



Neural Oxidant-stress by Azadirachtin Induces Anti-oxidative Enzymes Evincing Biomarker Potential in Paddy Pest, *Spathosternum prasiniferum prasiniferum* (Orthoptera:Acridoidea)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors participated to design the experiment. Author BM performed experiments. Author BM wrote the first draft and authors SM and AD checked. Author SM performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Azadirachtin ($C_{35}H_{44}O_{16}$ /AZT) develops antifeedancy/growth-regulation/fecundity-suppression/sterilization/oviposition/repellence and deformity in insect via biochemical/cellular changes and causes their death. Agricultural productivity/quality/eco-sustainability is concerned to this issue. ROS are cytotoxic-factors generated in invertebrates in stress-conditions. The present in-vivo/in-vitro study aimed to investigate the impact of dose dependant AZT toxicity on oxidative-stress-marker (alkaline-phosphatase/ALP; thiobarbituric-acid-reactive-substances/TBARS; non-protein-

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soluble-thiols/NPSH; acetyl-cholinesterase/AChE) and antioxidant-enzyme activity (superoxide-dismutase/SOD; catalase/CAT; glutathione-peroxidase/GPx; amylase) in brain/hemolymph of *Spathosternum prasiniferum prasiniferum* (Walker, 1871) (Orthoptera: Acridoidea). Acridids are highly abundant and bio-indicator of grassland-ecosystem. During cultivation, insects are exposed (dose/time dependant) to AZT. AZT developed restlessness, jerky-movements and swarming-movements in the insects. It promoted oxidative-stress-marker in brain/hemolymph in both sexes but female had significantly stimulated antioxidant-enzymes to overcome cellular-stress. Increase of brain TBARS, antioxidant-enzymes and decrease in NPSH by AZT indicates oxidative-stress induction in this species. In several instances damage to the brain DNA was noticed. In general female insect responded more intensely with some prominent adaptive strategies.

Keywords: *Azadirachtin; Orthoptera: Acridoidea; brain; hemolymph; reactive oxygen species; antioxidant defence.*

ABBREVIATIONS

AZT : *Azadirachtin*
 AD : *Approximate Digestibility*
 ECD : *Efficiency of Conversion of Digested Food*
 ECI : *Efficiency of Conversion of Ingested Food*
 ROS : *Reactive Oxygen Species*
 MDA : *Malondialdehyde*
 TBARS : *Thiobarbituric Acid Substances*
 NPSH : *Non-protein Thiols*
 ALP : *Alkaline Phosphatase*
 AChE : *Acetyl cholinesterase*
 SOD : *Superoxide Dismutase*
 CAT : *Catalase*
 GPx : *Glutathione Peroxidase*
 DNA : *Deoxyribonucleic Acid*

1. INTRODUCTION

All beneficial plants are moderately toxic in nature due to production of secondary metabolites to protect themselves against various predators [1]. Neem (*Azadirachtaindica*) is a natural source of insecticides. It is used as bio-control agrochemicals. Their toxicological properties are beneficial in way of killing harmful pest insects. Again, beneficial insects are also a member of ecosystem and its food chain and food web. So the massive loss or death of insect may result in an alarming situation to the biodiversity in that ecological niche. The antifeedant component, azadirachtin (AZT) is a tetranortriterpenoids present in neem which affects growth, development, behaviour, reproduction and metamorphosis in diverse insect taxa. AZT affects on insect's muscles, gut, central nervous system, immune system and finally results in death of insects [2]. Lepidopteran insects demonstrated enhanced antifeedant sensitivity against AZT exposure [3]. Mordueet al. [2] has correlated a significant

sensory response of chemo-receptors on the insect's mouth parts to antifeedant activity and stimulates specific deterrent cells in chemo-receptors. This suggests that AZT has direct and/indirect effects on dose dependant neural toxicity. But the biomarker potential of some antioxidant enzymes has not been revealed earlier in *Spathosternum prasiniferum prasiniferum*. Free radicals promote lipid peroxidation with loss of membrane integrity. In addition, degeneration of mitochondrial membrane can initiate a cascade of free radical reactions [4]. Nervous system is at a high risk to free radical-induced injury because it is rich in polyunsaturated fatty acid [5]. Literatures are inadequate on AZT-induced oxidative damage in insect's brain. Considering the wide use of AZT, we decided to investigate the acute effects of AZT on oxidative threat in the nervous system of *S. pr.prasiniferum*.

2. MATERIALS AND METHODS

2.1 Laboratory Culture of Insects

Adult female and male insects *S. pr. prasiniferum* were collected from agricultural fields from Midnapore district, West Bengal, India. And maintained in laboratory conditions (30±2°C, 70±5% relative humidity, 500±200 lux light intensity and 12L:12D photoperiod). Requisite number of fresh adults were transferred to clean, transparent plastic-jar (5 litres) with moist sterilized sand bed. In each jar fresh young grass leaves (*Cyperus kyllingia*: Cyperaceae) were provided.

2.2 Preparation of AZT Solution and Its Susceptibility Tests

Commercially available Nimbecidine (C₃₅H₄₄O₁₆, Azadirachtin) manufactured by T. Stanes & Company Limited was purchased from the local

market. Different concentrations (1, 5, 10, 15 and 20 ppm in water) of AZT were prepared and sprayed on their food plants. The rate of mortality of the insects was recorded. Percent mortality was calculated according to Abbott's formula.

$$\text{Abbott's corrected mortality} = \frac{\% \text{ mortality in treatment} - \% \text{ mortality in control}}{100 - \% \text{ mortality in control}} \times 100$$

2.3 *In vivo* AZT Treatment and Evaluation of Food Indices, Behaviour and Mortality Rate of the Insect

After the treatment period, hemolymph was pooled (from multiple insects) separately from male and female from different groups. Phenoloxidase inhibitor (phenylthiourea) was added to the hemolymph to prevent its changes in physic-chemical properties. To determine food indices, leaf weight, insect's weight and excreta weight were recorded before and after the experiment. The data were calculated to evaluate the consumption index (CI), approximate digestibility (AD), efficiency of conversion of digested food (ECD) and efficiency of conversion of ingested food (ECI) as per the protocol of Sharma and Norris [6].

2.4 *In vitro* Treatment Sample Preparation and Biochemical Assays

Newly born fresh adult female and male insects were utilized like previous experiment, their heads were dissected. Thereafter, their brains were incubated with different dilutions (1 to 20 ppm) of AZT or without AZT for 6 h and stored in -20°C. Frozen brains were homogenized in 5x phosphate buffer (0.1 mol/L, pH 7.4) followed by centrifugation at 10,000 x g in 4°C for 8 min. The supernatant was kept at -20°C.

2.4.1 Estimation of total protein

Protein concentration was measured by [7] Lowry et al. where BSA was used to generate the standard curve. The blue colour was determined after 30 min at 750 nm. The protein concentrations were expressed as µg/mg of wet tissue or µg/µl of hemolymph.

2.4.2 Estimation of lipid peroxidation and non-protein-soluble thiol (NPSH)

Malondialdehyde (TBARS) level (index of lipid peroxidation) were evaluated with thiobarbituric acid (TBA) reagent [8]. The extinction coefficient

of TBARS-TBA chromophore at 532 nm ($\epsilon=1.56 \times 10^5 \text{ mol}^{-1} \text{ cm}^{-1}$). The level of TBARS was expressed as nmol/mg of wet tissue or µmol/µl of hemolymph. The NPSH in homogenate were determined by formal 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) method [9]. The protein was precipitated by 5% Trichloroacetic acid (TCA). The transparent cytosol was added to Tris-HCl buffer containing 5 mM DTNB. The level of NPSH was assessed against a GSH standard curve and the concentrations were expressed as µg/mg of wet tissue or µg/µl of hemolymph.

2.4.3 Estimation of alkaline phosphatase (ALP) and acetyl cholinesterase (AChE)

Alkaline phosphatase activity was estimated according to the method of [10] Lima-Oliveira et al. Acetyl cholinesterase activity was examined by the method described by Ellman et al. [11].

2.4.4 In-gel assay of catalase (CAT), super oxide dismutase (SOD), glutathione peroxidase (GPx) and amylase

Gel zymogram assay was performed following the protocol described by Christine and Joseph 2010 [12]. Finally the gel was washed with distilled water to visualize the protein bands on the gel. Super oxide dismutase activity was determined in polyacrylamide gel (12%) according to the method of [12] Christine and Joseph 2010. Gels were finally washed in deionised water and illuminated under fluorescent light. Native polyacrylamide gel electrophoresis (8%) was carried out at 100 V for 2.5 h according to [13] Moreno et al. and [12] Christine and Joseph method. Gels after running with proteins were soaked in 0.008% cumin-hydro-peroxide for 10 min, rinsed with water, and stained in 1% potassium-ferricyanide followed by 1% ferric chloride for the development of colour. Non-denaturing polyacrylamide gel (8%) electrophoresis was performed as described by [14] Andrades and Contreras. Gel with resolved protein was incubated in 2.5% (v/v) Triton X-100 for 30 min at room temperature with gentle agitation. Washed gel was incubated at 30°C for 60 min in suitable buffer containing 1% (w/v) soluble starch. The gel was stained with Lugol solution (I₂ 0.33% and KI 0.66%).

2.4.5 DNA fragmentation analysis

Tissues were homogenised with 500 µl of lyses buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM

Table 1. Summary statistics for quantity biochemical variables in *S. pr. prasiniferum* after AZT toxicity. (P<0.001, significantly different from control group, 10 animals in each group, ANOVA analysis at df 5)

<i>In vivo</i> female	F	P	<i>In vivo</i> male	F	P	<i>In vitro</i> female	F	P
Brain_Protein	2711.215	.000	Brain_Protein	394.099	.000	Brain_Protein	1.624	.228
Brain_MDA	145.544	.000	Brain_MDA	457.248	.000	Brain_MDA	4.078	.021
Brain_NPSH	405.059	.000	Brain_NPSH	945.160	.000	Brain_NPSH	7.127	.003
Brain_AChE	5.642	.007	Brain_AChE	150.850	.000	Brain_AChE	2.355	.104
Brain_ALP	5829.368	.000	Brain_ALP	692.781	.000	<i>In vitro</i> male		
Hemolymph_Protein	438.797	.000	Hemolymph_Protein	25.581	.000	Brain_Protein	47.323	.000
Hemolymph_MDA	245.028	.000	Hemolymph_MDA	280.182	.000	Brain_MDA	90.389	.000
Hemolymph_NPSH	1513.440	.000	Hemolymph_NPSH	1373.509	.000	Brain_NPSH	223.561	.000
Hemolymph_ALP	10207.130	.000	Hemolymph_ALP	5224.813	.000	Brain_AChE	221.245	.000
Hemolymph_AChE	96.230	.000	Hemolymph_AChE	17.100	.000			

NaCl, 1% SDS) at 4°C. And the other steps were followed as described by [15] Garcia-Martinez et al. 1993. Finally, agarose gel (0.8%) was run at 5V for 5 min at 100V. The gel bands were documented in gel documentation system.

2.5 Statistical Analysis

All tests were performed in triplicate. Results were expressed as means \pm SD (n = 20). Mean and standard error values were determined for all the biochemical parameters and the results were expressed as means \pm SE (n = 3). The data were analysed (by SPSS 17 software) by one-way variance (ANOVA) followed by Fisher's least significant difference (LSD) multiple comparison test. The results were considered statistically significant when $P < 0.001$.

3. RESULTS

In treated group of grasshoppers abnormal behaviour of restlessness, sudden quick and jerky movements were observed at low concentration of pesticide whereas, increased movements accompanied with swarming movements and loss of equilibrium were observed in concentration group. Percentage mortality of insect was dose (AZT) and time dependently higher (Fig. 1e). Hemolymph protein

and *In vivo* but not *in vitro* brain protein from both sexes were significantly ($P < 0.001$) increased in treated group (Fig. 2a and Fig. 2f, 3a). Insects exposed to *in vivo* AZT treatment showed higher LPO in brain and hemolymph both sexes (Fig. 2b, 3b and 6b). Increased levels of NPSH were found *in vivo* both sexes brain tissues after AZT toxicity (Fig. 2c), but it significantly decreased significantly in *in-vitro* male brain (Fig. 2h).

ALP activity increased in male brain (Fig. 2d). Acetyl cholinesterase activity significantly decreased *in vivo* male brain and increased *in vivo* female brain (Fig. 2e).

SOD was significantly higher in *in vivo* and *in vitro* brain from both sexes (Fig. 4). SOD activities of AZT treated male hemolymph were also significantly higher. The CAT activity in *in vivo* brain of both sexes increased and that in *in vitro* male brain decreased with dose dependent manner (Fig. 4). *In vivo* and *in vitro* female brain showed increased α -amylase activity whereas moderately activity decreased in male brain of both experimental groups with the increasing AZT doses (Fig. 4). No inhibition of starch hydrolysing activity found in hemolymph of both sexes (Fig. 4). DNA stability was found to be decreased in *In vivo* and *In vitro* brain of both sexes (Fig. 5).

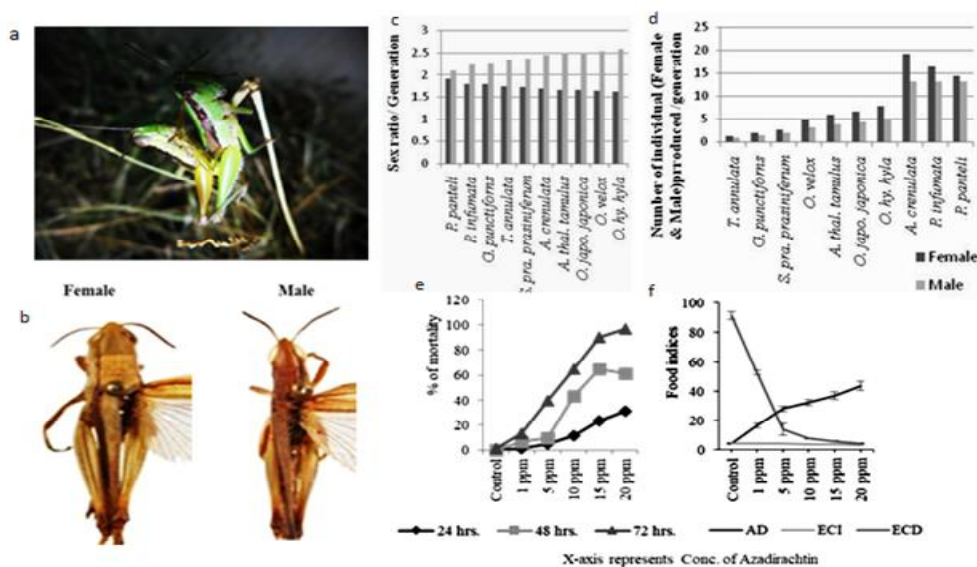


Fig. 1. (Left panel a, b) *Spathosternum prasinerum prasinerum* (Walker, 1871). Right panel: c. Sex ratios per generation of available acridids from West Bengal, India. d. Reproduction rate of common acridids. e. Cumulative mortality percentage (%) after AZT treatment. (Data represent the means \pm SD, n=20). f. Food indices after AZT treatment. Data represent the means \pm SD of triplicate analysis. Means within lines are significantly different ($P < 0.001$, ANOVA)

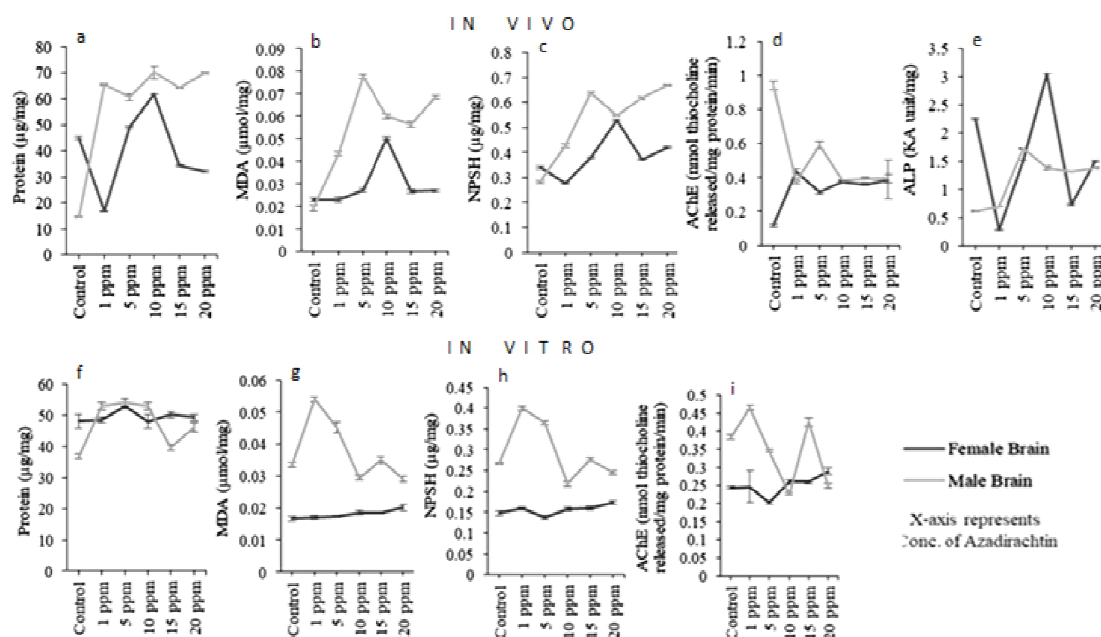


Fig. 2. Total protein (a), TBARS (b), NPSH (c), AChE (d), ALP (e) levels are shown in the brains of insects after AZT treatment. Data represent the means \pm SE of triplicate analysis. Total protein (f), TBARS (g), NPSH (h), AChE (i) content of *in vitro* brain are shown. Data represent the means \pm SE of triplicate analysis. Means within lines are significantly different ($P < 0.001$, ANOVA)

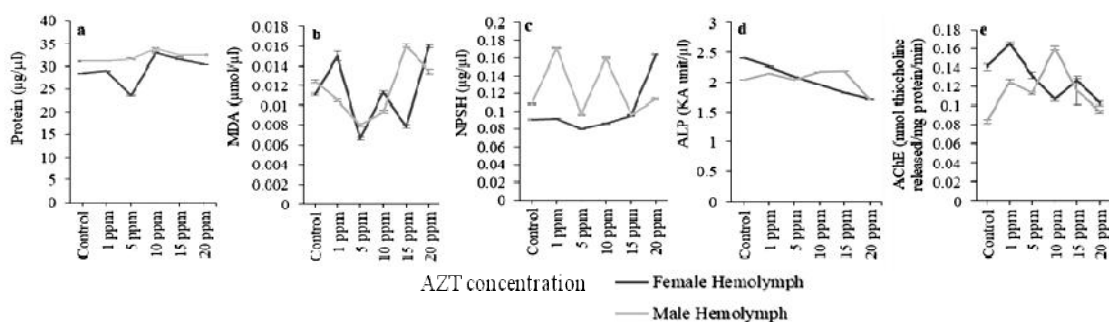


Fig. 3. Total protein, TBARS, NPSH, ALP, AChE content of hemolymph are demonstrated after AZT treatment to the insect. Data represent the means \pm SE of triplicate analysis. Means within lines are significantly different ($P < 0.001$, ANOVA)

4. DISCUSSION

The increase of the insect mortality was noticed in the current study in with the increase of azadirachtin (AZT). The increase of AZT in the insect's body impaired their metabolism and resistance against the toxic effects of AZT. And for that reason insects become susceptible to degenerative tissue damages. Food ingestion (contaminated with AZT) and preliminary digestion were significantly ($P < 0.001$) increased

at higher doses of the pesticide. In general male are found to be more sensitive to toxic exposure in relation to brain tissues. This is reflected in terms of ALP activities and gross peroxidation outcome. Oxidative stress is initiated by pesticides and it plays a significant role in the cellular toxicity [16]. AZT strongly induces mitochondrial superoxide anion other ROS and H_2O_2 [17]. Increase in neural protein content indicates that AZT may interfere with the protein synthesis or post translational phenomenon [18].

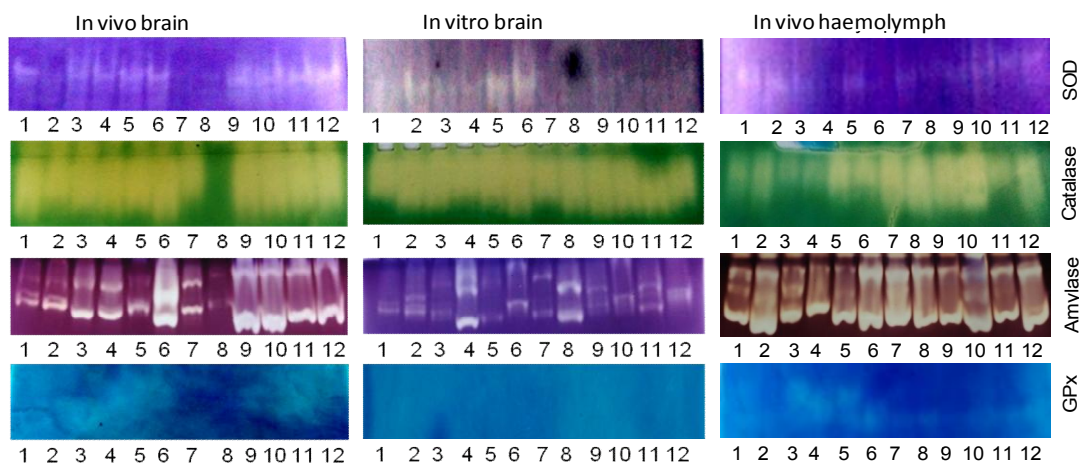


Fig. 4. Effect of AZT on specific activity of marker enzymes on brain and hemolymph of *S. pr. prasiniferum* showing on a polyacrylamide gel. lane distribution of each panel: Lane 1-6 female brain (1-control, 2-1ppm, 3-5ppm, 4-10ppm, 5-15ppm, 6-20ppm AZT) and Lane 7-12 male brain (as previous)

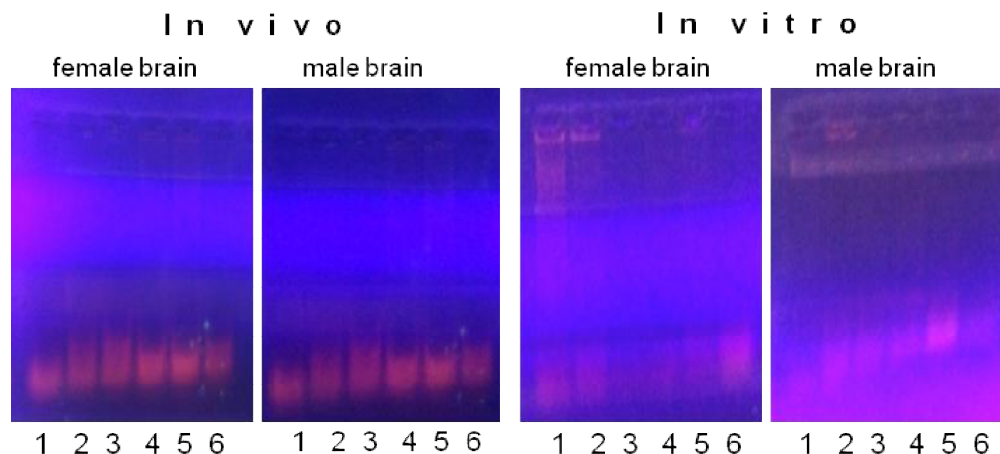


Fig. 5. Effect of AZT are shown on neural DNA of *S. pr. prasiniferum*. lane distribution: Lane 1-6 (1-control, 2-1 ppm, 3-5ppm, 4-10 ppm, 5-15 ppm, 6-20 ppm of AZT)

Lipid peroxidation (LPO) products are considered to be an important marker of the cellular oxidative stress [19]. Malondialdehyde, is a distinct representative of the (TBARS) cellular oxidation product. It is reported that pesticides can induced LPO by the enhancement of TBARS production [20]. In our observations, increased levels of LPO were found in the insect brain of both sexes exposed to AZT (Fig. 2b). This fact was justified by the results of increased rate of insect mortality at higher AZT doses. In addition, brain is reported to have low antioxidant defence system [21], that's why ROS could induce a certain level of toxicity. Protective and adaptive

roles of thiol substantial against oxidative stress induced toxicity [22]. Thus, NPSH level in tissues are considered a critical determinant of the cellular protective measures against toxicity [23]. Our results show that AZT toxicity increased the ALP activity in *in vivo* male brain (Fig. 2d). The toxicity was more distinct in higher doses [18].

Acetyl cholinesterase, neurotransmitter is mainly distributed in the brain and central nervous system [24]. Due to impaired AchE, disconnection in neural signal generates toxicity symptoms and dies in severe condition [25]. AZT binding with AchE is shown to be non-specific. In

this study AChE activity significantly decreased in *in vivo* male brain (Fig. 2e). That eventually induces impairment of recycling ACh and disruption of normal nerve conduction. Significant alterations in SOD and catalase activities in the insects suggest that AZT has stimulated the antioxidant mechanisms to overcome the oxidative stress. SOD is responsible for the dismutation of superoxide radicals to H₂O₂. The CAT system primarily defences against the oxygen related toxicity and this enzyme is used as biomarkers of oxidative stress [26]. Variable responses in catalase activity may be related to the differential production of ROS influenced by the metabolic and cellular respiratory rate. These rates are dependent on gender and neuro-endocrinal regulations of physical functions. Report reveals that CAT activity was decreased in mammalian brain intoxicated with different pesticides [27]. Report reveals that variable levels of superoxide radicals (O₂^{·-}) might be produced by different doses of pollutants [28].

The decreased activities of SOD and CAT attributed increased oxidative stress in male insect. This suggests decline in the enzymatic function might have impaired cellular glutathione and thus decreased the antioxidant capacity of the nervous tissue. This speculation was confirmed after estimating the levels of non-protein thiols. Glutathione peroxidase however, showed no change in activity after exposure to AZT. Thus we may explain that AZT exerts its toxicity by generating excessive oxidative stress via increased ROS and compromised antioxidant defence mechanism. AZT form stable complexes with α -amylase which could inhibit the formation of substrate enzyme complex [29]. By this way it inactivates digestive enzymes and cause poor nutrition, growth-retardation and death. Report revealed the inhibition of insect-pest α -amylase by plant derived inhibitors [30].

Extensive DNA laddering shown here might be due to the high level of oxidant molecules as demonstrated in high level of TBARS. Free radicals are known to damage the DNA stability. This is also consistent with previous studies where DNA fragmentation was induced by pesticide in mammalian lymphocytes [31] and in mammalian brain tissues [32]. Pesticides may also cause damage to proteins, lipids and DNA by oxidation. Elevation of TBARS and oxidative redox parameters like SOD, CAT in female brain suggests that female can respond in better way to overcome the oxidative stress against such pesticide.

5. CONCLUSION

The neuro-toxic effects of AZT are due to oxidative pathway attributed by the excessive production of ROS. Sex dimorphic pattern of AZT function via oxidative stress has been noticed in the current experimental species. Previous studies have been done on coleoptera species but scanty reports are available on orthoptera. The present results may be regarded as the foundation for further studies on other orthopteran insect's pest using such pesticides. However, an extensive research work should be undertaken on AZT for its better utilization in sustainable agriculture practices.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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