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Effect of Crude Neem (*Azadirachta indica)* Powder and Azadirachtin on the Growth and Acetylcholinesterase Activity of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

Amtul Jamil Sami,[1*] Sehrish Bilal,[1] Madeeha Khalid,[1] Farah Rauf Shakoori,[2] Fayyaz ur Rehman and A.R Shakoori[2]

[1]*Institute of Biochemistry and Biotechnology, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan*

[2]*Department of Zoology, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan*

[3]*School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan*

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Authors' Contribution
AJS conceived and designed the experiments. AJS and ARS wrote the article. SB and MK performed the experiments. FUR performed the computational analysis. FRS arranged the bioassays.

Key words
***Azadirachta indica*, Azadirachtin, *Tribolium castaneum*, Biopesticides, AChE.**

ABSTRACT

Adult and larval stages of *Tribolium castaneum* were treated with Neem leaf powder and azadirachtin (AZA) purified from Neem. Growth of insects decreased and mortality increased in a dose dependant manner. Acetylcholinesterase (AChE) activity was also decreased. AChE enzyme was treated with AZA and lower level of inhibition was observed. Computational analysis confirmed the results, as the molecule of AZA binds with the surface of enzyme rather than the active site residues. The results presented in the current study provide evidence that AChE which is an important enzyme involved in sensory perception can be inhibited by neem derived compounds thereby controlling pest attack by biopesticides.

INTRODUCTION

Considering hazardous effects of chemical pesticides on target organisms and development of resistance in the pests due to improper doses of insecticidal sprays, biopesticides are becoming more popular for pest control. Neem (*Azadirachta indica*) is the most investigated plant because of the diversity of natural insecticidal chemicals that can be extracted from it (Mordue *et al.*, 2010; Nisbet, 2000). The insecticidal, repellent and antifeedant properties of neem derivatives have been known for three decades (Mordue *et al.*, 2010) though farmers have been using this plant for centuries.

There are three main groups of compounds produced by neem plant (Schmutterer, 1990; Ascher, 1993). The neem seed extract contains several potentially insecticidal active ingredients that have been proven to cause repellence, growth and developmental abnormalities, disorders of metabolism, defects in ecdysis, enzymatic inhibition, fecundity and other aberrations. Coleoptera, Lepidoptera and Orthoptera are the most studied organisms in this respect (Morgan, 2009; Mordue *et al.*, 2010; Kavallieratos *et al.*, 2007).The tetranortriterpenoid azadirachtin (AZA) is the principal active ingredient in most formulations of neem (Senthil-Nathan *et al*., 2005; Senthil-Nathan *et al*., 2006). Krishnan *et al*. (2012) published on the genome and transcriptomes of *A. indica*. In our previous studies, we have shown that the neem derived compounds can inactivate glycohydrolases including amylases and cellulases (Sami, 2014; Sami and Shakoori, 2014).

Senthil-Nathan *et al.* (2007) had studied the effect of AZA on acetylcholinesterase (AChE) activity and histology of the brown planthopper *Nilaparvata lugens.* Robertson *et al*. (2007) reported binding of AZA with a binding complex primarily composed of heat shock proteins that caused nuclear disruption in *Drosophila* Kc167 cells. Moreover, it is capable of inducing apoptosis in SF9 cell lines by acting on some lysosomal pathways inducing natural cell death, indicating a possible mechanism of insecticidal action of neem (Wang *et al*., 2015). In this study we have determined the toxic effect of leaf powder of *A. indica* treated diet on mortality and the development of *Tribolium castaneum* adult and larval stages under laboratory conditions.

MATERIALS AND METHODS

Insect rearing

Red flour beetle, *Tribolium castaneum* insects were collected from the local vegetation and food stores and

* Corresponding author: amtuljamilsami@yahoo.com
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were reared in the laboratory on their basic diet, whole wheat flour for up to several generations. The cultures were maintained at 37±1ºC and 65±5% relative humidity for up to 30 days under standard conditions (Haliscak and Beeman, 1983).

Extraction of azadirachtin

Azadirachtin (AZA) was extracted from neem seed kernel using organic extraction as described previously (Sami and Shakoori, 2014).

Assessment of feeding behavior of insects to AZA

For the determination of antifeedant behavior of insects in response to AZA live *T. castaneum* larvae were used. A filter paper disc of 1 inch diameter was cut and soaked in inhibitor and dried for 30 mins. A petri plate was marked in 4 equal halves and the inhibitor soaked disc was placed in one quadrant. A total of 20 larvae were introduced in the quadrant containing filter paper disc at zero time and calculated the movement of insects with a time interval of 30 secs for 7 mins.

Bioassays

Dried neem leaf powder (100 mg) was mixed with 2 g of wheat flour in two clean glass vials. Two controls vials were prepared in the same way without neem powder. Thirty 3rd instar larvae and 30 adult insects were selected from the stock culture and placed in the treated glass vials (2.5 cm diameter and 5.5 cm height) containing the treated and control diet. Each glass vial was examined after two days interval for up to 8 days and percentage mortality, and average weight was calculated.

AChE activity assay

The AChE activity was measured according to the methods of Ellman *et al.* (1961) with some modifications. The reaction mixture contained 100 µl of homogenate and 2.6 ml of 0.1M phosphate buffer (pH 8). To this 0.01 M of the 5: 5-dithiobis-2-nitrobenzoic acid (DTNB) and 0.075 M Acetylthiocholine (ACh) was added. The reaction mixture was incubated at 30°C for 20 min, and the absorbance was taken at 412nm using the UV-Visible spectrophotometer.

RNA isolation

RNA was extracted from the insect with the help of Trizol reagent (bioteke) according to standard Trizol method to check the level of total RNA in treated samples. Extracted RNA was analyzed on 1.5% agarose gel.

Computational studies

For elucidation of docking of AZA with AChE of *T. castaneum* the structure for AChE *T. castaneum* (accession number EMBL EEZ99262.1) was generated based on homology modeling. The sequence was submitted for modeling to Swiss Model at ExPASy (Expert Protein Analysis System) bioinformatics resource portal of the Swiss Institute of Bioinformatics (SIB), an automated homology modeling serve, where BLAST was used to search the ExPDB (Expert Protein Data Bank) database for templates. AChE from *Drosophila melanogaster* was used as a template, as it shares 62.2% homology with *T. castaneum* AChE. After completing sequence alignment and its manual refinement, the catalytic site of AChE along with active site residues was docked with the inhibitor using Docking Server, a web based program that handles all aspects of molecular docking from ligand and protein set-up (Bikadi and Hazai, 2009; Morris, 1998).

Statistical analysis

The difference between larval weight, mortality, and repellency in control and treatment was determined by One-way ANOVA and Post Hoc was performed by LSD using SPSS 16.0 software. A significant level of 0.05 was used for all statistical data analysis.

RESULTS AND DISCUSSION

Effect on behavior of insect

Figure 1 shows behavioral sensitivity of *T. castaneum* larvae in the presence of AZA. Larvae demonstrated antifeedant properties in response to AZA exposure (Sami and Shakoori, 2014). Lepidoptera usually show enhanced sensitivity against AZA whereas Coleoptera, Hemiptera and Homoptera are less sensitive to AZA (Mordue *et al.*, 2010).

Mordue *et al*. (1998) has correlated the sensory response of chemoreceptors on the insects' mouth parts to antifeedant activity. AZA stimulates specific deterrent cells in chemoreceptors and blocks the firing of sugar receptors cells for stimulation of feeding (Blaney *et al*., 1990; Simmond *et al*., 1992; Mordue *et al*., 1998). Mordue *et al.* (2010) have reported the result generated by working on field insects and various growth defects.

Effect of neem on growth and mortality of insects

Insects and larvae exposed to crude neem powder showed a significant reduction in weight (Fig. 2) and increase in mortality rate (Fig. 3). Mortality rate of treated insects (adults and larvae) also decreased in a dose dependant fashion (Fig. 3A, B) which signifies the effect of inhibitors on insects. Similar report was

published by Mordue *et al*. (2010) which established that compounds from neem act on growth regulation including cuticle development and ecdysis, moreover it acts on cell's biosynthetic machinery thereby disregulating various metabolic pathways important for growth and development.

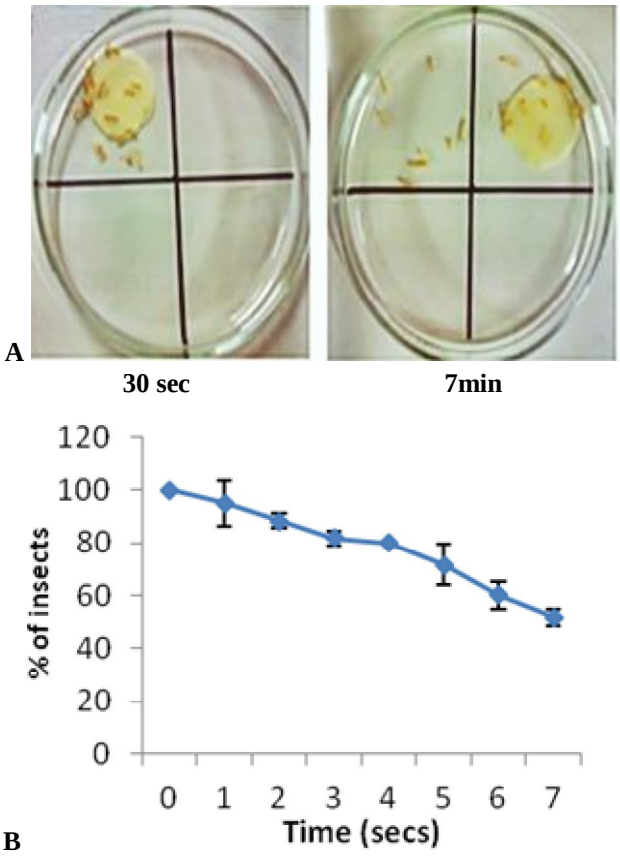

Fig. 1. A, Plates showing antifeedant behavior of live *T. castaneum* larvae in response to AZA; B shows percentage of larvae present in the zone containing inhibitor on the Petri dish after every minute. The graph shows a successive decrease in the number of insects around inhibitor with the passage of time (P=0.001). Each point represents mean of three replicates and error bars indicate SD.

The weight of control larvae and adults was 2.5±0.1mg and 23±4.0mg, respectively which after 8 days of treatment was reduced to 1.1±0.1 mg and 12.3±0.2 mg in larvae and adults, respectively (Fig. 2).

The mortality of insects increased upto approximately 37% and 10% in treated larvae and adults, respectively, compared to control insects (Fig. 3).

Disruption of growth (Mordue *et al*., 2010) and reproduction (Mordue *et al*., 1998) are considered to be the mains reasons of toxicity.

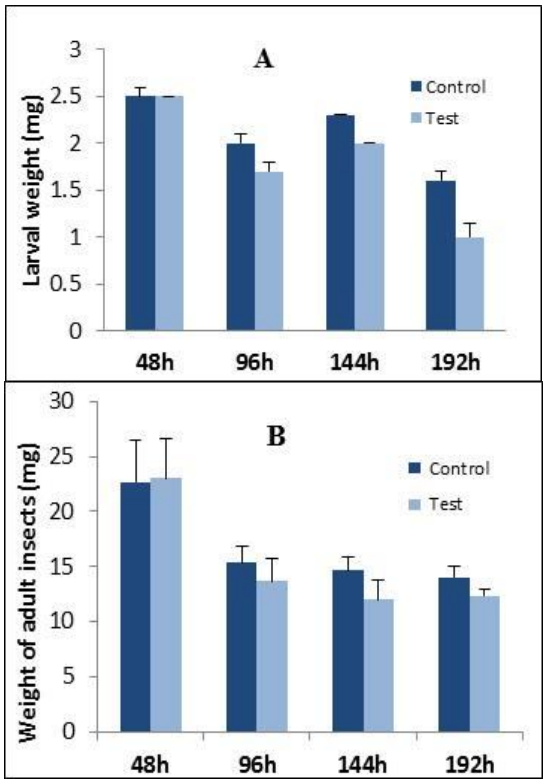

Fig. 2. Effect of neem life powder on the weights of larvae (A) and adults (B) of *T. castaneum.*

Effect on AChE activity

Figure 4 shows effect of neem leaf powder on total and specific activities of AChE of larvae of *T. castaneum.* The control insects showed increase in total AChE activity, reaching maximum level on 4th day of development. The total AChE activity gradually decreased during the subsequent developmental period. The specific AChE activities though maintain the same trend in the control larva, but its peak reached 144 h after the start of development. The activity increased 2 days after treatment and then decreased subsequently. Figure 4 shows that both the total and specific AChE activities of larvae increased but as the exposure to the inhibitor increased the activity of enzyme was reduced compared to control.

Figure 4 also shows mRNA levels of AChE genes which is a reflection of level of expression of AChE protein. The expression of the enzyme was higher at initial stage after 48 h as compared to normal subjects. Later the expression decreased. The specific activity of

the control and treated larvae was also comarabale to the expression studies. It shows that the insects reared on neem leaf powder had lower levels of proteins expressed as compared to the control subjects.

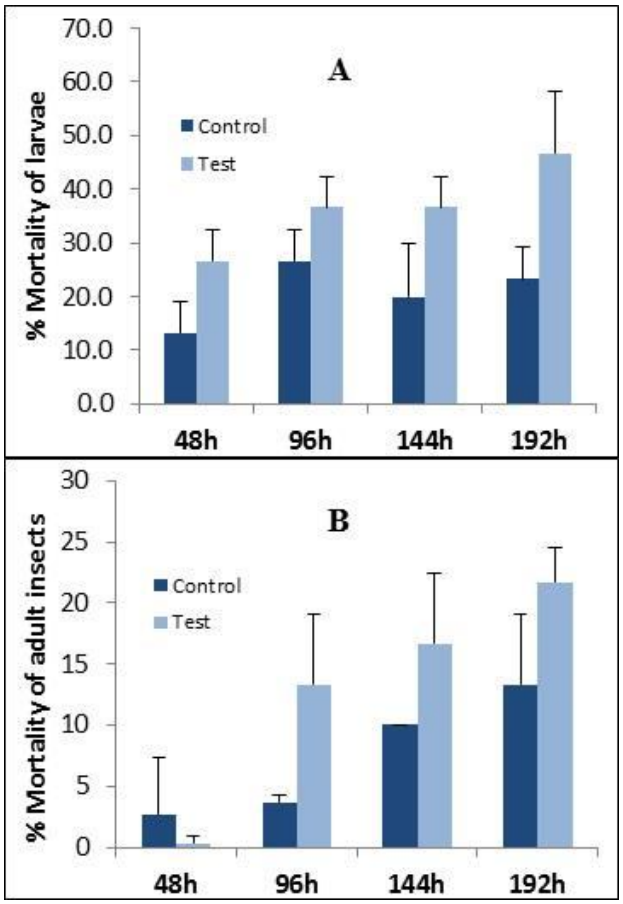

Fig. 3. Effect of neem leaf powder on larval and adult mortality. Number of dead insects was counted after every 2 days for 8 days.

Docking of AZA with AChE

Computational analysis revealed that binding of AZA with AChE is non-specific. AZA occupies various sites at the surface of AChE molecule (Fig. 5) rather than binding with the active site (Mordue *et al.*, 2010).

Activity assay indicated about 20% decrease in larval AChE with the increase in AZA concentration. The rates of enzyme activity cannot be determined specifically due to non-specific binding interactions of AZA with the enzyme as confirmed by computational analysis (Fig. 5). It was noticed that a higher concentration of AZA may be required for the inhibition. AChE activity was measured and considerable reduction was observed as compared to the controls, the specific activity also decreased. The level of RNA was also comparable to enzyme activity in treated insects (Lynn *et al*., 2012).

The docking results showed that in case of AZA it binds non-specifically on the molecule surface and also binding seems not so good. Its best fitting is not in cavity but on surface.

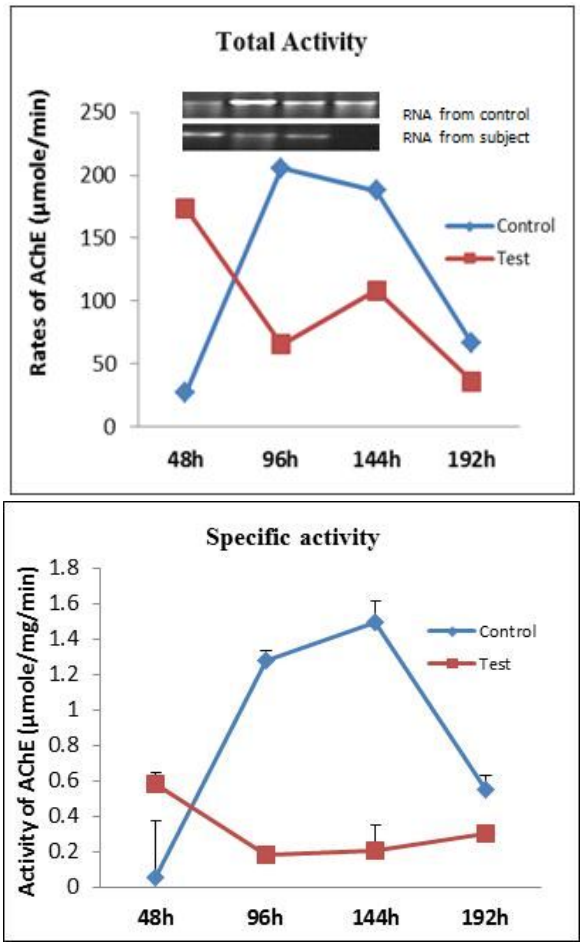

Fig. 4. Effect of neem leaf neem powder on AChE activity of larva recorded after every two days. A shows total activity while B shows the specific activity of AChE.

These results are in accordance with the experimental findings that AZA binds at various places on the surface of the molecule non-specifically. This phenomenon occurs majorly due to the extensive structure of AZA.

So in small volumes AZA has no considerable inhibitory activity but in larger amounts it inhibits the enzyme by blocking the access of substrate to the enzyme.

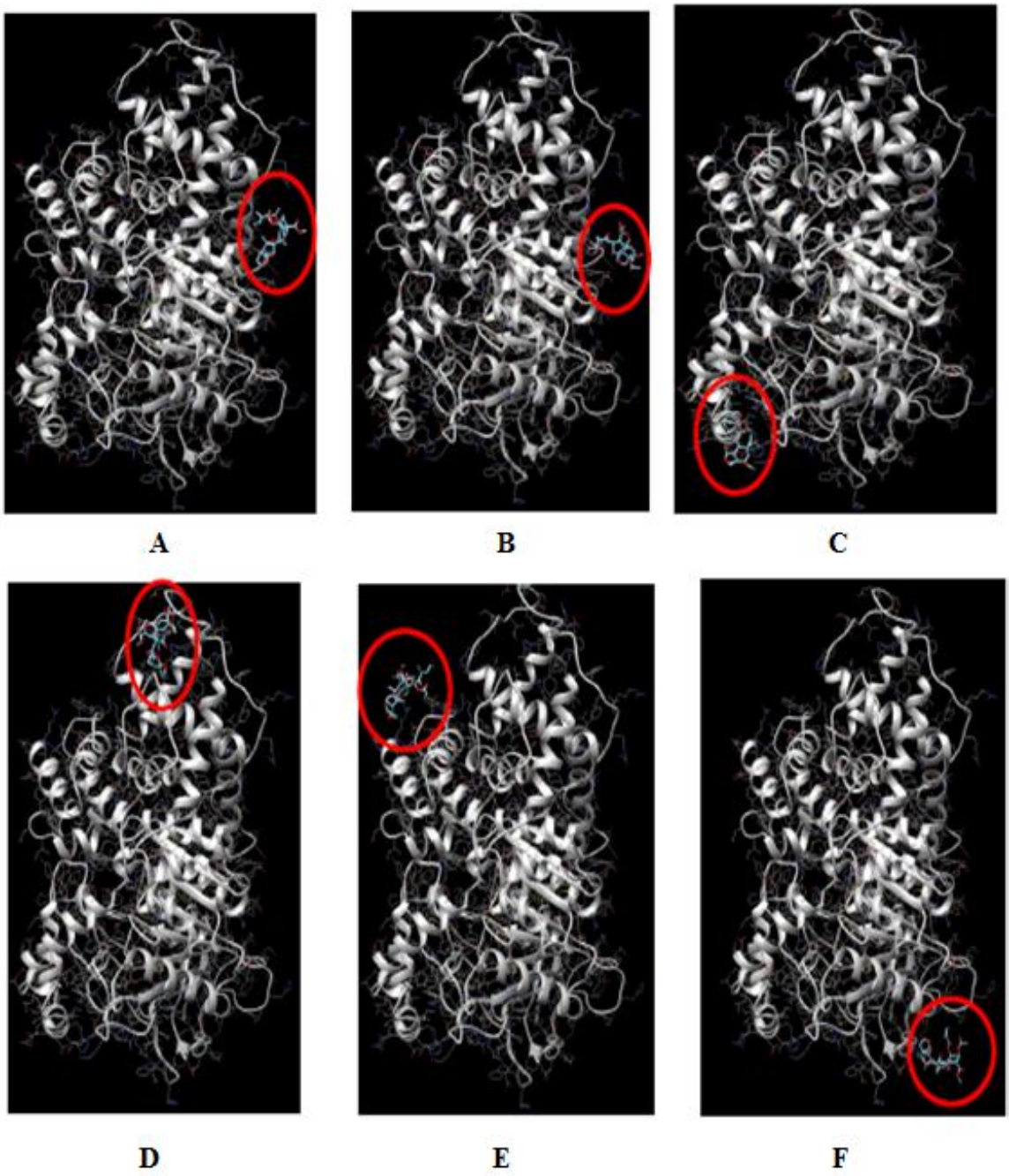

Fig. 5. Docking of AZA molecule on homology model AChE molecule, shows that the AZA molecule give it's best binding on the different positions on the surface of the enzyme molecule (A-F). Different positions of AZA molecule on the enzyme molecule are shown in red circle.

CONCLUSIONS

The results generated from this study are a platform for our further studies to investigate the molecular basis of *Azadirachta indica* compounds as biopesticides. Moreover, previous studies have been done on species of Coleoptera but no investigation has been done on stored grain pests of this order. These results represent the foundation for further studies on inhibition of stored grain insects using botanical pesticides.

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Statement of conflict of interest

The authors declare that there are no competing interests.

REFERENCES

Ascher, K.R.S., 1993. Nonconventional insecticidal effects of pesticides available from the Neem tree, *Azadirachta indica*. *Arch. Insect Biochem. Physiol.*, **22**: 433-449.

Bikadi, Z., and Hazai, E., 2009. Application of the PM6 semi-empirical method to modeling proteins enhances docking accuracy of AutoDock. *J. Cheminform.*, **1**:15.

Blaney, W. M., Simmonds, M. S. J., Ley, S. V., Anderson, J.C. and Toogood, P.L., 1990. Antifeedant effects of azadirachtin and structurally related compounds on lepidopterous larvae. *Ent. exp. Appl.*, **55**: 149-160.

Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**: 88-95.

Haliscak, J.P. and Beeman, R. W., 1983. Status of malathion resistance in five genera of beetles infesting farm-stored corn, wheat and oats in the United States. *J. econ. Ent.*, **76**: 717–722.

Kavallieratos, N. G., Athanassiou, C. G., Saitanis, C. J., Kontodimas, D. C., Roussos, A. N., Tsoutsa, M. S., and Anastassopoulou, U. A. 2007. Effect of two azadirachtin formulations against adults of *Sitophilus oryzae* and *Tribolium confusum* on different grain commodities. *J. Fd. Protect.*, **70**: 1627-1632.

Krishnan, N. M., Pattnaik, S., Jain, P., Gaur, P., Choudhary, R., Vaidyanathan, S., and Varghese, L., 2012. A draft of the genome and four transcriptomes of a medicinal and pesticidal angiosperm *Azadirachta indica*. *BMC Genomics*, **13**: 1.

Lynn, O.M., Kim, J.E. and Lee, K.Y., 2012. Effects of azadirachtin on the development and gene expression of fifth instar larvae of Indianmeal moth, *Plodia interpunctella*. *J. Asia-Pacif. Ent.*, **15**:101-105.

Morgan, D.E., 2009. Azadirachtin, a scientific gold mine. *Bioorg. med. Chem.*, **17**: 4096-4105.

Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K. and Olson, A. J., 1998. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.*, **19**: 1639-1662.

Mordue, A. J. L., Morgan, E. D., and Nisbet, A. J., 2010. Azadirachtin, a natural product in insect control. In: *Comprehensive molecular insect science* (eds. L.I. Gilbert, K. Iatrou and S.S. Gill), Elsevier, Amsterdam, pp. 117-134.

Mordue, A.J., Simmonds, M. S. J., Ley, S. V., Blaney, W. M., Mordue, W., Nasiruddin, M. and Nisbet, A. J., 1998. Actions of azadirachtin, a plant allelochemical, against insects. *Pestic. Sci.*, **54**: 277-284.

Nisbet, A.J., 2000. Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. *Anals Soc. Ent. Brasil*, **29**: 615-632.

Robertson, S.L., Ni, W., Dhadialla, T.S., Nisbet, A.J., McCusker, C., Ley, S.V. and Mordue, W., 2007. Identification of putative azadirachtin-binding complex from Drosophila Kc167 cells. *Arch. Insect Biochem. Physiol*. **64**: 200-208.

Sami, A.J., 2014. Azadirachta indica derived compounds as inhibitors of digestive alpha-amylase in insect pests: Potential bio-pesticides in insect pest management. *Europ. J. exp. Biol.*, **4**: 259-264.

Sami, A.J. and Shakoori, A. R., 2014. Potential of azadirachtin and neem (*Azadirachta indica*) based saponins as biopesticides for *in vitro* insect pests cellulase (Beta-1, 4-Endoglucanase) enzyme inhibition and *in vivo* repellency on *Tribolium castaneum*. *Br. Biotechnol J.*, **4**: 904.

Schmutterer, H., 1990. Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. *Annu. Rev. Ent.*, **35**: 271–297.

Senthil-Nathan, S., Choi, M. Y., Paik, C. H., Seo, H. Y., Kim, J. D. and Kang, S. M., 2007. The toxic effects of neem extract and azadirachtin on the brown planthopper, *Nilaparvata lugens* (Stål) (BPH) (Homoptera: Delphacidae). *Chemosphere*, **67**: 80-88.

Senthil-Nathan, S., Kalaivani, K., Murugan, K. and Chung, P.G., 2005. The toxicity and physiological effect of neem limonoids on *Cnaphalocrocis medinalis* (Guenee) the rice leaffolder. *Pestic. Biochem. Physiol*., **81**: 113-122.

Senthil-Nathan, S., Kalaivani, K., Chung, K. and Murugan, K., 2006. Effect of neem limonoids on lactate dehydrogenase (LDH) of the rice leaffolder, *Cnaphalocrocis medinalis* (Guene´e) (Insecta: Lepidoptera: Pyralidae). *Chemosphere*, **62**: 1388-1393.

Simmonds, M. S., Simpson, S. J. and Blaney, W. M. 1992. Dietary selection behaviour in *Spodoptera littoralis*: the effects of conditioning diet and conditioning period on neural responsiveness and selection behaviour. *J. exp. Biol.*, **162**: 73-90.

Wang, Z., Cheng, X., Meng, Q., Wang, P., Shu, B., Hu, Q. and Zhong, G., 2015. Azadirachtin-induced apoptosis involves lysosomal membrane permeabilization and cathepsin L release in *Spodoptera frugiperda* Sf9 cells. *Int. J. Biochem. Cell Biol.*, **64:** 126-135.