CHAPTER V

DISCUSSION

1. Acute toxicity of neem *Azadirachta indica* seed extract on Nile tilapia *Oreochromis niloticus*

In this study, the LC_{50} value of neem seed extract for 96 hr on Nile tilapia was determined at 36.25 mg/l (ppm). As the 96-hr LC_{50} of the stem bark extract of neem was evaluated in fish *Aphyosemon giardneri* at 15.1 mg/l (Osuala and Okwuaosa, 1993). Also, the 96-hr LC_{50} value of azadirachtin for juvenile salmon was greater than 4 mg/l and LC_{50} of neem extract which was the most toxic neem material to young salmon was 7±3 mg/l (Wan et al., 1996). However, the toxicity of neem-based products to the fish was depended on the solvents and emulsifiers that were used in formulating the materials and also depended on the genome of the neem tree from which the seed were collected. Moreover, the alcoholic extraction, increase in temperature within the optimal range, increase in acidity of aquatic medium and cold storage could improve the potency of the extract while boiling and room storage could reduce the activity (Osuala and Okwuosa, 1993). Therefore, neem was toxic to fish and the toxicity was related to the species difference and parts of neem tree and also depended on both concentration and duration of exposure.

2. Nile tilapia peripheral blood cells.

The descriptions of Nile tilapia *Oreochromis niloticus* peripheral blood cell in this study have virtually been confined to making with mammalian cells by referring to morphological similarities. Seven types of cell were obviously identified in this present study which included erythrocyte, thrombocyte, lymphocyte, monocyte, neutrophil, basophil and eosinophil. In contrast, there were some reports on fish haematology that could not identified some types and reported as unidentified cells (Murad and Houston, 1988). Types of leukocyte were species differences. For example, the peripheral blood of fish *Oreochromis mossambicus* comprised of lymphocyte, thrombocyte,

monocyte and granulocyte (Doggett et al., 1987). Ferguson (1976) noted that the plaice *Pleuronectes platessa* had three types of leukocyte including lymphocyte, monocyte and neutrophil. However, Onada (1934) found a couple types of *Cyprinus cyprinus* white blood cell ; lymphocyte and granulocyte.

Erythrocyte of Nile tilapia was an ellipsoidal cell with centrally located nucleus. Most of fish erythrocytes had the same structure (Watson et al., 1963, Christensen et al., 1978, Blaxhall and Daisley, 1973). However, the cell size of erythrocyte was different along species of fish. In this study, it measured about 9 x 6 μm while red blood cell of the goldfish *Carassius auratus* was about 8.8 x 13.4 μm in diameter (Watson et al., 1963) and red blood cells of the brook trout *Salvelinus fonlinalis* was about 14.5 x 10.2 μm (Christensen et al., 1987). Thrombocyte of Nile tilapia had a deep purple-staining nucleus and a relatively scanty, pink-staining rim cytoplasm. Morphologically, these cells compared favorably with these of fish *Oreochromis mossambicus* (Doggett et al., 1987) and the channel catfish *Ictalurus punctatus* (Williams and Warner, 1976). Thrombocyte was distinguished in form in different species of fish whatsoever. Thrombocyte of Nile tilapia was observed in three forms including round, spindle- and teardrop-shaped. On the other hand, Williams and Warner (1976) noted that there were two forms of thrombocyte in the channel catfish *Ictalurus punctatus* blood ; round and spindle-shaped. While Doggett et al.(1987) described two forms of thrombocyte in the peripheral blood of fish *Oreochromis mossambicus* ; an oval and teardrop-shaped thrombocytes.

Lymphocyte was numerous leukocyte in most fish. It had a spheroid nucleus with staining purple blue. The narrow cytoplasm margin had numerous fine pseudopodia extending. A similar structure was described in the cichlid *Oreochromis mossambicus* (Doggett et al., 1987) and the channel catfish *Ictalurus punctatus* (Williams and Warner, 1976). Somehow, these cells were variable in size from the very small cells (about 4 μm ϕ) to the larger cells (about 7 μm ϕ) in Nile tilapia. In the channel catfish *Ictalurus punctatus* blood, lymphocyte ranged from 5.6 to 9.0 μm (Williams and Warner, 1976). Also, Doggett and co-workers (1987) reported that lymphocyte of the cichlid *Oreochromis mossambicus* was 3 to 6.6 μm. From this study monocyte of Nile tilapia was usually round in shape with a diameter of

8 to 12 μm. The cells had the magenta-coloured eccentrically-located nucleus which was polymorphic and ranging from round, bilobed or kidney shaped. The large cytoplasm stained pale blue and vacuoles were present. The monocyte was also found in the cichlid *Oreochromis mossambicus* (Doggett et al., 1987), the channel catfish *Ictalurus punctatus* (Williams and Warner, 1976) and *Tilapia zillii* (Ezzat et al., 1974). However, this cell was not found in the circulation of goldfish *Carassius auratus* (Watson et al., 1963), brown trout *Salmo trutta* (L.) (Blaxhall and Daisley, 1973) and rainbow trout *Salmo gairdneri* (McCarthy et al.,1973). The morphological structure of monocyte was not different in most species of fish, particularly in the cichlid *O. mossambicus* and the channel catfish *I. punctatus* (Williams and Warner, 1976). The size of cell was significantly reported in different species of fish. Such the cichlid *O. mossambicus* monocyte ranged from 11.4 to 19.1 μm (Doggett et al.,1987) and also ranged from 8.3 to 65.0 μm in channel catfish *I. punctatus* (Williams and Warner, 1976).

Neutrophil of Nile tilapia was rare in the blood smear as same as fish *Tilapia zillii* (Ezzat et al., 1974), the channel catfish *Ictalurus punctatus* (Williams and Warner, 1976) and *Cyprinus carpio* (Onada, 1934). However, there were many researchers that did not report this cell type in their experimental fish such in case of fish *Salmo namaycush* (Lieb et al., 1953), *Salmo gairdneri* (McCarthy et al., 1973), *Cyprinus cyprinus* and *Misgurnus fossilis* (Onada, 1934). In this study, neutrophil was round to oval with containing an eccentric nucleus of variable form (round, intented, kidney-shaped or lobed) and the cytoplasm stained pale blue with numerous fine granules staining dark violet. A similar description of this cell type was given in the channel catfish *I. punctatus* (Williams and Warner, 1976). Difference in fish species was related to the size of neutrophil. The cell size ranged between 9 and 12 μm ϕ in Nile tilapia. Nevertheless this cell size was different in fish *T. zillii* which was between 10.8 and 12.6 μm and the nucleus was only oval (Ezzat et al., 1974). Basophil were infrequently found in the peripheral blood of Nile tilapia *Oreochromis niloticus*. There was basophil in the peripheral blood of fish *Tilapia zillii* (Ezzat et al., 1974) and channel catfish *Ictalurus punctatus* (Williams and Warner, 1976). By contrast, several workers have been unable to find basophil in the blood of fish *Cyprinus cyprinus*, *C. carpio*, *Misgurnus fossilis* (Onada, 1934), *Perca flavescens*

(Yokayama, 1947), *Salmo namaycush* (Lieb et al., 1953), *Anguilla japonica* (Sano, 1957), *Prosopium williamsoni* (McKnight, 1966), the brown trout *S. trutta* (Blaxhall and Daisley, 1973), the plaice *Pleuronectes platessa* (Ferguson, 1976) and the goldfish *Carassius auratus* (Fujimaki and Isoda, 1990). In this present study, basophil ranged about 7 to 11 μm in diameter. the eccentric located nucleus was large and sometimes oval, round or lobed in shape. A similar morphological structure of basophil was described in all fish which was reported finding this cell in their blood. However, the cell size of basophil was variable in different species of fish (Ezzat et al., 1974).

Eosinophil was rarely seen in the blood of Nile tilapia. A similar statement was mentioned in the haematological study of the channel catfish *Ictalurus punctatus* (Williams and Warner, 1976) and *Tilapia zillii* (Ezzat et al., 1974). Also, their absence in fish blood was reported in fish *Cyprinus cyprinus*, *C. carpio*, (Onada, 1934), the plaice *Pleuronectes platessa* (Ferguson, 1976), the brook trout *Salvelinus fontinalis* (Christensen et al., 1978) and the goldfish *Carassius auratus* (Fujimaki and Isoda, 1990). Nile tilapia eosinophil was round and oval in shape with an eccentric spheroid to oval nucleus. The small nucleus stained deep purple. The eosinophilic red-orange granules dispersed throughout the colorless cytoplasm. Difference to other fish species, Nile tilapia eosinophil was very fragile as compared to other leukocytes. Variable in size of eosinophil was observed in different species. In this study, the cell size of eosinophil was approximately 10 x 12 μm to 15 x 20 μm in diameter. It was the largest when compared with the channel catfish *Ictalurus punctatus* (Williams and Warner, 1976) and *T. zillii* (Ezzat et al., 1974).

3. Effect of neem seed extract on Nile tilapia blood cells

In this study, pathological changes of erythrocytes showed earlier than white blood cells. Anomalies of red blood cell occurred in both of immature and mature erythrocytes which comprised of eight patterns. The numerical increase of immature erythrocytes in blood circulation was found from the first until seventh month but the most severe effect was reported in the sixth month of exposure. A similar change was reported in coho salmon *Oncorhynchus kisutch* which exposed to various concentrations of treated wastewater containing total residual chlorine (Buckley, 1977), in the diseased silver salmon *O. kisutch.*

(Katz, 1950) and the flounder *Pleuronectes flesus luscus* maintained in water with low oxygen content (Soldatov, 1996). There were two factors which were the most commonly associated with induction of erythropoiesis in fish including decreases in red cell mass and ambient O_2 tentsion. Usually, reduction in cell numbers by bleeding or use of hemolytic agents acted as a powerful stimulus (Houston and Murad, 1992). Moreover, exposure to toxicants has speeded up the breakdown of the red blood cells, after which a compensatory erythropoiesis has often occured (Nikinmaa, 1992). In these studies, red blood cells with the extrusion of nuclear material in both immature and mature erythrocyte were observed in all treated fish. However, these effects were very marked alteration the second month exposed fish. A similar evidence of alteration was seen in fish *Puntius conchonius* (Gill and Pant, 1985), coho salmon *O. kisutch* (Buckley, 1977) and in the goldfish (Houston and Murad, 1992). These materials were sometimes called Heinz body inclusions (Buckley, 1977).

In mature erythrocytes, poikilocytosis and increasing of nuclear interchromatin space were manifested in the peripheral blood of all treated groups. The same effect was also reported in a freshwater fish *Punctius conchonius* after exposure to cadmium chloride (Gill and Pant, 1985), coho salmon *O. kisutch* early exposure to $TRCl_2$ (Buckley, 1977) and the diseased silver salmon *O. kisutch* (Katz, 1950). Vacuolated erythrocyte appeared in all groups of treatment which was demonstated that it was in the pathological status that caused by toxic agents or xenobiotic organisms. Hypochromic erythrocyte was manifested in treated fish throughout the experiment as same as in coho salmon *O. kisutch* exposed to chlorinated wastewater (Buckley, 1977).

The gray or basophilic cytoplasm appeared in all groups of treatment. These effects were probably due to thickening of cell or cell membrane and other cytoplasmic changes which resulted in altered staining properties. Similar effect was reported in the diseased fish (Katz, 1950) and coho salmon (Buckley, 1977). An appearance of a "ragged" cytoplasmic membrane or echinocyte or spiculated erythrocyte was observed in all groups of treatment. Also there was similar description in aberration of erythrocyte in the diseased silver salmon (Katz, 1950) and coho salmon (Buckley , 1977). Also, the evidence of dividing erythrocyte was early observed in the peripheral blood of treated fish which has been called amitotic division by Houston and

Murad (1992). However, the division of mature erythrocytes within the circulation has been shown an increase in the number under conditions of respiratory stress, high temperature, phenylhydrazine HCI-induced anaemia and chronic exposure to heavy metals such as cadmium (Soldatov, 1996). Also, it may indicated that neem seed extract is toxic to Nile tilapia by causing anemia.

From the result, anomalous monocyte and neutrophil were only seen in experimental fish. Monocyte with hyperthrophic nucleus was sometimes found in treated fish, espectially from the fourth until the seventh month of experiment. Early exposure to a sublethal concentration was not seen this kind of aberration (1-3 months). The alteration of neutrophil was noticed by the vacuolization in cytoplasm of exposed fish. Occasionally, it was found in fish blood exposed for 3-7 months. Howerver, this abnormality of neutrophil was relatively less evidence in this study. Also, in other species of fish, the same pathological monocyte and neutrophil were not available for comparison.

4. The effect on differential leukocyte count

Percentage of lymphocyte in treated fish increased significantly ($p \leq 0.05$) when compared with control group. Increasing of lymphocyte number may be due to an accelerated lymphopoiesis and or an efflux of lymphocytes from the lymphopoietic loci. Lymphocytosis might be also a repercussion of toxicant-induced cell and tissue damage or an acceleration lymphopoiesis. Cadmium chloride exposed fish showed the elevation of these cells (Gill and Pant, 1985). Rao and colleagues (1990) reported that five species of fish from the polluted areas had a significant increase of lymphocyte which was due to the inducement of immune defense. The immunomodulatory effect of neem oil against lymphocyte was examined by Upadhyay et al.(1992) by showing a significantly higher in lymphocyte proliferation in treatment when compared to control. Therefore, it is reasonable to ascribed to neem seed extract stimulated an accelerated lymphopoiesis in this present study.

The percentage of monocyte was significantly lower in the first, third and fourth month of Nile tilapia exposed to neem seed extract. Correspondingly, Ruparelia and colleagues (1990) reported that monocytes showed a reduction of number in tilapia *Oreochromis mossambicus* after exposed to cadmium. Alteration in the percentage of monocyte might concern with phagocytosis because Ellis and co-workers (1976) reported that this cell had a phagocytic property against carbon partical. Therefore, such cells increased rapidly in a localised area of injury espectially in necrotic tissues (Ellis, 1977). Thus, a fall in monocyte number may be due to these cells migrate from blood circulation to damaging tissue which was harmed by neem seed extract. The percentage of neutrophil was significantly higher in the second month of fish exposed to neem seed extract. Similar to this present study, tilapia *Oreochromis mossambicus* showed a significant higher in neutrophil number after exposure to cadmium (Ruparelia et al., 1990). Correspondingly, Alkahem (1994) noted that a significant elevation ($p<0.001$) exhibited in Nile tilapia *O. niloticus* after exposed to 1.5, 3.0 and 5.0 mg/l nickel chloride for 4 days. Furthermore, a significant increase in neutrophil number was found in the same species of fish that exposed to a lethal concentration (80 ppm) of neem seed extract for 96 hr (Tangtong and Wattanasirmkit, 1997). Neutrophilia in fish exposed to toxicants may be attributed to the increase longevity in circulation, release from storage sites or to differential sensitivity of lympho-and granulopoietic cells (Alkahem, 1994). Hence, similar consideration may also apply to neem seed extract that affects following all causes of neutrophilia.

The average percentage of basophil ranged from 0.12 to 1.00 % and 0.18 to 0.95 % for the control and treated fish. The difference was significantly increased after 3 months. Gill and Pant (1985) reported that fish *Puntius conchonius* exposed to 0.63 and 0.84 mg/l cadmium chloride for 96 hr presented an increase in basophil number. Also, a significant increase ($p<0.01$) in basophil count was reported in the peripheral blood of fish *O.niloticus* exposed to 1.5 and 3.0 mg/l nickel chloride for 96 hr (Alkahem, 1994). Murad and Houston (1988) noted that the basophil number of goldfish *C. auratus* was significantly elevated ($p<0.01$) after exposure to Cd^{2+}. For the effect of neem on basophil number in fish was reported a significant higher in Nile tilapia exposed to a lethal concentration of neem seed extract (Tangtong and Wattanasirmkit, 1997). Basophilia in fish exposed to toxicants may be

attributed to the increase longevity in circulation, release from storage sites or to the differential sensitivity of lympho-and granulopoietic cells (Alkahem, 1994). Hence, the increase in this cell in fish exposed to neem seed extract may be ascribed to having similar causes.

In normal blood of Nile tilapia, the mean percentage of eosinophil ranged between 0.12-0.86 %. While it ranged from 0.60-1.00 % for treated fish. A significant increasing was showed in fish exposed to neem seed extract for 3 months. The same result was reported in the similar species after exposure to sublethal concentrations (1.5, 3.0 and 5.0 mg/l) nickel chloride for 96 hr (Alkahem, 1994). Consistent with this, Murad and Houston (1988) stated that eosinophil number showed a significant higher ($p<0.01$) in goldfish *C. auratus* after exposure to Cd^{2+}. Furthermore, Tangtong and Wattanasirmkit (1997) mentioned that there was a significant ($p\leq0.05$) increase in eosinophil number in Nile tilapia exposed to a lethal concentration. The explanation of eosinophilia in fish exposed to toxic agents might be ascribed to being longer life span of this cell in peripheral blood, release from storage sites or to differential sensitivity of lympho- and granulopoietic cells. Thus, the effect of neem seed extract on the percentage of eosinophil may deal with the previous hypotheses.

5. Effect of neem seed extract on haematological parameters

The haematological parameters showed pathological condition by increasing in total leukocyte count of the fish after exposure to 25.07 ppm neem seed extract. The present result is in accordance with the finding of Rao and colleagues (1990) who studied about hematological effects in five species of mullet, catfish and mackerel from complex polluted waters of Visakhapatnam harbour (in India) that showed significantly higher. Moreover, Alteration in total leukocyte number was also observed in blood of fish that exposed to cadmium chloride (Murad and Houston, 1988), toxicants (detergent, ammonium sulphate, fertilizer and Metasystox) (Van Vuren, 1986) and an organophosphorus pesticide (Sampath et al.,1993). Allen (1994) studied about changes in the haematological profile of the cichlid *Oreochromis aureus* (Steindachner) during acute inorganic mercury intoxication. The presence of elevation in leukocyte number was appeared in fish exposed to mercury. The toxic effect of other substances was also examined in Nile tilapia *Oreochromis niloticus*. Furthermore, Lai et al.

(1990) mentioned that leukocytosis often appeared in neonates and infants administrated orally with small amounts of Margosa oil, an extract of the seed of the neem tree. So this indicates that neem seed extract affects on the increasing in the total leukocyte number that correlated to the number of lymphocyte, neutrophil, basophil and eosinophil.

Total red blood cell count did not exhibited significant difference in all groups of experimentation. However, it tended to be lower in treated fish when compared with control group. A significant reduction in the number of RBC was also observed in fish exposed to Ekaluk, an organophosphorus pesticide (Sampath et al, 1993) and detergent (Van Vuren, 1986). Erythropenia was manifested in the fish *Puntius conchonius* that was indicative of anemia (Gill and Pant, 1985). Rao and colleagues (1990) studied about haematological effects from complex polluted waters in India. Ruparelia and colleagues (1990) reported that the effect of cadmium on blood of tilapia *Oreochromis mossambicus* during prolonged exposure was resulted a decrease in red blood cell counts when compared to control and discussed that the anemia developed by tilapia. In this study, haematocrit value showed a significant decrease in the seventh month of exposure period. This was correlated to the result of total red blood cell count that seemed to be lower in treated fish when compared between control. The decline in haematocrit values was also reported in fish exposed to pulp and paper mill effluent (Jeney et al., 1996), detergent (Van Vuren, 1986) and an organophosphorus pesticide (Sampath et al.,1993). Conversely, Alkahem (1994) investigated the toxicity of nickel and the effects of sublethal levels on haematological parameters of fish *O. niloticus* but the result showed a significant ($p<0.01$) increase in the percentage of haematocrit. This demonstrates that various substances caused differently effects on blood parameters. However, similar to this present study, it was stated by other works, too. Analogously, Gill and Pant (1985) noted that the cyprinid fish *Puntius conchonius* exposed to cadmium chloride exhibited a significant ($p<0.01$) decrease in the haematocrit value.

In case of the mean cell volume (MCV), it was undifferent between control and treatment since the alteration in the value of MCV in fish blood was due to several causes such as exposure to polluted waters (Rao et al., 1990), heavy metals (Gill and Pant, 1985),

organophosphorus pesticides (Sampath et al., 1993), detergent, fertilizer and ammonium sulphate (Van Vuren, 1986). The effect of nickel chloride was also shown by changing in MCV in fish *O.niloticus* exposed to 1.5, 3.0 and 5.0 mg/l nickel chloride (Alkahem, 1994). Moreover, Ibrahim et al.(1992) stated that Brown Hisex chicks which were fed diets containing neem leaf showed a sign of macrotic anemia by increasing of the mean cell volume.

6. Effect of neem seed extract on blood biochemistry

Nile tilapia blood glucose level showed a significant decrease in the second, fifth and seventh month. Analogously, there was a report about antidiabetic effects of neem on mammals (Pillai and Santhakumari, 1984; Dixit et al.,1986). This suggests that neem may cause these effects by several mechanisms including increase peripheral glucose utilization, increased release of insulin and or inhibition of proximal tubular reabsorption mechanism for glucose in the kidney (Sharma et al., 1983). However, a similar report was in accordance with the finding of Peter and Peter (1997) who also reported that exposure of perches *Anabas testudineus* to a neem-based pesticide had lower glucose concentration in serum, thereby indicating hypoglycemic effect of the neem-base pesticide that was due to its direct metabolic effect on tissue and or due to increase in insultin secretion. Hypoglycemia is caused by damage to glucagon cells or hypopituitarism in mammals (Srivastava and Narain, 1985). Signs of anemia was also indicated by decreasing in blood glucose level (Srivastava and Narain, 1985). Janart (1997) reported about the accumulation of glycogen in liver of Nile tilapia after long-term exposure to neem seed extract. Hence, the possible effect of neem against Nile tilapia can ascribe to these hypotheses which cause by several mechanisms. However, it is able to mentioned that neem had a hypoglycemic effect in both fish and mammals.

Glutamic pyruvic transaminase (GPT) level showed an increase in all fish that exposed to a sublethal concentration of neem seed extract. But significant differences between control and treatment were exhibited in the fifth until the seventh month. Accordingly, Akah et al. (1992) examined the effect of neem leaf extract in rabbits and reported that the dose of the extract to 2,328 mg/kg resulted in a significant rise of GPT level that dealed with hepatotoxicity of this extract. On the contrary,

alkaline phosphatase (ALP) level and glutamic oxaloacetic transaminase (GOT) level were not found any significant differences in both control and treatment groups. Thus, neem seed extract may also affect liver function of Nile tilapia.