certain forms of plant or animal life that are considered to be pests. The importance of controlling pests have led to the development of a variety of insecticides that prevent agricultural loss and spreading of diseases. The irrational use of these insecticides causes some serious hazards including alteration in the environmental condition and biomagnifications in higher organisms. To overcome these drawbacks more effective and specific insecticides were developed. One among this category is chlorantraniliprole, a ryanodine receptor agonist useful against lepidopteran pests. The mode of action and toxic effect of chlorantraniliprole needs detailed investigation and the present work is an attempt to evaluate the same on the fat body and haemolymph of the final instar larvae of silk insect, *Bombyx mori*. Total protein, urea and aminotransferases in the fat body and haemolymph were analysed. Reduced protein level indicates the reduction in protein synthesis due to toxic stress. Increased urea levels are seen due to the detoxification of chlorantaniliprole. The activity of aminotransferases were high in treated groups compared to the normal, indicates the physiological stress condition due to the insecticide.

KEYWORDS: *Bombyx mori*, Chlorantraniliprole, fat body, haemolymph, aminotransferases, urea.

INTRODUCTION

The use of pesticide in agriculture has significantly increased during the past three decades. The use of insecticide is believed to be one of the major factors behind the increase in agricultural productivity in the 20th century. The importance of controlling pests have led to the development of a variety of insecticides and its irrational use causes serious hazards like alteration in the environmental condition and biomagnifications in the higher trophic levels. The increased and indiscriminate use of these insecticides resulted ill effects in the non-target organisms including beneficial organisms and the natural enemies of major pests. To manage these problems, a new chemical category of insecticides called diamides were developed. One among this category is chlorantraniliprole, 3-bromo-*N*- [4-chloro-2-methyl 6-(methylcarbamoyl) phenyl] -1-(3-chloropyridin-2-yl)-1H-pyrazole- 5-carboxamide, which is developed by Nihon Nohyaku Co. Ltd (Tokyo, Japan) in 1998. It is a ryanodine receptor agonist and is mainly used against lepidopteran pests, which are the major pests of many crops. The chemistry is conformed to control insects by activations of ryanodine receptor, which leads to uncontrolled calcium release in muscle and contracts permanently, leading to death (Lahm *et al*., 2009). There is less evidence reporting the toxic effect of chlorantraniliprole on non-target organisms. The effects of chlorantraniliprole were observed on four species of beneficial insects and found to have no apparent adverse effect on them (Larson *et al*., 2014). Chlorantraniliprole exhibits a high activity against all lepidopteran insects and the treated larvae showed symptoms, such as loose muscle control, stop feeding and exhibit a pronounced contraction paralysis of the whole body. Chlorantraniliprole has been shown to be effective on resistant strains of beet-army worm and *Helicoverpa armigera* (American Bollworm) (Lahm *et al*., 2009). The toxic effects of chlorantraniliprole were analyzed on the silkworm *Bombyx mori* and symptoms like incomplete ecdysis, thin shelled cocoons etc were observed (Munhoz *et al*., 2013). Several studies were reported on the effects of insecticides on fat body metabolism of insects. In *Spodoptera exigua* and *Tenebrio molitor* larvae which exposed to fenitrothion (organophosphate) resulted in decrease in weight of fat body (Adamsk *et al*., 2012). In another study of IGR, Ni-nitro-N-methylurea injected in the abdomen of *Periplaneta americana*, the protein contents of ovary of treated insects and the protein contents of fat body of treated ones significantly decreased and reduced the lipid contents of the fat body (Jain *et al*., 2003). In the carbaryl treated larvae of the *Bombyx mori*, alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT) in the fat body and haemolymph showed higher magnitude compared to the normal (Sebastian, 2002). The applications of juvenile hormone analogue in the diet with high levels of methyl parathion significantly increased the activities of AlAT and AsAT in greater wax moth, *Galleria melonella* larvae (Ender *et al*., 2005). Studies revealed that exposure to chlorantraniliprole impair the development of the freshwater invertebrate *Chironomus riparius* at environmental relevant concentrations (Rodrigues *et al*., 2015). Haemolymph is the circulating body fluid that serves as an excellent barometer in determining the biochemical status of a developing insect. Insect fat body

is mesodermal in origin and in adults it appears as a scattered mass of tissue in haemolymph and is the source of several proteins found in haemolymph. Numerous studies have focused on the effects of insecticides on *Bombyx mori*, including toxicity, retardation of development and growth, fecundity, mortality, food utilization and economic parameter. The present study was undertaken with the aim to investigate the morphological and biochemical changes in the protein level, urea level, Alanine aminotrasferase (AlAT) and Aspartate aminotransferase (AsAT) levels in the fat body and haemolymph of the fifth instar larvae of *Bombyx mori*, treated with chlorantraniliprole.

MATERIALS & METHODS

Experimental organism

The silkworm is the caterpillar of the domesticated silkmoth, *Bombyx mori*, is an economically important insect, being a primary producer of silk and belongs to the Phylum Arthopoda, Class Insecta and Order Lepidoptera. The bivoltine silkworm hybrid, Elite –CSR 2x4 of uniform size and age was used. Newly hatched larvae were collected immediately after their brushing from Central Silk Board, Pallatheri, Palakkad, Kerala with the help of well grown mulberry plantation. The present work was done on the fifth instar larvae, beginning from the first hour after its fourth moult and continued till the last day of the instar, at 24 hour intervals.

Administration of pesticide

The test pesticide, chlorantraniliprole was purchased under the trade name Takumi from Rallis India Limited. For determining LC_{50} concentration; a series of concentration (in distilled water) of insecticide was prepared on the active ingredient based on ppm by diluting. The LC_{50} in this experiment found as 5ppm/L. A formulation of 2.5ppm/L chlorantraniliprole solution in distilled water was used in the present study for the analysis of toxicological effects. The mulberry leaves dipped in pesticide solution was drained in air for half an hour and was exposed to a set of larvae. Leaves washed with distilled water served as control treatment and was maintained with normal feeding. The fifth instar larval period is divided into five chronologically identified stages: *i.e*., 0h, 24h, 48h, 72 h, and 96h.

Biochemical analysis

Tissues were collected from sufficient number of larvae of control and insecticide treated groups soon after the administration of food. Larvae were dissected along the mid dorsal line in ice-cold insect ringer and the fat bodies were removed immediately. The tissue was homogenized and diluted to appropriate volume with water for all assays except enzymes. Pooled haemolymph samples were extracted from appropriate number of both normal and treated larvae separately. One of the thoracic legs of anaesthetized larvae was amputated and the haemolymph oozed out was immediately collected and its volume found out. The analysis was carried out at 24 hour intervals in on the basis of unit volume in the case of haemolymph.

Total protein was estimated by the method of Lowry *et al*. (1951) using crystalline bovine serum albumin (Fraction V, Stigma) as standard. The blue colour developed is measured against a reagent blank at 540 nm in a spectrophotometer. Fearon reaction, modified by Beale and Croft (1961) was used for the estimation of urea, and the colour developed was read at 535nm. The activity of AsAT and AlAT were estimated following the method of Reitman and Frankel (1957) using pyruvic acid as standard and the colour developed was read after 10 min at 520 nm. One unit enzyme activity corresponds to the formation of 1mole of keto acid per minute at 37ºC under the experimental conditions. The results obtained from the biochemical analyses were exhibited in tables and figures. The values obtained in each experiment were subjected to statistical analysis using student't' test in order to verify the significance of variation between normal and treated larvae.

RESULTS

Effects of chlorantaniliprole on morphology

The result of present investigation indicate that Chlorantraniliprole affect the general growth of silkworm larvae with a reduction in the body size and exhibiting general symptoms of slow movement, permanent insect body contration, leading to rapid cessation of feeding *etc*.

The larval stages of *Bombyx mori* were identified by their size and feeding characteristics. The larvae began feeding immediately after 12 hour of its fourth moult. The *Bombyx mori* larvae treated with chlorantraniliprole showed lack of feeding and hence notable reduction in body size was seen. Other changes observed after the treatment were discolouration of whole body cuticle into creamy white colour, which was evident 24 hours post treatment. Afterwards, larvae became sluggish in movement with rupturing of the body cuticle and oozing out of haemolymph was noted. The amount of faecal matter was reduced compared to the normal larvae and the excreta were in the form of fluid nature instead of its original pellet form. However in the later stages the reduction in body size was very prominent, when compared to the control.

Effects of chlorantaniliprole on physiology of fat body and haemolymph

The fresh weight of the fat body gradually increases throughout the final instar larval days, but in pesticide treated larvae the reverse is the condition. Contradictory to this, the volume of haemolymph gradually decreases with the developmental ages of larvae. But in treated larvae, the decrease in the haemolymph volume was more, as compared to the control. The haemolymph gradually became denser with the developmental ages of larvae. But, in treated larvae haemolymph was less dense, as compared to controls. Also, the volume of haemolymph was less on first day, then steadly rise to a peak on respective days. But, treated larvae showed a reduction in the volume of haemolymph.

Effects of chlorantaniliprole on biochemisty of fat body

The amount of total protein in the fat body increased with larval age in the normal insect whereas in treated larvae, the same decreased gradually till 96 h. In 96 h and 24 h the treated larvae show a sharp decrease in protein level. There were significant decrease in the 48 h and 72 h of treated larvae. The concentrations of protein in the fat body of normal and treated larvae during its development are depicted in Fig. 1a.

The amount of the urea in the fat body of normal larvae increased steadily. The 48hour and 72hour developmental stages show significant variation in the urea level of the larvae treated with pesticide compared with normal larvae. The levels of urea in the fat body of the normal and chlorantraniliprole treated larvae during its developmental stages in the fifth instar are given in Fig.2b.

In the fat body of normal larvae the AsAT activity steadily increased with larval development and show a peak value at 48 hour and declined up to 96 hours .But in treated larvae the variation of AlAT activity exhibited similar pattern but with a high magnitude. A highly significant variation found at the 48 hour treated larvae and the variation recorded in Fig.3a. In normal larvae the activity of AlAT increased up to 72hour and then gradually decreased. But in treated larvae the activity increased with high magnitude and in 72hour and 96hour treated larvae,the activity show twofold increase compared to normal. The levels of Alanine amino transferase activity in the fat body of the normal and chlorantraniliprole treated larvae during its developmental stages in the fifth instar are given in Fig. 4a.

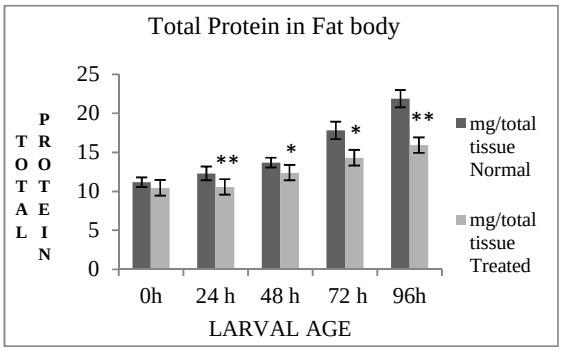

FIGURE 1a: Total protein levels in fat body

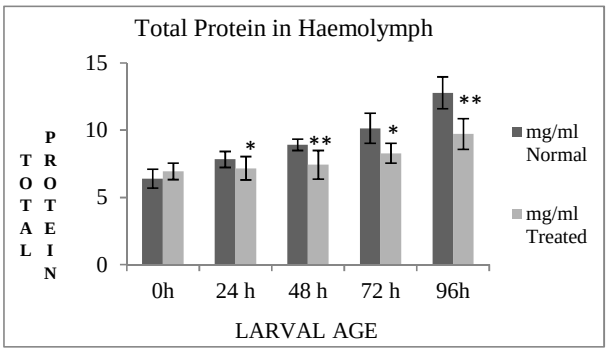

FIGURE 1b: Total protein levels in haemolymph

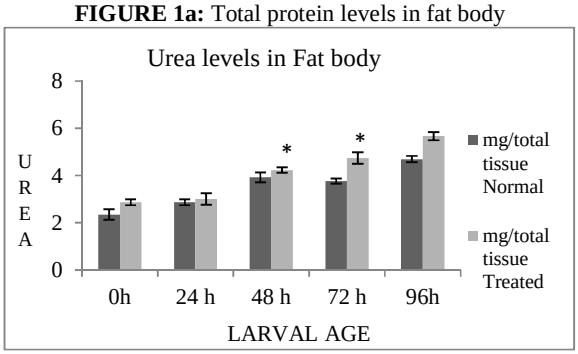

FIGURE 2a: Urea levels in fat body

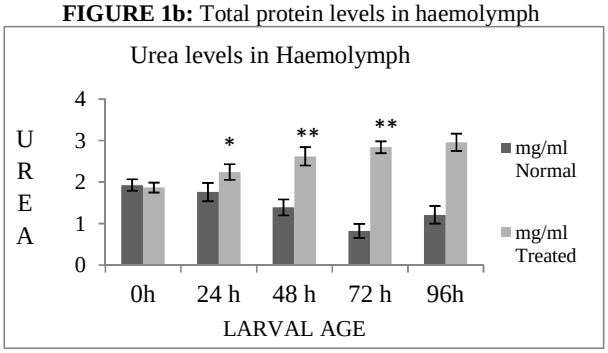

FIGURE 2b: Urea levels in haemolymph

Effects of chlorantaniliprole on biochemistry of haemolymph

The total haemolymph protein increased with larval age in the normal (control) larvae of *Bombyx mori*. But, in the treated larvae the protein level is reduced. The amount of total protein in *Bombyx mori* larva at 24hour after treatment does not show significant difference with control but, in 96 hour larva, total protein was reduced considerably. The levels of total protein in haemolymph of control and Chlorantraniliprole treated larvae, during its developmental stages in the fifth instar larvae are depicted in Fig.1b. The urea level in the haemolymph decreased with larval age in the normal but, in the treated larvae, urea level was considerably increased during respective larval ages. The data obtained revealed that in 48hour and 72hour larva, when treatment with Chlorantraniliprole showed a sharp increase in urea level. In 72hours, there was a threefold increase in the urea level of treated larvae, as compared to normal. The concentration of urea in the haemolymph of normal and treated larvae of *Bombyx mori* during it developmental stages in fifth instar are presented in Fig.2b.

Aspartate aminotransferase and alanine aminotranferase content of haemolymph was measured. The treated larvae, at 24hour, does not show significant difference with normal (control) larvae, in the AsAT activity .But, in the treated larvae at 48 hour, 72 hour and 96 hour at larval stages, the level of AsAT was highly increased. The level of AsAT activity in the haemolymph of the normal and Chlorantraniliprole treated larvae are given in Fig 3b. The treatment of Chlorantraniliprole in *Bombyx mori* larvae caused an increased AlAT activity in larval haemolymph. A significant variation was noted in 24 hour and 48hour of treated larvae as compared to normal. Whereas in 72hour the AlAT activity in treated larvae were two-fold in increase and was highly significant. The level of alanine amino transferase activity in the haemolymph of the normal and Chlorantraniliprole treated larvae are depicted in Fig.4b.

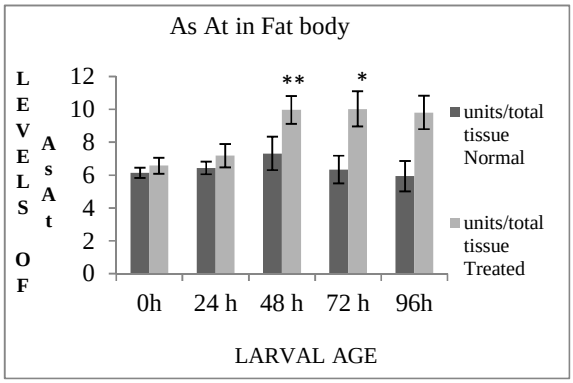

FIGURE 3a: levels in fat body

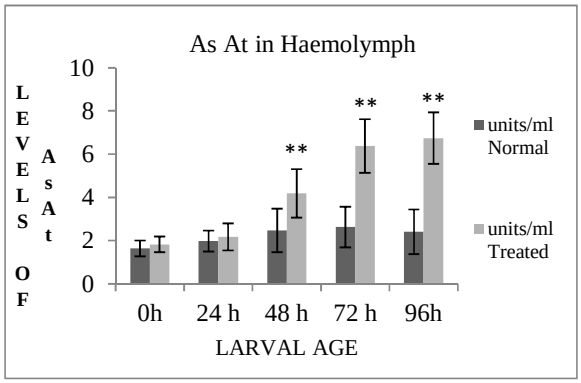

FIGURE 3b: levels in haemolymph

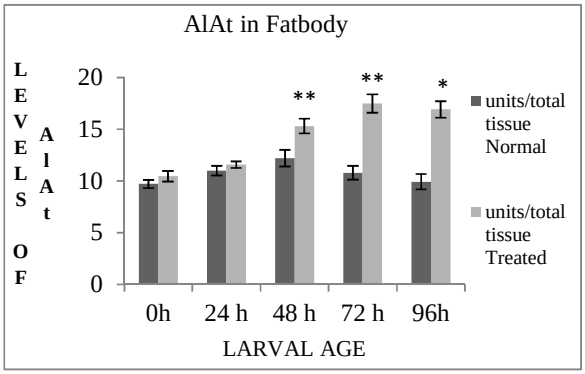

FIGURE 4a: AlAt levels in fat body

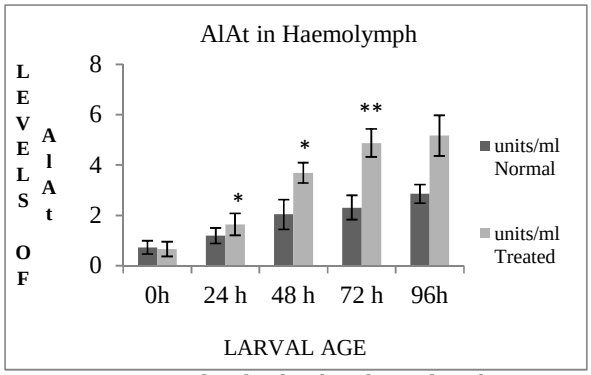

FIGURE 4b AlAt levels in haemolymph

All values are the means of 5 determinations

*The values are significant at P<0.05 against the normal for n=5,

**The values are highly significant at P<0.01 against the normal for n=5.

DISCUSSION

Several studies were evaluated on the toxicity effects of different insecticides in lepidopterans. The result of present investigation indicate that Chlorantraniliprole affect the general growth of silkworm larvae with a reduction in the body size and exhibiting general symptoms of slow movement, permanent insect body contraction, leading to rapid cessation of feeding *etc*. Recently, It has been reported that the mode of action of Chlorantraniliprole, were checked for their efficacy and poisoning characteristic by injection in to the larvae of *Spodoptera litura.* It is been suggested that, Ryanodine which bind to calcium release channel present in the sacroplasmic and endoplasmic reticulum is long known to cause, the contraction of insect skeletal muscle leading to paralysis (Nauen, 2006). Protein, being the key constituent, could be expected to play a role in the compensatory mechanism of silkworm during stress. There is evidence that chronic exposure to sub-lethal concentrations of CAP decrease levels of proteins, glycogen and total lipids in fish (Bantu *et al*., 2013). Findings suggest that RNA in both ovary and fat body was more sensitive to insecticides in *Nilaparvata lugens* and that the insecticide-induced changes in RNA content in turn influence protein synthesis in ovary and fat body (Lin *et al*., 2009). It was reported that Pyriproxyfen did not affect the protein band pattern in treated insects, although it affected the amount of protein concentration (Aribi *et al*., 2006). An insecticide, Phoxim exposure resulted in a significant reduction in total protein and increase in free amino acid and protease activity in the haemolymph of fifth instar *Bombyx mori* (Bing Li *et al*., 2012). Studies revealed that sub-lethal concentration of the phytopesticide nimbecidine on *Sphaerodema rusticum* (true bug) caused a significant decline in the contents of glycogen, protein and lipid and an increase of glucose and amino acid contents of fat body, testis and seminal vesicle (Shoba *et al*., 2011). So the result obtained in the Chlorantraniliprole treated silkworm larvae shows decreased haemolymph protein is correlated with other findings.

Urea is a significant excretory product of insects and plays a significant role in osmoregulation in animals. In normal condition, the urea level in 0 hour is high and then gradually decreases due to excretion. After 0h, the active feeding and active excretion occur hence the urea level is decreased. In the 96hour of the fifth instar larvae urea level increase because of the pre-pupation stage. In Chlorantraniliprole treated insect the urea level show increased magnitude compared to normal. High magnitude is mainly due to the result of toxic compounds that persists in the body of insect. The elevated ammonia level is reported in the liver and the muscle tissue of the *Clarius batrachus* (Begum, 2003). The lambda cyhalothrin treated kidney of the rats showed elevated blood urea (Hamadi *et al*., 2009). The urea elevated in treated larvae mainly for to overcome the osmotic imbalance as a result of dehydration. Aminotransferases are connecting links between carbohydrate and protein metabolic pathways. The protein synthesis requires a balanced amino acid pool

and transamination is one of the chief mechanisms which functions as a regulator of the same (Reddy *et al*., 1991). The silkworm larvae show increase aminotransferase activity is due to increase rate of feeding. The results of the present study when Chlorantraniliprole treated larvae showed a similar pattern of changes in the activities of AlAT and AsAT, but with a higher magnitude compared to the normal. This can be attributed to the reduced food intake of the larvae since feeding reflect aminotransferase activity levels. The larvae were found to feed a lesser quantity of treated leaves that bring the animal condition similar to partial starvation. Similar observations were seen that the carbaryl treatment will induce a high aminotransferase activity in the silkworm body tissue (Sebastian, 2002). The diet with high level of methyl parathion significantly increases the activities of aminotransferase in *Galleria mellonella* larvae (Ender *et al*., 2005).

CONCLUSION

Very few works have been reported on the effect of Chlorantraniliprole on the biochemical parameters, especially on the haemolymph of the lepidopteran pests. The present study was an attempt to elucidate the toxicological effects of Chlorantraniliprole in the fatbody and haemolymph of the fifth instar larvae of *Bombyx mori*. The results revealed that Chlorantraniliprole exerts significant decrease in the biochemical parameters of the larvae. Further it caused a noted increase of aminotransferase activity levels in the in both fat body and haemolymph of the fifth instar larvae of *B. mori.* In the view of such observations it can concluded that Chlorantraniliprole is a promising compound in the field of chemical pest control

REFERENCES

Adamsk, Kazimierz Ziemnicki., Katorzyna Fila., Rodoslav, V. Zikic., Andress Stajn (2012) Effect of long term-exposure to Fenitrothion on Spodoptera exigua and Tenebrio molitor larval development and antioxidant enzyme activity. Crop Protection. 15: 80-99.

Aribi, N., Smagghe, S., Lakbar, N., Soltani, N. (2006) Effect of pyriproxyfen, a juvenile shormone analog on development of the mealworm, Tenebrio molitor ,pestic. Biochem. Physiol. 84, 55-62.

Bantu, N., Vakita, V., Karra, S., (2013) Effect of chlorantraniliprole on biochemical and certain biomarkers in various tissues of freshwater fish Labeo rohita (Hamilton). Proteins. 1, 205–215.

Beale, R.N. and Croft, D. (1961) A sensitive method for the colorimetric determination of Urea. Journal of Clinical. Pathology, 14(4): 418-424.

Begum, Ghousia (2003) Carbofuran insecticide induced biochemical alternations in liver and muscle tissues of the fish clarias batrachus (Linn. and recovery response. Aquatic Toxicology, 66: 83-92.

Bing . Li., Zhe Cheng and Jie Cheng (2012) Cerium Chloride improves protein and carbohydrate metabolism of fifth instar larvae of Bombyx mori under Phoxim toxicity. Springer Science, 2, 38-51.

Ender, I., Ferah, A., Kemal, B. and Ahmet, G. (2005) Biochemical stress indicators of greater Lahm, P., Daniel Cordova, and James D. Barry. (2009) New and selective Ryanodine receptor activators for insect control. Bioorganic and Medicinal Chemistry, 17, 4127-4133.

Hamadi Fetoui, Mohamed Makni., Mouldi Garoui, E I., Najiba Zeghal. (2009) Toxic effects of lambda cyhalothrin, a synthetic pyrethroid pesticide, on the rat kidney: Involvement of oxidative stress and protective role of ascorbic acid. Experimental and Toxicological Pathology. 62, 593-599.

Jain., Janak Ahi., Sulekha Raji., Namitha Jain (2003) Ni-nitro-N methylurea induced changes in protein level of ovary and fat body of the insect Periplaneta americana. Pesticide Biochemistry and Physiology. 75: 40-52.

Lahm, P., Daniel Cordova, and James D. Barry (2009) New and selective Ryanodine receptor activators for insect control. Bioorganic and Medicinal Chemistry, 17, 4127-4133.

Larson J. L, Redmond C T and Potter D A (2014). Impacts of a neonicotinoid, neonicotinoid–pyrethroid premix, and anthranilic diamide insecticide on four species of turf inhabiting beneficial insects. Ecotoxicology, 23:252–259.

Lin-Quan Ge, Jun-Huan Hu, Jin-Cai Wu, Guo-Qing Yang, and Hainan Gu. (2009) Insecticide-induced changes in protein, RNA, and DNAcontents in ovary and fat body of female *Nilaparvata lugens* (Hemiptera: Delphacidae). Journal of Economic Entamology, 102(4): 1506-1514.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall, R.J. (1951) Proteins measurement with the Folin-phenol reagent. The Journal of Biological Chemistry, 193, 265-275.

Munhoz R.E.F., Bignotto T.S., Pereira N.C., Saez C.R.N., Bespalhuk R., Fassina V.A., Pessini G.M., Baggio M.P.D, Ribeiro L.F.C., Brancalhão R.M.C., Mizuno, S., Aita W S and Fernandez M A (2013) Evaluation of the toxic effect of insecticide chlorantraniliprole on the silkworm *Bombyx mori* (Lepidoptera: Bombycidae). Open Journal of Animal Sciences, 3:4, 343-353.

Nauen, R., Konanz, S., Hirooka, T., Nishimatsu, T. and Kodama,H. (2007) Flubendiamide, a unique tool in resistance management tactis for pest Lepidoptera difficult to control. Pflanzenschutz- Nachrichtenbayer, 60, 247-262.

Reddy, K. V. R., Remadevi, O. K., and Benchamin, K. V. (1991) Impact of the uzifly parasitization on the body growth, silk gland tissue somatic index and haemolymph properties of silkworm, Bombyx mori. Indian Journal of Sericulture, 39**,**113-120.

Reitman, S. and Frankel, S. (1957) A colorimetric method for the determination of serum glutamic oxalacetic and

glutamic pyruvic transaminases. American Journal of Clinical Pathology, 28(1): 56-60.

Rodrigues, A.C.M., Gravato ,C., Quintaneiro, C., Golovko, O., Žlábek, V., Barata, C., Soares, A.M.V.M., Pestana, J.L.T. (2015) Life history and biochemical effects of chlorantraniliprole on *Chironomus riparius*. Science of the Total Environment, 508: 506–513.

Sebastian, C.D. (2002) Effects of carbaryl on the protein metabolism of the final instar larva of Bombyx mori. Ph. D. Thesis, University of Calicut.

Shoba, V., Elanchezhiyan, C., Hemalatha, S., Selvisabanayakam (2011) Sub- lethal effect of phytopesticide nimbecidine on biochemical changes in the adult male insect *Sphaerodema rusticum* (Heteroptera: Belostomatidae).International Journal of Research in Pharmaceutical Sciences. 2(1), 12-17.