CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS :

For examining the insecticidal effect of neem oil the full grown adults of cockroach and grasshopper were employed for the present study. The cockroaches were collected from a single source (bakery) while the grasshoppers were obtained from the grass (Cynodon dictyolon) near the sugarcane fields around the city of Akalkot. The culture of these insects was maintained separately in the laboratory under constant conditions of temperature (28 ± 2). The glass jars of the size 15x20x30 cm. were used for the culture. To avoid variations in the tissue components of the body due to variations in the diet, the cockroaches were fed with bakery bread whereas the grasshoppers with the grass. Both insects were cultured in batches, each batch consisting of five insects per jar. While feeding the cockroaches with bread, care was taken to see that the bread was free of any fungal contamination etc. Similarly for grasshoppers fresh grass was added from time to time. After every twentyfour hours the glass jars were changed to avoid any unhygenic conditions caused due to excretion etc.

In order to determine the effect of neem extract on the feeding activity of the insects understudy, the plant chemcial was administered through their respective diets. The neem seed oil was obtained from Prof. C.M. Ketkar, Pune. Different concentrations of this oil ranging from 50 µl to 500 µl per ml were prepared in acetone. Known quantity of bread (in the form of sliced pieces of equal size) and grass were mixed with these different grades of neem oil and were fed to cockroaches and grasshoppers respectively. At least five dietary replicates each of 50 µl, 100 µl, 300 µl, 400 µl and 500 µl were allowed to feed. For each of these concentrations five insects were tested for their feeding efficiency. The

feeding activity was observed for 6 hr, 12 hr, 18 hr, 24 hr, 36 hr and 48 hr. for the above mentioned concentrations. Both the insects adminstered with lower dietary concentrations i.e. at 50 µl were found to be active and did not have any deterrant activity after 24 hrs. but reduced their feeding after 36 hrs. However, at the higher dietary concentrations i.e. at 200 µl, 300 µl, 400 µl and 500 ml reduced compeltely (Completely) the amount of diet consumption at 24 hrs, 18 hrs, 12 hrs, and 6 hrs respectively and affected the survivility of the insects. Insects exposed to 100 µl dietary concentrations exhibited slight reduction in diet consumption became inactive or sluggish after 24 hrs treatment. The dietary effect of neem oil at this concentration was more or less found to be the same in both the insects. Therefore, insects fed with 100 µl neem oil and exposed for 24 hrs were employed for the further experimental work. The untreated insects were taken as normal or control. The following tissues/organs i.e. haemolymph, fat body and different parts of the alimentary tract (foregut, midgut, hepatic caeca, Malpighian tubules and hindgut) from both the treated and untreated cockroaches and grasshoppers were employed for the study of free amino acids and proteins.

Amino acids :

The amino acid composition of the haemolymph, fat body and different parts of the alimentary canal (i.e. foregut, midgut alongwith hepatic caeca and hindgut) was determined by the unidimensional ascending chromatography (Block et al.,1955). Haemolymph from both the insects was obtained by cutting the coxae of the legs. In all, 100 µl of haemolymph was pooled and employed for the study. Extracts of the fat body and different regions of the gut were prepared in equal volume of 80% alcohol and chloroform. The tubes were then centrifuged for five minutes at 3000 g. The supernatant water layer

containing amino acids was employed for the analysis. Extracts were applied as compact spots with the help of micropipettes on Whatman No.1 paper sheet (40 x 40 cm.). About 100 µl samples of each tissue was loaded at each spot and the paper was subjected to aseending chromatography. The chromatography chromatograms were developed in a solvent system of n-butanol : glacial acetic acid : distilled water (80:20:20) for more than 10 hrs. The spots were developed by ninhydrin and identified with the corresponding Rf values of the standard amino acids run alongwith the samples. The amino acids were further confirmed by their different colours developed after spraying 0.2% isatin in acetone (Smith,1953).

Proteins :

During the course of present investigation both qualitative as well as quantitative analyses of proteins were made. Qualitative analysis of the protein patterns was made by employing disc electrophoresis on polyacrylamide gels while quantitative estimation was carried out by bioassay method of Lowery et.al.(1951)

Electrophoretic Method :

Proteins from the different tissues/organs of the two insect species understudy were fractionated on polyacrylamide gels following the methods of Ornstein(1964) and Davis (1964) with slight modifications. The appartus used for conducting electrophoresis was of 'shandon' type. Separating (running) gel having 5% acrylamide concentrations was employed. Glass tubes of (110 mm length and 6 mm width were used for layering the gel of standard stock solutions. Gel was prepared in Tris-borate buffer having a pH of 8.9 (0.1 M Tris and 0.025 M boric acid). The same buffer was employed for electrophoresis. 70 mg/ml of ammonium persulphate and 70 µl TEMED were used for

polymerization of gels (Foigen et.al.,1980).

Samples of the different tissues/organs were prepared by homogenizing different organs in the above buffer (100 mg/ml). The homogenates were centrifuged, for five minutes at 3000 g. The clear supernatants of each sample (100 µl) were mixed with a solution of 50% sucrose : 0.25% bromophenol blue (tracking dy) at a ratio of 4 parts of sample and 1 part of sucrose-dye solution. The samples were then electrophoresed, at 150V. Electrophoresis was carried out initially for 5 to 10 minutes at 1.5 mA/tube, until the sample entered the gel. It was then continued further for about 90 minutes at 3 mA/tube. The current was stopped after reaching the tracking dye at the bottom of the gels. All operations were carried out in the cold. At least three replicates of each sample were made to established the reproducibility of the resultant electrophoretic patterns.

After electrophoresis, the gels were removed from the tubes. Total length of the gels and the distance travelled by the dye front was measured. The gels were then stained for general proteins in 1% Amido Black in 7% acetic acid for one hour. Excess stain was removed by transferring gels to 7% acetic acid for 24 hrs.

The relative mobility (Rm) of the fractions was calculated using the following formula :

$$Rm = \frac{DTB}{DTD} \times \frac{LB}{LA}$$

where DTB = Distance of each band, DTD = Distance travelled by tracking dy, LB = Length of the gel before stainінng and LA = Length of the gel after staining.

Bioassay Method :

Biochemical estimation of proteins was carried out according to the method described by Lowry et al, (1951). A sexwise study of the protein content from different tissues/organs of both the insects was made.

A)Preparation of Reagents :

Following reagents were prepared :

1) Standard protein solution : 13 mg bovine alubmin dissolved in 250 ml distilled water and made alkaline.

2) Lowry's A solution - 2% Na-carbonate in 0.01 M NaOH

3) Lowry's B_1 solution- 2% Na-citrate in distilled water.

4) Lowry's B_2 solution- 1% $CuSO_4$ in distilled water

5) Lowry's (solution - To 1 ml of Lowry's B_1 solution added 1 ml of B_2 solution and 100 ml of Lowry's A solution and mixed well. This solution was prepared just before the use and used within 15 minutes.

B) Preparation of Samples :

Homogenization of the different tissues/organs understudy was carried out by using clean glass mortor pestle chilled previously. Chilled water was used for homogenization and required dilution (5 mg/ml).

C) Chemical Reaction :

For the bioassay of proteins 7 test tubes were taken. In first five test tubes 0.0, 0.2, 0.4, 0.6 and0.8 ml of homogenates were added. Two control test tubes contained 0.2 and 0.4 ml standard protein solution. To each of the tube, distilled water was added to adjust the volume to 1.5 ml and then added 3 ml. of Lowry's solution. The mixture in each tube was mixed well and kept for 15

minutes to form copper protein complex. After this time interval 0.5 ml Folin's phenol reagent was added to each tube. Then they were allowed to develop colour for one hr. at room temperature & the reading were taken at 660 mu specol colorimeter.

D) Calculations :

The final colour production is a result of biuret reaction of protein with copper ions in an alkaline medium and reduction of the phosphomolydic-phosphotungstic reagent by the tyrosine and typtophan present in treated protein.

The amount of protein was calculated in term of mg/gm wet weight of the tissue using the following formula :

$$\frac{\text{O.D. of unknown x standard protein x dilution}}{\text{O.D. of standard protein x wt. of sample}} = \text{mg protein/gm wet wt. of tissue.}$$

All the chemicals and reagents employed during the course of present investigation were of AR grade.