Neural oxidant-stress by Azadirachtin induces anti-oxidative enzymes evincing biomarker potential in paddy pest, *Spathosternum prasiniferum prasiniferum* (Orthoptera:Acridoidea)

Running title: brain oxidant stress by Azadirachtin in Orthoptera

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-phosphatise/ALP; thiobarbituric-acid-reactive-substances/TBARS; non-protein-soluble-thiols/NPSH; acetyl-cholinesterase/AChE) and antioxidant-enzyme activity (superoxide-dismutase/SOD; catalase/CAT; glutathione-peroxidise/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/hemolymphin 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.

Key words:

Azadirachtin, Orthoptera:Acridoidea, brain and hemolymph, reactive oxygen species, antioxidant defence

Abbreviations:

azadirachtin (AZT), approximate digestibility (AD), efficiency of conversion of digested food (ECD), efficiency of conversion of ingested food (ECI), reactive oxygen species (ROS), malondialdehyde (MDA), Thiobarbituric acid substances (TBARS), non-protein thiols (NPSH), alkaline Phosphatase (ALP), acetyl cholinesterase (AChE), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), deoxyribonucleic acid (DNA).

1. Introduction:

All beneficial plants are moderately toxic in nature due to production of secondary metabolites to protect themselves against various predators (Wink, 1988). 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 loss of insect leads threatened to biodiversity. 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 (Mordueet al.,2010). Lepidopteran insects demonstrated enhanced antifeedant sensitivity against AZT exposure (Sami and Shakoori, 2014). Mordueet al. (2010) 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 *S. pr. 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. Nervous system is at a high risk to free radical-induced injury because it is rich in polyunsaturated fatty acid. (Halliwell,1992). 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^{o}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 (*Cyperuskyllingia*: Cyperaceae) were provided.

2.2. Preparation of AZT solution and its susceptibility tests:

Commercially available Nimbecidine ($C_{35}H_{44}O_{16,}$ 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.

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

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 group and gender-wise. 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 evaluate 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 (1991).

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 -20ºC. 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 Lowry et al. (1951) 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) wereevaluated with thiobarbituric acid (TBA) reagent (Buege and Aust, 1978). The extinction coefficient of TBARS-TBA chromophore at 532 nm (ε=1.56×105 $mol^{-1}cm^{-1}$). 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 (Forman, 2009). The protein was precipitated by 5% Trichloroacetic acid (TCA). The transparentcytosol 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 Lima-Oliveira et al. 2016. Acetyl cholinesterase activity was examined by the method described by Ellman et al. (1961).

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). 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 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 Moreno et al. (1990) and Christine and Joseph 2010 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 Andrades and Contreras, (2017). 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 30ºC for 60 min in suitable buffer containing 1% (w/v) soluble starch. The gel was stained with Lugol solution (I_2 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 NaCl, 1% SDS) at 4°C. And the other steps were followed as described by 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.

2.4.6. Statistical analysis:

All tests were performed in triplicate. Results were expressed as means ± SD (n = 20). Mean and standard error values were determined for all the biochemical parameters and the results were expressed as means ± 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 significant 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).

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 (Stevenson et al., 1995). AZT strongly induces mitochondrial superoxide anion other ROS and H_2O_2 (Huc, 2007). Increase in neural protein content indicates that AZT may interfere with the protein synthesis or post translational phenomenon (Senthil Nathan et al., 2004).

Lipid peroxidation (LPO) products are considered to be an important marker of the cellular oxidative stress (Hoek and Pastorino, 2002). 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 (Catalgol et al., 2007). 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 (Ward et al. 1994), that's why ROS could induce a certain level of toxicity. Protective and adaptive roles of thiol substantial against oxidative stress induced toxicity (Otto and Moon, 1995). Thus, NPSH level in tissues are considered a critical determinant of the cellular protective measures against toxicity (Meister & Tate 1976). 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 (Senthil-Nathan et al., 2004).

Acetyl cholinesterase, neurotransmitter is mainly distributed in the brain and central nervous system (Kim et al., 2006). Due to impaired AchE, disconnection in neural signal generates toxicity symptoms and dies in severe condition (Tang, 2000). 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 H_2O_2. The CAT system primarily defences against the oxygen related toxicity and this enzyme is used as biomarkers of oxidative stress (Pandey et al., 2003). 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 (Mekail and Sharafaddin, 2009). Report reveals that variable levels of superoxide radicals ($O_2.^-$) might be produced by different doses of pollutants (Monteiro et al., 2006).

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 (Sami and Shakoori, 2010). 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 (Sivakumar et al., 2000).

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 (Sharma et al., 2010) and in mammalian brain tissues (Hussien et al., 2013). 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 otherorthopteran insect's pest using such pesticides. However, an extensive research work should be undertaken on AZT for its better utilization in sustainable agriculture practices.

References:

Andrades A., Contreras L.M., Amylase Zymography. 2017. Methods Mol Biol. 1626, 301-308.

Buege J.A., Aust S.D.,1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302-10.

Catalgol, B.K., Ozden, S., Alpertunga, B., 2007. Effects of trichlorfon on malondialdehyde and antioxidant system in human erythrocytes. Toxicol. In Vitro 21, 1538-1544.

Christine J.W., Joseph J.C., 2010. Measurement of superoxide dismutase, catalase, and glutathione peroxidase in cultured cells and tissue. Nat Protoc. 5(1), 51–66.

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

Forman, H.J., 2009. Critical methods in free radical biology & medicine. Free Radic. Biol. Med.47, 207.

Garcia M.V., Macias D., Fau G.Y., Ganan Y., Fau-Garcia-Lobo J.M., Garcia-Lobo JmFau - Francia M.V., FranciaMvFau-Fernandez-Teran M.A., Fernandez-Teran Ma Fau-Hurle J.M., Hurle J.M., (1993). Internucleosomal DNA fragmentation and programmed cell death (apoptosis) in the interdigital tissue of the embryonic chick leg bud. Journal of Cell Science, 106: 201-208.

Halliwell, B., 1992. Reactive oxygen species and the central nervous system. J. Neurochem.59, 1609-1623.

Hoek, J.B., Pastorino, J.G., 2002. Ethanol oxidative stress and cytokine induced liver damage. Alcohol 27, 63-68.

Huc, L., Tekpli, X., Holme, J.A., 2007. c-Jun NH2-terminal kinase-related Na^+/H^+ exchanger isoform 1 activation controls hexokinase II expression in benzo(a) pyrene induced apoptosis. Cancer Res. 67, 1696.

Hussien, H.M., Abdou, H.M., and Yousef, M.I., 2013. Cypermethrin induced damage in genomic DNA and histopathological changes in brain and haematotoxicity in rats: The protective effect of sesame oil. Brain Res. Bull. 92, 76-83.

Kim, J.I., Jung, C.S., Koh, Y.H., Lee, S.H., 2006. Molecular, biochemical and histochemical characterization of two acetyl cholinesterase cDNAs from the German cockroach *Blattellagermanica*. Insect Mol. Biol. 15, 513-522.

Lima-Oliveira, A.P., Alevi, K.C., Anhê, A.C., Azeredo-Oliveira, M.T.,2016. Alkalinephosphatase activity in salivary gland cells of *Rhodniusneglectus* and *R. prolixus*(Hemiptera, Triatominae). Genet. Mol. Res. 15(3). doi: 10.4238/gmr.15038339. PubMed PMID: 27525888.

Lowry, O.H., Rosebrough, M.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Meister, A., Tate, S.S., 1976. Glutathione and related gamma-glutamyl compounds: Biosynthesis and utilization. Ann. Rev. Biochem. 45, 599-604.

Mekail, A., Sharafaddin, Z.A., 2009. A study of the activity of catalase and glutathione S-transferase in wearling and adult rats intoxicated with diazinon, carbaryl and lambda-cyhalothrin. J. Duhok. Uni. 12, 138-145.

Monteiro, D.A., Almeida, J.A., Rantin, F.T., and Kalinin, A.L. 2006. Oxidative stress biomarkers in the freshwater characid fish *Bryconcephalus*, exposed to organophosphate insecticide folisuper 600 (Methyl parathion). Comp. Biochem. Physiol. 143, 141-149.

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

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

Mordue-Luntz, A.J., Nisbet, A.J., Nasiruddin, M. Walker, E., 1996. Differential thresholds of azadirachtin for feeding deterrence and toxicity in locusts and an aphid. Entomol. Exp. Appl. 80, 69-72.

Moreno, O.A., Vazquez-Duhalt, R., Nolasco, H., 1990. Extracellular accumulation of high specific-activity peroxidase by cell suspension cultures of cowpea. Plant Cell Rep. 9, 147- 150.

Otto, D.M., Moon, T.W., 1995. 3,3′,4,4′-tetrachlorobiphenyl effects on antioxidant enzymes and glutathione status in different tissues of rainbow trout. Pharmacol. Toxicol. 77, 281-287.

Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., Raisuddin, S., 2003. Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallagoattu*(Bl. &Schn.). Sci. Total Environ. 309, 105-115.

Sami, A.J., Shakoori, A.R., 2010. Cellulase activity inhibition and growth retardation of associated bacterial strains of *Aulacophorafoviecollis* by two glycosylated flavonoids isolated from *Mangiferaindica* leaves. J. Med. Plant Res.5,184-190.

Sami, A.J., Shakoori, A.R., 2014. Potential of azadirachtin and neem (*Azadirachtaindica*) based saponins as biopesticides for in vitroinsect pest's cellulase (Beta-1,4- endoglucanase) enzyme inhibition and in vivorepellency on *Triboliumcastaneum*. Br. Biotechnol. J. 4, 904-917.

Senthil-Nathan S., Chung, P.G., Murugan, K., 2004. Effect of botanicals and bacterial toxin on the gut enzyme of *Cnaphalaocrosismedinalis*. Phytoparasitica 32, 433-443.

Sharma, D., Saxena, P., Singh, V., Sharma, R., 2010 Assessment of DNA degradation in lymphocytes of albino rat (*Rattusnorvegicus*) under lambda cyhalothrin stress. World Appl. Sci. J. 11 (1), 24-28.

Sharma, H.C., Norris, D.M., 1991. Comparative feeding preference and food intake and utilization by the cabbage looper (Lepidoptera: Noctuidae) on three legume species. Environ. Entomol. 20, 1589-1594.

Sivakumar, S., Mohan, M., Franco, O.L., Thayumanavan, B.,2006. Inhibition of insect pest α-amylases by little and finger millet inhibitors. Pest. Biochem. Physiol.85, 155-160.

Stevenson, D.E., Kehrer, J.P., Kolaja, K.L., Walborg, E.F., Klaunig, J.E., 1995. Effect of dietary antioxidants on dieldrin induced hepatotoxicity in mice. Toxicol. Lett. 75, 177-183.

Tang, Z.H., 2000. Research status and perspectives of insect resistance to insecticides in China. Entomol. Knowledge 37, 97-103.

Ward, R.J., Kuhn, L.C., Kaldy, P., Florence, A., Peters, T.J., Crichton, R.R., 1994. Control of cellular iron homeostasis by iron responsive elements in vivo. Eur. J. Biochem. 220, 927-931.

Wink, M., 1988. Plant breeding importance of secondary metabolites for protection against pathogens and herbivores. Theor. Appl. Genet. 75, 225-233.

Figure Legends

Fig. 1. (Left panel a, b) *Spathosternum prasiniferum prasiniferum* (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 ± SD, n=20). f. Food indices after AZT treatment. Data represent the means ± 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 ± SE of triplicate analysis. Total protein (f), TBARS (g), NPSH (h), AChE (i) content of in vitro brain are shown. Data represent the means ± 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 ± SE of triplicate analysis. Means within lines are significantly different ($P < 0.001$, ANOVA).

Fig 4. Effect of AZT on specific activity of marker enzymes on brain and hemolymph of *Spathosternum 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 *Spathosternum pr. prasiniferum*. Lane distribution: Lane 1-6 (1-control, 2-1ppm, 3-5ppm, 4-10ppm, 5-15ppm, 6-20ppm of AZT)

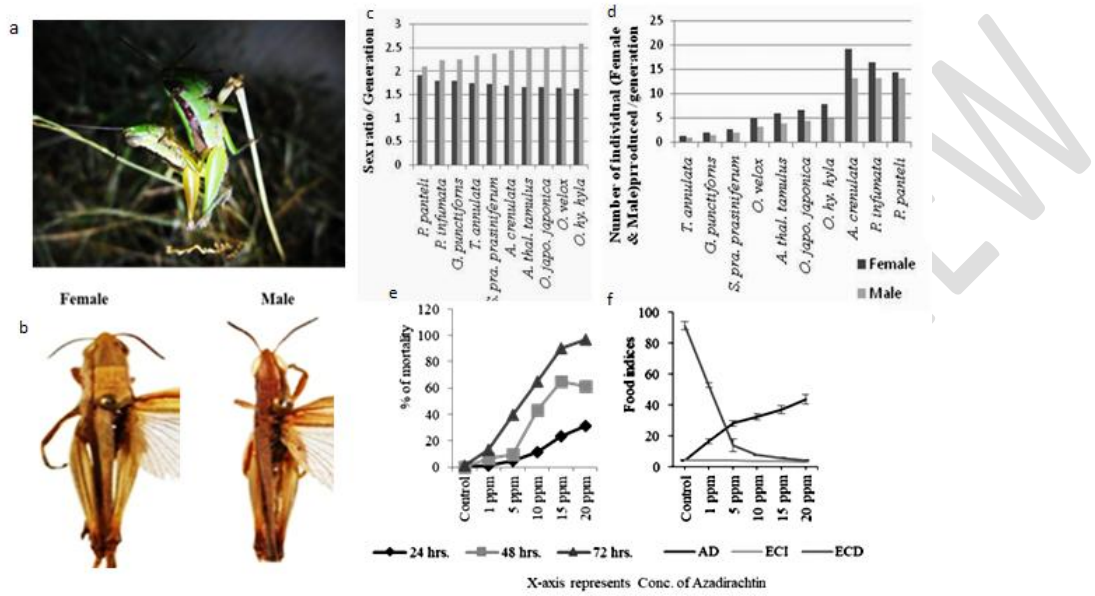

Fig 1

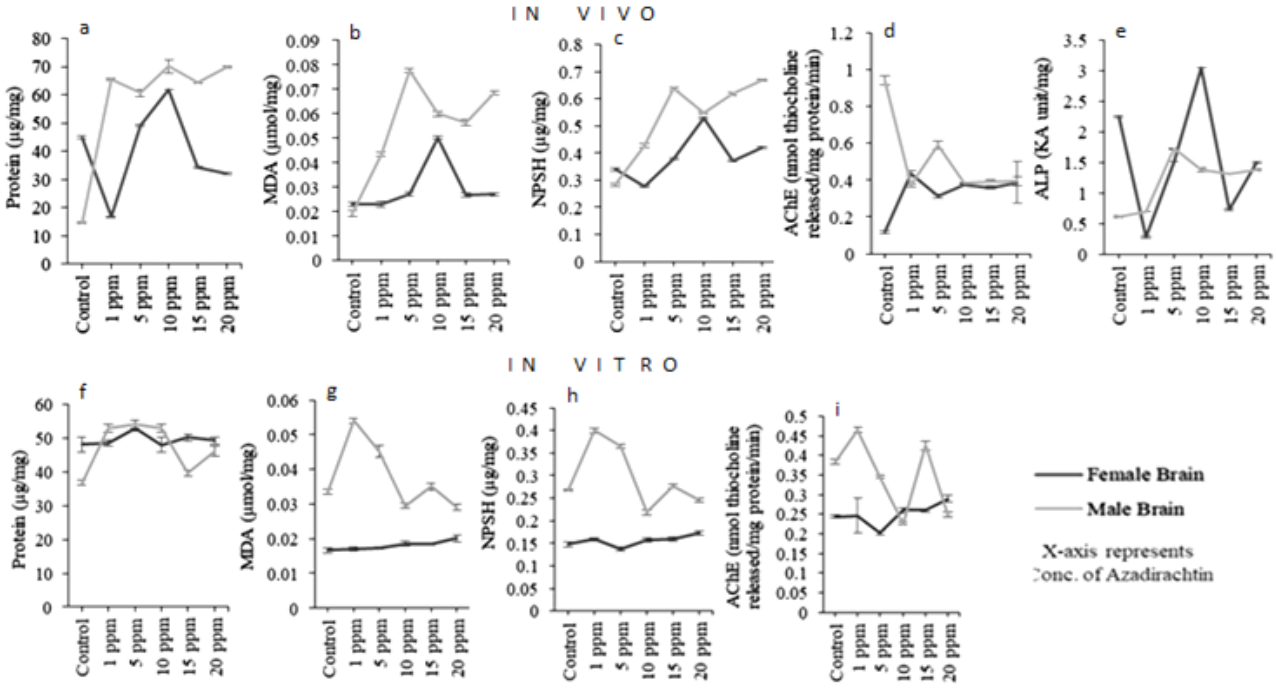

Fig 2

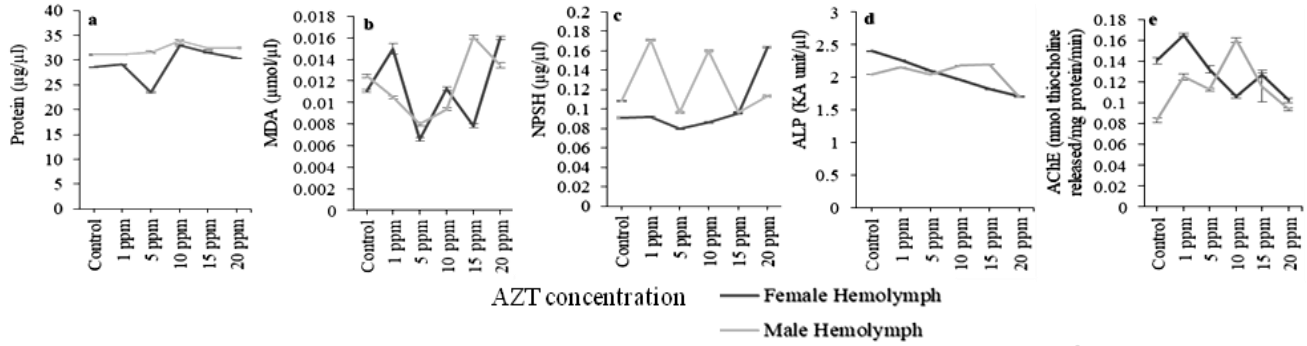

Fig. 3

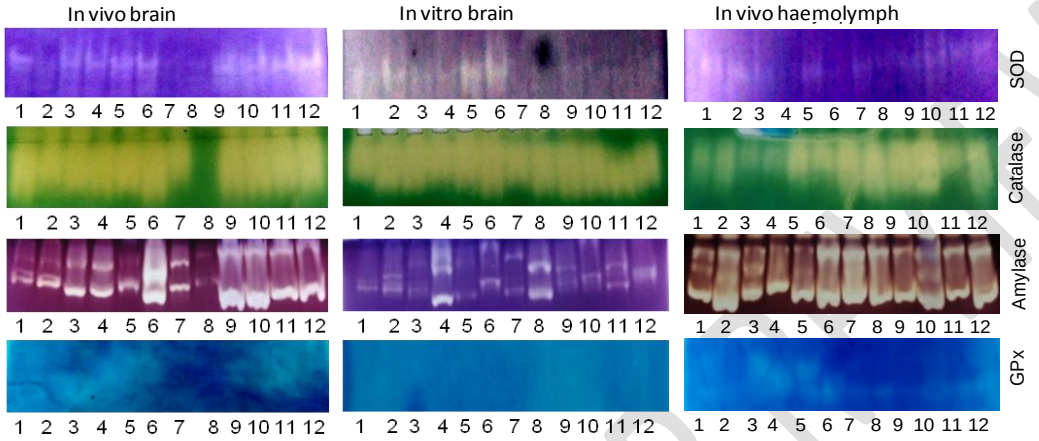

Fig. 4

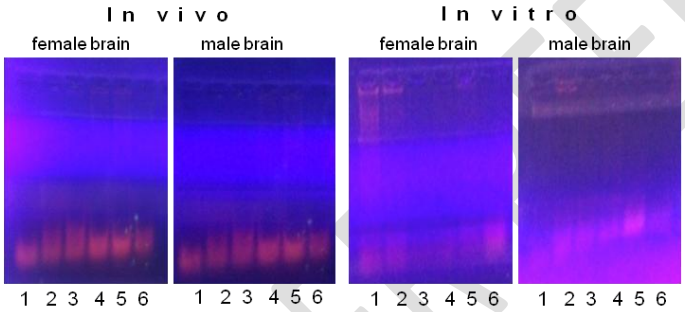

Fig. 5

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

In vivo female	F	P	In vivo male	F	P	In vitro 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	**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			