APPENDIX

Conference proceedings

International level

- Balaram Manna, Dipsikha Ghosh, Amlan Das. Entomology Laboratory, Department of Zoology, University of Calcutta. "Prospects of insect protein in livestock management". International Conference on INTZOOCON 2018, 1st - 3rd February, 2018, Department of Zoology, University of Calcutta.

National level

- Balaram Manna, Amlan Das, Smarajit Maiti. Cell and Molecular Therapeutics Laboratory, Department of Biochemistry, Oriental Institute of Science and Technology, Vidyasagar University and Entomology Laboratory, Department of Zoology, University of Calcutta. "Toxicological consequences of botanical pesticide (Azadirachtin) on short horn grasshopper *Spathosternum prasiniferum prasiniferum* (Orthoptera; Acridoidea)". National Seminar on ENVIRONMENTAL CHANGE: ADAPTATION CHALLENGES BY SUSTAINABLE DEVELOPMENT, 13th - 14th July, 2018, Department of Biological Sciences, Midnapore City College.

LIST OF PUBLICATIONS

Research articles

Manna B, Maiti S, Das A (2019) Neural oxidant-stress by azadirachtin induces anti-oxidative enzymes evincing biomarker potential in paddy pest, *Spathosternum prasiniferum prasiniferum* (Orthoptera: Acridoidea). Asian Journal of Environment & Ecology. 10(2):1-10. DOI: 10.9734/ajee/2019/v10i230111

Manna B, Maiti S, Das A (2020) Bioindicator potential of *Spathosternum prasiniferum prasiniferum* (Orthoptera: Acridoidea) in pesticide (azadirachtin)-induced radical toxicity in gonadal/nymphal tissues; correlation with eco-sustainability. Journal of Asia-Pacific Entomology. 23(2):350-357. DOI: 10.1016/j.aspen.2020.02.007

Manna B, Maiti S, Das A (2020) Sex dimorphic adaptive responses against Azadirachtin toxicity in gut tissues of *Spathosternum prasiniferum prasiniferum* (Orthoptera; Acridoidea). International Journal of Tropical Insect Science. DOI: 10.1007/s42690-020-00180-1

Manna B, MaitiDutta S, Dalapati S, Maiti S (2020) Oxidative stress induced toxicity and DNA stability in some agri-field based livestock/insect by widely used pesticides. Combinatorial Chemistry & High Throughput Screening. DOI: 10.2174/1386207323666200415110745

Reprint of Published Paper

Asian Journal of Environment & Ecology

10(2): 1-10, 2019; Article no.AJEE.50980
ISSN: 2456-690X

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.

Article Information

DOI: 10.9734/AJEE/2019/v10i230111
Editor(s):
(1) Prof. Daniele De Wrachien, Professor of Agricultural Hydraulics, Department of Agricultural and Environmental Sciences, State University of Milan, Italy.
(2) Dr. Sarfraz Has, Assistant Professor, Department of Agricultural Engineering, Muhammad Nawaz Shareef University of A, Multan, Agriculture, Multan, Pakistan.
Reviewers:
(1) Seweta Srivastava, Lovely Professional University, India.
(2) Joshua I. Raji, Florida International University, USA.
Complete Peer review History: http://www.sdiarticle3.com/review-history/50980

Original Research Article

Received 22 June 2019
Accepted 24 August 2019
Published 09 September 2019

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-

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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.

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 ($C_{35}H_{44}O_{16}$, 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 (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) wereevaluated with thiobarbituric acid (TBA) reagent [8]. 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 [9]. 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 [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 (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

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)

***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	***In vitro* 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 ± 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).

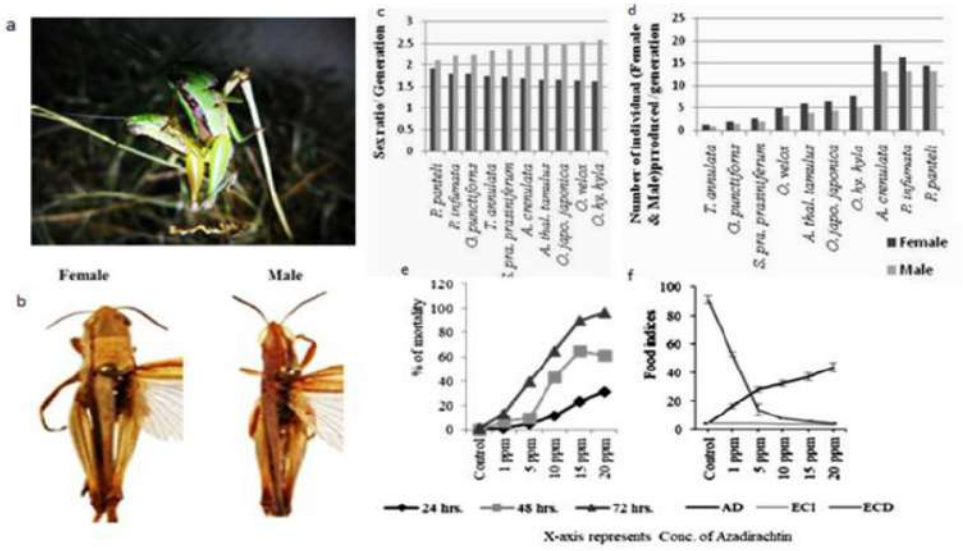

Fig. 1. (Left panel a, b) *Spathosternum prasiniferum prasiniferum* (Walker, 1871). Right panel: c. Sex ratios per generation of available acridids from West Bengal, India. d. Reproduction rate of common acridids. e. Cumulative mortality percentage (%) after AZT treatment. (Data represent the means ± 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)

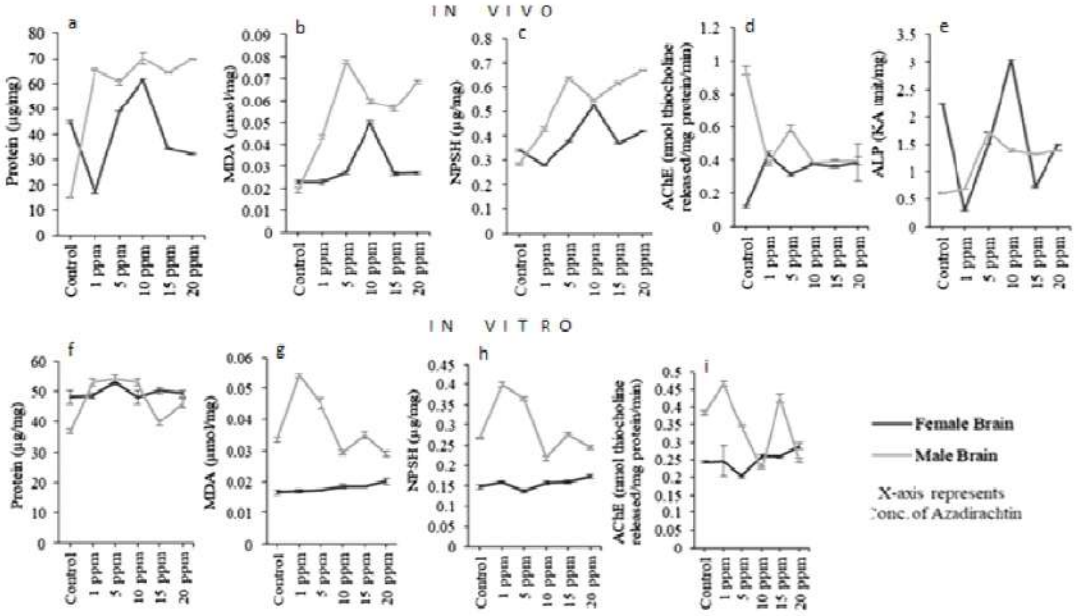

Fig. 2. Total protein (a), TBARS (b), NPSH (c), AChE (d), ALP (e) levels are shown in the brains of insects after AZT treatment. Data represent the means ± SE of triplicate analysis. Total protein (f), TBARS (g), NPSH (h), AChE (i) content of *in vitro* brain are shown. Data represent the means ± SE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

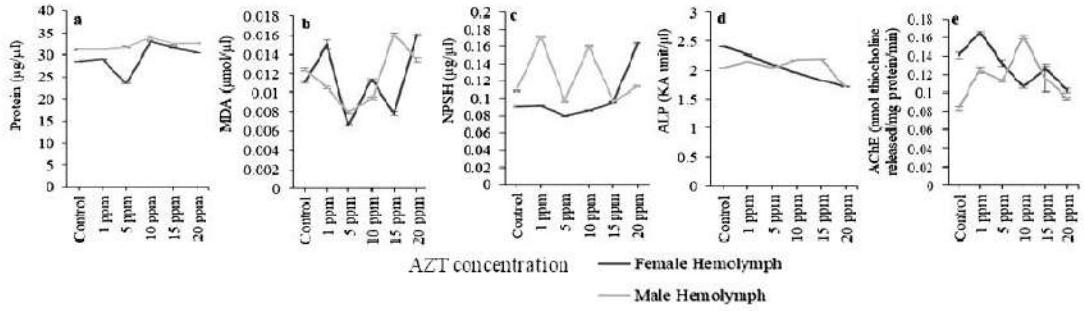

Fig. 3. Total protein, TBARS, NPSH, ALP, AChE content of hemolymph are demonstrated after AZT treatment to the insect. Data represent the means ± SE of triplicate analysis. Means within lines are significantly different (P < 0.001, ANOVA)

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].

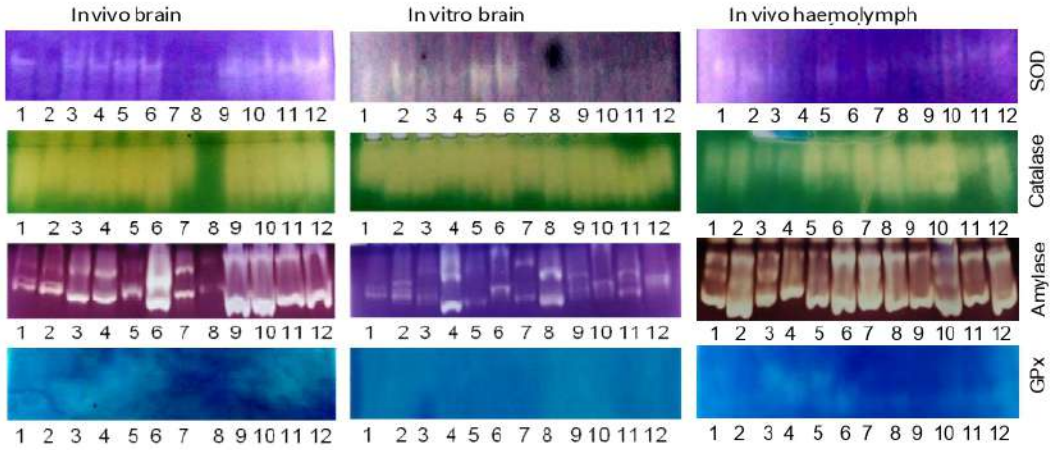

Fig. 4. Effect of AZT on specific activity of marker enzymes on brain and hemolymph of *S. pr. prasiniferum*showing on a polyacrylamide gel. lane distribution of each panel: Lane 1-6 female brain (1-control, 2-1ppm, 3-5ppm, 4-10ppm, 5-15ppm, 6-20ppm AZT) and Lane 7-12 male brain (as previous)

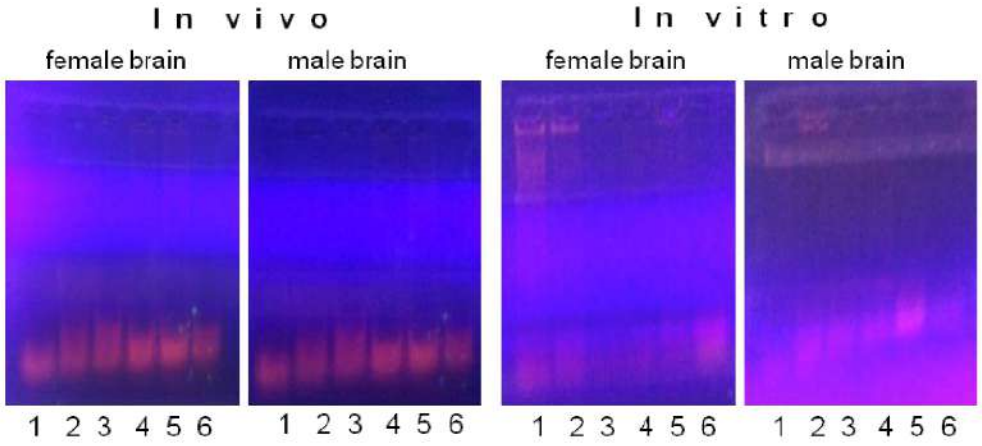

Fig. 5. Effect of AZT are shown on neural DNA of *S. pr. prasiniferum*. lane distribution: Lane 1-6 (1-control, 2-1 ppm, 3-5ppm, 4-10 ppm, 5-15 ppm, 6-20 ppm of AZT)

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 H_2O_2. 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 ($O_2^{\cdot-}$) 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.

ACKNOWLEDGEMENTS

Authors are grateful to Science & Engineering Research Board (SERB), Govt. of India for Financial assistance (SR/FT/LS-174/2012) and the Director, Zoological Survey of India (Kolkata) for identification of the acridid species.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/50980

Journal of Asia-Pacific Entomology 23 (2020) 350–357

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Full length article

Bioindicator potential of *Spathosternum prasiniferum prasiniferum* (Orthoptera; Acridoidea) in pesticide (azadirachtin)-induced radical toxicity in gonadal/nymphal tissues; correlation with eco-sustainability

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ARTICLE INFO

Keywords:
Azdirachtin
Gonads
Juvenile tissue
Oxidative biomarkers
Reactive oxygen species
Antioxidant enzymes

ABSTRACT

Acridids are highly abundant living-organism/major-component in agricultural field, globally. It may act as a dependable bio-indicator species in response to environmental-stress. Antioxidants protect insects by scavenging free radicals. Here, we investigated the dose dependant azadirachtin ($C_{35}H_{44}O_{16}$ AZT) toxicity on oxidative-biomarker; Alkaline-phosphatase (ALP), malondialdehyde (MDA), non-protein-soluble-thiol (NPSH) and anti-oxidants like superoxide-dismutase (SOD), catalase (CAT), glutathione-peroxidase (GPx) and amylase of gonads from both sexes and juvenile tissues of common grasshopper, *Spathosternum prasiniferum prasiniferum* (Walker, 1871). The newly hatched adults (female: male = 1:1) are exposed to 1 to 20 ppm AZT for 48 h and have compared to control and 6 h incubation with same concentration of azadirachtin for in vitro experiments. Both in vivo and in vitro experiment demonstrated significant influences on oxidative biomarkers with increasing antioxidant enzymatic activities in either sex. The male gonads represents decreasing antioxidant enzyme activities compared to female gonads. Lesser protection by CAT and SOD are noticed in male than female in response to AZT exposure. This experiment suggests that azadirachtin increased the major biomarkers with decreasing antioxidant enzyme activities resulting in more free-radicals related threat in adult male gonads. Variable dose responses were noticed on ALP, AchE and MDA in either gender suggesting multiphasic action of the pesticides. Higher mortality rate is noticed in male with lower nymphal life span. Moreover, nymph IInd is more susceptible than nymph IVth in vitro intoxication of azadirachtin. Possible life threat of vast representatives of agricultural ecosystem by pesticide should be avoided to maintain different bio-geo cycle and eco-sustainability.

Introduction

The growth regulatory effects of azdirachtin (AZT) and other products of *Azadirachta indica* are of extensive theoretical and practical interest. Treatment of insects by AZT causes larval growth inhibition, malformation, mortality leading to death. This activity has been proved in different species including lepidoptera and diptera which alter ecdysteriod and juvenile hormones from the brain corpus cardiacum complex (Ascher, 1993). Extracts from various parts of the *A. indica* have different degrees of insect's growth regulator activity.

AZT can modify ecdysteriod by inhibiting the release of prothoracotropin hormone and have adverse effects on ovarian development, fecundity and fertility (Mordue-Luntz and Blackwell, 1993). Reports reveal that the sex behaviour of insects in mating in response to sexual pheromones (Dorn et al., 1987) and spermatogenesis in males (Shimizu, 1988) are affected by AZT treatment. When oviposition sites are treated with AZT, oviposition repellency, deterrency and inhibition occurred in several agriculture-field insects (Dhar et al., 1996).

Azdirachtin changes the biological fitness including low absorption of nutrients and reduced lifespan, (Wilps, 1989), high mortality (Dorn et al., 1987). AZT has direct effects on different tissues and cells especially, those have rapid growth and mitotic behaviour. But, in general AZT toxicity has not been tested at enzymatic, biomarker and anti-oxidants levels in the reproductive organs of *S. pr. prasiniferum*. In the current study we have evaluated dose dependent AZT toxicity on both female and male gonads by investigating oxidative stress marker, antioxidant enzymes activities and genotoxicity in nymph tissues of this species.

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https://doi.org/10.1016/j.aspen.2020.02.007
Received 19 June 2019; Received in revised form 8 February 2020; Accepted 14 February 2020
Available online 22 February 2020

Materials and methods

Chemicals and Reagents: Bovine serum albumin (BSA), thiobarbituric acid (TBA), reduced glutathione (GSH), 5-5′-dithiobis-2-nitro benzoic acid (DTNB), ammonium molybdate, ascorbic acid, nitro blue tetrazolium (NBT), agarose (low melting point), acrylamide, N,N′-methylene-bis-acrylamide, ammonium per sulphate, and xanthine, tetramethylethylenediamine (TEMED) were purchased from Sigma chemicals (St. Louis, MO). Sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), ethylenediamine tetraacetic acid (EDTA) were supplied from SRL, India or MERCK, India. For alkaline phosphatase (ALP) assay. kits were purchased from either from Ranbaxy Laboratories Limited, India or MERCK India, Worli, Mumbai 400018.

Laboratory mass culture of insects

All collected wild acridids are acclimatized in specially designed cages made of nylon net gauge on wooden frame measuring 125 × 75 × 50 cm under laboratory conditions ranging from 30 °C to 35 °C temperature, 70 to 80% relative humidity, 500 lx light intensity and 12L:12D photoperiod. Plastic trays measuring 30 × 20 × 10 cm filled with fine and sterilized moist sand are kept inside the cages for insect oviposition. Specimens are sent to Zoological Survey of India, Kolkata for taxonomic identification. Species (Fig. 1a and b) selection is made depending on association with grass and occurrences throughout the year.

Collection of insecticide

Commercially available Azadirachtin which brand name Nimbecidine Plus 10,000 ppm manufactured by T. Stanes & Company Limited (Fig. 1e) purchased from the local retailers of West Bengal was used in the present study. Different concentrations are prepared by dilution with distilled water.

In vivo experiment

Dose dependant AZT were uniformly applied independently to 25 g of food plants placed in water containing conical flask in plastic jars. Then, newly hatched adult of 1:1 female and male insects of same age are placed in each jar and covered with mosquito net. Four replicates have used for each concentration in comparison to control where grasses without pesticide are used. Percentages (%) of mortality were estimated after 24, 48 and 72 h post-treatment and the values have corrected by Abbott's formula (Finney, 1964) (Fig. 1f). Alive insects from each treatment have dissected and fat bodies free gonads have taken separately from female and male individuals and store in −20 °C.

In vitro experiment

Fresh fat bodies free gonads from both sexes and instar wise whole body juvenile tissue are incubated with different dilutions of AZT prepared for the experimental purposes in comparison to control for 6 h at room temperature and separately store in −20 °C for sample preparation.

Preparation of sample for biochemical analysis

The pooled tissues from each treatment are homogenized in phosphate buffer (pH7.4) and centrifuged at 10,000 rpm for 8 min. The 20% supernatant is transferred to new eppendorf and preserved at −20 °C until onset of biochemical assay.

Biochemical analysis

At the beginning of each analysis, the samples are allowed to equilibrate to room temperature and all activities are measured by using standard method.

Assay of total protein contents

Total protein content of different tissues was estimated by the

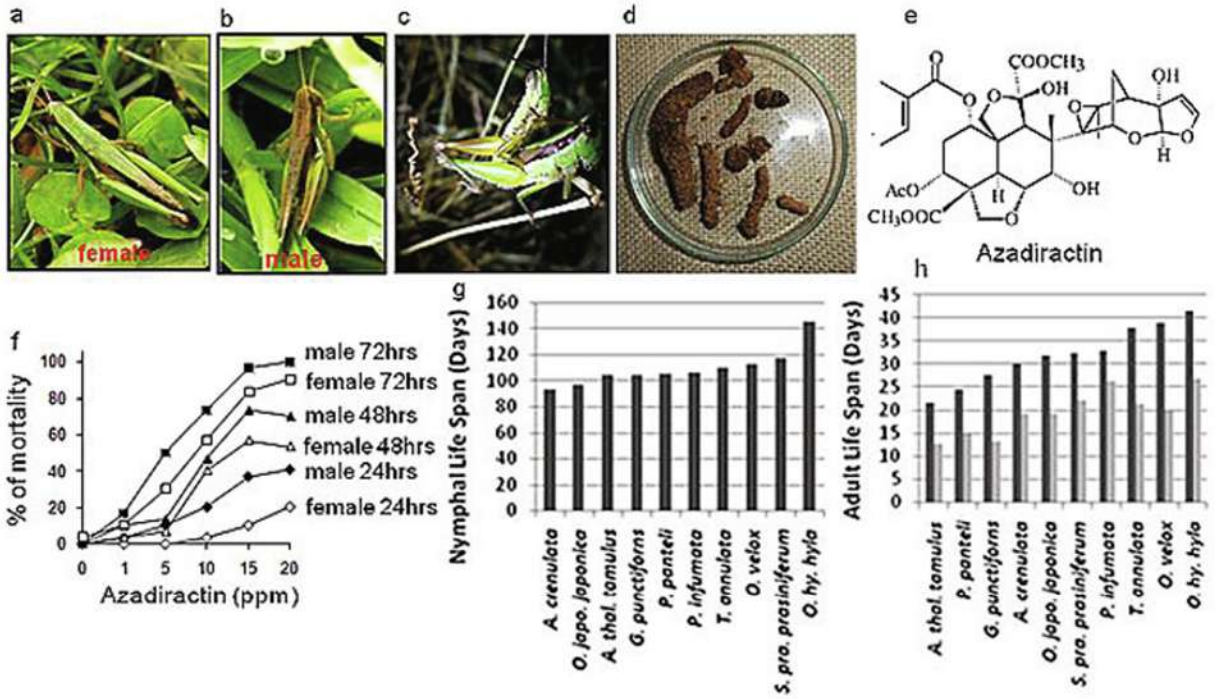

Fig. 1. a) Female and b) Male *Spathosternum prasiniferum prasiniferum* (Walker), c) Copulated species, d) Egg pod laid by insect, e) Chemical structure of azadirachtin and f) Dose, time and sex dependent mortality percentage (data represent the means, n = 20). (control vs. treated groups), g) Nymphal life span and h) Adult life span of common acridids under laboratory condition.

method of Lowry et al. (1951). The blue colour product is measured after 30 min at 750 nm against the blank. Protein concentrations are expressed as μg/mg wet tissue.

Estimation of malondialdehyde (MDA)

The supernatant is used for the estimation of MDA determined from the reaction of thiobarbituric acid (Okhawa et al., 1979). The amount of MDA formed is measured by taking the absorbance at 530 nm ($\varepsilon = 1.56 \times 105\ mol^{-1}\ cm^{-1}$) and the concentrations were expressed as μmol/mg wet tissue.

Estimation of non-protein soluble thiol (NPSH)

The NPSH in homogenate is determined by standard DTNB (5,5′-dithiobis-2-nitrobenzoic acid) method with a slight modification (Forman, 2009). In brief, the protein is precipitated by 5% trichloroacetic acid (TCA) and the clear cytosol is added to Tris-HCl buffer containing 5 mM DTNB. The level of NPSH is determined against a GSH standard curve and the concentrations are expressed as μg/mg wet tissue.

Assay of alkaline phosphatase activity (ALP)

Alkaline phosphatase activity in homogenate is estimated according to Belfield and Goldberg (1974). To measure the activity of ALP, 0.1 ml of cytosol is incubated at 37 °C in presence of a mixture of Tris–HCl (pH 8.0) and p-nitrophenyl phosphate. The activity is measured at 405 nm and the concentrations are expressed as KA unit/mg wet tissue.

Estimation of acetyl cholinesterase (AChE)

Acetylcholinesterase activity is examined by the method of Ellman et al. (1961). The incubation mixture consisted in a final volume of 0.2 ml, 20 m M phosphate buffer (pH 7.6), 8 mM acetylcholine iodide and 20 μl of crude enzyme preparation. The incubation is carried out at room temperature of 28 °C ± 1 °C for 30 mm with constant shaking. The reaction is stopped by adding 1.8 ml of DTNB phosphate ethanol reagent. The colour is read immediately at 412 nm. The results are expressed as nmol thiocholine released/mg protein/min.

In-gel assay for catalase (CAT)

In-gel CAT assays are followed the principles described by Zerbetto et al. (1997) and Weydert and Cullen (2010). The non-denaturing (8%) acrylamide gel is washed with distilled water for 10 min containing 25 μg proteins. The gel is shaken in 100 ml distilled water with 100 μl H_2O_2 for 10 min & then it is washed with distilled water for 5 min. An identical gel is stained with 30 ml 2% ferric chloride ($FeCl_3$) and 30 ml 2% potassium ferricyanide [$K_3Fe(CN)_6$] were poured onto the gel at the same time. When the gel becomes yellowish green then the stain solution is removed. Finally, the gel is washed with distilled water and protein bands appear on the gel.

In-gel assay for superoxide dismutase (SOD)

Superoxide dismutase activity gel is performed according to the method described by Beauchamp and Fridovich (1971) and Weydert and Cullen (2010) with slight modifications. One hundred fifty μg protein/lane is electrophoresed through a non-dissociating riboflavin gel consisting of 5% stacking gel (pH 6.8) and a 12% running gel (pH 8.8) at 4 °C. The gels are first incubated in 2.43 mM nitro blue tetrazolium (NBT) in deionized water for 15 min and then in 0.028 mM riboflavin/280 mM N,N,N′,N′-tetramethyl ethylene diamine (TEMED) in 50 mM potassium phosphate buffer (pH 7.8) for 15 min in the dark. Gels are then washed in deionized water and illuminated under fluorescent light until zones of SOD activity are distinctly evident.

In-gel assay for peroxidase (GPx)

Native polyacrylamide gel electrophoresis (8%) is carried out at 100 V for 2.5 h according to the method reported by Moreno et al. (1990) and Weydert and Cullen (2010) without SDS. First, an equal amount of protein (100 μg) extracts is applied and then gels are 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 colour development.

In-gel assay for amylase

Non-denaturing 8% polyacrylamide gel electrophoresis (PAGE) is carried out as described by Davis (1964). An equal amount of protein (100 μg) from pooled sample is applied electrophoresis is performed with 100 V at 4 °C. Afterward the gel is incubated in 2.5% (v/v) Triton X-100 for 30 min at room temperature with gentle agitation. Then, the gel is rinsed with deionized water and washed gel is incubated in fresh buffer containing 1% (w/v) soluble starch at 30 °C for 60 min. After it is washed with distilled water, the gel is subjected to staining with Lugol's solution (I_2 0.33% and KI 0.66%) at ambient temperature until the appearance of clear zones in protein bands with α-amylase activity against a dark blue background.

The density of all the in-gel enzymatic activity bands were evaluated by the ImageJ software and the numerical data are plotted as bar-diagram to compare AZT effects with comparison to that of control.

DNA fragmentation

Tissue is homogenised with 500 μl of lysis buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS) at 4 °C and centrifuged in cold at 12,000 rpm for 10 min. The supernatant is extracted with mixture of phenol: chloroform: isoamyl alcohol (gentle agitation for 5 min followed by centrifugation) and precipitated in two equivalence of cold ethanol and one tenth equivalence of sodium acetate. After spinning down and decantation, the precipitation is re-suspended in 30 μl of TE buffer and 5 μl of loading buffer for 30 min at 37 °C. The 0.8% agarose gel with ethidium bromide is run at 5 V for 5 min before increasing to 100 V and documented in gel documentation system (Compton, 1992). In these conditions, damaged DNA appears as a ladder consisting of DNA fragments. A suitable DNA ladder (EZ Load 500 bp Molecular Ruler #1708354, Life Sciences, BioRad) was also run to assume the damage pattern.

Analysis of data

Data were subjected to one way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) multiple comparison two tailed *t*-test (SPSS17) software to determine the effects of AZT. Mean and standard error values are determined for all the biochemical parameters and the results are expressed as means ± SE (n = 3) and the results are considered statistically significant when $P < 0.001$.

Results

General observation

An abnormal behaviour like restlessness, sudden quick, jerky movements are observed in the insects at low concentration of AZT whereas increased movements accompanied with swarming, loss of equilibrium and abnormal oviposition are observed in higher concentration of AZT exposed insects. We watched insect mortality increases with the doses of AZT treatment in both sexes. It is noticed that male is more sensitive to the AZT and female is more tolerant to AZT. The mortality increases almost linearly and after the 5 ppm dose, mortality rate becomes higher (Fig. 1f). From the Fig. 1g it can be suggested that the present experimental species and other similar species have high nymphal life span (= > 80 days), but the present sp. has ~100 days life span. But in Fig. 1h it is clear that adult female has appreciably higher life span than its male counterpart and this is true for all the primarily studied organism from that habitat. This finding is in parity with our biochemical and molecular biological data; that is female has a greater adaptability it terms of oxidant stress and antioxidant activities.

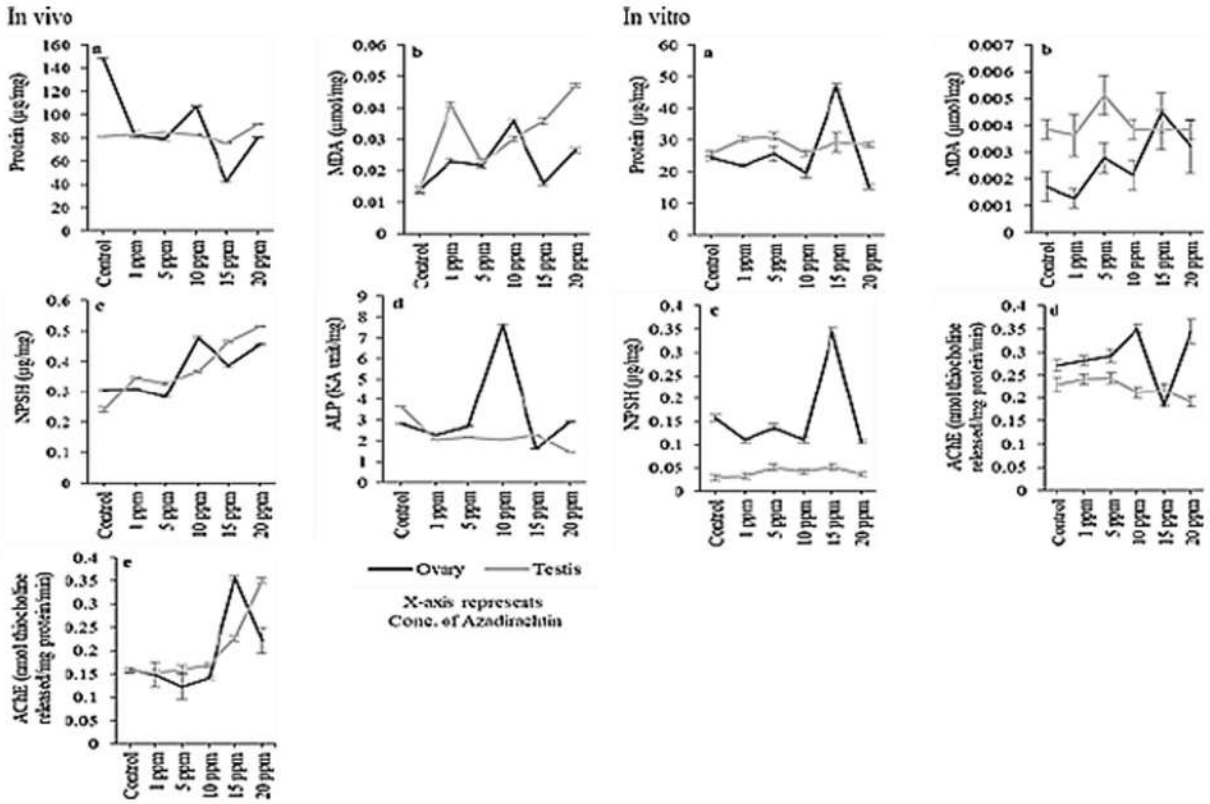

Fig. 2. Total protein, MDA, NPSH, ALP, AChE content of in vivo and in vitro gonads after AZT treatment with different concentrations in *S. pr. prasiniferum*. Data represent the means ± SE of triplicate analysis. Means within lines are significantly different ($P < 0.001$, ANOVA).

Effect on cellular protein

In vivo female gonad protein significantly ($P < 0.001$) and periodically decreased but no significant change was noticed in case of male gonad protein (Fig. 2a, in vivo). Especially in female the protein concentration was found to be variable in relation to the AZT concentration. And this suggests the dose dependant multiphasic activity of the current pesticides. These trends of changes were also noticed in case of other parameters. Multiphasic drug response is reported earlier. No significant changes were noticed in *in vitro* gonads from both sexes when compared to that of control (in vitro, Fig. 2a) except in female at 15 ppm dose where the protein was found to be approximately double than that of the control. Instar-II and instar-IV tissue protein are significantly ($P < 0.001$, Table 1) increased in comparison to that of control (Fig. 4a). The ANOVA result suggests that dose dependant changes of NPSH, MDA and AChE in both sex were highly significant ($p < 0.001$, Table 1). And the densitometry data of catalase and SOD suggest their gradual increase in both male and female except catalase in female.

Effect on lipid peroxidation

In case of instar-II MDA increased at initial AZT concentration (0.013 pmole to 0.022 pmole). At higher dose this group showed multiphasic responses possibly done to the variable adaptive responses. The instar-IV showed a better adaptive responses due to the maturation in tissue protective system.

In vivo AZT treated gonads show higher MDA level for both sexes except in female at 15 ppm dose. In vivo males the values reach the highest level of MDA, both at 1 and at 20 ppm of AZT. And the levels were higher only in female in in vitro condition only at 15 ppm dose but not in all concentrations. The dose dependant changes are analyzed by ANOVA in table 1 which was found to be highly significant ($p < 0.001$). Significant ($P < 0.001$) increased MDA level were

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	F	P	In vitro	F	P	In vitro	F	P
female			*female*			Nymph-II-Protein	111.618	0.000
Ovary-Protein	2624.345	0.000	Ovary-Protein	69.954	0.000	Nymph-II-MDA	16.891	0.000
Ovary-MDA	89.031	0.000	Ovary-MDA	3.091	0.051	Nymph-II-NPSH	47.355	0.000
Ovary-NPSH	462.802	0.000	Ovary-NPSH	175.317	0.000	Nymph-II-AChE	96.281	0.000
Ovary-ALP	28348.160	0.000	Ovary-AChE	18.147	0.000	Nymph-IV-Protein	40.860	0.000
Ovary-AChE	20.430	0.000				Nymph-IV-MDA	6.009	0.005
male			*male*			Nymph-IV-NPSH	15.535	0.000
Testis-Protein	18.745	0.000	Testis-Protein	2.288	0.111	Nymph-IV-AChE	48.942	0.000
Testis-MDA	171.919	0.000	Testis-MDA	0.857	0.537			
Testis-NPSH	438.852	0.000	Testis-NPSH	2.845	0.064			
Testis-ALP	4561.863	0.000	Testis-AChE	2.449	0.095			
Testis-AChE	184.389	0.000						

observed in vivo female and moderately higher in male gonads (Fig. 2b). In vitro female gonad showed higher MDA after AZT treatment (Fig. 2b, in vivo). In case of instar-II MDA increased at initial AZT concentration (0.013 pmole to 0.022 pmole). At higher dose this group showed multiphasic responses possibly done to the variable adaptive responses. The instar-IV showed better adaptive responses due to the maturation in tissue protective system (4b).

Effect on NPSH

An increased level of NPSH is found in vivo both sexes gonads after AZT treatment (Fig. 2c). Moreover, a significant decreased NPSH level is observed in only instar-IV whereas the level increased in instar-II (Fig. 4c). It is noticed that in vitro treatment with AZT does not affect the level of NPSH in males and that in females it produces a polyphasic response between 10 and 20 ppm (Fig. 2c).

This is reported that transcriptional/translational regulation is in several cases show multiphasic responses with different doses of different drugs. Notwithstanding, enzymatic activity and its Km, Vmax, are also variable in different conditions and it may be due to different isoenzymatic responses.

Effect on ALP activity

AZT toxicity significantly depresses ALP activity in in vivo male gonad but increases in in vivo female gonad (Fig. 2d).

Effect on AChE activity

The in vitro experimental result suggests that AChE activity decreased from 0.24 to 0.2 nmole/mg protein/min from control to 10 ppm AZT dose and this value was 0.17 nmole/mg protein/min at 20 ppm dose. In female gonad it slightly increased from 5 to 10 ppm doses and drastically decreased at 15 ppm but came closer to the normal value at next higher dose. Acetyl cholinesterase activity significantly increases in invivo female and male goads (Fig. 2e). In vitro male AChE activity decreased from 0.24 to 0.2 nmole/mg protein/min from control to 10 ppm AZT dose and this value was 0.17 nmole/mg protein/min at 20 ppm dose. In female gonad it slightly increased from 5 to 10 ppm doses and drastically decreased at 15 ppm but came closer to the control value at higher next dose. (Fig. 2d). The AChE activity of Nymph-II is significantly depressed (from 5 to 20 ppm) after AZT treatment than that of corresponding doses in Nymph-IV (Fig. 4e). But when the dose effects are compared to the corresponding control then the Nymph II is only seen to have increased with 1 ppm AZT, but all other values are approx. the same, while the AChE activity of nymph IV increased with all the doses, being the highest at 10 ppm and that between 5 and 15 ppm a multiphasic behaviour was presented again.

Effect on SOD activity

Super oxide dismutase activity in in vivo gonads from both sexes after treatment is significantly higher than that of control (Fig. 3) and showed decreased activity in in vitro gonads. SOD activity of both instar tissues shown significantly higher level of SOD activity in comparison to control (Fig. 4).

Effect on catalase activity

In in-vivo gonad tissues of both sexes, CAT activities increased and in in-vitro male gonads CAT activities decreased gradually with the increase of AZT dosage (Fig. 3). In vitro the activity in males increased significantly to 5 ppm AZT and then fell to near the control value at 20 ppm of AZT dose.

CAT activity increased in instar-IV whereas instar-II showed moderate change with the increase of dosage with comparison to the control value (Fig. 4). In detail, Nymph II (except with 1 ppm AZT) there seems to be no significant differences.

Effect on amylase activity

In vivo and in vitro female gonads showed increased α-amylase activity whereas decreased in in vivo and in vitro male gonads with the increase of dosage in comparison to control (Fig. 3). Inhibition of starch hydrolysing activity is found in instar-IV and increased activity is found in the group of treated instar II (Fig. 4).

Effect on GPx activity

In gel GPX activity in in vivo and in vitro brain (Fig. 3) of both sexes and also in juvenile tissues (Fig. 4) is not significantly varied in response to AZT toxicity in comparison to controls.

Densitometry data of band strength of all enzymatic activities were performed in gonad tissues of both sex (Fig. 3) and in nymphal tissues (Fig. 4). The densitometry data of in vivo and in vitro investigation suggest dose dependant increase is higher in female in case of all enzymes except in vivo GPx activity. We did not find appreciable GPx band in in vitro condition. In case of in vitro condition enzyme was almost unresponsive to AZT in case of male gonad.

In case of nymphal tissues AZT dose dependant enzymatic activity change was only noticed in case of ctalase of 4th inster nymph. In case of amylase and SOD activity irregular increase was noticed in only 2nd inster nymph.

Effect on DNA

In vivo and in vitro gonads of both sexes and DNA laddering of in vitro juvenile tissues induced by AZT doses are observed in comparison to that of control (Fig. 5). Differential responses were noticed in case of AZT dependant DNA laddering pattern. In case of nymph more DNA laddering was noticed in II versus IV inster.

Discussion

It is evident that AZT toxicity strongly interferes with the production of total cellular protein of gonads (Fig. 2a) and juvenile tissue (Fig. 4a) and during gonadal maturation and reproduction period (mating, copulation, pregnancy, egg-laying and hatching), insects remain very susceptible to environmental stress after exposure (Senthil-Nathan et al., 2004).

Pesticides toxicity lead to oxidative stress by generation of free radicals, changes in lipid peroxidation (LPO) and antioxidants levels (Ender and Onder, 2006). Pesticides does not directly initiates lipid peroxides but generates numerous radicals indirectly like superoxide radical (O_2^-) and hydroxyl radical (OH·) that damage cellular proteins, lipids and DNA by oxidation (Kale et al., 1999). Our result shows that gonadal MDA level increases with the increased dose both in in vivo and in vitro experiment. Lipid peroxidation arises from the reaction of free radicals with lipids and this is regarded as an important sign of cellular injury (Hoek and Pastorino, 2002). Sometimes the AZT effect on MDA levels seems biphasic to multi-phasic and higher in male (Fig. 2b). Fetoui et al. (2008) reported that concentration dependent pesticides toxicity resulted in a significant increase in MDA production justifying our results. Increased ROS production may thus be associated with the AZT metabolism leading to the peroxidation of membrane lipids of the gonads finally causing to cell damage and hampering reproduction.

Higher increase of NPSH in female than male (Fig. 2c) suggests their better protective response to nullify MDA level keeping ovum in healthier physiological state. Previous investigators have also reported the significant decrease in NPSH level is concentration and time dependent (Sharma and Ansari, 2013) by elevation of MDA. In vivo experiment in

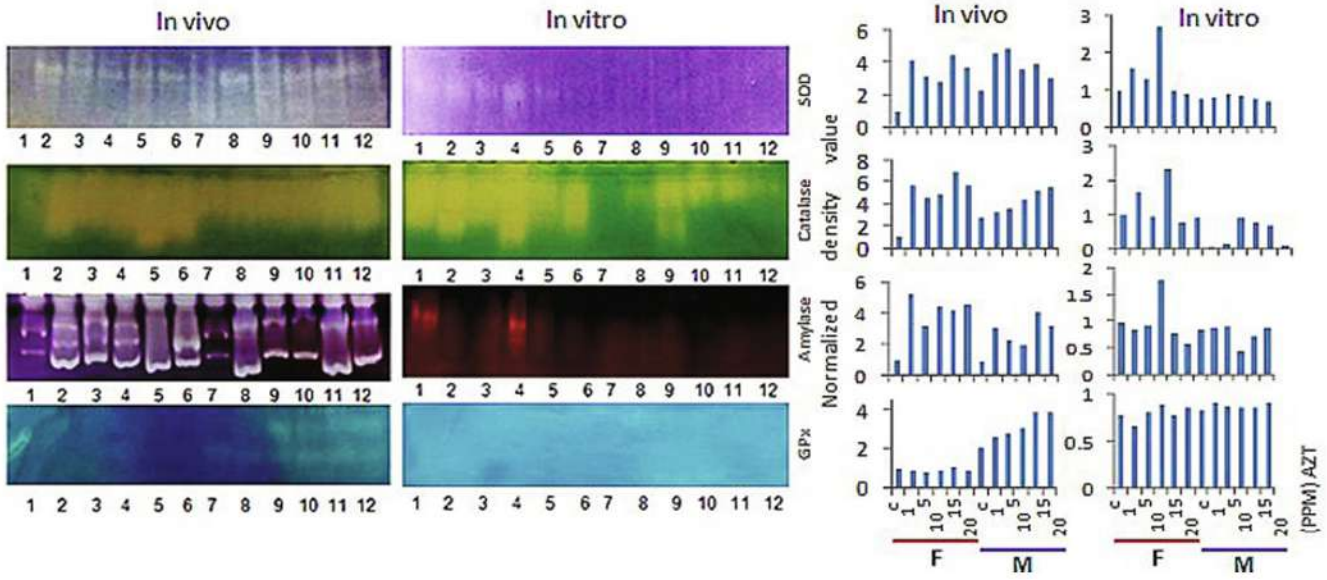

Fig. 3. Enzyme activities are shown on a polyacrylamide gel. Each panel (both in vivo and in vitro experiment) – Lane distribution: Lane 1–6 female gonad (1-control, 2–1 ppm, 3–5 ppm, 4–10 ppm, 5–15 ppm, 6–20 ppm) Lane 7–12 male gonad (7-control, 8–1 ppm, 9–5 ppm, 10–10 ppm, 11–15 ppm, 12–20 ppm). Panel a – SOD, Panel b – Catalase, Panel c – Amylase, Panel d – GPx.

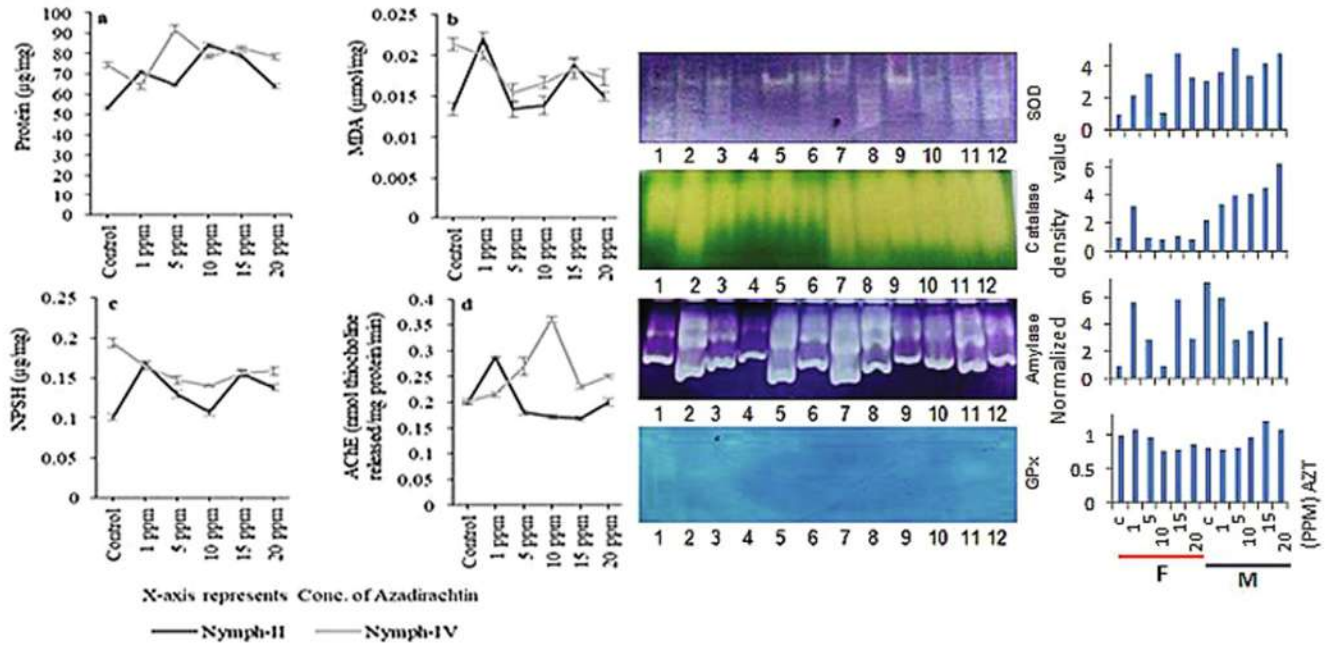

Fig. 4. Total protein, MDA, NPSH, AChE content of in vitro juvenile tissue after AZT treatment with different concentrations in *S. pr. prasiniferum*. Data represent the means ± SE of triplicate analysis. Means within lines are significantly different ($P < 0.001$, ANOVA). In vitro nymph enzyme activities are shown on a polyacrylamide gel. Each panel (in vitro experiment)-Lane distribution: Lane 1–6 nymph-II tissue (1-control, 2–1 ppm, 3–5 ppm, 4–10 ppm, 5–15 ppm, 6–20 ppm) Lane 7–12 nymph-IV tissue (7 – control, 8–1 ppm, 9–5 ppm, 10–10 ppm, 11–15 ppm, 12–20 ppm). Panel a – SOD, b – Catalase, c – Amylase, d – GPx.

female shows stronger adaptive responses (Fig. 3). The densitometry data of all enzymatic activities were analyzed (Figs. 3 and 4). These data suggests that acute exposure even in isolated tissue is responsive in case of female but not in male. Higher adaptability in female may be governed by its specific pattern of hormonal metabolism which needs to be further explored (McCabe et al., 2017; Gochfeld, 2017).

The strong increase/activation of amylase suggests an induction of hyper-metabolic state for enhanced energy catabolism and energy utilization to maintain the metabolic integrity is higher in female than male. Nymph-II is found to be more sensitive with dose dependent AZT toxicity. The higher level of NPSH and MDA at this stage emphasizes the occurrence of toxicity and thiol mediated adaptive responses against it. The decreased NPSH level of in vitro nymph-IV indicates that protection against the ROS is more required. Interestingly, higher acetyl cholinesterase (AChE) in nymph-IV promote depletion of active neurotransmitter acetylcholine (Fig. 4d) might impair proper neural transmission and adaptive organ-wise communication. Nymph-IV represents decreased AChE activity indicating acetylcholine (ACh) is not broken and accumulates within synapses, causing overall decline in neural and muscular control (Dutta and Arends, 2003) and initiates accumulation of free radicals leading to lipid peroxidation (Yang et al., 1996).

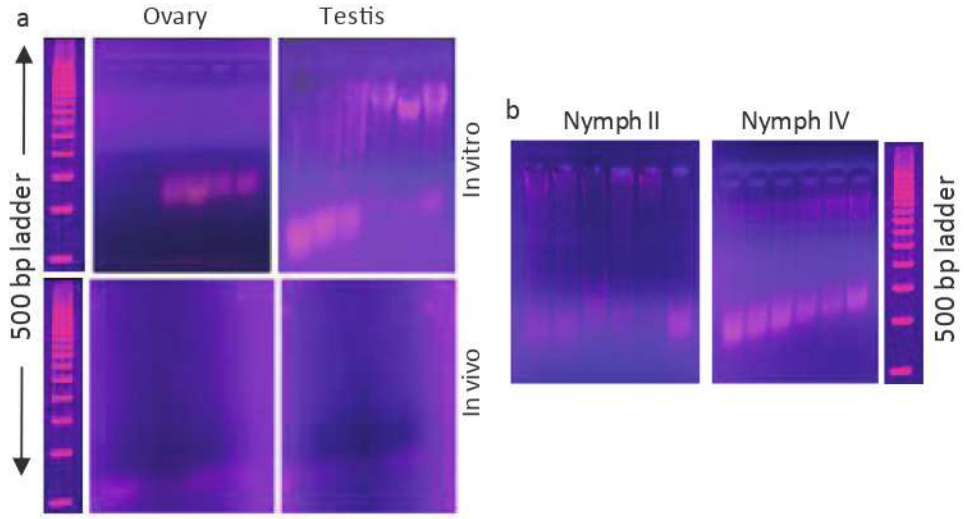

Fig. 5. Effect of AZT on gonadal DNA and juvenile DNA of *S. pr. prasiniferum*. Lane distribution: Lane 1–6 (1-control, 2–1 ppm, 3–5 ppm, 4–10 ppm, 5–15 ppm, 6–20 ppm).

Antioxidant protection consists of many enzymatic and non-enzymatic factors which maintain the physiological level of reactive oxygen (Ondreicka et al., 1998). Superoxide dismutase plays a major role in the first line antioxidant defence system by catalysing the dismutation of superoxide radical to form H_2O_2 and molecular O_2 (Ho et al., 1998). Tsan (1993) found that SOD activity increased during hypoxia. Catalase is a ubiquitous enzyme present in cells of all aerobic organism, which converts two molecules of the strong oxidant, H_2O_2 to molecular O_2 and two molecules of H_2O (Laszlo et al., 1991). Kono and Fridovich (1982) found that superoxide anion inhibited CAT activity and the presence of H_2O_2 inhibited the action of dismutase. Increased CAT activity in female gonads is not essentially related to reduction of MDA levels. In other words, the MDA level is elevated in in vivo female gonads when CAT activity reached maximum in this tissue. The increased MDA level indicates an enhancement of lipid peroxidation by prooxidant substances which react with unsaturated fatty acids of biological membrane during AZT intoxication. These results suggest the possibility of peroxidation process of AZT intoxication at higher dose. In this study we observe decreased CAT activity in in vitro female and male as well as in in vitro nymph-IVth of AZT intoxication. This indicate a reduced ability of the tissue to protect itself against H_2O_2, whereas increased SOD activity indicates that protection against superoxide radical is more prominent.

Both SOD and CAT activities of in vitro male gonads (Fig. 3) and nymph IInd (Fig. 4) decreased significantly and that evidently shows that AZT impairs the male reproductive function in grasshopper and instar specific juvenile tissue. The CAT activity was reduced by pesticides induced enhancement of superoxide radicals (Yu, 1994) or might be due to binding of AZT to catalase or by inhibiting catalase synthesis. In the present study, low dose of AZT cannot adversely affect reproductive functions of both gonads. Dose dependant higher rate of DNA degradation is noticed in all cases of in vivo and in vitro studies (Fig. 5a). Especially, in nymph the DNA degradation is found to be more in both stages after being treated with higher concentration of AZT (Fig. 5b) which is the normal consequence of oxidative stress and the same is demonstrated through elevation in MDA and reduction in antioxidant enzymes. This is also consistent with previous studies where DNA fragmentation is induced by pesticide in rat lymphocytes (Sharma et al., 2010) and in mammalian brain (Hussien et al., 2013).

According to Zhang et al. (2004) severe oxidative stress suppresses antioxidant defence enzyme activities due to oxidative damage and loss of compensatory mechanisms. In our study, ovary and nymph IV of grasshopper exhibiting a recovery response by catalase activity is observed which was adjustable and effective to protect from cellular injury. However, AZT effect on this species making them more sensitive to environmental changes and the parameters evaluated can be used to monitor pesticide toxicity in environment. More toxicity in male than female finally ends up in less net reproducibility of the species in toxicity. But protective strategies in female may be of interests to study the adaptive mechanisms which could have been implemented to augment the adaptive behaviour in either sex of this species or other species. Periodic changes of activities were noticed in almost all enzymatic action and in other biological parameters also in the current study. This is revealed from the zymographic analysis and the corresponding densitometry data. This type of drug action has been reported earlier (Martinez et al. 2013; Belcarz et al. 2013).

In conclusion, the survival and fitness of a species is determined by its fecundity and reproducibility. The oxidative-antioxidative balance in both sexes is of great interests in maintaining its gene-pool and existence/participation of other representatives of the particular ecological niche. The interpretation of these results will be more complete after further investigation of more biochemical and molecular determinants in this or other species. Azadirachtin has a potential use as phyto-pesticides in insect pest management. The current study represents the first report dealing with the sex and age dependent impairment of enzyme activities by AZT.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study is part of Ph.D. thesis research work by Balaram Manna and is supported by the Department of Biochemistry, Vidyasagar University, Midnapore-721102, West Bengal, India. The Author is thankful to the Secretary Ph.D. committee, Department of Biochemistry, Vidyasagar University for providing necessary support and the Director, Zoological Survey of India (Kolkata) for authentic identification of the acridid species. The author is thankful to Mr. Raju Manna, M.A. (Vidyasagar University), West Bengal for grammatical and syntactical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aspen.2020.02.007.

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Sex dimorphic adaptive responses against Azadirachtin toxicity in gut tissues of Spathosternum prasiniferum prasiniferum *(Orthoptera; Acridoidea)*

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International Journal of Tropical Insect Science

e-ISSN 1742-7592

Int J Trop Insect Sci
DOI 10.1007/s42690-020-00180-1

Springer

International Journal of Tropical Insect Science
https://doi.org/10.1007/s42690-020-00180-1

ORIGINAL RESEARCH ARTICLE

Sex dimorphic adaptive responses against Azadirachtin toxicity in gut tissues of *Spathosternum prasiniferum prasiniferum* (Orthoptera; Acridoidea)

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Received: 27 January 2020 / Accepted: 29 May 2020

Abstract

Acridids (short-horned-grasshopper) are distributed largely in the agricultural-field, grass-lands, and forests. It may act as a dependable bio-indicator species in response to ecological-stress. Among the acridids found in West Bengal, *Spathosternum prasiniferum prasiniferum* (Walker, 1871) are multivoltine in nature and were considered for the present study. Azadirachtin is a tetranortri terpenoids present in neem *tree (Azadirachta indica),* which develops antifeedancy/ growth-regulation/ fecundity-suppression/ sterilization/ oviposition/ repellence and deformity in insect via oxidative stress by the production of free radicals and causes their death. Antioxidants protect insects by scavenging free radicals. This study explored Azadirachtin toxicity on food-indices/biochemical/physiological influences on mid-gut of *Spathosternum prasiniferum prasiniferum.* Commercially available Azadirachtin was applied (1–20 ppm) on *S. pr. prasiniferum* for in vivo/in vitro experiment, where gut-tissues were analysed to determine oxidative-stress-markers (ALP/MDA/NPSH/AChE), antioxidant-markers (SOD/CAT/GPx). Food-weight/insect-weight/excreta-weight were recorded before/after the experiment to evaluate the consumption-index(CI), approximate-digestibility(AD), efficiency of conversion of digested-food(ECD) and efficiency of conversion of ingested-food(ECI). Nutrient metabolizing-enzymes (cellulase/amylase) were screened. Azadirachtin (>7 ppm) decreased ECD and restricted food-consumption that increased insect-mortality (50–80%). The gut MDA significantly increased with an impairment of soluble-thiols. The antioxidant-enzymes were variably impaired resulting in tissue-damages more in male than female. Finally, Azadirachtin influenced nutrient-metabolizing-enzymes and antioxidant-enzymes indicating these parameters to be good stress-markers. This finding might be extrapolated for further evaluation of the ecological impact of Azadirachtin on the food chain/web in a composite-ecosystem.

Keywords Azadirachtin · Gut · Food indices · Oxidative stress · Antioxidant defence

Abbreviations

AD	Approximate digestibility
CI	Consumption index
ECD	Efficiency of conversion of digested food
ECI	Efficiency of conversion of ingested food
ROS	Reactive oxygen species
MDA	Malondialdehyde
TBA	Thiobarbituric Acid
NPSH	Non-protein soluble-thiols
ALP	Alkaline phosphatase
AChE	Acetyl cholinesterase
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
DNA	Deoxyribonucleic acid

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Introduction

Pesticides induce oxidative stress which leads to the generation of free radicals, changes in antioxidants levels and lipid

Published online: 14 June 2020

peroxidation (Ender and Onder 2006) causing damage to proteins, lipids and DNA (Kale et al. 1999). Oxidative stress has been shown to play a crucial role in the cellular toxicity generated by a large number of pesticides (Stevenson et al. 1995). Free radicals especially reactive oxygen species (ROS) are essential for different metabolic functions including the cell signalling and other physiological processes (Li et al. 2016).Toxic effects of neem extracts and pure Azadirachtin on mosquito larvae have been demonstrated (Zebitz 1987). Azadirachtin is generally considered less harmful to the environment than other commonly used pesticides (Mordue and Blackwell 1993) that is highly oxidized with many reactive functional groups in proximity to each other (Ley et al. 1993).

Physiological and biochemical effects of an insecticide and its mode of action can be obtained from both in vivo and in vitro toxicity studies. There is wide variety of synthetic and natural insecticides. Most of the active principles of terpenoids found in neem products like Azadirachtin that acts as an anti-feedant and insecticidal compound (Isman 1997). Azadirachtin was first isolated by Butterworth and Morgan (1968); its chemistry (Ley et al. 1993) and biological activity (Mordue and Nisbet 2000) have been studied.

Thetoxicoutcome on insects comes about either by direct effects of Azadirachtin or by indirect effects via the disruption of endocrine system and neuro-endocrine tissues.Feeding behaviour depends upon both neural input sensory code of the insect. Azadirachtin stimulates specific deterrent chemoreceptors and blocks the sugar transporter, which stimulate feeding (Blaney et al. 1990). Scanty work has been done on this field. However, no previous work explored the mechanism of feeding impairment and growth retardation.

Azadirachtin has been shown to specifically bind on the membrane of the insect tissue (Nisbetet al. 1995). Dose dependent Azadirachtin caused reduction in body weight of final instar nymphs of *Schistocerca gregaria* and at the highest dose did not cause absolute feeding inhibition (Rao and Subrahmanyam 1986). Such treatments reduced the feeding rate, growth and utilization of food to body mass. Similarly, a physiological dose into female *Locusta migratoria* did not cause starvation, though food consumption was reduced without significant loss or addition to body weight (Subrahmanyam et al. 1989). Beside antifeedant effect, Azadirachtin affects metamorphosis (Ruscoe 1972) and these effects were observed in several orders on *Dysdercus koenigii* (Koul 1984), *Bombyx mori* (Koul et al. 1987), *Locusta migratoria* (Sieber and Rembold 1983), and *Rhodnius prolixus* (Garcia et al. 1986).

Azadirachtin binding in cellular site on insect tissues (Nisbet et al. 1995) shows a high level of specific binding of tritiated dihydro-azadirachtin to cell membranes that binding is time dependent and dissociation of the Azadirachtin membrane complex is incomplete showing very tight binding is not affected by Azadirachtin except at very high doses (Nisbet et al. 1996). Azadirachtin binding complex integrity within membranes is essential for its activity (Mordue et al. 1999). It means Azadirachtin alters or prevents the formation of new assemblages of organelles as well as cytoskeleton resulting in disruption of cell division. At ecological level, a higher rate of bioaccumulation and biomagnifications of the toxicants in grass land insect and in the upper level of food pyramid is detrimental for the composite agricultural habitat.

Spathosternum prasiniferum prasiniferum (orthoptera; acrididae) is a multivoltine short-horned-grasshopper with ecological, economical, nutritional and medicinal importance. Natural habitat of this species often faces the risk of contamination by diverse xenobiotics including azadirachtin based pesticide.

In this background, the aim of the current research study is to evaluate the free radial toxicity and DNA protection strategies in mid-gut of Azadirachtin-exposed to *Spathosternum prasiniferum prasiniferum* in vivo and in virto condition (Walker, 1871). Present study will help to extrapolate the pesticides effects on grassland ecosystem and to find a suitable biomarker for toxicity study Tables 1, 2 and 3.

Methods and materials

Rearing of insects

Wild grasshopper species were collected from grassland and acclimatized in specially designed cages made of nylon net gauge on wooden frame measuring 125 × 75 × 50 cm. Laboratory conditions(26 ± 1 °C, 70 ± 5% RH, 500 lx light intensity and 12 L:12D photoperiod) was maintained for insect oviposition. Specimens were sent to Zoological Survey of India, Calcutta, for authentic identification (Fig. 1a).Species selection is made depending on association with grass and occurrences throughout the year.

Preparation of insecticide

Commercially available (Azadirachtin) which brand name Nimbecidine 10,000 ppm (purity >99%) manufactured by T. Stanes & Company Limited purchased from the local retailers of West Bengal was used in the present study. Different concentrations are prepared by dilution with distilled water.

In vivo *treatment and* susceptibility test of Azadirachtin

Dose dependant Azadirachtin (1, 5, 10, 15 and 20 ppm in water) was uniformly applied to 25 g of food plants placed in water containing conical flask. Newly emerged adult of 1:1 female and male insects were placed in jars (11 × 11 × 26) cm and covered with mosquito net. Three replicates were used for each dose in comparison to control where grasses without

Table 1 Food indices of common acridid species studied in semi laboratory condition. (Data represent the means, $n = 20$)

Sl.No	Species	Food Consumption/ individual (mg)	Faecal matter production (mg)	Mean Weight (mg)	Duration of feeding period (Days)	Consumption Index (CI)	Approximate Digestibility (AD)	Efficiency of Conversion of Digested food (ECD)	Efficiency of Conversion of Ingested food (ECI)
1	*Acrida exaltata*	2524.74	2011.61	516.25	29.5	144.27	20.32	399.08	8.76
2	*Aiolopus thalassinus tamulus*	2073.08	1783.17	207.5	21.5	214.80	13.98	257.42	8.81
3	*Atractomorpha crenulata*	2042.47	1702.34	181.25	30	338.06	16.65	106.65	2.64
4	*Aulacobothrus luteipes infernus*	3427.95	2466.51	78.75	20.5	892.35	28.04	86.41	1.22
5	*Gasonula punctiforns*	1832.27	1650.69	107.5	27.75	472.92	9.90	75.09	3.07
6	*Oxya fuscovittata*	3336.30	2935.80	230	34.25	496.81	12.00	113.58	1.44
7	*Oxya hyla hyla*	2682.60	2035.15	145	41.5	767.78	24.13	129.13	2.61
8	*Phlaeoba infumata*	1926.22	1818.17	158.75	33	400.4	5.60	69.83	1.72
9	*Sphatosternum prasiniferum prasiniferum*	2800.18	2508.42	66.25	32.5	1373.67	10.41	142.84	0.57

pesticide are used. Percent of mortality was calculated after the treatment. Only alive insects from each treatment were dissected and the guts (Fig. 1b) from both sexes were stored in −20 °C. Corrected mortality was calculated according to Abbott (1925).

$$\text{Corrected Mortality} = \frac{\%\text{mortality in treatment} - \%\text{mortality in control}}{100 - \%\text{mortality in control}} \times 100$$

The data such as leaf weight, insect's weight and excreta weight before and after the treatment were recorded for consumption index (CI), approximate digestibility (AD), efficiency of conversion of digested-food (ECD) and efficiency of conversion of ingested-food (ECI) as used by Sharma and Norris (1991).

Invitro treatmentand preparation of gut extracts for activity assay

Fresh gut (Fig. 1c) tissueswere incubated with different dilutions of Azadirachtin (1–20 ppm) with suitable control without Azadirachtin. After 6-h incubation, tissues were stored in −20 °C.Whole guts were homogenized in 0.1 M chilled phosphate buffer (pH 7.4), centrifuged in 10,000 rpm/4 °C and the supernatant were stored at −20 °C.

Biochemical assays of oxidative stress

Protein content was estimated according to Lowry et al. (1951). Malondialdehyde level (index of lipid peroxidation) was determined from the reaction of thiobarbituric acid (TBA) reagent (Ohkawa1979). The amount of MDA-TBA chromophore was measured by taking the

Table 2 Live biomass of common acridid species from nearby agriculture field of West Bengal, India. (Data represent the means, n = 20)

Sl. No	*Species name*	Biomass after one generation (mg)		Total biomass/generation (a + b)
		M (a)	F (b)	(mg)
1	*Acrida exaltata*	6528.95	14,381.6	20,910.5
2	*Aiolopus thalassinus tamulus*	726.94	1356.63	2083.58
3	*Atractomorpha crenulata*	1318.03	4964.47	6282.5
4	*Aulacobothrus luteipes infernus*	87.6316	191.84	279.47
5	*Gasonula punctiforns*	128.84	282.1	410.947
6	*Oxya fuscovittata*	623.68	2169.08	3457.5
7	*Oxya hyla hyla*	1276.18	1435.79	2059.47
8	*Phlaeoba infumata*	119.47	2735.53	4011.71
9	*Sphatosternum prasiniferum prasiniferum*		205.52	325

Table 3 Summary statistics for quantity biochemical variables in *Spathosternum pr. Prasiniferum* after Azadirachtin toxicity. ($P < 0.001$, significantly different from control group, 10 animals in each group, ANOVA analysis at df 5)

Invivo	F	P	Invivo	F	P
female			female		
Gut-Protein	1692.025	.000	Gut-Protein	1560.169	.000
Gut-MDA	76.000	.000	Gut-MDA	24.894	.000
Gut-NPSH	462.444	.000	Gut-NPSH	10.422	.000
Gut-ALP	26,775.782	.000	Gut-AChE	488.850	.000
Gut-AChE	72.802	.000			
male			male		
Gut-Protein	482.278	.000	Gut-Protein	191.866	.000
Gut-MDA	29.547	.000	Gut-MDA	57.167	.000
Gut-NPSH	89.850	.000	Gut-NPSH	206.874	.000
Gut-ALP	26,126.022	.000	Gut-AChE	86.667	.000
Gut-AChE	161.937	.000			

absorbance at 530 nm ($\varepsilon = 1.56 \times 105$ mol^{-1} cm^{-1}). The NPSH was determined by standard DTNB (5,5′-dithiobis-2-nitrobenzoic acid) method of Forman (2009). Alkaline phosphatase activity was estimated according to Belfield and Goldberg (1971).The activity is measured at 405 nm and the concentrations are expressed as KA unit/mg wet tissue.

Acetyl cholinesterase activity was evaluated by Ellman et al. (1961) method with some modification. The colour was read at 412 nm. The results were expressed as nmol thiocholine released mg protein^{-1} min^{-1}.

Antioxidant enzyme assay

The superoxide dismutase (SOD)and the catalase (CAT) activity was performed in gel zymogramaccording to the method of Weydert and Cullen (2010).To visualize SOD activity, the first, gels incubated Nitro Blue Tetrazolium riboflavin mixed with (TEMED) in potassium phosphate buffer.

For CAT assay the non-denaturing (8%) acrylamide gel is shaken in 100 ml distilled water with 100 µl H_2O_2.Then it is stained by 2% Ferric Chloride ($FeCl_3$) and 30 ml 2% Potassium Ferricyanide [$K_3Fe(CN)_6$]according to the method of Zerbetto et al. (1997).

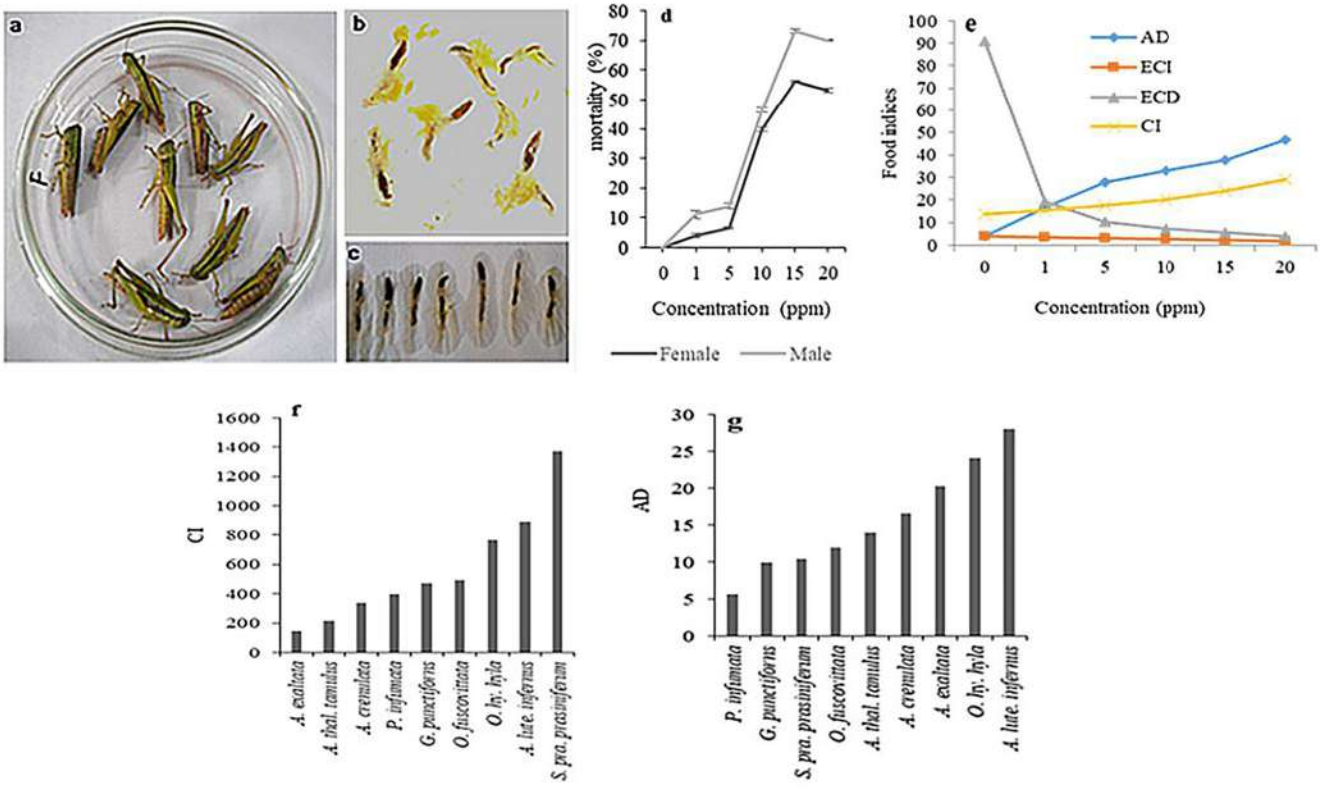

Fig. 1 a. Treated female and male *S. pr. prasiniferum* b. Dissected whole gut tissue after invivo treatment c. Desected whole gut tissue for invitro treatment. d. Sex dependant percent mortality after Azadirachtin treatment with different doses in *Spathosternum pr. prasiniferum*. (Control vs. treated groups). e. Food indices after Azadirachtin treatment with different doses in *Spathosternum pr. prasiniferum*. Means within lines are significantly different ($P < 0.001$, ANOVA). (Control vs. treated groups).f. Consumption Index (CI) of some common acridids under laboratory condition. Approximate Digestibility (AD) of some selected acridids under laboratory condition

Native polyacrylamide gel (8%) electrophoresis was carried out without SDSfor peroxidase activity in zymogram staining (Weydert and Cullen 2010).

Nutrient metabolizing enzyme assay

For amylase assay Non-denaturing 8% polyacrylamide gel electrophoresis (PAGE) was carried out as described by Andrades and Contreras (2017).After washing the gel was subjected to staining with Lugol's iodine (I_2 0.33% and KI 0.66%) until the appearance of clear zones in protein bands with α-amylase activity.

In-gel assays were performed using non-denaturing PAGE for visualising cellulase activities. The substrate 0.2% CMC were incorporated into the separating gels. After 20 min destaining, 1% glacial acetic acid was added to the gel for better visualisation (Willis et al.2010).

DNA fragmentation

Tissue was homogenised in lyses buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS) at 4 °C and centrifuged. The 0.8% agarose gel with ethidium bromide was run at 5 V for 5 min before increasing to100 V and documented in gel documentation system (Compton 1992). In these conditions, damaged DNA appears as a ladder consisting of DNA fragments.

Statistical analysis

Results from all the dose groups were exploited for multiple comparisons one way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) multiple comparison two tailed t-test using (SPSS 17) software. Mean and standard error values are determined for all the biochemical parameters and the results are expressed as means ± SE ($n = 3$) and the results were considered statistically significant at the level of $P < 0.001$.

Results and discussion

Studied grasshoppers represent abnormal behaviour with restlessness and jerky movements at low dose of Azadirachtin but increased movements accompanied with swarming movements and loss of equilibrium at higher dose. Dose dependant mortality was observed after Azadirachtin treatment compared to that of control (Fig. 1a).Food ingestion, digestion and consumption index (CI) which were increased in higher doses, were statistically significant ($P < 0.001$) (Fig. 1e),and the approximate digestibility (AD) was not significantly increased (Fig. 1.e) and ECD and ECI were reduced after the drug treatment.

The ANOVA results indicate that dependence between any two of the above mentioned parameters is highly significant ($P < 0.001$) (Fig. 1e) for all comparisons. When multiple comparison two tailed t-test was performed, it was noticed significant ($P < 0.001$ *to* $P < 0.01$) after the dose dependant response of Azadirachtin (Fig. 2).In insect the gut protein contents in vivo and in vitro of both sexes have been significantly ($P < 0.001$) different compared to that of control (Fig. 2a). A significant ($P < 0.001$) increased levels of lipid peroxidation (LPO) were found in vivo female and male gut after Azadirachtin administration (Fig. 2b). Azadirachtin reduced ALP activity in vivo of both sexes gut cells (Fig. 2e). Dose dependent in vivo and in vitro gut SOD activity was significantly higher of both sexes after Azadirachtin treatment (Fig. 3).

Increased α-amylase activity were observed in female gut whereas inhibition effect was observed in male gut at increased dose of Azadirachtin and decreased cellulase activity was observed in female gut (Fig. 3).Oxidative stress induced gut of both sexes DNA laddering were observed in comparison to control (Fig. 4).

The increase in CI and AD was stimulating for enzymes activities during feeding. Comparison of CI and AD among several field insects suggests that when the consumption index (CI) is higher in this insect the approximate digestibility is very much lower with comparison to other similar field insects. This suggests they damage plants more than they are actually capable to digest it. Nevertheless, this hypo-metabolic state increases the mortality of the insect as a result of Azadirachtin toxicity. Azadirachtin interferes with growth via digestive impairment by inhibiting the secretion of proteinases from gut cells. Decreased level of ECD suggested that Azadirachtin have toxic effects on *S. pr. prasiniferum* like other conventional pesticides. Azadirachtin also reduced weight gain and nutritional indices in an insect. This is possibly due to the reduction in digestive enzymes found in the larval mid-gut (Nouri-Ganbalani et al. 2016).

Since the alimentary tract confronts major amount of pesticide entering through oral route, oxidative stress was performed to observe any tissue damage in the gut of Azadirachtin -exposed insect. An increased level of lipid peroxidation (LPO) was found in vivo female and male gut (Fig. 2b). Azadirachtin increased the MDA production (El-Demerdash 2007) due to increased reaction of free radicals and lipids. In the present study, an increased level of NPSH was found in gut cells which suggest possible adaptive strategies against Azadirachtin toxicity (Fig. 2c). This biomolecule can serve as a sink for free radicals and reactive species against oxidative stress induced toxicity (Otto and Moon 1995).

Alkaline phosphatase is a primary hydrolytic enzyme of insect's gut (Sakharov et al. 1989) that hydrolyses phosphormonoesters under alkaline conditions and its activity was decreased by Azadirachtin (Senthil Nathan 2006). Azadirachtin

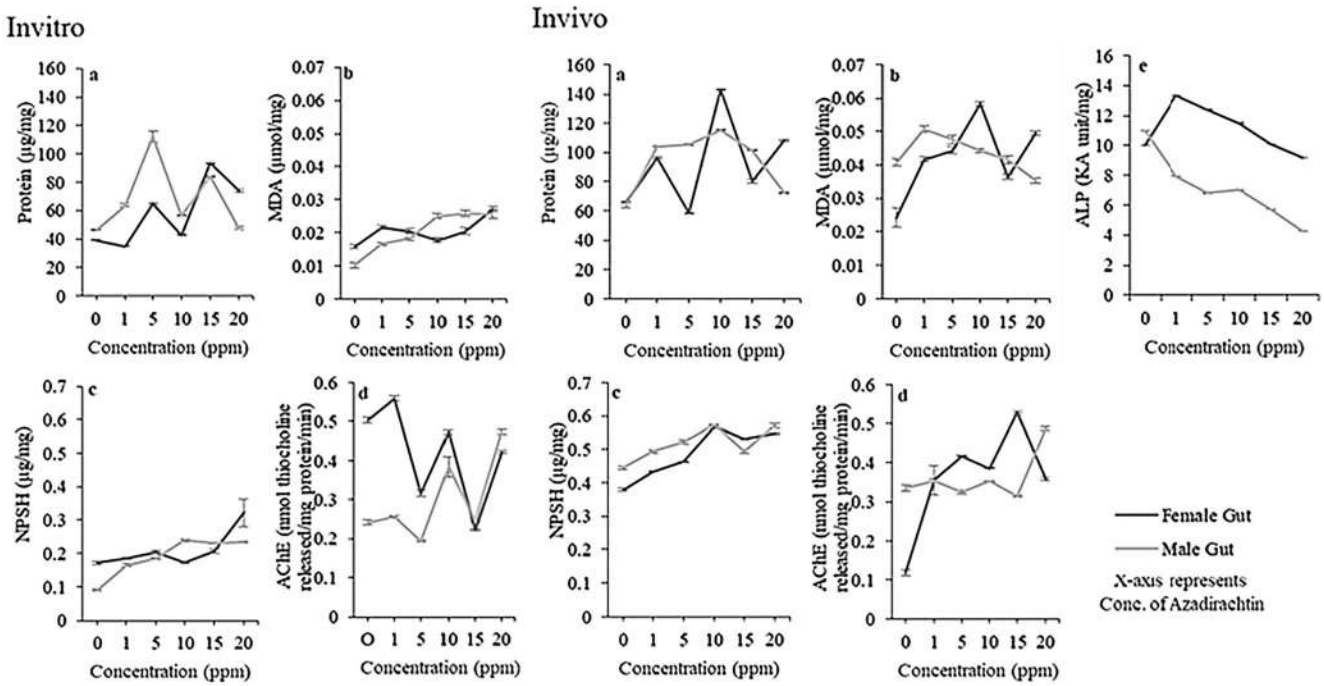

Fig. 2 Invitro: Total protein (**a**), MDA (**b**), NPSH (**c**), AChE (**d**) content of invitro gut after 48-h Azadirachtin treatment with different doses in *Spathosternum pr. prasiniferum.* Means within lines are significantly different ($P < 0.001$, ANOVA). In vivo: Total protein (**a**), MDA (**b**), NPSH (**c**), ALP (**d**), AChE (**e**) content of in vivo gut after 48-h Azadirachtin treatment with different doses in *Spathosternum pr. prasiniferum.* Means within lines are significantly different ($P < 0.001$, ANOVA)

dose dependently reduced the ALP activity in vivo gut cells of both sexes (Fig. 2e).Most aromatic plant products have inhibitory activity on acetylcholinesterase (AChE) (Shaaya and Rafaeli 2007). Acetylcholinesterase activity were significantly decreased in female by non-specific binding with Azadirachtin other than active site therefore, acetylcholine cannot be degraded and insects would die with toxic symptoms (Mordue et al. 2010), whereas, male represent higher enzymatic activity (Fig. 2d).

Sex dimorphic role of some of the adaptive response is evident in the current study such as increase of CAT activity in female but decrease in male insect. Report suggests that pesticide intoxicates tissues by increasing oxidative stress leading to generation of free radicals that change in antioxidants levels and LPO (Ender and Onder 2006). The degeneration of mid-gut tissue was accompanied by the transcriptional regulation of mitogen-activated protein kinase (MAPK) and calcium apoptotic signalling (Shu et al. 2018). Heavy metal induced MAPK P38 kinase activation is found to be linked.

Inhibition of insect α-amylase by plant derived inhibitors is reported (Sivakumar et al. 2006). It was observed that increased α-amylase activity in female gut at higher dose of Azadirachtin is most probably due to consumption and utilization of large quantities of food. Whereas, Azadirachtin inhibition of this enzyme in male gut (Fig. 3) suggest that this pesticide acts as sex dependant manner under the influence of specific hormone. It manifests insecticidal effect because they

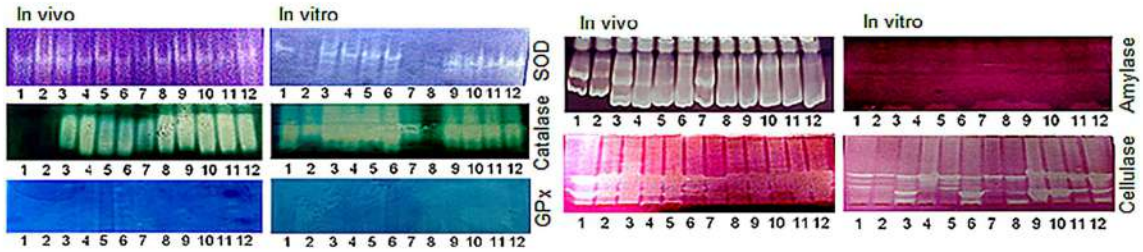

Fig. 3. Effect of Azadirachtin on specific activity of marker enzymes on gut of *Spathosternum pr. prasiniferum*showingon a polyacrylamide gel. Each panel (invivo and invitro experiment)-Lane distribution: Lane 1–6 female tissue (1-control, 2–1 ppm, 3–5 ppm, 4–10 ppm, 5–15 ppm, 6–20 ppm) Lane 7–12 male tissue (7-control, 8–1 ppm, 9–5 ppm, 10–10 ppm, 11–15 ppm, 12–20 ppm). Panel a- SOD, Panel b- CAT, Panel c- GPx. Effect of Azadirachtin on specific activity of digestive enzymes on gut of *Spathosternum pr. prasiniferum* on a polyacrylamide gel. Each panel (in vivo and in vitro experiment)-Lane distribution: as mentioned above. Panel a- amylase, Panel b- cellulose

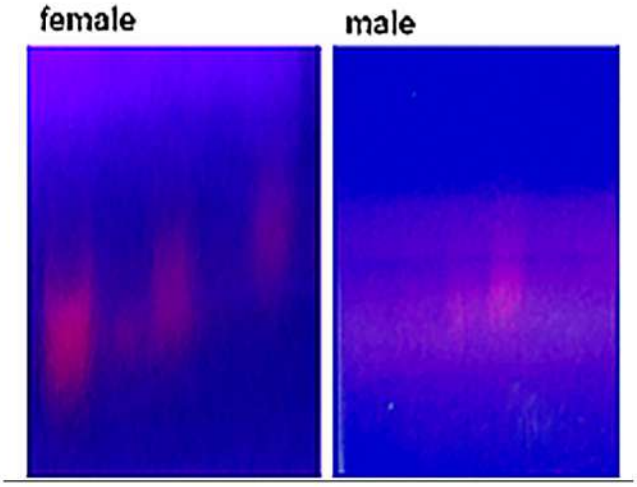

Fig. 4 Effect of Azadirachtin on gut DNA of *Spathosternum pr. prasiniferum*. Lane distribution: Lane 1–6 (1-control, 2-1 ppm, 3-5 ppm, 4-10 ppm, 5-15 ppm, 6-20 ppm)

form stable complexes with digestive enzymes which could inhibit the formation of enzyme-substrate complex. Thus, blocking of digestive enzymes resulted in poor nutrition, growth retardation and death of the insect (Bezzar-Bendjazia et al. 2017). Neem extract has an ability to inhibit the cellulase activity and inhibition of cellulase by some of its phenolic compounds is evident. Decreased activity of cellulase in female gut (Fig. 3) suggests that some plant derived molecules may block the enzyme activity (Sami and Shakoori 2011). Present work indicates that, oxidative stress induces lower DNA stability (Fig. 4) and less mRNA/protein production. Macromolecular disruption by Azadirachtin-induced MDA elevation has shown in some insect species (Sharma et al. 2010).

Based on the findings it can be concluded that beside antifeedant property, the toxic effects of Azadirachtin may be sex dimorphic. The most serious effects involve changes in behaviour, nutritional indices and antioxidant status that can affect diversity, community structure and even loss of this ecologically important species. Bioaccumulation and magnification of pesticides/toxicant in the food cycle have the most deleterious effects on the ecological system and that may also impair the agricultural productivity. A large number of communities worldwide consume different types of insects including grasshopper as the source of high protein-source food. So, pesticides toxicities in lower organisms have also detrimental effects on human health.

Acknowledgements This study is a part of Ph.D. thesis research work by Balaram Manna and is supported by the Department of Biochemistry, Vidyasagar University, Midnapore-721102, West Bengal, India. The author is thankful to the Director, Zoological Survey of India (Kolkata) for authentic identification of the acridid species. The author is thankful to Ms. Prativa Pradhan, Assistant Technology Manager, Department of Agriculture, Govt. of West Bengal, for species selection and technical assistance.

Compliance with ethical standards

Declaration of conflicting interests The author(s) declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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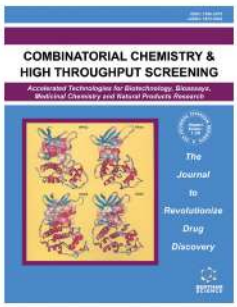

Research Article

Oxidative Stress-Induced Toxicity and DNA Stability in Some Agri-field Based Livestock/Insect by Widely Used Pesticides

(E-pub Ahead of Print)

Author(s): Balaram Manna, Sangita MaitiDutta, Subrata Dalapati, Smarajit Maiti*

Journal Name: Combinatorial Chemistry & High Throughput Screening

Accelerated Technologies for Biotechnology, Bioassays, Medicinal Chemistry and Natural Products Research

DOI : 10.2174/1386207323666200415110745 (https://doi.org/10.2174/1386207323666200415110745)

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Abstract:

Aim and Objectives: Human continuously uses pesticides in the field to control pest population and weeds for considerable agricultural productivity. Side-by species like grazing-animals, insects and other species are adversely affected by or become resistant to pesticides. Insect, bird and cattle are highly abundant dwellers of the agriculture-field and represent three distinct phylum having versatile physiological features. Beside higher agricultural-productivity, protection to several species will maintain ecological/environmental balance. Studies on the effect of widely used pesticides on their DNA-stability and important enzymaticactivities are scanty.

Materials and Methods: Antioxidant-activity (Superoxide-dismutase; SOD/Catalase- by gel-zymogram-assay) and DNA-stability (fragmentation-assay) in hepatic/gut tissues were studied here after in-vitro exposure of Chlorpyrifos, Fenvalerate, Nimbicidine or azadirachtin to goat/cow/poultry-hen/insect.

Results: In general, all pesticides were found to impair enzymatic-activities. However, lower organisms are affected more than higher vertebrate by azadirachtin-treatment. DNA fragmentation was found more in insects/poultry-birds than that of the cattle in hepatic/gut tissues. Inversely, toxicity/antioxidant marker-enzymes were more responsive in insect gut-tissues. However, mitochondrial-toxicity revealed variable effects in different species. It is noticed, chlorpyrifos to be the most toxic pesticide followed by Fenvalarate/Nimbicidine (azadirachtin, AZT). Nevertheless, AZT revealed its higher DNA-destabilizing effects in the field-insects than the other animals.

Conclusion: Field-insects are highly integrated to their ecosystem and the local bio-geochemical cycle, which may be impaired. Pesticides may have toxic effects on higher vertebrate and it may sustain in the soil after metabolized to its different derivative. Some of the sensitive biochemical parameters of this organism may be used as biomarker for pesticide toxicity.

Keywords: Pesticides, oxidative stress toxicity, livestock, insect, agriculture, DNA stability

Article Details

(E-pub Ahead of Print)
DOI: 10.2174/1386207323666200415110745
(https://doi.org/10.2174/1386207323666200415110745)
Price: $95

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Combinatorial Chemistry & High Throughput Screening, 2020, *23*, 0-0

RESEARCH ARTICLE

Oxidative Stress-Induced Toxicity and DNA Stability in Some Agri-field Based Livestock/Insect by Widely Used Pesticides

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Abstract: ***Aim and Objective*:** Humans continuously use pesticides in the field to control the pest population and weeds for considerable agricultural productivity. Side-by species like grazing-animals, insects and other species are adversely affected by or become resistant to pesticides. Insects, birds and cattle are highly abundant dwellers of the agriculture-field and represent three distinct phyla having versatile physiological features. Besides higher agricultural-productivity, protection to several species will maintain ecological/environmental balance. Studies on the effect of widely used pesticides on their DNA-stability and important enzymatic-activities are insufficient.

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ARTICLE HISTORY

Received: December 15, 2019
Revised: March 17, 2020
Accepted: March 25, 2020

DOI:
10.2174/1386207323666200415110745

Keywords: Pesticides, oxidative stress toxicity, livestock, insect, agriculture, DNA stability.

1. INTRODUCTION

The liver is the largest organ in the vertebrate body and it is the major site of xenobiotics metabolism. Hepatic injury is a common pathological outcome, which exists in many liver diseases. Environmental toxicants have adverse effects on a large community of vertebrates; mammals and non-mammals. Chemical fertilizers and pesticides are used for plant growth promotion; high-yield and protecting crops, which may have adverse effects on agri-field consumers and humans also. Cows *(Bos taurus)*, goats (*Capra aegagrus hircus*) and poultry birds such as hen (*Gallus gallus domesticus*) have been recognized as the most effective livestock for promoting health and economy, worldwide. These animals are predominantly common in the agriculture field and consume those products.

Pesticides have adverse effects on animal and human health [1]. Chemical pesticides are classified as organophosphates, organochlorines, carbamates, *etc* [2]. These substances mostly remain un-metabolized compounds in the ecosystem. The toxicity of pesticides is categorized in relation to their ability to produce lipid peroxidation, to impair antioxidant

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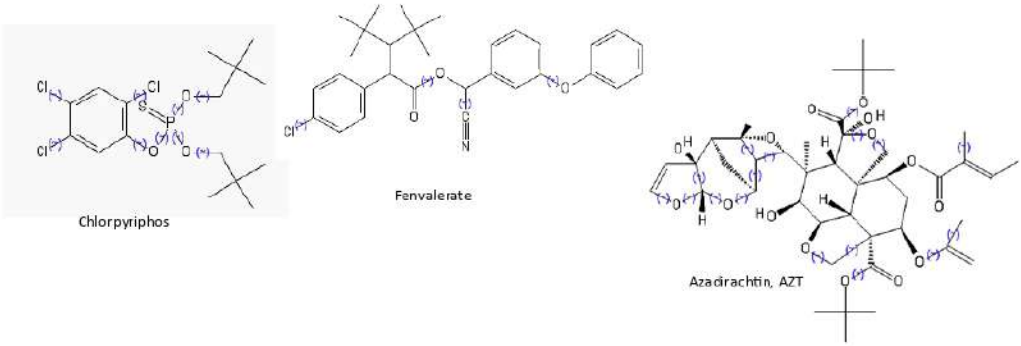

Fig. (1). Chemical structure of Chlorpyrifos, Fenvalerate (TATAfen) and Nimbecidine (Azadirachtin, AZT).

status, mitochondrial/DNA stability. Stimulation of free-radical production, induction of lipid-peroxidation and disruption of the antioxidant components by pesticides may develop toxicity in the living system. Reactive oxygen species (ROS) are the main causative agents for oxidative stress, which cause several diseases in human, like Alzheimer's, diabetes, cancer *etc*. [3,4]. In many cases, ROS production is an integral part of the pathophysiological mechanism that helps in damaging major macromolecular and cytoskeletal structures. Degradation in the DNA structure and the mitochondrial membrane instability drastically damage the cellular structure. Necrotic and/or apoptotic death of the cells is the result of the toxicity [4]. Moreover, the mechanism of toxicity and its impact depend on the complexity of the exposed organisms. The complexity of the organism depends on its systematic position in the phylogenetic tree. There is an impact of evolution on the adaptive nature of the organism.

In the current study, four different living systems were selected from distinct phylogenetic positions. All are highly abundant agri-field dwellers and consumers. Therefore, the toxicity generated by pesticides on their metabolic system/organ will be due to their differences in physiological characteristics and systematic positions in animal kingdom. Sustained exposure of pesticides may affect animal health making them more sensitive to environmental-factors and especially the effects on agricultural field-insect are more detrimental. The purpose of this investigation is to provide a brief analysis of the toxicity effects of several pesticides on some common cattle, birds and insects and their comparative analysis. Biochemical/molecular parameters are evaluated here, which may represent their biomarker potentials. Further investigations may be designed to establish some toxicity indicator species.

2. MATERIALS AND METHODS

2.1. Pesticides used in this Study and their Chemistry (Fig. 1)

The chemical structures were drawn by the NCBI software PubChem Sketcher V2.4 utilizing the web address https://pubchem.ncbi.nlm.nih.gov/edit3/index.html

Chlorpyrifos: *O,O*-Diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate($C_9H_{11}Cl_3NO_3PS$), molar mass;350.57g·mol^{-1}, density; 1.398 gcm^{-3} (43.5°C), solubility in water; 2 mg/L, chemical nature; combustible, reacts strongly with amines, strong acids, caustics (Fig. **1**).

Fenvalerate: (*RS*)-*alpha*-Cyano-3-phenoxybenzyl(RS)-2-(4-chlorophenyl)-3-methylbutyrate ($C_{25}H_{22}ClNO_3$), molar mass; 350.57 g·mol^{-1}, density; 1.175 gcm^{-3}, solubility in water; 2 µg/L, chemical nature; fenvalerate is most toxic to bees and fish. It is found in some emulsifiable concentrates, ULV, soluble powders, slow-release drug formulations, insecticidal fogs and in granules (Fig. **1**).

Nimbecidine: Azadirachtin AZT; Dimethyl (2a*R*,3*S*,4*S*,*R*, *S*,7a*S*,8*S*,10*R*,10a*S*,10b*R*)-10-(acetyloxy)-3,5-dihydroxy-4-[(1*S*,2*S*,6*S*,8*S*,9*R*,11*S*)-2-hydroxy-11-methyl-5,7,10-trioxatetra cyclo[6.3.1.$0^{2,6}$.$0^{9,11}$]dodec-3-en-9-yl]-4-methyl-8-{[(2*E*)-2-methylbut-2-enoyl]oxy}octahydro-1*H*-furo[3',4':4,4a]naphth o[1,8-*bc*]furan-5,10a(8*H*)-dicarboxylate($C_{35}H_{44}O_{16}$); molar mass; 720.72 g·mol^{-1}, chemical nature; this compound is found in the seeds (0.2 to 0.8 percent by weight) of the neem tree, *Azadirachta indica* (Fig. **1**).

2.2. Animal Tissue

Fresh liver tissue of adult specimens from common healthy livestock's goat (*Capra aegagrus hircus*) and cow (*Bos taurus* and *Bos indicus*), poultry bird as hen (*Gallus gallus domesticus*) were collected from the local Govt. registered market. Those were kept in plastic containers in the cold room at the temperature of about 4°C in darkness and experiments were immediately performed with the permission of the Institutional Ethical Review Board.

2.3. *In vitro* Treatment of Animal Tissues

Fresh liver tissues from different livestock's were incubated (for three hrs at room temperature) with different dilutions (w/v) of Chlorpyrifos such as 0.20%, 0.27%, 0.40%, 0.80%, 4.00%, or Fenvalerate 0.08%, 0.10%, 0.13%, 0.20%, 0.40% or Nimbecidine (Azadirachtin) 100 ppm, 133 ppm, 200 ppm, 400 ppm. 2000 ppm. The control marked dishes were treated with the vehicle. The biochemical studies were carried out with the experimental tissues after the incubation period.

2.4. *In vivo* Treatment of Insect Tissues

To determine LC_{50}, serial concentrations of AZT (Azadirachtin $C_{35}H_{44}O_{16}$, 1 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm) were applied uniformly and separately to 25 grams of food plants placed in water containing conical flask in plastic jars (11×11×26) cm covered with mosquito net fixed by

rubber bands at room temperature. Then, same-aged male female and male (1:1) insects (Orthoptera: Acridoidea) from newly hatched adults were placed in each jar and covered. Four replicates were used for each concentration (including control) and all jars were kept at 26±1°C and 70±5% relative humidity. Alive insects from each treatment were dissected and fat-bodies free guts were taken separately from female and male insects and stored in -20°C until use.

2.5. Preparation of Tissue Homogenate

Immediately after the treatment period, livers of decapitated livestock and gut tissues from insects were taken, weighed, cut into pieces and homogenized in 0.1 M chilled phosphate buffer, pH 7.4. The volume of the buffer was adjusted to obtain a 20% (w/v) homogenate. The homogenization was performed with the use of the Teflon homogenizer of the Potter-Elvehjem type. Next, the homogenates were centrifuged at 10,000×g for 30 min at 4°C in order to obtain supernatants, which were used to measure the enzyme activities.

2.6. Assay of Total Protein Contents

The total protein content of different tissues was estimated by the standard method of Lowry *et al.* 1951 [5]. Protein concentration was calculated and expressed as µg/mg wet tissue.

2.7. Determination of Antioxidant Enzymes

For the antioxidant status of SOD, CAT activity was determined using gel zymography.

2.7.1. Assay of Catalase Activities by Gel Zymography

The non-denaturing (8%) acrylamide gel was washed with distilled water for 10 minutes containing 25 µg proteins. The gel was shaken in 100 ml distilled water with 100 µl. H_2O_2 for 10 minutes and then it was washed with distilled water for 5 minutes. The control similar gel was stained with 30 ml, 2% Ferric Chloride ($FeCl_3$) and 30 ml 2% Potassium Ferricyanide [$K_3Fe(CN)_6$]. It was poured onto the gel at the same time. When the gel becomes yellowish green then the stain solution was removed. Finally, the gel was washed with distilled water and enzyme-activity bands appeared on the gel [6].

2.7.2. Assay of Super Oxide Dismutase Activities by Gel Zymography

SOD activity was performed according to the method described by Christine and Joseph, 2010 [7]. Supernatant from tissue homogenate was electrophoresed in riboflavin gel at 4°C. To visualize SOD activity, gels were first incubated in 2.4 mM nitro blue tetrazolium (NBT) in deionized water for 15 min and then in 0.028 mM riboflavin/280 mM N,N,N′,N′-tetramethyl-ethylenediamine (TEMED) in 50 mM potassium phosphate buffer (pH 7.8) for 15 min in the darkroom. After washing, the gel was illuminated under fluorescent light to get a distinct SOD activity band.

All the zymogram gel bands were analyzed by ImageJ software and the data was used for statistical analysis.

2.8. DNA Fragmentation Analysis

Liver tissues and gut tissues were treated with 500 µl of lysis buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS) and centrifuged at 12,000×g for 30 min. The supernatant was extracted with a 1:1 mixture of phenol: chloroform and then precipitated in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. After spinning down and decantation, the precipitate was re-suspended in TE buffer and 5 µl of loading buffer. The 0.8% agarose gel was run and the band density was evaluated by the gel documentation system and the percentage of DNA stability was expressed as bar diagram [8]. A suitable DNA ladder (EZ Load 500 bp Molecular Ruler #1708354, Life Sciences, Bio-Rad) was also run to assume the damage pattern.

2.9. Mitochondrial Membrane Stability Assay by Fluorescence Microscopy

Mitochondria from the drug added liver tissues of cow, goat and poultry bird-hen were isolated and incubated at 37°C in a medium composed of 5 mM K_2HPO_4, and 5mM $MgCl_2$, 135 mM KCl, 20 mM MOPS, at pH 7.0. Incubations also contained Rhodamine123 (R123) [9]. R123 and all other chemicals were from Sigma Chemical Co. (St. Louis, MO). Fluorescent measurements of mitochondria and extracts were made using a fluorescence microscope (Nikon, Eclipse LV100 POL), with the VisComet (Impulse Bildanalyse) software.

2.10. Statistical Analysis

Statistical analysis with the raw data was performed in SPSS 17 to evaluate the level of significance and differences between the two groups (Student's t-test). One way ANOVA was performed to characterize the significance of differences between and within groups of cattle and insects or different drug-dose treated groups. Values in the bar diagrams are the mean ± SE from several independent experiments (3 to 5).

Levels of significances (P values) are presented as the SPSS software calculates it. $P<0.05$ or less than this value has been considered to be significant in the current experiment.

3. RESULTS AND DISCUSSION

In the current study, the catalase activity in goat liver decreased after *in vitro* incubation with pesticides. Besides, this inhibition was prominent in the case of chlorpyrifos (Fig. **2a**) and AZT (Fig. **2c**). Nevertheless, no significant alteration was noticed in response to fenvalerate treatment (Fig. **2b**). The activity of superoxide dismutase (SOD) in goat liver distinctly decreased after chlorpyrifos intoxication (Fig. **2f**). However, the statistical calculation did not present significant changes. However, in the case of gut tissues in the present experimental insect, both catalase and SOD activities increased after *in vivo* and *in vitro* AZT exposure, which is found to be highly significant (Fig. **2d** and **e**; Fig. **2i**

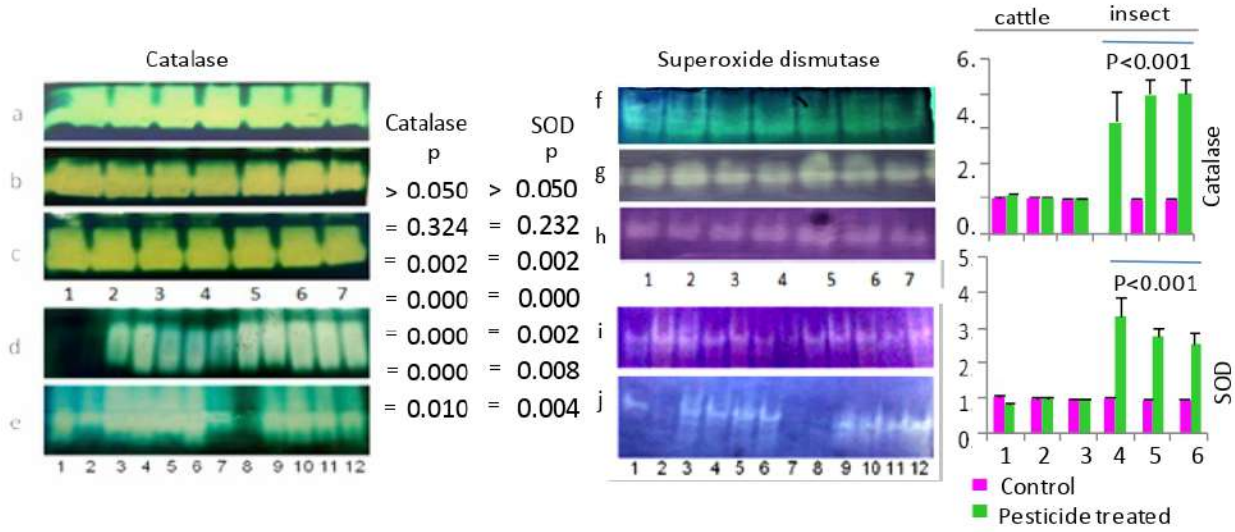

Fig. (2). Left panel: Goat (*Capra aegagrus hircus*) Liver (treated with Chlorpyrifos) catalase activity in gel zymogram. **-gel a-** Lane (1- 2 control, 3- 0.20 %, 4- 0.27 %, 5- 0.40 %, 6- 0.80 %. 7- 4.00 %) **gel b-** Catalase activity in Goat Liver (treated with Fenvalerate) : Lane (1- 2 control, 3- 0.08 %, 4- 0.10 %, 5- 0.13 %, 6- 0.20 %. 7- 0.40 %) **gel c-** Catalase activity in Goat Liver (treated with Nimbecidine) Lane (1- 2 control, 3- 100 ppm, 4- 133 ppm, 5- 200 ppm, 6- 400 ppm. 7- 2000 ppm) **gel d-** Catalase activity is shown in the gut of the insect *in vivo* treated with Nimbecidine. Lane distribution: Lane 1- 6 female gut (1- control, 2- 1 ppm, 3- 5 ppm, 4- 10 ppm, 5- 15 ppm, 6- 20 ppm.) and Lane 7- 12 male gut (7- control, 8- 1 ppm, 9- 5 ppm, 10- 10 ppm, 11- 15 ppm, 12- 20 ppm.) **gel e-** Catalase activity is shown in the gut of the insect *in vitro* treated with Nimbecidine. Lane distribution: Lane 1- 6 female gut (1- control, 2- 1 ppm, 3- 5 ppm, 4- 10 ppm, 5- 15 ppm, 6- 20 ppm.) and Lane 7- 12 male gut (7- control, 8- 1 ppm, 9- 5 ppm, 10- 10 ppm, 11- 15 ppm, 12- 20 ppm). Right panel: SOD activity is shown on a polyacrylamide gel- lane distribution in **gel f, g, h, i** and **j** are similar to gel a, b, c, d and e in the right panel respectively. Data in the last panel is the mean ± SE. Statistical data-P values of comparison between each drug-dose group of cattle and insects. Right most figure of bar diagram comparison of all drugs together in cattle versus insect. Statistical data shows that insects are more influenced and affected by pesticide exposure. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

and **j**). Moreover, the extent of increase of enzymatic activities with comparison to control is higher in female insect (lane 1-6) than that of male insects (lane 7-12). Small or negligible changes are noticed in Fig. (**2g**) and (**2h**). This may suggest that these pesticides were less effective in the livestock and birds to generate radical toxicity, so the adaptive responses were less prominent. However, in the case of insects, the toxicity and the adaptive responses both were significant. Oxidative stress has been shown to play a crucial role in the cellular toxicity generated by a large number of pesticides [10]. Free radicals especially reactive oxygen species (ROS) are essential for different metabolic functions including the cell signaling and other physiological processes [11]. The role of AZT has been already reported as a potential inducer of mitochondrial superoxide anion radicals and H_2O_2 [12]. This has been clearly noticed in our present study.

The impairment in antioxidant defense mechanism is evident by the cellular damage caused by pesticides [13]. This has been reflected in the mitochondrial membrane degeneration studies (Fig. **4**). This degeneration is responsible to develop a free radical cascade possibly at the cytosolic level. Plasma membrane and organelle membrane stability is a determinant of cytotoxicity. Earlier it has been suggested that altered membrane fluidity can be responsible for the decreased activity of pesticide in mouse [14] and in the experimental chicken model [15]. Therefore, the pesticide may impair the physiological and biochemical processes across the species and that may be influenced by cellular physiology. The catalase activity was impaired in the experimental organisms after exposure to different concentrations of pesticides *i.e.*, Chlorpyrifos, Fenvalerate, Nimbecidine. It might be due to the binding of the pesticide residue to catalase or by inhibiting this enzyme synthesis [16]. An appreciable level of decrease in SOD activity was noticed in the liver of cattle (Fig. **2f** and **2g**). Nevertheless, a significant dose-dependent increase of SOD and catalase activity was noticed after both *in vitro* and *in vivo* AZT exposure to the insect of both sexes (bar diagram in the right panel of Fig. **2**). This suggests that antioxidant enzymes are more responsive (P<0.001) in environmental stress in the lower organisms and it may be selected as a biomarker in a variety of oxidative stress. A significant decrease in catalase activity was observed in the brain, liver and kidney tissues of *Channa punctatus* exposed to a different pesticide, triazophos [17]. Tripathi and Singh (2013) [18] also noticed that the catalase activity reduced in the brain, gill, liver and skeletal muscles of α-methrin treated *Channa punctatus*. The degree of effects of Chlorpyrifos, Fenvalerate and Nimbecidine on catalase and other protein profile was variable in different livestock-tissues because it may depend on the location and metabolic involvement of the tissues [19]. Tissue-specific antioxidative responses were noticed to take place during stress exposure.

In the present study, we have established that pesticides influence antioxidative enzymes in the liver of higher organisms and gut of the insects and, therefore, liver injury

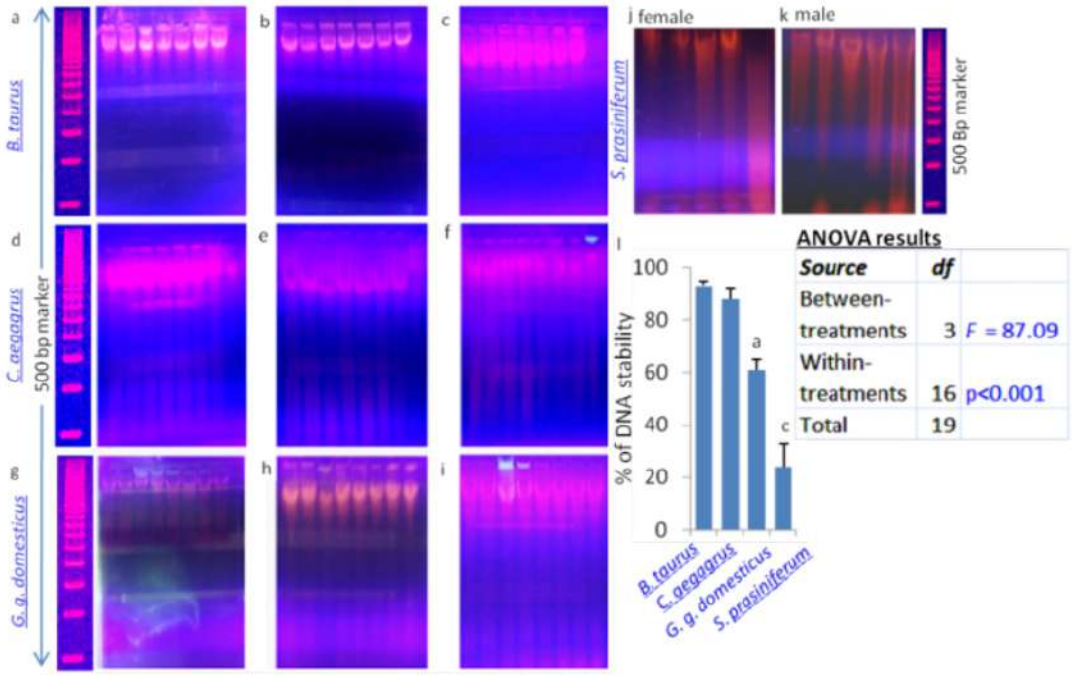

Fig. (3). (a, b, c) DNA fragmentation result is shown in the liver of cow *(Bos taurus) in vitro* treated with Chlorpyrifos (a). Lane (1- 2 control, 3- 0.20 %, 4- 0.27 %, 5- 0.40 %, 6- 0.80 %. 7- 4.00 %); Flavaralate (b). Lane 1- 7 liver tissue (1- 2 control, 3- 0.08 %, 4- 0.10 %, 5- 0.13 %, 6- 0.20 %. 7- 0.40 %); Nimbecidine (AZT) (c). Lane (1- 2 control, 3- 100 ppm, 4- 133 ppm, 5- 200 ppm, 6- 400 ppm. 7- 2000 ppm). **(d, e, f)** DNA fragmentation result is shown in the liver of goat (*Capra aegagrus hircus*) *in vitro* treated with Chlorpyrifos (d). Lane (1- 2 control, 3- 0.20 %, 4- 0.27 %, 5- 0.40 %, 6- 0.80 %. 7- 4.00 %). Fenvalerate (e). Lane: (1- 2 control, 3- 0.08 %, 4- 0.10 %, 5- 0.13 %, 6- 0.20 %. 7- 0.40 %). Nimbecidine (AZT) (f). Lane (1- 2 control, 3- 100 ppm, 4- 133 ppm, 5- 200 ppm, 6- 400 ppm. 7- 2000 ppm). **(g, h, i)** DNA fragmentation result is shown in the liver of poultry bird, hen (*Gallus gallus domesticus*) *in vitro* treated with Chlorpyrifos (g). Lane (1- 2 control, 3- 0.20 %, 4- 0.27 %, 5- 0.40 %, 6- 0.80 %, 7- 4.00 %). Fenvalerate (h). Lane (1- 2 control, 3- 0.08 %, 4- 0.10 %, 5- 0.13 %, 6- 0.20 %. 7- 0.40 %). Nimbecidine (i). Lane (1- 2 control, 3- 100 ppm, 4- 133 ppm, 5- 200 ppm, 6- 400 ppm. 7- 2000 ppm). DNA fragmentation result is shown in in vivo gut treated with Nimbecidine (AZT) **(j)** female Lane (1- control, 2- 1 ppm, 3- 5 ppm, 4- 10 ppm, 5- 15 ppm) **(k)** male gut (1- control, 2- 1 ppm, 3- 5 ppm, 4- 10 ppm, 5- 15 ppm). Bar diagram results (5 independent experiments) and students t-test values suggest that insects are the most affected and then the birds' group in terms of their DNA stability. Level of significances are shown; **a.** $P<0.05$ and **c.** $P<0.001$. ANOVA result suggests that between and within groups, the differences in the DNA stability are significantly different at $P<0.001$($F = 87.09$). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

associated with this insecticide may be due to oxidative tissue damage. There is little evidence of change in the antioxidant system after intoxication with organophosphate to agri-field dweller organisms. Hai *et al.* (1997) [20] observed an increase of SOD and catalase activities in the liver of dichlorvos-treated carps. They also found elevated levels of malondialdehyde in the livers of dichlorvos (DDVP)-treated fish. Fish is the organism of the aquatic ecosystem and exposure *via* contamination in water might have an instant effect. The mechanism of intoxication might suggest the pattern of toxicity in our present experimental organism. Yang *et al.* (1996) [21] suggested that the organophosphates and carbamates, besides their inhibitory effect on acetylcholinesterase, initiate the accumulation of free radicals leading to lipid peroxidation on such organism. Mitochondrial stability and function have not been observed in those previous experiments. In the current investigation, we evaluated mitochondrial membrane potential in cattle and birds and considered that pesticides have strong destabilizing activity. Due to the very low yield of mitochondria, the signal was undetectable in the insect. Nevertheless, direct evidence of antioxidative responses (catalase, SOD) and DNA instability was noticed in the insect after AZT exposure.

Antioxidant protection is accomplished by many enzymatic and non-enzymatic factors, which maintain the physiological level of reactive oxygen metabolites [22]. The antioxidant defense system includes superoxide dismutase, catalase and glutathione peroxidase enzymes. We measured the activity of two antioxidative enzymes, SOD and catalase. The SOD plays an important role in the first line of the antioxidant defense system by catalyzing the dismutation of superoxide radicals to form hydrogen peroxide and molecular oxygen. Further, this hydrogen peroxide undergoes a free-radical cascade reacting with transition metals or lipid-peroxidation products [23].

In the present study, a significant DNA laddering was found in the experimental insect induced by AZT (Fig. **3j** and **3k**). A moderate DNA laddering was also noticed in the poultry bird (Fig. **3g**, **3h** and **3i** for three pesticides). Nevertheless, in the higher vertebrate, DNA was found to be more stable (Fig. **3a** to **3f**). The pesticide treatment induces DNA fragmentation which is one of the criteria of necrotic or apoptotic cell death. This finding would be related to the magnitude of the exposure to pesticides and was directly involved in DNA fragmentation. Therefore, the major changes observed in DNA fragmentation of the liver may

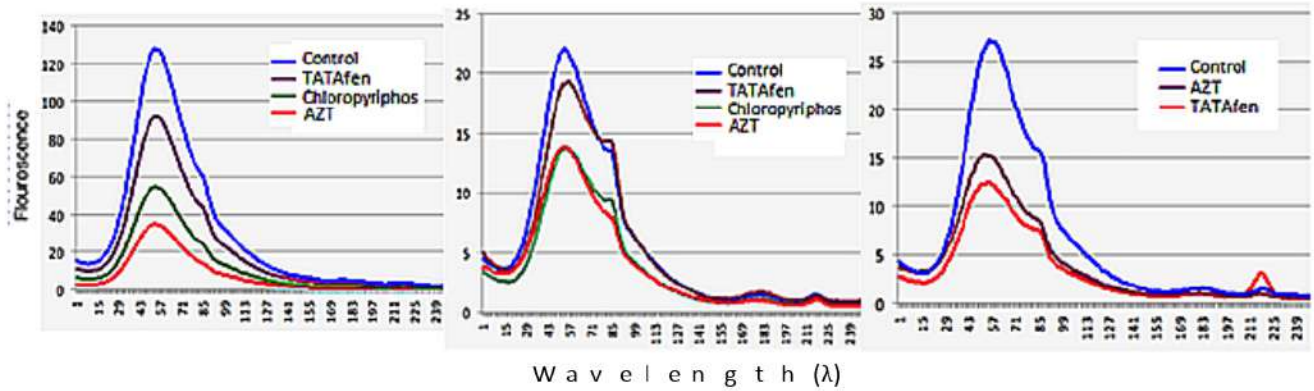

Fig. (4). Mitochondrial membrane potential/stability assays (Rhodamine method) by fluorescence microscopy. Depending on the sample availability, this experiment was conducted. Left panel: cow, middle panel: goat and right panel: poultry bird. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

suggest a direct effect of the pesticides. The percent of DNA stability calculated from the densitometry analysis data (Fig. **3i**) suggests that cattle DNA is more protected than the poultry bird DNA damage ($P<0.05$) and insect DNA damage ($P<0.001$). Fungicide-induced DNA damages were caused by oxidative stress and might be responsible for the higher occurrence of apoptotic cell death [24]. Similar to our present study, the electrophoretic analysis confirmed the potential role of epoxiconazole in inducing DNA fragmented ladder that is regarded as the marker of apoptosis [25].

The present study was conducted to investigate the pesticides in alleviating oxidative stress in liver injury and DNA fragmentation. The liver is the site of biotransformation by which a toxic compound is transformed into a less harmful form to reduce toxicity [26]. However, during these processing hepatic cells may itself be damaged to some extent and develop chronic hepatotoxicity. Pesticides induce oxidative stress, which leads to the generation of free radicals, changes in antioxidants levels and lipid peroxidation [27] thus causing damage to proteins, lipids and DNA [28]. The report reveals that dimethoate induced DNA damage in the hemocytes of the insect *C. biguttulus* [29]. Some critical analyses have been shown earlier in *Drosophila* sp., but not in the present experimental grasshopper species. Dichlorvos (DDVP), an organophosphate pesticide was reported to induce DNA damage and also affect pre and post replication repair mechanisms in *Drosophila* sp. [30]. The normal error rectification processes were thus distorted and a certain degree of mutagenicity was generated at the level of biotransformation by the application of the pesticides.

DNA fragmentation observed in the present study has a very wider significance. It is the normal consequence of oxidative stress that was demonstrated through the inactivation of antioxidant enzymes (catalase and SOD) in the metabolic organs. This is also consistent with previous studies where DNA fragmentation was induced by pesticides in rat lymphocytes [31] and in rat brain by cypermethrin [32]. Not during the single generation period, even maternal pesticide (fenvalerate) exposure during pregnancy-period may impair growth and brain development in mouse offspring [33]. Hampering in the brain behavior is shown to trigger Parkinson-like symptoms through the initiation of autophagy and p38 MAPK/mTOR signaling pathway [34]. Moreover, interferences by fenvalerate in the expression of pro-apoptotic Bcl-2 family proteins increase the carcinogenicity potential of this pesticide [35]. The differences in the DNA stability of different organisms may be due to differences in DNA sequences between lineages, which noticed in our current study. These facts influence the stability, mutability and error-correction efficiency of the DNA. In lower organisms, the DNA was found to be less stable in response to xenobiotic/pesticide exposure (Fig. **3j** and **3k**). The fluorescent experiment by Rhodamine 123(R123) shows a notable decline in membrane potentials in any pesticides treated liver tissues compared to that of control groups of all species (Fig. 4). R123 is one of the most dependable signaling molecules to estimate mitochondrial damage and apoptotic signaling. Fenvalerate (TATAfen) is noticed here to be the most damaging to mitochondrial structure (Fig. **4**). Mitochondrial membrane damage may generate a significant toxicity cascade and energy depletion in the cell and in the whole organism.

The insects originated between the Cambrian period (Euarthropoda, 535 MY) and the Devonian period (385 MY). During this period and further, during the long tenure of speciation, insects have gone through less diversification (Fig. **5**). Therefore, the adaptive modification and DNA stabilization are less likely to happen here. In contrast, Aves originated 135 MY ago in the first part of the Cretaceous period and went through stages of more diversification (Fig. **5**). Comparatively, less DNA fragmentation is noticed in *G. g. domesticus*, the poultry hen (Fig. **3g**, **3h** and **3i**). The report reveals that in prokaryotes like bacteria, environmental stress and other 'mutants' have influenced the selection pressure. Nevertheless, those have lower DNA repair efficiency, hence a higher mutation rate [36]. In cattle, very less amount of DNA-fragmentation is noticed (Fig. **3a** to Fig. **3f**). Extensive diversification and adaptive modification are noticed in Mammals, which originated in the Cenozoic period, 65 MY. Moreover, these mammals diverged in rodents, primates, cattle and others in a very short (Cenozoic) time (Fig. **5**). Adaptation in higher eukaryote may be governed more by natural selection-pressure than mutation associated forced error on DNA sequences/stability. Even species can differ in rate of copy

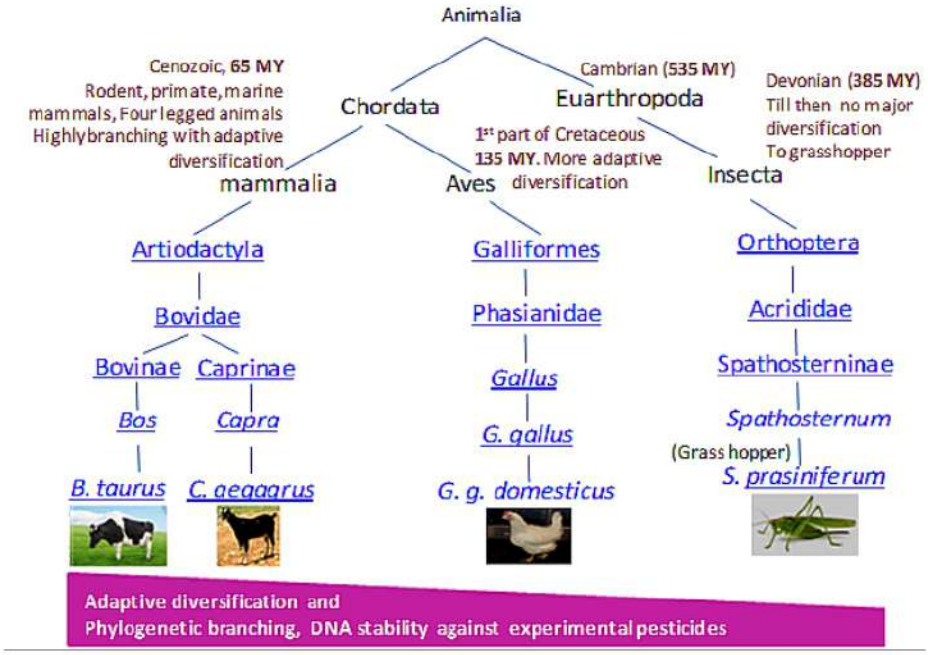

Fig. (5). Respective position and lineage diversification of the four living systems investigated in the current study; cow, goat, poultry-hen and an insect-grasshopper. DNA stability has been shown to be related to the relative phylogenetic position of the organism. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

error detection and repair. Number of DNA replications per generation can also vary with population structure and mating system [37]. Beside the toxicity and adaptation studies, and their comparative analysis in different living system, there may have some wider messages in our study. This work may resemble a reference model of environmental stress and its ecological/evolutionary impact dictated by the xenobiotic-interfered DNA stability. As for example, Daphnia hemoglobin (Hb) has been established as the widely recognized respiratory pigment. As a reliable biomarker, it can predict the water contamination with bisphenol A (BPA), benzo[a]pyrene (B[a]P), chlorpyrifos [38]. Azadirachtin has been shown to suppress the growth and development of *Bactrocera dorsalis* by releasing cathepsin [39] and induce apoptosis in *Bombyx mori* by releasing extra Ca^{2+} [40]. Lower organisms are more social and actively participate in the formation of a composite niche/ecosystem. Therefore, any kind of changes or deterioration in the ecosystem may affect their fecundity, reproductive ability, life span and mortality. The changes in their DNA structure/stability may modulate their phenotypic behavior and their interaction with neighboring organisms.

CONCLUSION

From the present study, we can infer that pesticide intoxication may induce oxidative stress more in the lower organisms. However, the effect of subchronic exposure of pesticides on livestock leading to oxidative stress requires further evaluation at the molecular level. After an acute exposure, the risk assessment should be performed to quantify the exposure of humans to that particular contamination. For a sustained or chronic exposure, the risk assessment is important at a community level with a focus on its long-term ecological and environmental impact.

LIST OF ABBREVIATIONS

AZT	=	azadirachtin
CAT	=	catalase
DDVP	=	dichlorvos
ppm	=	parts per million
ROS	=	reactive oxygen species
SOD	=	superoxide dismutase

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

For all animal experiments, proper permissions were obtained from the Institutional (Oriental Institute of Science and Technology) Review Board.

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are the basis of this research. All the animal experiment procedures followed were in accordance with the standards set forth in the eighth edition of "Guide for the Care and Use of Laboratory Animals" (grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals_prepub.pdfpublished by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

All ethical norms and maintain requisite regulatory affairs. The firm house is a Government accredited (CPCSEA-Committee for the Purpose of Control and Supervision of Experiments on Animals: Reg. no 1A2A/PO/BT/S/15/CPCSEA. <http://cpcsea.nic.in/Auth/index.aspx>) organization under the Dept. of Animal Husbandry and Dairy, Ministry of Agriculture and Farmer's Welfare, Govt. of India.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

Received from the Institution.

AVAILABILITY OF DATA AND MATERIALS

The dataset used and/or analyzed during the current study are available from the corresponding author [SM] on a reasonable request.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

Institutional members.

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