Chapter: 5
MATERIALS AND METHODS

5. MATERIALS AND METHODS

The present work was taken up to evaluate the acute in-vitro toxicity of chlorpyrifos, fenvalerate, azadirachtin were studied against common agri-field animals and in-vivo and in-vitro toxicity of the azadirachtin (AZT) was studied in selected acridid-pest.

5.1. Pesticides used for screening and their chemistry

A. Chlorpyrifos

O,O-DiethylO-3,5,6-trichloropyridin-2-ylphosphorothioate($C_9H_{11}Cl_3NO_3PS$), molar mass ; 350.57g·mol^{-1}, density; 1.398 g/cm^3 (43.5°C), solubility in water; 2 mg/L, chemical nature: Combustible, reacts strongly with amines, strong acids, caustics.

B. Fenvalerate

(RS)-Alpha-Cyano-3-phenoxybenzyl(RS)-2-(4-chlorophenyl)-3methylbutyrate($C_{25}H_{22}ClNO_3$), molar mass; 350.57 g·mol^{-1}, density; 1.175 g/cm^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.

C. Nimbecidine

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

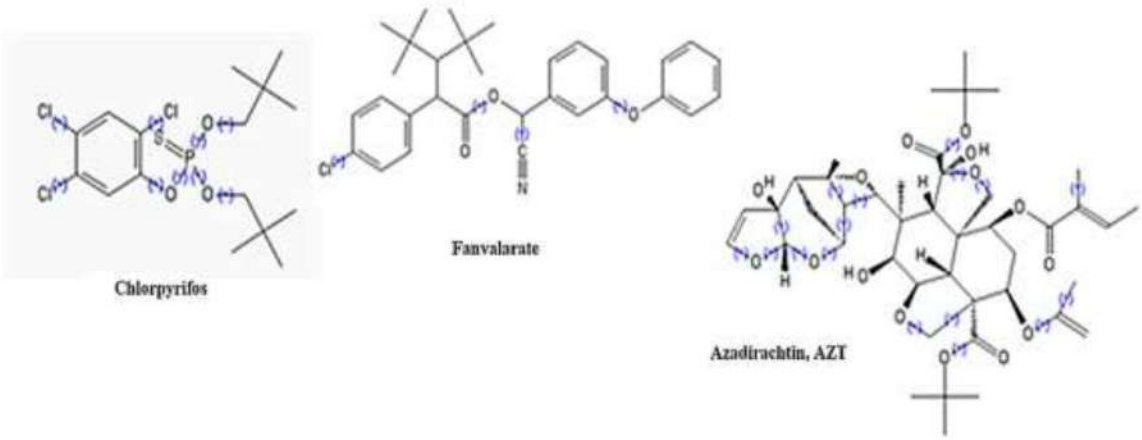


Plate 11. Structural formula of chlorpyrifos, fanvalarate (TATAfen) and nimbecidine (azadirachtin; AZT). The chemical structures were drawn by the NCBI software Pub Chem Sketcher V2.4 utilizing the web address https://pubchem.ncbi.nlm.nih.gov/edit3/index.html.

5.1.1. Insecticide selected

Azadirachtin is a natural pesticide used throughout the world to control insect-pests especially and have less toxic effects on non-target/beneficial organisms. It is highly degradable, non-persistent nature and low mammalian toxicity. Technical grade AZT includes align, azadirachtin and turplex. The physical properties and other details of AZT are as follows:

- Common name: Nimbecidine Plus 10,000 ppm (azadirachtin)
- Chemical name: Tetranortriterpenoids
- Empirical formula: $C_{35}H_{44}O_{16}$
- Molecular weight: 720.7
- Melting point: 165°C
- Physical state: It is yellow green powder with a strong garlic sulphur odour
- Solubility in water: 0.00005 mg/L
- CAS Number: 11141-17-6

5.2. Experimental animals

5.2.1. Animal tissue other than insect

Fresh tissue of adult healthy specimens from common agri-field animal's cow (*Bos taurus and Bos indicus*), goat (*Capra aegagrus hircus*) and 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 permission of the Institutional Ethical Review Board.

5.2.2. In-vitro treatment of animal tissue

Fresh liver tissues from different agri-field animals were incubated with different dilutions of chlorpyrifos such as 0.20%, 0.27%, 0.40%, 0.80%, 4.00%; fenvalerate 0.08%, 0.10%, 0.13%, 0.20%, 0.40% and nimbecidine (azadirachtin) 100 ppm, 133 ppm, 200 ppm, 400 ppm, 2000 ppm was prepared for the experimental purposes compare with control for 3-hrs at room temperature. The biochemical studies were carried out with the experimental tissues after the incubation period.

5.3. Insect's collection

Short horned wild grasshoppers were collected from different zones of Midnapore (East and West) district, West Bengal, India by net sweeping method were chosen with the intension of survey amongst different habitats which includes open grasslands, bushy vegetation with grass land patches, ground surfaces and cultivated areas grasshopper diversity was carried out.

5.3.1. Ecological study of insects

The primary work dealt with morphology, taxonomy and ecology of some grasshoppers in and around the Medinipur district. Total 38 grasshopper's species have been recorded of the Family-Pyrgomorphidae and Acrididae. A list of distribution of each of these spe-

cies has also been given. The ecological studies have been conducted in some chosen localities within this district to study their behaviour in the field and also their population fluctuations in relation to some physical parameters like humidity and temperature. The number of species encountered from these areas were nine, of which the species *Spathosternum pr. prasiniferum* were most dominant (39.98%) in order of dominance (Fig. 4; Table 4). The maximum population was obtained during the month of September and minimum population was encountered in the month of May.

5.3.2. In-house rearing of insects (grasshoppers)

Collected insects were poured in aerated plastic bags and transported to the laboratory where they were reared in specially designed cages made of nylon net gauge on wooden frame (125×75×50) cm under laboratory conditions (30±2°C, 70±5% relative humidity, 500±200 lux light intensity and 12L:12D photoperiod). Moist sterilized sand filled plastic cups (300 ml) were placed at the floor of the cage for oviposition and *ad-libitum* food plants were provided for their diet. Adults were allowed to copulate and oviposit and after oviposition, egg pod contain ovipositing cups were collected and transferred to the incubator at 38±2°C until hatching. The newly born hatchlings were reared until adult on similar settings.

5.3.3. Species identification

Adult specimens are sent to Zoological Survey of India, Kolkata for taxonomic identification. Species (Plate 18) selection for this study is made depending on association with grass, occurrences and from life table prepared throughout the year (Table 3 and Plate 15).

Plate 12. Wild grasshopper collection for in-house rearing. **(a)** and **(b)** Random collection of grasshoppers from their natural habitat. **(c)**, **(d)**, **(e)** and **(f)** Insectarium set-up for in-house rearing and maintaining of grasshoppers with photoperiod (12L:12D) and humidity (70±5%) throughout experiments.

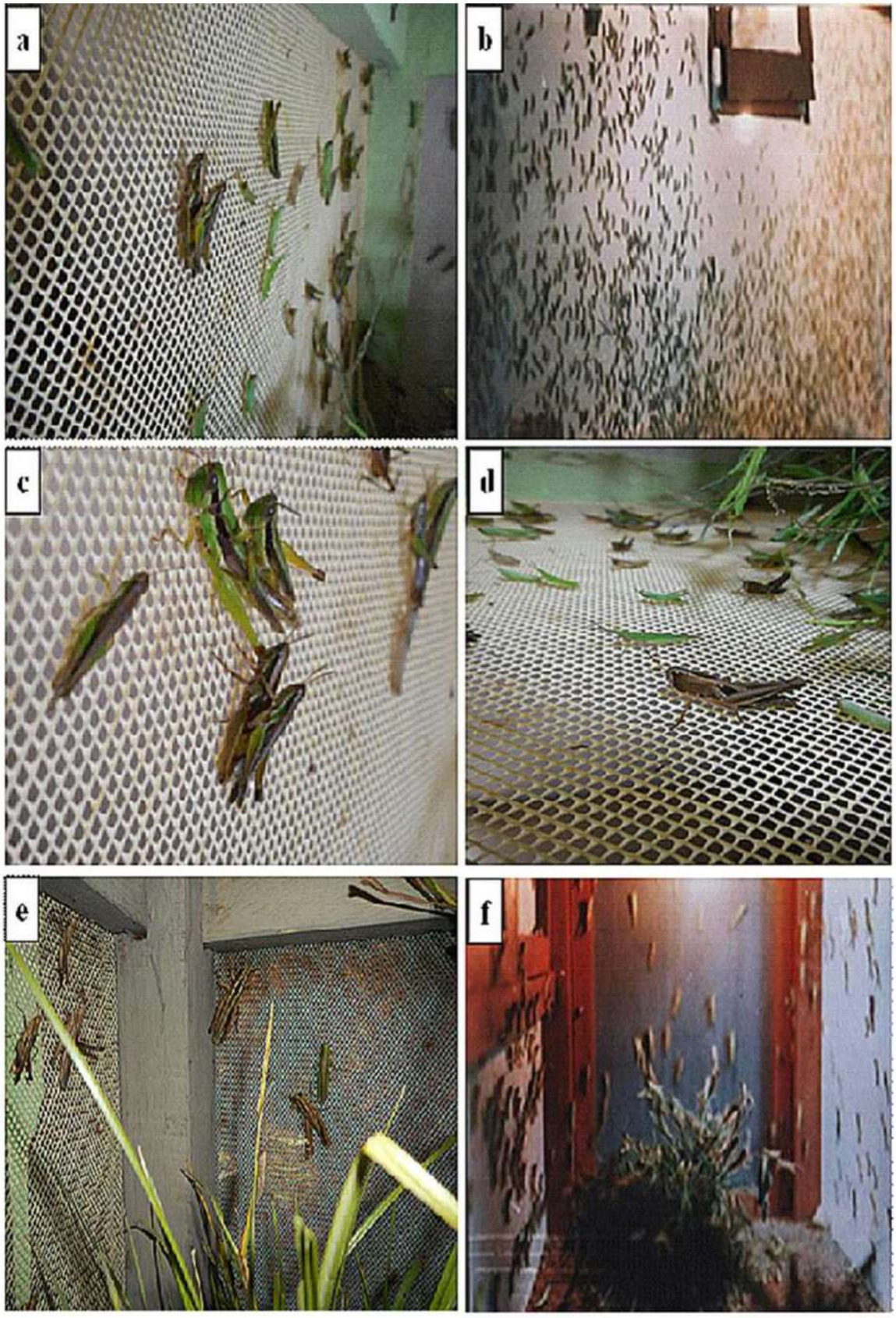


Plate 13. In-house rearing of grasshoppers. **(a)**, **(b)**, **(c)**, **(d)**, **(e)** and **(f)** Insects are allowed for copulation in insectarium.

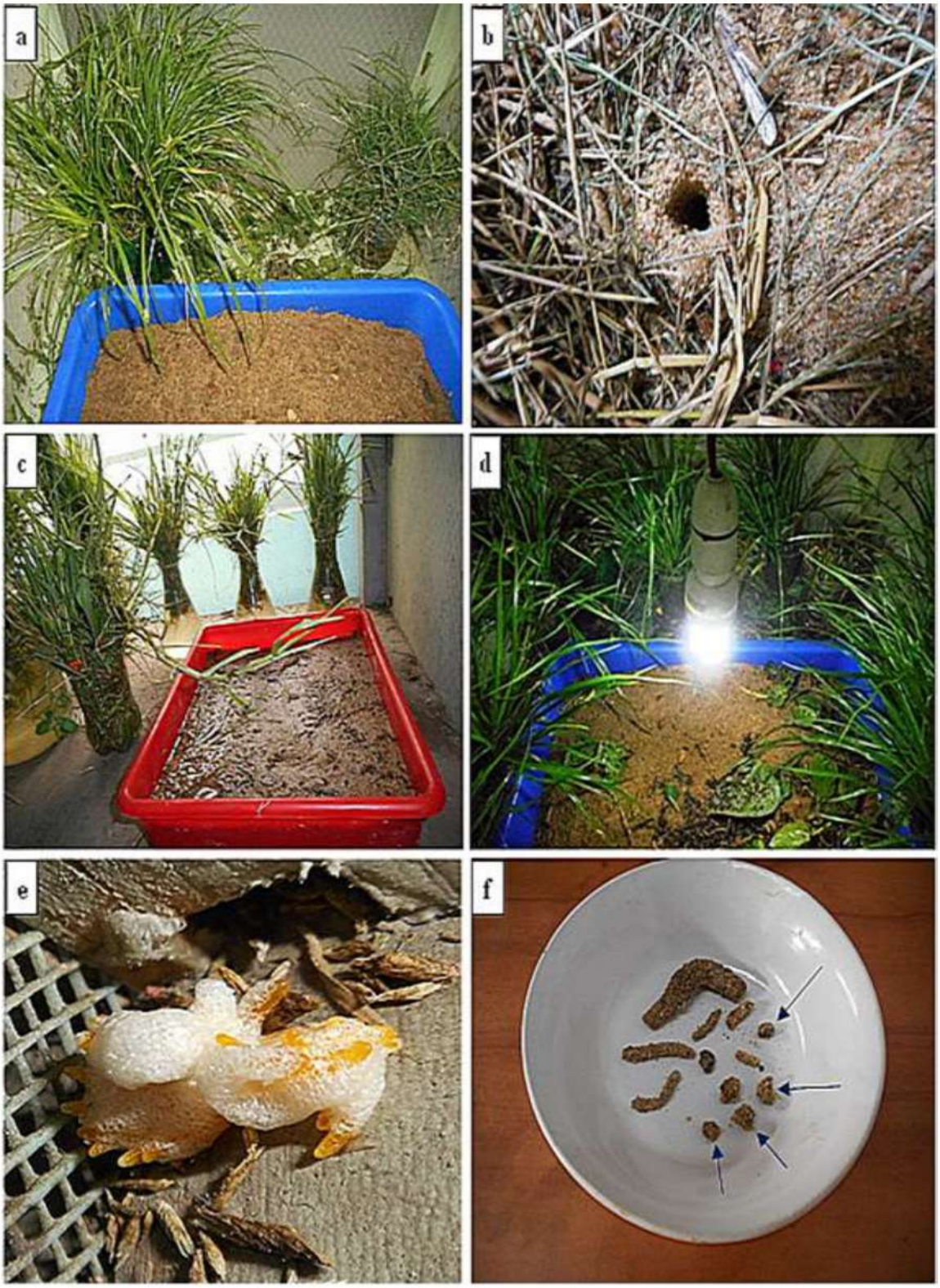
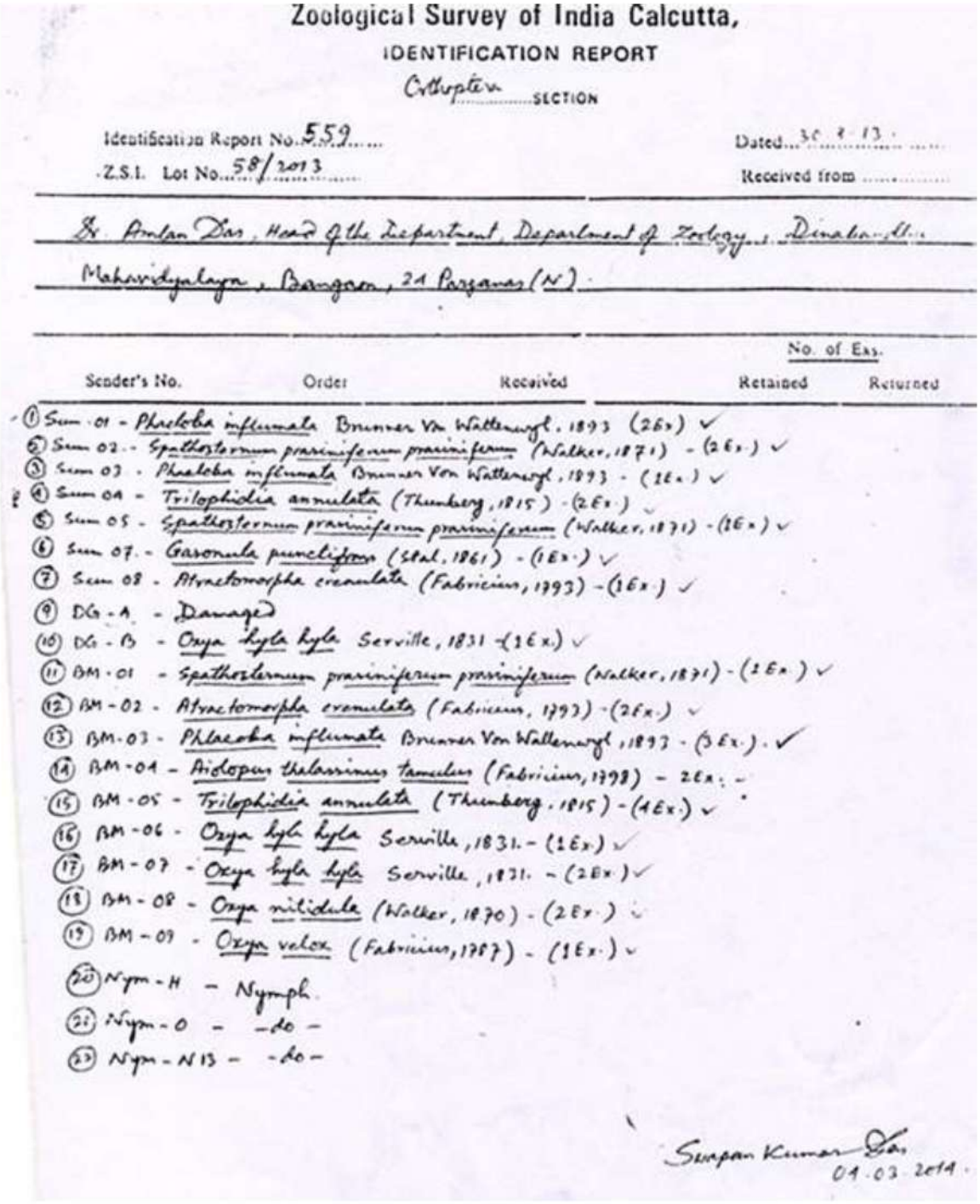


Plate 14. In-house oviposition and random egg pods collection for production of mass hatchlings. **(a)**, **(b)** and **(c)** Oviposition site in laboratory, **(d)** Temperature maintenance throughout rearing, **(e)** Eggs with frothy material, **(f)** Collected egg pods from oviposition sites.

Plate 15. Initial screening of experimental species. **(a)** Grossly studied grasshopper species. **(b)** Selected species for experimental purpose.



Zoological Survey of India Calcutta,

IDENTIFICATION REPORT

Orthoptera SECTION

Identification Report No. 559 Dated 30.9.13

Z.S.I. Lot No. 58/2013 Received from

Dr. Anjan Das, Head of the Department, Department of Zoology, Dinabandhu Mahavidyalaya, Bongaon, 24 Parganas (N).

Sender's No.	Order	Received	No. of Exs. Retained	Returned
(1) Sem-01	Phaloba inflexata	Brunner Von Wattenwyl, 1893	(2Ex.) ✓	
(2) Sem-02	Spathosternum prasiniferum prasiniferum	(Walker, 1871)	- (2Ex.) ✓	
(3) Sem-03	Phaloba inflexata	Brunner Von Wattenwyl, 1893	- (1Ex.) ✓	
(4) Sem-04	Trilophidia annulata	(Thunberg, 1815)	- (2Ex.) ✓	
(5) Sem-05	Spathosternum prasiniferum prasiniferum	(Walker, 1871)	- (1Ex.) ✓	
(6) Sem-07	Gasonula punctifrons	(Stal, 1861)	- (1Ex.) ✓	
(7) Sem-08	Atractomorpha crenulata	(Fabricius, 1793)	- (1Ex.) ✓	
(9) DG-A	Damaged			
(10) DG-B	Oxya hyla hyla	Serville, 1831	- (1Ex.) ✓	
(11) BM-01	Spathosternum prasiniferum prasiniferum	(Walker, 1871)	- (1Ex.) ✓	
(12) BM-02	Atractomorpha crenulata	(Fabricius, 1793)	- (2Ex.) ✓	
(13) BM-03	Phlaeoba inflexata	Brunner Von Wattenwyl, 1893	- (3Ex.) ✓	
(14) BM-04	Aiolopus thalassinus tamulus	(Fabricius, 1798)	- 2Ex. -	
(15) BM-05	Trilophidia annulata	(Thunberg, 1815)	- (4Ex.) ✓	
(16) BM-06	Oxya hyla hyla	Serville, 1831	- (1Ex.) ✓	
(17) BM-07	Oxya hyla hyla	Serville, 1831	- (2Ex.) ✓	
(18) BM-08	Oxya nitidula	(Walker, 1870)	- (2Ex.) ✓	
(19) BM-09	Oxya velox	(Fabricius, 1787)	- (1Ex.) ✓	
(20) Nym-H	Nymph			
(21) Nym-O	-do-			
(22) Nym-N13	-do-			

Swapan Kumar Das
04.03.2014

Plate 16. Identification report of experimental species from wild species by the Zoological Survey of India, Kolkata.

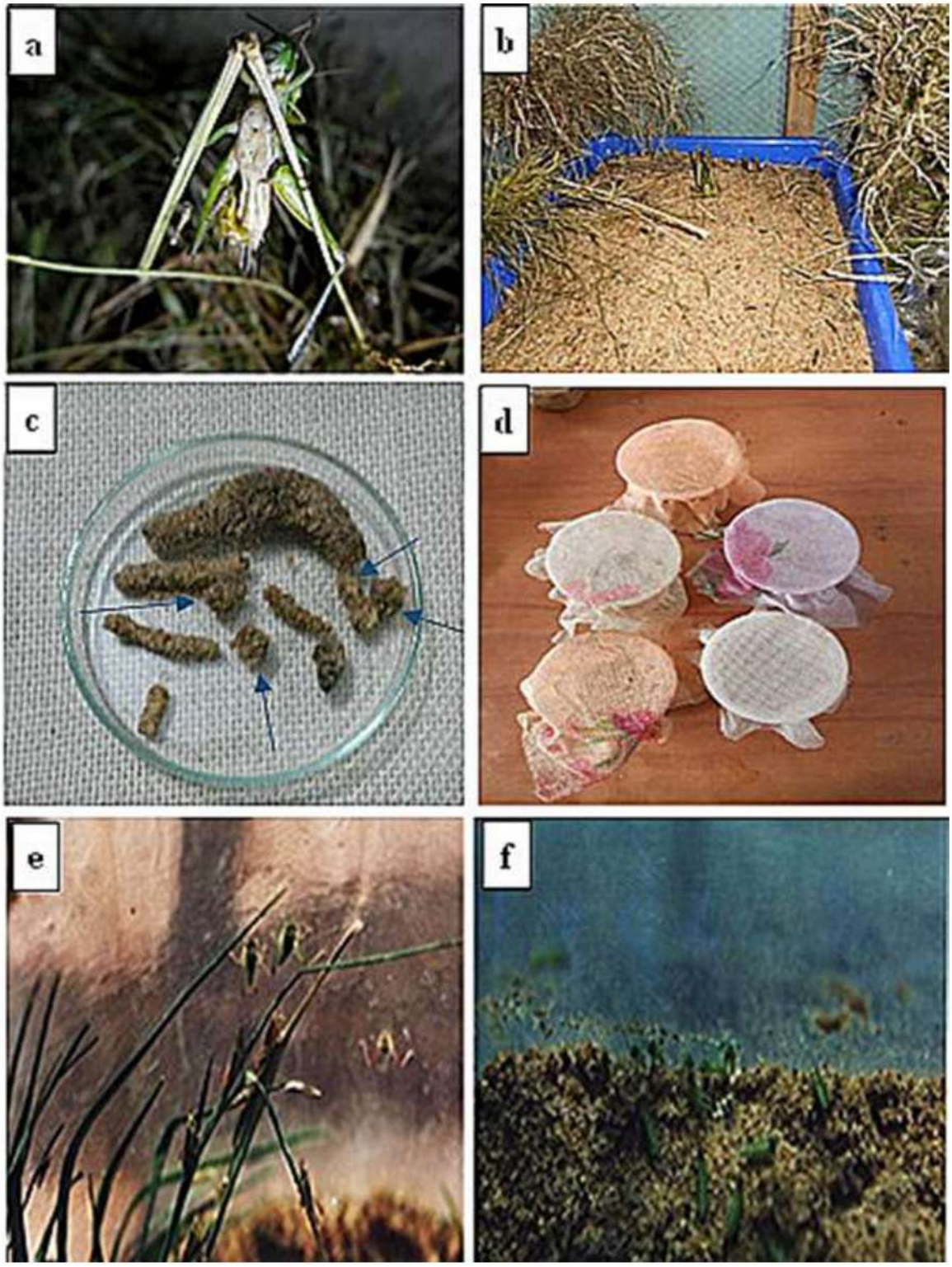


Plate 17. In-house rearing of *Spathosternum prasiniferum prasiniferum* (Walker 1871). **(a)** Copulation of selected species, **(b)** In-house oviposition site, **(c)** Egg pods collection, **(d)** Egg pods and eggs are allowed for production of hatchlings, **(e)** and **(f)** Emergence of hatchlings.

5.3.4. Grasshopper species of interest for toxicity study (model organism)

Multivoltine acridids are more suitable as they could yield higher annual biomass than the univoltine ones. Among the acridids found in West Bengal, *Spathosternum prasiniferum prasiniferum* (Walker) were found to be multivoltine in nature and were considered as model organism for the present study.

a. Morphology

Species are small and slightly greenish with two dark greenish bands run behind the lower part of the eyes in appearance. Females are about 17.9 mm long having 4.8 mm long antennae and males are about 14.4 mm long with antennae of about 3.9mm. They can complete four generations per annum.

b. Distribution

India (West Bengal, Andhra Pradesh, Arunachal Pradesh, Bihar, Goa, Himachal Pradesh, Jammu and Kashmir, Kerala, Madhya Pradesh, Maharashtra, Orissa, Rajasthan and Tamil Nadu), Myanmar, China, Thailand and Vietnam.

c. Place of *Spathosternum prasiniferum prasiniferum* in the animal kingdom

Kingdom- Animalia
Phylum- Arthropoda
Subphylum- Hexapoda
Class- Insecta
Subclass- Pterygota
Infraclass- Neoptera
Order- Orthoptera
Suborder- Caelifera
Infraorder- Acrididea
Superfamily- Acridoidea
Family- Acrididae
Subfamily- Spathosterninae
Genus- Spathosternum
Species- *Spathosternum prasiniferum prasiniferum*

Plate 18. Sex differentiation of *Spathosternum prasiniferum prasiniferum* (Walker 1871).

Plate 19. Selection of pesticide used in toxicity studies. **(a)** Insecticides available in markets, **(b)** Insecticide used for experimental purpose, **(c)** Instrument used for aerial application of insecticide, **(d)** Dissect out of targeted tissues from exposed insects.

5.4. Preparation of test insecticide solution

Commercially available nimbecidine (azadirachtin) manufactured by T. Stan sand Company Limited, purchased from the local market of West Bengal, India were used for the present study. Different concentrations (1 ppm, 5 ppm, 10 ppm, 15 ppm and 20 ppm) of AZT were prepared by dilution with distilled water (where, 1 ppm=1 mg per liter).

5.5. In-vivo treatment

Dose dependant AZT were uniformly applied separately to 25 grams of fresh tender grass leaves (*Cyperus kyllingia*: Cyperaceae) were provided in 100 ml water filled conical flasks for their food in clean, transparent plastic jar (11×11×26) cm with moist sterilized sand bed. Then, same aged 1:1 female and male insects from just newly hatched adult were placed in each jar and covered with mosquito net fixed by rubber bands. Three replicates were used for each concentration and all jars were kept under laboratory conditions in comparison to control where grasses without pesticide were used. Percentage of mortality was estimated after post treatment.

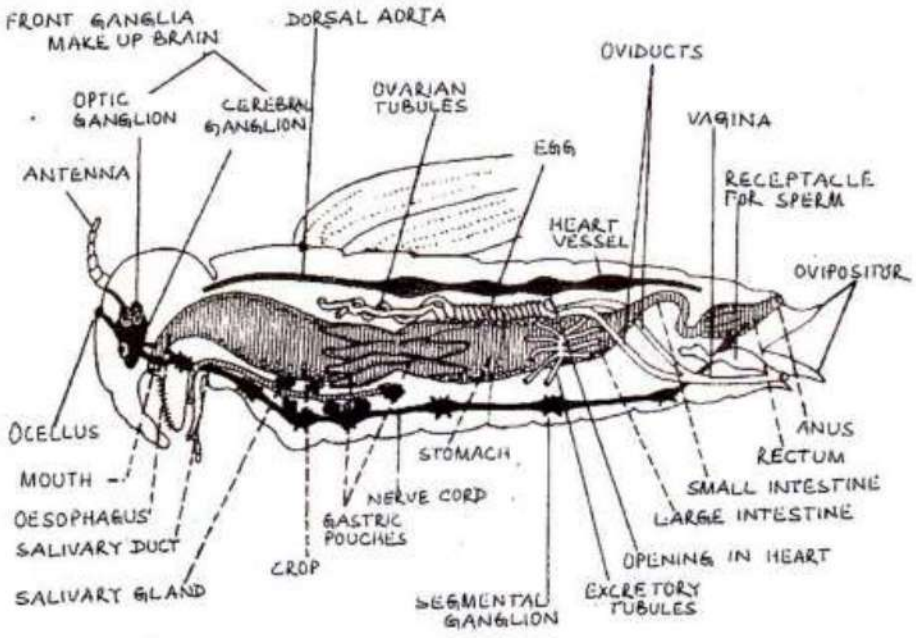


Plate 20. Anatomical structure of a female insect.

All the insects in each jar of each set were allowed to feed for 48-hrs. Only alive insects after the treatment period of 48-hrs I pooled haemolymph from each female insects from each jar of a set. And likewise, haemolymph of male insects were also pooled up for each treatment of a set and also from control collected separately into eppendorf-pipette containing phenoloxidase inhibitor (phenylthiourea) to prevent tanning or darkening and then diluted 5× with 0.7% insect's saline. During assays, the diluted haemolymph was frozen to rupture the haemocytes and then centrifuged at 2000 rpm for 5 min. Only the supernatants were used for assay directly or frozen (-20ºC) until further use. Subsequently dissected and collected fat bodies free gut, brain and gonads (Plate 26) from each treatment of both sexes were removed and separately stored in -20ºC for biochemical analysis. Each AZT-concentration was replicated three times included AZT-free controls.

5.5.1. Susceptibility test of azadirachtin

Sex dependant toxic mortality in comparison with controls was recorded after 48-hrs of the experiment. Corrected mortality was calculated according to Abbott (1925).

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

5.5.2. Determination of nutritional-indices

The data such as leaf weight, insect's weight and excreta weight before and after the experiment were recorded for the calculation of consumption index (CI), approximate digestibility (AD), efficiency of conversion of digested-food (ECD) and efficiency of conversion of ingested-food (ECI) as per the formula of Waldbauer (1968) and as used by Sharma and Norris (1991).

Plate 21. In-house topical application of AZT. **(a)**, **(b)** and **(c)** Dose and time dependent in-vivo experimental set-up, **(d)** Feeding behaviour of insects after AZT application.

5.6. In-vitro treatment

Fresh fat bodies free gut, brain and gonads from both sexes and instar wise whole body juvenile tissue were incubated with different dilutions of AZT prepared for the experimental purposes in comparison to control for 6-hrs at room temperature and separately store in -20ºC for sample preparation.

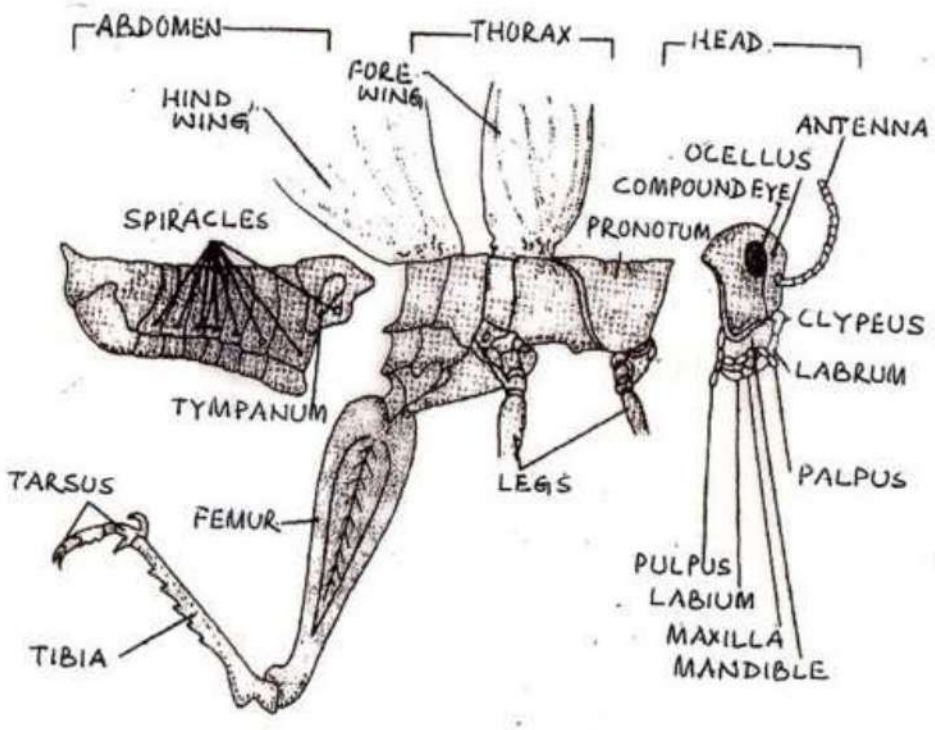


Plate 22. Anatomical structure of a male insect.

5.7. Preparation of sample for biochemical analysis

The pooled tissues from each treatment and control are separately homogenized in 5 times volume (w/v) within 0.1 M chilled phosphate buffer (pH 7.4) and centrifuged at 10,000 rpm for 8 min. The homogenization was performed with the use of the Teflon homogenizer of the Potter-Elvehjem type. The 20% supernatant is carefully separated from the pellet and transferred to new eppendorf and preserved at -20°C until onset of biochemical assay.

5.8. 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.

5.8.1. Estimation of total protein

Protein concentration of the samples was measured following the method of Lowry et al. (1951) with minor modifications where Bovine Serum Albumin (BSA, HiMedia, India) was used to generate the standard curve. The blue colour product is measured after 30 min at 750 nm against the blank. Protein concentrations are expressed as µg/mg wet tissue or µg/µl of haemolymph.

5.8.2. Estimation of malondialdehyde (MDA)

Malondialdehyde (MDA) level (index of lipid peroxidation) was evaluated with thiobarbituric-acid (TBA) reagent (Okhawa 1979) with minor changes. The amount of MDA formed is measured by taking the absorbance at 530 nm (ε=1.56×105$mol^{-1}cm^{-1}$) and the concentrations were expressed as µmol/mg wet tissue or µmol/µl of haemolymph.

5.8.3. Estimation of non-protein-soluble-thiols (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 or µg/µl of haemolymph.

5.8.4. Estimation of alkaline-phosphatase (ALP)

Alkaline-phosphatase activity in homogenate is estimated according to Belfield and Goldberg (1971). 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 (King-Armstrong) KA unit/mg wet tissue or KA unit/µl haemolymph.

5.8.5. Estimation of acetyl-cholinesterase (AChE)

Acetyl-cholinesterase activity was examined by the method of Ellman et al. (1961) with minor changes. The incubation mixture consisted in a final volume of 0.2 ml, 20 mM 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 min 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.

5.8.6. In-gel assay for catalase (CAT)

In-gel CAT assays are followed the principles described by Zerbetto (1997). The non-denaturing (8%) acrylamide gel is washed with distilled water for 10 min containing 25 µg protein. The gel is shaken in 100 ml distilled water with 100 µl H_2O_2 for 10 min and then was 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.

5.8.7. In-gel assay for superoxide-dismutase (SOD)

Superoxide-dismutase activity gel is performed according to the method described by Beauchamp and Fridovich (1971) with slight modifications, where 150 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 ribo-

flavin/280 mM N,N,N',N'-tetramethylethylenediamine (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.

5.8.8. In-gel assay for glutathione-peroxidase (GPx)

Native polyacrylamide gel electrophoresis (8%) is carried out at 100 V for 2.5-hrs according to the method reported by Moreno et al. (1990) without SDS. First, an equal amount of protein (100 µg) extracts is applied and then gels are soaked in 0.008% cumin hydroperoxide for 10 min, rinsed with water and stained in 1% potassium ferricyanide followed by 1% ferric chloride for colour development.

5.8.9. 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 30ºC for 60 min. After it is washed with distilled water, the gel is subjected to stained with Lugol's solution (I_2 0.33% and KI 0.66%) at ambient temperature until the appearance of clear zones of protein bands with amylase activity against a dark blue background.

5.8.10. In-gel assay for cellulase

In-gel assays were performed using non-denaturing PAGE for visualising cellulase activities. The enzyme sample was partially denatured at 70°C for 3 min for increasing the gel resolution. Following heating, the samples were briefly centrifuged (Oppert et al.2010) and then loaded in 6% stacking and 12% separating polyacrylamide gel. The substrate 0.2% carboxymethylcellulose (CMC) was incorporated into the separating gels. After

electrophoresis, the gels were washed in phosphate buffer (0.1 M) for 30 min at 60°C. Then the gels were stained with Congo red (0.1%) for 10-15 min at room temperature. The gels were de-stained by washing in 50 ml of 1 M NaCl until cellulase bands became obvious as clear zones where CMC had been ruined due to the enzymatic activity. After 20 min de-staining, 1% glacial acetic acid was added to the gel for better visualisation (Willis et al. 2010).

5.8.11. Detection of oxidative DNA-fragmentation analysis

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 and the percentage of DNA stability was expressed as bar-diagram. A suitable DNA ladder (EZ Load 500 bp Molecular Ruler #1708354, Life Sciences, BioRad) was also run to assume the damage pattern.

5.8.12. Mitochondrial membrane stability assay by fluorescence microscopy

Mitochondria from the commonly used pesticides added liver tissues of common agri-field animals were isolated and incubated at 37°C in a medium composed of 5 mM K_2HPO_4, and 5 mM $MgCl_2$, 135 mM KCl, 20 mM MOPS, at pH 7.00, incubations also contained R123 (Sakamuru et al. 2016). The R123 and all other chemicals were from Sigma Chemical Co. (St. Louis MO). Fluorescent measurements of mitochondria and ex-

tracts were made using a fluorescence microscope (Nikon, Eclipse LV100 POL), with the VisComet (Impulse Bildanalyse) software.

5.9. Statistical analysis

Data were performed in triplicate. Mean and standard deviation (SD) values were determined for other than biochemical test and the results were expressed as means ± SD (n=3). Mean and standard error (SE) values were determined for all the biochemical parameters and the results were expressed as means ± SE (n=3). The data were analysed using analysis of one-way variance (ANOVA) followed by Fisher's least significant difference (LSD) multiple comparisons. All the statistical analyses were performed using (SPSS 17) software and the results were considered statistically significant when $P<0.001$. 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.