Chapter 3

Materials and Methods

3.1 Subjects

The *O. niloticus* L. broodstock used in this study were obtained from Pathumtani Breeding Station, Department of Fisheries, the Ministry of Agriculture and Cooperatives of Thailand. The fry (at the age of 2 days) were initially acclimated and observed in 325-L glass aquaria for 4 weeks prior to exposure. The fish was fed commercial pellets (CP Company) twice daily at approximate 3-5 % body weight throughout the study. Figure 3-1 shows young and mature Nile tilapia *O. niloticus* used in this study.

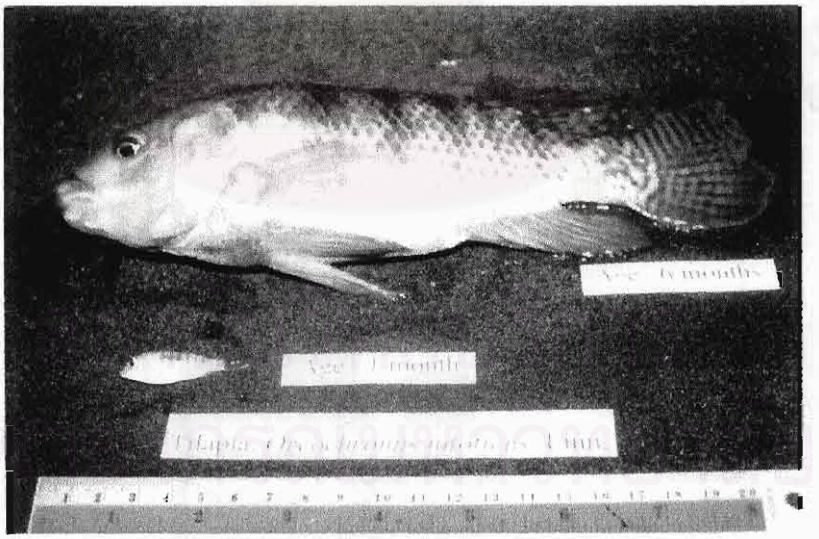

Figure 3-1 Nile tilapia *Oreochromis niloticus* Linn. at the age of 1 month and 6 months.

3.2 Holding conditions

Tap water was filtered through carbon-resin filter, aerated and finally used as holding water in this experiment. Water conditions including temperature, dissolved oxygen, hardness and pH were measured routinely throughout the test.

Water temperature in control and treatment aquaria were similar, ranged between 24 and 27 °C. Dissolved oxygen ranged between 7 and 7.4 mg/l in both groups as well as hardness that range between 66 and 112 mg/l as $CaCO_3$. The pH value in control water ranged between 5.4 and 5.9 while in treatment water ranged between 5.4 and 5.8.

3.3 Test material

The test material used in this experriment was an alcoholic neem *A. indica* A. Juss. seed extract (Neemix®; Phuetphunthammachat Company, Thailand). The extract was dissolved directly into holding water to yield the desired concentrations. Figure 3-2 shows the commercial extract used in this experiment.

Figure 3-2 Neemix®, an alcoholic *A. indica* seed extract from commercial source.

3.4 Acute toxicity test

The acute static toxicity bioassay (ASTM, 1980; FAO No.185, 1982) was carried out to determine the LC_{50} value at 95% confidence intervals of the neem seed extract after 96 hours. The fish at the age of 4 weeks were divided into experimental and control group and they were fasted prior to the exposure. The details of each testing protocol are presented below.

3.4.1 Range-finding test

The test was conducted in 14-L glass jars containing 10 litres of different concentrations of the extract solution. Five jars were filled with the treated solution at 0.01, 0.1, 1, 10 and 100 ppm while one jar was filled with holding water and left as control. All experimental conditions were triplicated and 10 fishes were used for each condition. The fish of undetermined sex were randomized from the holding aquaria and assigned to the experimental jars. Mortality was observed and recorded every 24 hours throughout the test. The percentage of mortality observed in each concentration is presented in table 3-1.

Table 3-1 Percent of mean mortality of *O. niloticus* at various neem seed extract concentrations in range-finding test (n=30).

Exposure time	Mean mortality (%) at neem seed extract concentrations (ppm)					
	0.01	0.1	1	10	100	Control
24-hour	0	0	0	0	100	0
48-hour	0	0	0	0	100	0
72-hour	0	0	0	0	100	0
96-hour	0	0	0	0	100	0

3.4.2 Definitive test

The preliminary range-finding test indicated that the concentration of 100 ppm neem seed extract was the lethal concentration, yielding 100% mortality in young tilapia while the concentration of 10 ppm extract was the maximum nonlethal concentration, yielding 0% mortality in the fish. Base on this pilot test, a definitive study was carried out with concentration of 30, 35, 40, 45, 50, 55, 60, 65 and 70 ppm of the extract and the test protocol was as described in the previous test. The percentage of mortality observed in each concentrations is shown in table 3-2.

3.4.3 Analysis for LC_{50} values

The median lethal concentration (LC_{50}) values and its corresponding 95% confidence intervals of each exposure time were calculated by Probit Analysis (Finney, 1971). The calculation was performed on computer by Probit Analysis program and the results are shown in table 3-3.

Table 3-2 Percent of mean mortality of *O. niloticus* at various neem seed extract concentrations in definitive test (n=30).

Exposure	Mean mortality (%) at neem seed exrtract concentrations (ppm)									
time	30	35	40	45	50	55	60	65	70	Control
24-hour	0	0	3.33	53.33	40.0	46.67	100.0	100.0	100.0	0
48-hour	0	26.67	73.33	80.0	66.67	86.67	100.0	100.0	100.0	0
72-hour	0	40.0	73.33	86.67	80.0	100.0	100.0	100.0	100.0	0
96-hour	0	46.67	80.0	100.0	100.0	100.0	100.0	100.0	100.0	0

Table 3-3 Neem seed extract median lethal concentration (LC_{50}) and 95% confidence intervals (in ppm) for *O. nilloticus*.

Exposure time (hours)	LC_{50}	95% confidence intervals		R^2
		Lower	Upper	
24	47.71	69.67	55.49	0.77
48	40.71	34.50	45.17	0.82
72	38.92	36.13	41.28	0.78
96	36.25	34.47	37.94	0.62

3.5 Determination of application factor(AF)

The range defined between the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) of neem seed extract was selected from the previous calculation of LC_{50} values. The LC_4 was chosen as a maximum acceptable toxicant concentration (MATC). So the MATC was 30.21 ppm (the LC of different probit values are shown in Appendix II). Base on these values, the application factor (AF) was calculated as presented below.

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

$AF = 30.21 / 36.25$

$AF = 0.83$

From the AF, the sublethal concentration of the extract used for subchronic toxicity test was determined at $0.83LC_4$. Therefore, the concentration of neem seed extract for subchronic exposure was 25.07 ppm.

3.6 Subchronic exposure

New *O. niloticus* brood stock was obtained from the same source and was acclimated for 4 weeks in 325-L glass aquaria, provided with aerated water supply prior to and during subchronic exposure to neem seed extract. The treatment aquaria was filled with 150 litres of 25.07 ppm neem seed extract while the control was filled with 150 litres holding water. The exposure was begun after one month of age and carried out continuously for seven months.

The static renewal system was used throughout the test. Holding water of both treatment and control group was renewed every two days. The fish (aged 4 weeks, average weight 1-2 g) were divided into control and treatment group and were removed into separate aquarias. Each aquaria contained approximate 250 fishes which were consequently separated into two aquarias when the fish grew up to the age of 4 months.

3.7 Sampling

During the exposure period, fish of both control and exposed group were sampled (n= 20) on the 3rd (control group only), 4th, 5th, 6th and 7th month. They were fasted for 24 hours prior to sampling during exposure period.

Sampling was initiated by dipnetting randomly selected fish from each aquaria. The length (cm) and weight (g) of each fish was measured. Then, they were cool shocked at 0 $^{\circ}$C. The abdomen of each fish was opened. The left and right gonads were removed and measured for weight (g) and approximate length (cm). The gonads were fixed in Bouin's fixative for 48 hours and then preserved in 70% ethanol.

3.8 Determination of Gonadosomatic index (GSI) and fecundity

Gonadosomatic index (GSI) of female fish were calculated by dividing the ovaries weight by the whole body weight and multiplying by 100 as shown below.

$$GSI = \frac{\text{ovaries weight (g)}}{\text{body weight (g)}} \times 100$$

The female reproductive potential, or fecundity were determined by analysis of the GSI and oocyte count. The oocytes were classified into 3 groups according to oocyte diameter and external characteristics such as shape and color. The diameters were determined upon the longest dimension, called horizontal diameter (n=80 in each group). The largest, ripe eggs and the intermediate, yolked oocytes were counted from subsample of ovary sample, and multiplied by the total weight of ovary, then divided by the subsample weight. The smallest, immature oocytes were estimated from dry weight of ovarian tissue left after the largest and intermediate oocytes were separated.

3.9 Histological analysis

The ovaries sample were fixed in Bouin's fixative for 48 hours and preserved in 70 % ethanol. Five ovaries from each experimental group were sampled for histological study. Ovarian tissues were divided into three parts: anterior, median and posterior, and processed according to standard histological techniques (Humason, 1979). All tissue blocks were sectioned at 7 μm and stained with hematoxylin and eosin. Histological study were performed by light microscopy following two aspects.

Routine paraffin method for ovarian tissues of *O. niloticus*

10% Glycerol in 70% ethanol (several days)
↓
90% Ethanol (60 min.)
↓
95% Ethanol (60 min.)
↓
95% Ethanol (30 min.)
↓
Ethanol absolute (60 min.)
↓
Ethanol absolute (180 min.)
↓
Ethanol absolute (180 min.)
↓
Ethanol absolute (180 min.)
↓
Xylene (30 min.)
↓
Xylene (30 min.)
↓
Xylene in paraplast 1:1 (60 min.)
↓
Paraplast (60 min.)
↓
Paraplast (60 min.)
↓
Embed in paraplast

Basic histology of *O. niloticus* ovary

Ovarian tissues of the control fish at the age of 4 to 8 months were investigated. Developmental stages of oocytes were determined upon the histological characteristics according to the stages classified by West (1990) as followed.

1. Chromatin nucleolar stage
2. Perinucleolar stage
3. Yolk vesicle (cortical alveoli) formation

4. Vitellogenic (yolk) stage
5. Ripe (mature) stage

Histological alterations of *O. niloticus* ovary

The histopathological changes in ovarian tissues of the neem treated fish from 4th, 5th, 6th and 7th month of exposure were examined in comparison with the tissues of the control fish from the same period.

3.10 Data analysis and statistical procedures

The LC_{50} values at 95% confidence intervals of each exposure time were determined on computer by Probit analysis program. (Finney, 1971)

General calculations of body weight, GSI, oocyte count and oocyte diameter were performed on computer by Microsoft Excel for Windows 95 version 7.0.

Statistical analysis of the data was performed on computer by SPSS for Windows release 7.5. Student's t-test was used to compared effects on body weight, GSI, oocyte count and fecundity. Significant difference was considered from probability of $p \leq 0.05$. Oneway ANOVA was used to determined significant differences at $p \leq 0.05$ of the diameter of three groups of oocyte.