



## Highlighted Article

# Effect of azadirachtin on acetylcholinesterase (AChE) activity and histology of the brown planthopper *Nilaparvata lugens* (Stål)<sup>☆</sup>

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## Abstract

The effect of azadirachtin (AZA), a tetranortriterpene from neem, on the mortality, acetylcholinesterase (AChE) activity, and the development of the ovaries of female brown planthopper (BPH), *Nilaparvata lugens* (Stål) was tested in laboratory experiments. The lethal concentrations were determined and applied topically to the adult female BPH (LC<sub>50</sub> = 0.47 ppm and LC<sub>90</sub> = 0.96 ppm). The treated insects exhibited toxic symptoms with a dose-dependent mortality. The LC<sub>50</sub> concentrations were tested against AChE activity of the BPH. The results indicate that AZA significantly inhibits the activity of AChE only at the high dose ( $P < .0001$ ) compared with control. Adult females that were exposed to AZA showed a significant reduction in weight (23%, 40%, and 64% for 0.1, 0.25, and 0.5 ppm, respectively). Fecundity was also significantly reduced in AZA treatments compared to the control. Histological study of ovary sections revealed abnormalities in follicular epithelial cells due to AZA treatment.

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**Keywords:** Azadirachtin; BPH; AChE; Enzyme; Histology; Ovary; Follicular epithelial cell

## 1. Introduction

Biopesticides with different modes of action may minimize insecticide resistance and pest resurgence problems while being safe and ecologically acceptable (Matthews, 1999; Copping and Menn, 2000; Senthil Nathan et al., 2006a,b). The insecticidal, repellent and antifeedant properties of neem (*Azadirachta indica* A. Juss) derivatives have

been known for three decades (Schmutterer, 1990; Ascher, 1993). Although several potentially insecticidal active ingredients occur in neem seed extract, the principal active ingredient in most formulations is the tetranortriterpene azadirachtin (AZA) (Sundaram, 1996; Senthil Nathan et al., 2005). But the action of AZA against the brown planthopper (BPH), with respect to acetylcholinesterase (AChE) activity, is unknown.

AChE-EC 3.1.1.7 is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter, acetylcholine, in the nervous system in various organisms (Oehmichen and Besserer, 1982; Wang et al., 2004). It is well known that the altered AChE is one of the main resistance mechanisms in many insect pests. Insensitivity of AChE observed in organophosphorous or carbamate-resistant insects has been well documented for various insect pests (Hama, 1983; Hemingway et al., 1986; Yoo et al., 2002; Wang et al., 2004). Organophosphorous insecticides, such as diazinon, target AChE and irreversibly

**Abbreviations:** BPH: brown planthopper; AZA: azadirachtin; ACh: acetylcholine; AChE: cetylcholinesterase; LC: lethal concentration; IGR: insect growth regulator; DAG: days after germination

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inhibit the enzyme by phosphorylating a serine hydroxyl group within the enzyme active site (Russell, 1980; Babu et al., 1989; Fournier and Mutero, 1994; Bretaud et al., 2000). Likewise AChE was affected by neem and other botanicals. For example, AChE activity is significantly inhibited in rat brain when treated orally with 80, 160, and 320 mg/kg of Vepacide, an active ingredient from neem (*A. indica*) seed oil (Rahman et al., 1999).

The BPH is an important insect pest of rice in Asian countries, causing damage by directly sucking phloem sap (Sogawa, 1980) in rice plants and causing economic damage directly by producing symptoms commonly referred to as 'hopperburn' (Bae and Pathak, 1970; Sogawa, 1980). The indiscriminate use of synthetic insecticides in rice fields may lead to outbreaks of the BPH due to their lethal effects on natural enemies (Fabellar and Heinrichs, 1986; Tanaka et al., 2002; Claridge et al., 2002). Also, field application of certain pesticides has been shown to induce resurgence of the BPH (Chelliah and Heinrichs, 1980; Hardin et al., 1995).

The objective of this study is to identify the effect of AZA on the adult female BPH using biochemical and toxicological (histological) methods.

## 2. Materials and method

### 2.1. Laboratory mass culture of BPH

The susceptible strain of *Nilaparvata lugens* has been maintained for more than 10 years in the laboratory of the Honam Agricultural Research Institute, Rural Development Administration (RDA), Iksan, South Korea, without any exposure to insecticide. These insects were maintained on *Oryza sativa* L. seedlings (9–11 days after germination (DAG) for first to third instar; 21 DAG for late third instar to adult) in acrylic cages at  $27 \pm 1$  °C, 40–60% RH, and a photoperiod of 16:8 (L:D) h.

### 2.2. Chemicals

Acetylthiocholine (ATCh) iodide, 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), phosphate buffered saline pH 7.4, and eserine salicylate were purchased from Sigma. All other chemicals used were of reagent grade. Pure AZA (purity >99%) was generously provided by Prof. Steve Ley, University of Cambridge, UK.

### 2.3. Bioassay

Test-material solutions (AZA—0.10, 0.25, 0.5, and 1 ppm) were applied by topical application to test insects, which were anesthetized by using CO<sub>2</sub>. One micro liter of test-material solution containing the appropriate concentration of AZA was applied to individual female adults by a standard digital syringe (Hamilton, USA, Model 7110) and the 24 h mortality was subjected to a probit analysis (Finney, 1971) to determine LC<sub>50</sub> and LC<sub>90</sub> values. Twenty females per concentration were used for all the experiments and each experiment was replicated five times. After the topical application, the BPH were transferred into an experimental cage (48 cm height and 34 cm width, two sides of which had 20 cm<sup>2</sup> mesh panels for aeration) containing four rice seedlings (21 DAG) for further observation. For the BPH weight experiment, five newly emerged females were used. Initial weight and final weight (72 h after treatment) were recorded. For the fecundity test, a 1 week-old once-mated female was caged for 72 h on a rice plant after treatment with AZA. The number of eggs in each replicate was recorded. All the experiments were replicated

five times. Mean body weight (mg) and mean numbers of eggs laid by the female are shown in figures after analysis with analysis of variance (ANOVA).

### 2.4. Estimation of AChE -EC 3.1.1.7 inhibition

The activity of AChE was determined by the method of Ellman et al. (1961) using ATCh iodide as a substrate. Twenty female adult BPH were homogenized in 20 ml of ice-cold 0.1 M phosphate buffer (pH 7.4). After filtering through cheese cloth, the homogenate was centrifuged at 10,000g for 20 min (Beckman Coulter, Optima L-100 XP ultracentrifuge). The supernatant was directly used as the AChE enzyme source. A series of LC<sub>50</sub> and below LC<sub>50</sub> concentrations of AZA (0.10, 0.25, and 0.50 ppm) were added to an incubation mixture consisting of 1 ml of enzyme solution (equivalent to one female), 2 ml 0.1 M phosphate buffer, 100 ml 0.1 mM DTNB was added and the samples were placed at 30 °C in a shaking water bath for 10 min. Then 20 ml of 0.075 mM acetylcholine iodide was added to the mixture. After incubation for 20 min at 30 °C, the reaction was stopped by adding 0.2 ml of 5 mM eserine salicylate. The AChE activity was spectrophotometrically measured at 412 nm (UV visible spectrophotometer, UV2450, Shimadzu). The enzyme activities were expressed as  $\mu\text{mol}/\text{min}/\text{mg}$  protein.

### 2.5. Histology

The effect of AZA on the ovary was investigated on female adults of BPH 72 h after topical application time. The samples were fixed in Bouin's solution. The specimens were embedded in an embedding medium (optimal cutting temperature (OCT), Tissue-Tek, Sakura, USA). The blocks were cooled to  $-27$  °C for 3 h and cut into 1.5  $\mu\text{m}$  ribbons with an ultra-cryo-microtome (Cryocut 1800, Leica, Germany). The ribbons were stained with Delafield's hematoxylin and counter-stained with eosin, and mounted after drying. The sections were observed and photographed under light microscope (Leica, DMRE, Germany).

### 2.6. Statistical analysis

Data from bioassay and enzyme activity were subjected to ANOVA of arcsine square root transformed percentages. Differences between the treatments were determined by Tukey's multiple range tests. Difference between means were considered significant at  $P \leq 0.05$  (Snedecor and Cochran, 1989; SAS Institute, 2001). The lethal concentrations (both LC<sub>50</sub> and LC<sub>90</sub>) were calculated using probit analysis (Finney, 1971) and values were expressed as means with standard deviation of five replicates. Mortality was corrected using Abbott's (1925) formula, if it was necessary.

## 3. Result

### 3.1. Mortality bioassay

In bioassay tests, AZA treatments applied topically on a dorsal surface of the BPH at concentrations of 0.25, 0.5, and 1.0 ppm tested affected the BPH and produced mortality. The LC<sub>50</sub> and LC<sub>90</sub> were 0.47 and 0.96 ppm, respectively. The effect of AZA on the mortality of BPH could be observed at the different concentrations (0.25, 0.5, and 1 ppm), and it was significantly different from control ( $F = 16108.4$ ,  $df = 19$ ,  $P = < 0.0001$ ) (Fig. 1).

### 3.2. Effect of AZA on AChE activity

Neem compound AZA inhibited AChE activity, over a wide range of concentrations even below the LC<sub>50</sub>. Doses

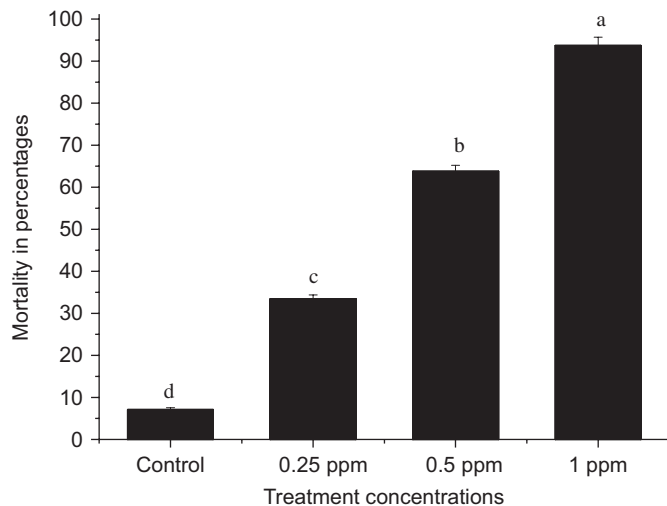


Fig. 1. Mean (SEM ±) percentage mortality of the adult female BPH after treatment with AZA. Means (SEM ±) followed by the same letters above bars indicate no significant difference ( $P < 0.05$ ) according to a Tukey test.

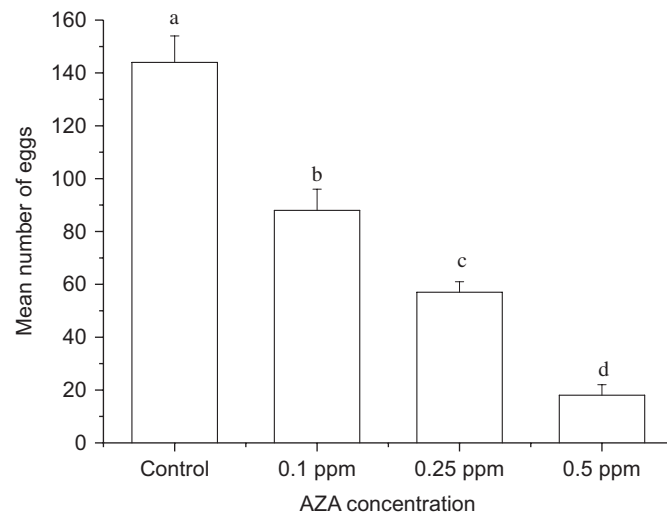


Fig. 3. Number of eggs laid by the female BPH after treatment with AZA (mean (SEM ±) fecundity of a single insect).

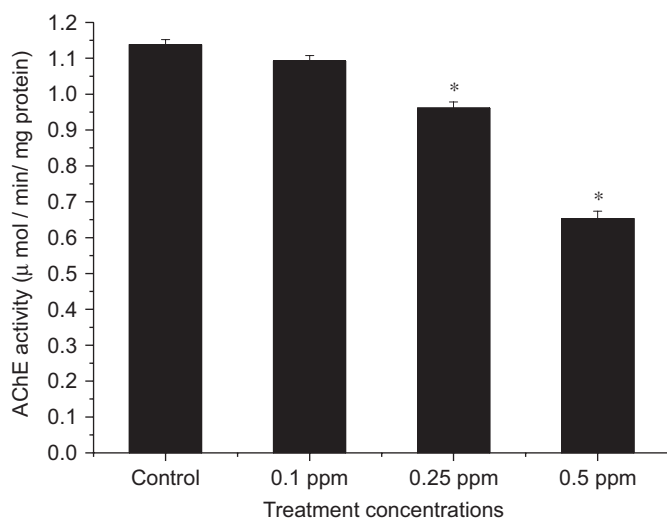


Fig. 2. AChE activity of adult female BPH after treatment with AZA. Asterisks indicate significant differences between treatment and control by a Tukey test at the 0.05 level.

equal to the  $LC_{50}$  and below were evaluated on enzyme activity in the adult female BPH (0.10, 0.25, and 0.50 ppm for AZA). The AChE activity varied with the dose of treatment. The AChE inhibition of the adult female BPH at various concentrations of AZA is shown in Fig. 2. The effect on AChE enzyme activities was concentration dependent. The AChE activity of adult female BPH was significantly inhibited by only 0.25 and 0.5 ppm ( $F = 817.55$ ,  $df = 19$ ,  $P = < 0.0001$ ).

### 3.3. Effect of AZA on the female BPH weight, fecundity and ovary

Among AZA treatments, the maximum number of eggs (88.4) laid by a female were obtained in the 0.10 ppm

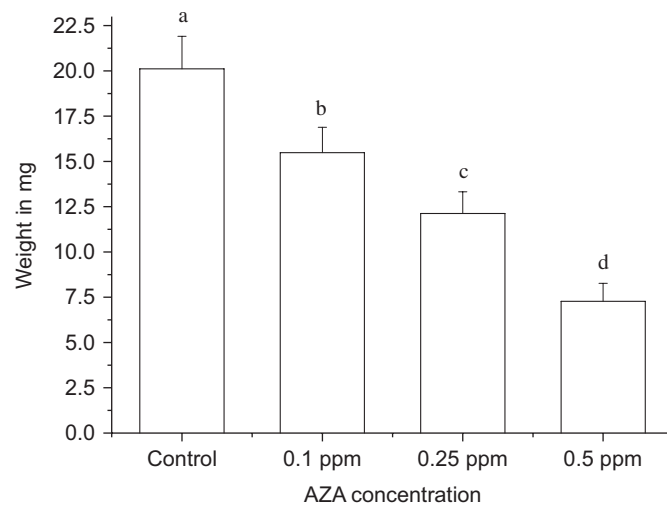


Fig. 4. Weight of adult female BPH after treatment with AZA (mean (SEM ±) weight of five insects).

(Fig. 3) treatment. The maximum (144.4) number of eggs was laid in the control treatment ( $F = 378.73$ ,  $df = 19$ ,  $P < 0.0001$ ). This indicated that treatment of adults with AZA has strong inhibitory effects on fecundity. The adult female body weight was 20.11 mg in control. At 0.25 ppm the female BPH weight fell to 12.12 mg (Fig. 4). It was further significantly reduced to 7.27 mg ( $F = 269.94$ ,  $df = 19$ ,  $P < 0.0001$ ) in the AZA treatment at 0.5 ppm. The results clearly show a gradual decrease in the adult weight of insects treated with AZA.

The effects of  $LC_{50}$  doses of AZA applied topically on the newly molted adult female BPH were investigated on the ovary structure after the exposure time of 72 h. The cross-sections of ovary from control and treated series were observed under light microscopy (Fig. 5). In controls, the follicle cells of the female appeared to be formed by large similar cells (Fig. 5A). AZA seemed to affect the structure

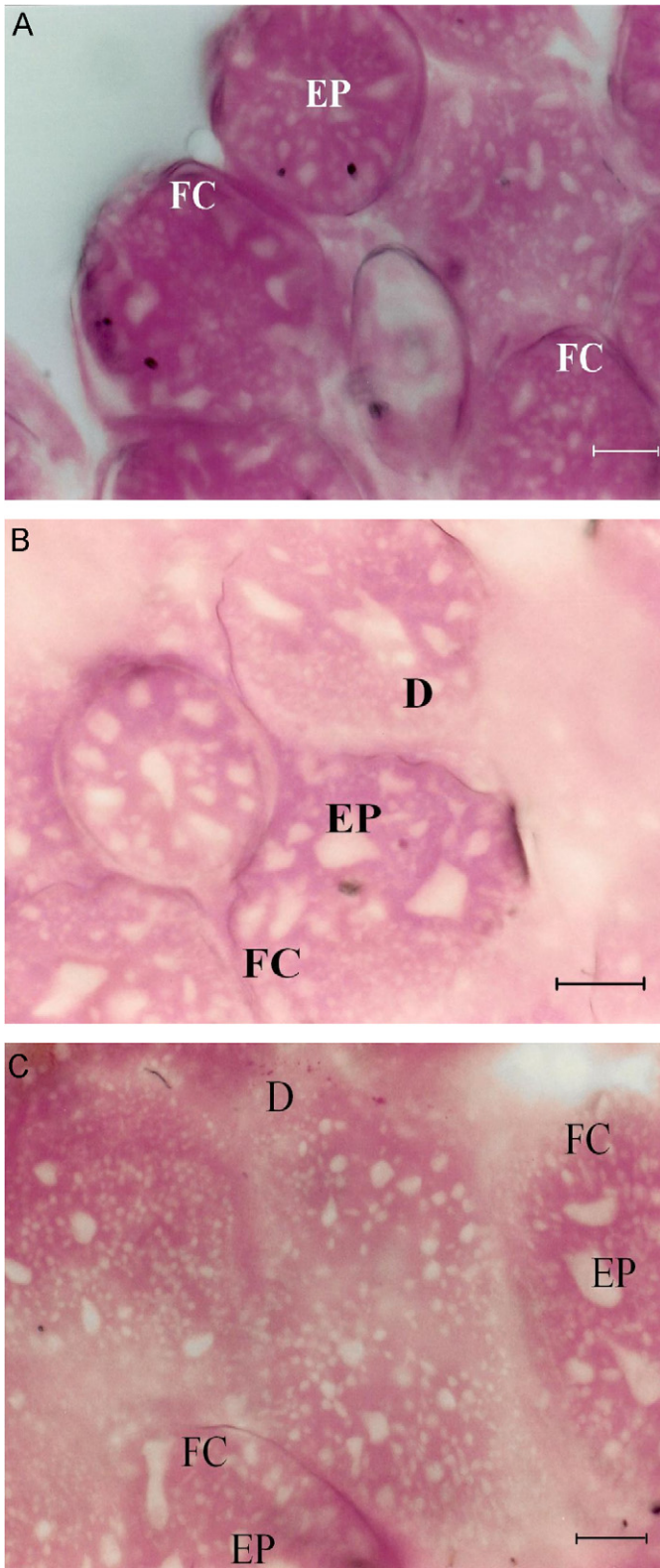


Fig. 5. Histological section of the ovary of the adult female BPH. (A) Control, (B) treated with 0.25 ppm AZA and (C) treated with 0.5 ppm of AZA. (EP—epithelial plug; FC—follicle cell; D—Damaged portion; bar = 4 μm.)

of follicle epithelial cell. At the  $LC_{50}$  concentration of AZA at 0.25 ppm the follicle epithelial cells were disrupted when compared with controls (Fig. 5B). At the  $LC_{50}$



Fig. 6. Morphological structure of the eggs of BPH. (A) Control and (B) treated with 0.25 ppm.

concentration of AZA at 0.5 ppm the follicle epithelial cells are completely destroyed (Fig. 5C). Also, the malformed egg structure was observed in a lower dose treatment (Fig. 6).

### 3.4. Morphological effect of AZA

All topical treatments at  $LC_{90}$  levels of adult female BPH resulted in some malformed adult females that were unusually dark in color (Fig. 7A–C). Body segments and structures located at the abdomen and leg parts were lost. The outer cuticle color changed to a brownish black. The ovipositor had a very thin cuticle, which swelled up, ruptured, and allowed the internal fluid of the female BPH to leak out in the 0.5 ppm AZA treatment. Deformities of the legs and mouth parts were observed in the 1 ppm treatment.

## 4. Discussion

The  $LC_{50}$  and  $LC_{90}$  treatments of AZA were topically applied in a single administration onto the adult female

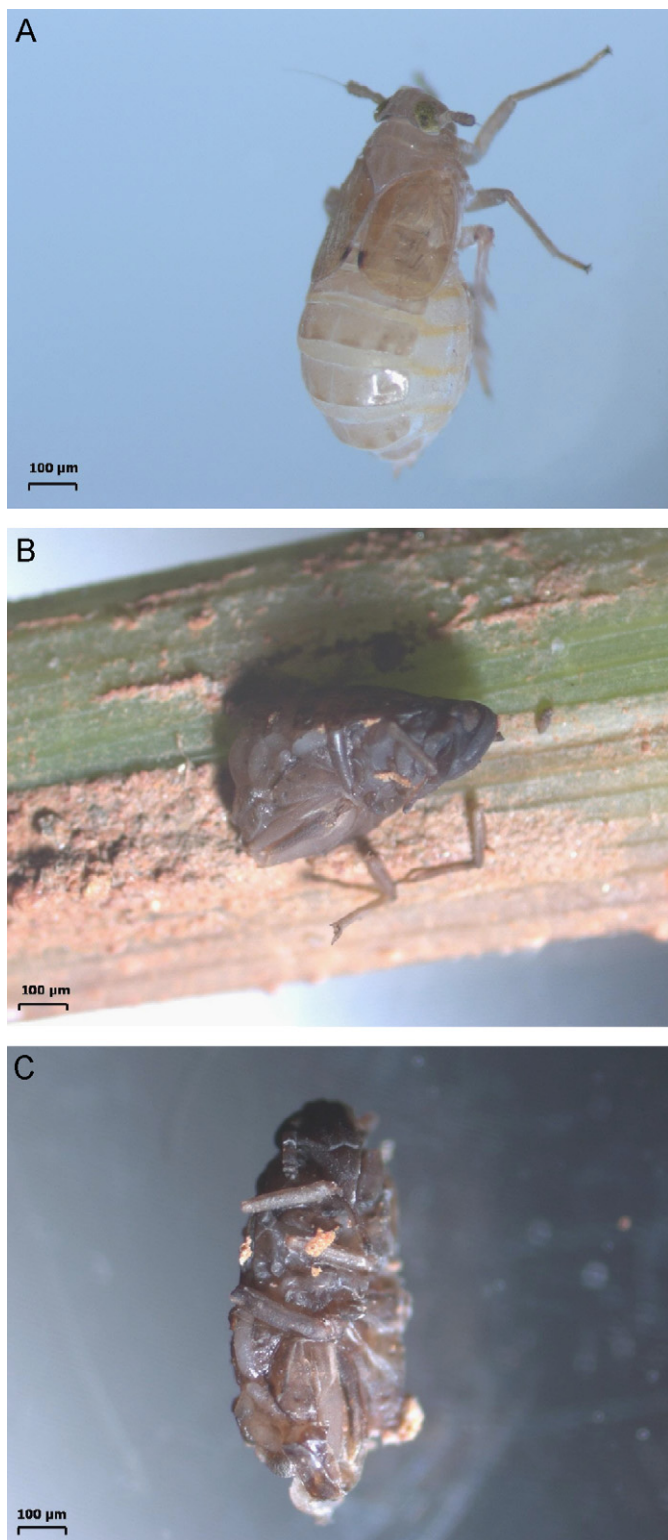


Fig. 7. Morphological structure of the adult brachypterous gravid female BPH. (A) Control and (B) treated with 0.5 ppm AZA and (C) treated with 1 ppm of AZA.

BPH within the 24 h of molt. As shown in Fig. 2, AZA produced mortality in a dose-dependent manner. AZA causes mortalities in insects as a result of its insect growth

regulatory (IGR) activity (Mordue et al., 1998; Sundaram, 1996). The main effects of neem and AZA in our experiment were the inhibition of the AChE activity in higher doses of AZA treatment. The alteration of AChE was observed in the cockroach, *Periplaneta americana* L., at 4 ppm of AZA (Shafeek et al., 2004) and the snail, *Limnaea acuminata* Lamarck, at 40% and 80% concentrations of neem oil (Singh and Singh, 2000). It was also observed that 25 g distilled water extracts of the botanicals *Punica granatum* L., *Thymus vulgaris* L., and *Artemisia absinthium* L., significantly inhibited the AChE activity of nematodes at 100% concentrations (Korayem et al., 1993). Several essential oils from aromatic plants, monoterpenes, and natural products have been shown to be inhibitors of AChE (Shaaya and Rafaeeli, 2007). Pulegone-1,2-epoxide, isolated from the Verbenaceae medicinal plant, *Lippia stoechadifolia* L. (Poleo), showed an irreversible inhibition of the AChE in house fly and Madagascar roach (Grundy and Still, 1985). Also, Ryan and Byrne (1988) reported that the six monoterpenes, Pulegone ( $0.12 \times 10^4$  ppm), gossypol ( $0.45 \times 10^4$  ppm), citral ( $1.5 \times 10^4$  ppm), linalool ( $2.5 \times 10^4$  ppm), (-)-bornyl cetate ( $2.7 \times 10^4$  ppm), and cineole ( $4.3 \times 10^4$  ppm), representing characteristic constituents of secondary plant metabolites were tested on AChE from the eel *in vitro* and, on toxicity, *in vivo*, in a stored product pest, *Tribolium castaneum* Herbst. All six terpenoids inhibited AChE. Similarly, Kostyukovsky et al. (2002) using AChE extracted from the stored product insect, *Rhizopertha dominica* Fab, showed that inhibition of AChE activity was obtained by  $10^{-3}$  M levels of the essential oils from the plants belonging to the Labiateae family.

The present finding also showed that reduced weight during egg maturation, with reduced growth in treated adult, confirms earlier findings (Senthil Nathan et al., 2007). Other studies noted depressed development of ovarian follicles of the milkweed bug, *Oncopeltus fasciatus* Dallas (Dorn et al., 1986) and increased mortality (Saxena and Khan, 1985) and decreased fecundity (Saxena et al., 1984) in the BPH by neem oil and cake. Studies carried out on AZA and some of its derivatives as insect feeding deterrents revealed that neither hydrogenation of  $\Delta$  double bonds nor deacetylation caused any change in effect, but blocking of hydroxyl groups affected the feeding inhibitory activity, while acetylation of AZA caused a 25% decrease in the activity; etherification with a bulky trimethylsilyl group eliminated it altogether (Devakumar and Dev, 1996; Roy and Saraf, 2006). Thus, the stereochemical environment around the hemiacetal region seemed to be critical for its activity (Roy and Saraf, 2006).

AChE is of interest because it is the target site for organophosphorous and carbamate insecticides in the central nervous system, and its role in cholinergic synapses is essential for insects and higher animals (Fournier and Mutero, 1994) Inhibition of AChE causes accumulation of ACh at the synapses, so that the post-synaptic membrane is in a state of permanent stimulation, which results in paralysis, ataxia, general lack of co-ordination in the

neuromuscular system, and eventual death (Singh and Singh, 2000; Aygun et al., 2002).

Although the adult female BPH treated with an LC<sub>50</sub> dose survived for as long as it took the control female to lay eggs, the treated females were unable to lay their eggs or laid a very few malformed eggs. Reduced fecundity and weight of BPH when treated with AZA suggests that egg development may have been hindered by low food intake, but there was a significant increase ( $P < 0.01$ ) in the fecundity in the control. But egg maturation in AZA-treated insects is often incomplete; chorion formation is not very frequent (Dorn et al., 1986). Also the time between onset of egg maturation and chorion deposition is longer than that in normal females which are comparable with the effect of AZA on the ovarian development of *O. fasciatus* (Dorn et al., 1986).

Histological examinations of the abdomen from treated adult female BPH revealed that topically applied AZA resulted in major cytological perturbations in relation to the formation of chorion. At the LC<sub>50</sub> dose of AZA the ovarian follicle epithelial cells appeared completely destroyed (Fig. 4).

## 5. Conclusion

To conclude, AZA inhibits AChE in the adult BPH only in higher doses. Results from weight, fecundity, and histological studies suggest that the neem compound AZA directly affects the ovarian follicles and vitellogenesis in BPH. Our histological and morphological observations support this hypothesis.

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## Disclaimer

We assure that the research does not involve any humans or other domestic animals in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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