CHAPTER III

MATERIALS AND METHODS

3.1 Experimental animal

The experiments were performed with the freshwater fish *Oreochromis niloticus* commonly known as Nile tilapia (Fig.3.1). Young tilapia at the age of 2 days, irrespective of sex, were obtained from Pathumtani Breeding Station, the Department of Fishery, the Ministry of Agriculture and Cooperatives. They were acclimated to the laboratory conditions for a period of about 4 weeks in 325-L glass aquaria before experimentation to ensure that they were disease-free and recovered from transportation stress. Fish were fed on a commercial pellet food twice a day. Thereafter, the test of toxicity and LC_{50} values were determined.

3.2 Chemicals

Neem *Azadirachta indica* A.Juss. seed extract was used for the experiments. It was an alcoholic extract of neem seed that was procured from commercial sources, a product of the Phuetphunthammachat Company. Its trade name was Neemix (Fig.3.2). The concentration solution was prepared by dissolving in water directly.

Chemical required for haematological and biochemical analysis are summarized in Appendix A.

3.3 Apparatus

Apparatus and equipment required for fish culturing, toxicity testing, blood collecting, haematological and biochemical analysis are summarized in Appendix B.

3.4 Acute toxicity test

3.4.1 Range-finding test

A toxicity range-finding test was a down-scaled, abbreviated static acute test in which group of fish were exposed to widely-spaced neem seed extract concentrations. This pilot study was carried out in 14-L glass jars containing 10-L of neem seed extract and using 4 weeks old tilapia (weighing 1-2 g). Ten fish were exposed to neem seed extract at concentrations of 0.01, 0.1, 1, 10 and 100 ppm. There was no addition of neem seed extract solution to water, in case of control. Tests were performed in triplicate for each concentration. The number of deaths at each concentration were recorded every 24 hr up to 96 hr. The percent mortality of this test was shown in Appendix C.

3.4.2 Definitive test

Base on the results of range-finding test, a definitive study was set up with concentration of 30, 35, 40, 45, 50, 55, 60, 65 and 70 ppm of neem seed extract. The tests were performed with ten fish per concentration and there were three replicates for each concentration.

Test procedures

The static acute toxicity bioassay (ASTM, 1980, FAO no. 185 1982) was performed to determined the LC_{50} values and 95% confidence limits of neem seed extract after 96 hr.

The experiments were carried out in 14-L glass jars containing 10 L of neem seed extract testing solution. In case of control fish there was no addition of neem seed extract solution to the water. Tests were performed in triplicate for each concentration. Each replicate consisted of ten fish.

Young Nile tilapia (*Oreochromis niloticus*) at the age of 4 weeks, weighing 1-2 g, 2-3 cm length were randomly assigned to the experimental glass jars, ten fish were tested at each concentration. All fish were fasted for 24 hr prior to experiments and during the test which continued for 96 hr. Mortality was recorded every 24 hr from the beginning of the test until the end of experiment.

3.4 Data analysis for LC_{50} values

The LC_{50} values and 95% confidence limits of neem seed extract after 24, 48, 72 and 96 hr were calculated by probit analysis (Finney, 1971).

3.5 Determination of application factor (AF)

The AF was intended to provide an estimate of the relationship between a test material's chronic and acute toxicity, which could then be applied to aquatic organisms for which a MATC, it was the numerical value of the ratio of the MATC to the incipient LC_{50} 96 hr estimated in dynamic acute toxicity test, that is,

$$AF = MATC / LC_{50}\ 96\ hr$$

The maximum acceptable toxicant concentration (MATC) was the estimated threshold concentration of a chemical within a range defined by the highest concentration tested at which no significant deleterious effect was observed (NOEC) and the lowest concentration tested at which some significant deleterious effect was observed (LOEC).

From the range defined between the NOEC and LOEC of neem seed extract, the LC_4 was chosen, the sublethal concentration for the long-term study was determined at 25.32 ppm (Calculating of this value was shown in Appendix C).

3.6 Sublethal toxicity test

Sublethal toxicity tests permitted evaluation of the possible adverse effects of neem seed extract under conditions of long-term exposure at sublethal concentrations. The static renewal tests were used for the experimentation.

Experimental procedures

The static renewal tests were employed for all subchronic toxicity test. The experiments were carried out in 325-L glass aquaria, containing 150-L of 25.32 ppm neem seed extract solution. The test medium was changed every two days.

One hundred and fifty young Nile tilapia (*Oreochromis niloticus*) aged 1 month, weighing 1-2 g, 2-3 cm length, were randomly assigned to each experimental glass aquaria with constant aeration at room temperature. There were six experimental glass aquaria that were divided into two groups of three replicates including treatment and control. The experiments were performed under natural light. They were fed on a commercial pellet food twice daily. Thereafter, fish from both control and treatment were sampled for blood collection monthly until the seventh month of experiment.

3.7 Blood collection

Sampling of blood was done monthly from fifty fish of each group, control and treatment, irrespective of sex. Prior to blood sampling, each fish was measured and weighed. Feeding was also stopped 24 hr before each sampling interval started. Blood samples were drawn into heparinized tuberculin syringes by cardiac puncture (Fig.3.3). The cardiac puncture was done especially in fish at the age of 5, 6, 7, and 8 months. Each blood sample was pooled from five specimens for haematological examinations, including the total number of erythrocytes, white blood cell counts, haematocrit values. Biochemical determinations consisted of three enzymes, alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT).

The caudal peduncle of each fish was also cut with a sharp blade (Fig.3.4) for preparation of blood smear for differential leucocyte count and blood cell type characteristic studies. These information was collected in each fish at the age of 2, 3, 4, 5, 6, 7 and 8 months. Furthermore, blood samples by caudal peduncle were used for estimating blood glucose levels of each fish at the age of 3, 4, 5, 6, 7 and 8 months.

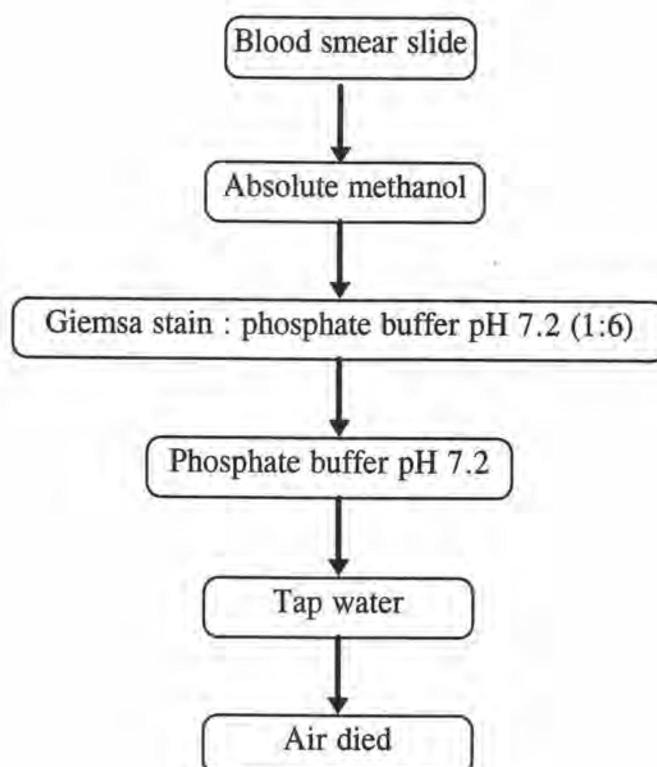
Note: Fish at the age of 2, 3 and 4 months were a small size, thus blood sampling was not adequate for haematological parameters (total blood cell counts and haematocrit values) and biochemical parameters (ALP, GOT and GPT).
For the glucose levels were begun to study in fish at the age of 3 months up to 8 months.

3.8 Haematological analysis

3.8.1 Morphological aberrations

Blood smear was prepared from non-heparinized fresh blood of caudal peduncle of each fish. Three slides per individual were prepared, air dried, fixed in absolute methanol and stained with Giemsa stain which diluted with phosphate buffer pH 7.2 (1 ml Giemsa/5 ml phosphate buffer pH 7.2) for 20 min. The slides were then rinsed with phosphate buffer pH 7.2 again and then with tap water and were then dried in air (Humason, 1979). Blood corpuscles were finally examined with a light microscope at 1000X magnification to identify different types of blood cell. Differential leukocyte counts were then studied.

Diagram for blood smear preparation



3.8.2 Total cell counts

The RBC diluting pipette and a 1:200 dilution (Fig.3.5) were used for erythrocyte counts. They were made in duplicate from each pooled sample using 0.75% sodium chloride solution for dilution. The methods used were then the visual counting of cells in a Bright-Line hemacytometer. The red cells in the smaller squares (Fig.3.5) were counted and multiplying the total count by 10^6 to determine the total number of erythrocytes per cubic millimeter of blood.

White blood cell counts were made by using Shaw's solution (Shaw, 1930). These solutions were prepared just prior to use. The standard WBC diluting pipette and a 1:20 dilution (Fig.3.5) were used for diluting. The WBC counting was then counted in a Bright-Line hemacytometer under a light microscope. The cells in the four large squares (Fig.3.5) were counted.The total number of cells counted was multiplied by 50 to determine the total number of leucocyte per cubic millimeter of blood.

3.8.3 Haematocrit determination

Haematocrit values were measured by using a microcapillary centrifuge and microcapillary reader (Larson and Sneiszko, 1961). The pooled blood samples was prepared in commercially heparinized capillary tubes and then centrifuged at a high speed for five min.

Red cell indice, MCV (Mean Corpuscular Volume) was calculated from the values of the total RBC count and haematocrit (Hesser, 1960). It followed as :

$$\text{MCV } (\mu m^3) = \text{Haematocrit } (\%) \times 10 \,/\, \text{RBC } (10^6 / cm^3)$$

3.9 Biochemical analysis

3.9.1 Blood glucose determination

Blood glucose was determined in whole blood from the caudal peduncle of each fish. It was then estimated by One Touch Basic Blood Glucose Meter (a Johnson & Johnson company).

3.9.2 ALP, GOT and GPT determinations

The pooled sample was centrifuged promtly after collecting haematological data at 1,500 rpm for 30 min. Plasma was separated from the remaining sample and stored at -70 °C. The enzymes including ALP, GOT and GPT were determined by the colorimetric method using commercial kits (Sigma Chemical Company).

ALP determination

Procedures

1. Pipetted into each tube of 0.5 ml 221 Alkaline buffer solution Catalog No. 221 and 0.5 ml stock substrate solution. Then placed each tube in a 37 °C waterbath to equilibrate.
2. Pipetted 0.1 ml water into tube labelled Blank and 0.1 ml plasma into tube labelled Test. Recorded exact time, mixed gently and replaced in waterbath promptly.
3. After exactly 15 min, added 10.0 ml 0.05 N NaOH to each tube and mixed thoroughly.
4. Read absorbance of Test and Blank as reference at 420 nm. Determined alkaline phosphatase activity from calibration curve.
5. Added 4 drops concentrated HCL to each tube and mixed.
6. Again read absorbance of Test using Blank as reference at the same wavelength. Determined alkaline phosphatase activity from calibration curve.
7. Substracted the alkaline phosphatase activity of step 6 from alkaline phosphatase activity of step 4, yielding corrected alkaline phophatase activity of plasma.

GOT and GPT determination

Procedures (GOT)

1. Pipetted 1.0 ml Sigma prepared substrate, Catalog No.505-1 into each tube and then placed in a 37 °C waterbath to warm.
2. Added 0.2 ml plasma and shaked gently to mix. Left in waterbath.

3. Exactly 1 h after adding plasma, add 1.0 ml Sigma color reagent, Catalog No. 505-2 to stop activity and start color reaction. Shaked gently and left at room temperature (18-26 °C).

4. 20 min after adding Color reagent, added 10.0 ml 0.40 N NaOH. Mixed thoroughly.

5. Waited at least 5 min more. Then, read and recorded absorbance at 490 nm wavelength., using water as Blank.

6. Determined GOT activity from calibration curve.

Procedures (GPT)

1. Pipetted 1.0 Alanine-α-KG substrate, Catalog No. 505-51 in to each tube and placed in a 37 °C waterbath to warm.

2. Added 0.2 ml plasma and shaked gently to mix. Left in waterbath.

3. Exactly 30 min after adding plasma, added 1.0 ml Sigma color reagent, Catalog No. 505-2. Shaked gently and left at room temperature (18-26 °C).

4. 20 min after adding Color reagent, added 10.0 ml 0.40 N NaOH. Mixed thoroughly.

5. Waited at least 5 min more. Then, read and recorded absorbance at 490 nm wavelength, using water as Blank.

6. Determined GPT activity from the calibration curve.

3.10 Statistical analysis for blood parameters

Means $\pm$ SE were calculated for each process per group. The data was analyzed for significance of differences by the student's t-test at $p \leq 0.05$.

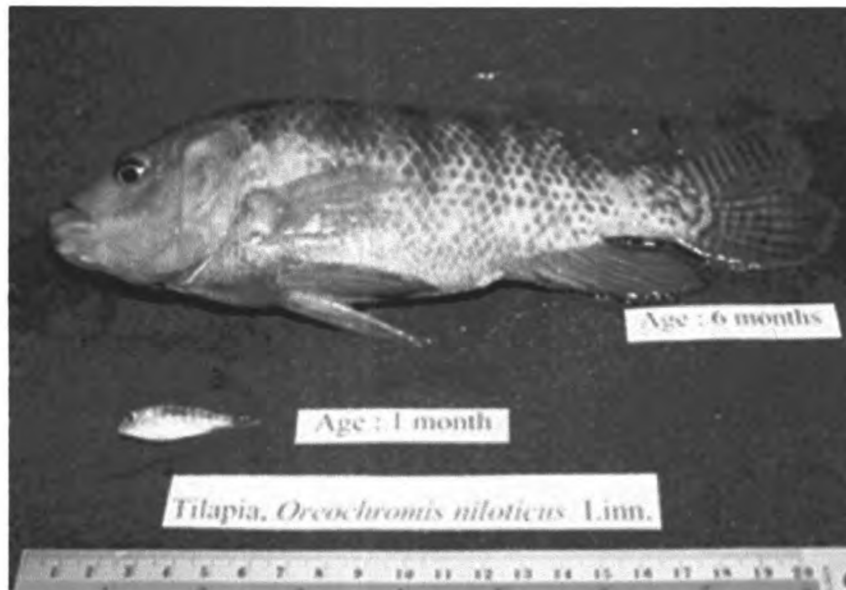


Fig.3.1 Photograph of Nile tilapia *Oreochromis niloticus* Linn. at the age of 1 and 6 months.

Fig.3.2 An alcoholic extract of neem *Azadirachta indica* seed extract from commercial source.

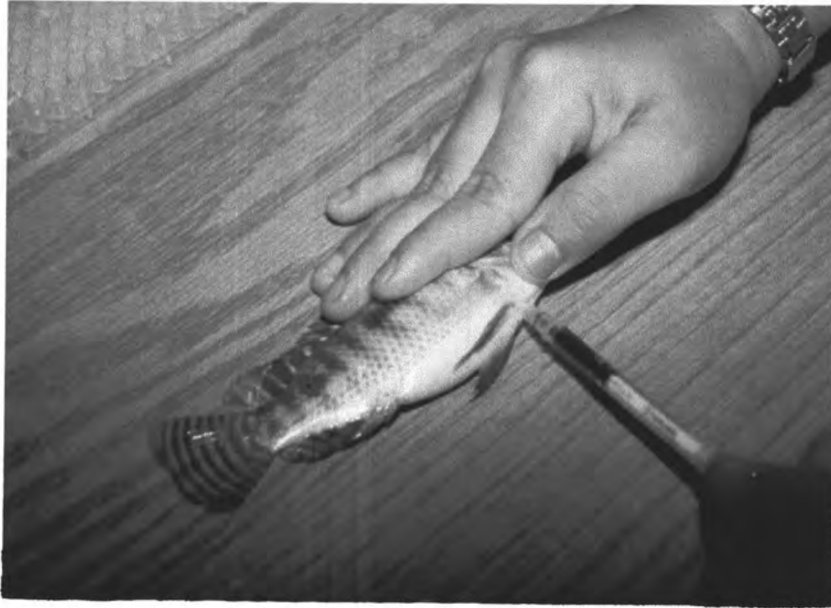
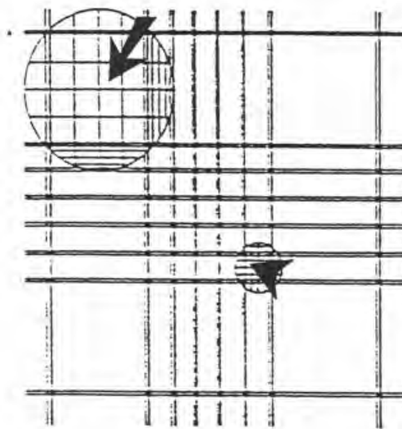
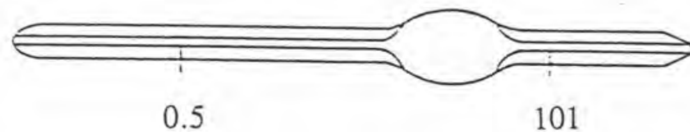


Fig.3.3 This photograph shows cardiac puncture technique for blood sampling.

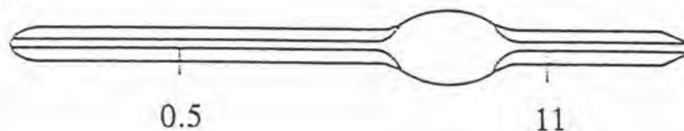
Fig.3.4 This photograph shows blood sampling by cutting caudal peduncle.



(a) Hemacytometer



(b) RBC diluting pipette



(c) WBC diluting pipette

Fig.3.5 (a) Hemacytometer shows the smaller squares for red blood cell count (arrow-head) and the four large aquares for white blood cell count (arrow) (b) RBC diluting pipette (c) WBC diluting pipette.